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Special Issue Reprint

Current and Future Trends in Cosmetics Research

The 10th Anniversary of Cosmetics - Volume II

Edited by
Enzo Berardesca

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Current and Future Trends in Cosmetics Research: The 10th Anniversary of Cosmetics—Volume II

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Guest Editor

Enzo Berardesca

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Guest Editor
Enzo Berardesca
Department of Dermatology
and Cutaneous Surgery
University of Miami
Miami
USA

Editorial Office
MDPI AG
Grosspeteranlage 5
4052 Basel, Switzerland

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Article

SVX Spider Silk-Inspired Biopolymer and Enhanced Cosmetics Efficacy

Konstantin Press, Noa Hadar, Ella Sklan, Alon Meir, Gregory Idelson, Tanya Karakouz, Miriam Gubelbank, Ali Abu Znaid and Shlomzion Shen *

Seevix Material Sciences Ltd., Jerusalem 9112001, Israel; kosta@seevix.com (K.P.)
* Correspondence: info@seevix.com; Tel.: +972-2-5407888

Abstract: The cosmetics industry is undergoing a shift towards sustainability and efficacy, driven by consumer demand for eco-friendly and safe products. This paper introduces SVX, a spider silk-inspired raw material intended to transform cosmetic formulations. Produced through fermentation, SVX is a biopolymer composed of self-assembled proteins characterized by a porous structure for delivering active ingredients safely to the skin. The study utilized in vitro and ex vivo methods to assess SVX's ability to protect against oxidative stress, enhance skin hydration, and support ingredient delivery. Safety assays, including the HET-CAM, patch test, and HRIPT, demonstrated that SVX is non-irritating and safe for topical application. Additionally, FTIR analysis confirmed SVX's capacity for sustained release of active ingredients, such as hyaluronic acid, over an 8 h period. Results showed that SVX significantly improved skin barrier protection and exhibited superior antioxidant properties compared to control formulations. Its biocompatibility, along with a vegan and biodegradable composition, aligns with the principles of sustainability, with over 60% biodegradability achieved within 10 days. Furthermore, SVX displayed antioxidant efficacy approximately 130 times greater than L-ascorbic acid, based on DPPH assay results. These findings suggest that SVX offers a versatile and sustainable solution for skincare formulations, combining environmental responsibility with benefits for skin health and performance.

Keywords: SVX; protein; spider silk; biotechnology; cosmetic innovation; sustainability; fermentation; vegan biopolymer; smart delivery system; green chemistry; biodegradability

1. Introduction

The cosmetics industry stands at a pivotal juncture, navigating a landscape defined by evolving consumer preferences, stringent regulatory standards, and an increasing emphasis on sustainability [1–8]. In this context, the quest for cosmetics that not only enhance beauty and longevity but also adhere to principles of environmental responsibility and safety is of great importance [9–12]. Consumers are seeking products that align with their ethical values, eschewing harmful chemicals and embracing eco-friendly formulations that promote both personal wellness and planetary health [13–15].

Against this backdrop, the cosmetics industry faces several unmet needs, prompting a paradigm shift towards solutions rooted in green chemistry and sustainability [16,17]. One pressing demand is for cosmetics crafted through eco-friendly practices, devoid of harmful chemicals, and compatible with vegan lifestyles [18–23]. SVX (INCI: *sr-(Tetrapeptide-74 Hexapeptide-40 Expression Vector pMBP-parallel 1 Polypeptide-1 Spider Polypeptide-1 Spider Polypeptide-5*), offers a potential solution to these unmet needs [24–28].

SVX, a novel raw material, represents a convergence of technology and sustainability [29–31]. Produced through a fermentation process utilizing biotechnology processes, SVX is a large biopolymer composed of about 470,000 protein monomers that self-assemble into particles of approximately 0.7 microns. This biopolymer, with its non-penetrative

yet porous structure, offers a novel approach to cosmetic formulation, providing safe and effective delivery of active ingredients without compromising skin integrity [32–36].

Moreover, SVX is intended to answer the industry's commitment to eco-friendly practices. As a sustainable and vegan biopolymer, SVX underscores the potential for cosmetics to be both effective and responsive to environmental concerns. Furthermore, SVX boasts antioxidant properties, surpassing conventional antioxidants such as vitamin C in efficacy. By forming a protective network on the skin, SVX acts as a shield against free radicals, oxidative stress, and environmental pollutants, offering multifaceted benefits for cosmetics applications [37].

Recombinant proteins, encompassing growth factors, chemokines, and structural and membrane proteins, are increasingly in demand due to their purity, their efficacy, and their ability to be produced in greater amounts. In cosmetics, they are prized for their ability to rejuvenate skin, repair wounds, and improve wrinkles, owing to their effect on collagen. In haircare, they are known to improve shine and health. The appeal of recombinant proteins in beauty products is further bolstered by their ability to be produced without animal sources, making them suitable for vegan formulations. These proteins can also be used in products sold in regions with stringent regulations, such as Europe, where materials isolated directly from human cells are forbidden in cosmetics [38].

In light of these developments, there is a growing need to explore the multifaceted potential of SVX in cosmetic formulations. This paper endeavors to unravel the properties and applications of SVX, shedding light on its potential role as a transformative raw material in the cosmetics industry. Through analysis and empirical evidence, this paper aims to elucidate the benefits of SVX, from its impact on skin physiology to its efficacy as a smart delivery system for sensitive ingredients [38]. By examining SVX through a scientific lens, this paper seeks to underscore its potential as a game-changer in cosmetic innovation, paving the way for a more sustainable and conscientious approach to skin and hair care [39–45].

2. Materials and Methods

2.1. SVX Production

Fermentation process: SVX's one-step fermentation process represents an advancement in biotechnology. Using recombinant DNA technology, a patented DNA code inspired by spider silk DNA is engineered into bacteria that synthesize SVX. Bacteria produce SVX in a controlled fermentation process. The downstream process includes the purification of SVX from the bacteria. This scalable and controlled process, with high batch-to-batch consistency and purity, eliminates the need for extracting or depleting natural resources, further solidifying SVX as a sustainable potential solution for the cosmetic industry [46].

2.2. SEM Image Analysis

Instrument: Analytical high-resolution scanning electron microscope Apreo 2S by Thermo Fisher Scientific (Waltham, MA, USA).

Cryo-SEM: The instrument is equipped with the system PP3010 (manufactured by Quorum Technology, Lewes, UK), permitting fast cooling of samples down to cryogenic temperatures, their fracture, coating, and SEM imaging at controlled temperatures.

Sample preparation: Prior to the SEM imaging, the samples were iridium-coated for 1 min using the Quorum Q150V S Plus Sputter Coater (Quorum Technology, UK). This coater is designed to produce fine-grain "cool" coatings, achieving thin coatings of 2–3 nm without charging effects in SEM. Iridium and gold-palladium coatings are available for different types of samples.

2.3. Biodegradability Test

Biodegradability assessment: The biodegradability of SVX was assessed by Bio-EC labs following OECD 301 B guidelines. This study aimed to determine the biodegradability

of raw material ingredients by evaluating the production of CO_2 or dissolved organic carbon (DOC) over a minimum of 28 days in a liquid environment [47].

Test conditions:

- Sample preparation: The test was performed on a 10% SVX (*w*/*w*) dispersion in water.
- Test system: The biodegradability of organic compounds by microorganisms in an aquatic medium was examined using a static test system. This test system included an inorganic medium, with the organic compound serving as the sole nominal source of carbon and energy (with a theoretical concentration of 10 to 20 mg/L of carbon), and a mixed inoculum from an urban wastewater treatment plant. The final blend of this test system contained less than 30 mg/L of suspended matter.
- Incubation: The mixture was stirred in test vessels exposed to a CO_2-free airflow for approximately 28 days at a temperature of 22 °C ± 2 °C. The test duration could be extended by two weeks if degradation had commenced but had not yet reached a plateau.

CO_2 measurement:

- CO_2 trapping: Carbon dioxide generated during microbial degradation was collected in external vessels containing a barium hydroxide solution and subsequently quantified via titrimetric analysis.
- CO_2 calculation: The measured CO_2 was compared to the theoretical amount (CO_2Th) and expressed as a percentage. CO_2 capture occurred in the first vessel containing the barium hydroxide solution, positioned close to the test blend. The remaining vessel was exchanged for one containing a freshly prepared barium hydroxide solution.
- Barium hydroxide volume: The volume of barium hydroxide in the vessels ranged from 250 to 300 mL, depending on the interval between the two measurements.

Final analysis:

- Post-incubation: On the 28th day, about 1 mL of concentrated hydrochloric acid was added to each test vessel to decompose carbonates and bicarbonates. The obtained values were referenced as the "28 bis" incubation day.

This detailed methodology ensured accurate and reliable measurement of the biodegradability of the 10% SVX (*w*/*w*) dispersion in water, reinforcing its suitability as a sustainable ingredient in cosmetic formulations.

2.4. Safety Assessments

Safety assessments of SVX were conducted by Bio-EC labs (Longjumeau, France) and include the following tests:

Patch test: Evaluated the cutaneous compatibility of SVX fibers. The fibers were applied to the external face of the arm for 48 h on 22 volunteers. Post-application, the skin condition was assessed.

Human repeat insult patch test (HRIPT): Determined allergic reactions to SVX biopolymer and SVX-based anti-wrinkle serum on 50 healthy subjects. Ten applications with a sealing patch were examined up to 48 h after removal.

Phototoxicity: An in vitro method was used to assess the phototoxic potential of SVX fibers (Phototoxicity testing methods and applications, Cosmetics Europe, 1997).

Ocular irritation: Assessment was conducted using the hen's egg chorioallantoic membrane (HET-CAM) method.

Ethical conduct of the study: The study adhered to the principles of good clinical practice as outlined in the ICH Topic E6 "Note for Guidance and Good Clinical Practice" (CPMP/ICH/135/95), along with the Helsinki Declaration (1964, WMA) and its subsequent revisions. It was conducted according to the sponsor's defined standard operating procedures and study protocol. All recorded study events were reported, with data accuracy and compliance with the protocol verified and confirmed by study participants.

Scope of tests compliant with:

- Regulation of the European Parliament and the Council (EC) No. 1223/2009 of 30 November 2009 on cosmetic products.
- Cosmetics Europe—The Personal Care Association (previously COLIPA) Guidelines "Product Test Guidelines for Assessment of Human Skin Compatibility 1997".
- Cosmetics Europe—The Personal Care Association (previously COLIPA) Guidelines for Evaluation of the Efficacy of Cosmetic Products 2008.
- "Appraisal of Safety of Chemicals in Foods, Drugs and Cosmetics" by J.H. Draize, published by the Association of Food and Drug Officials of the United States.

Ethical committee:

In line with the procedures of the investigating center, the protocol, informed consent form, and preclinical information concerning the investigational product SVX were submitted to the center's internal committee. The committee ensured that the project met the conditions of optimal scientific rigor, assessed its general relevance and the suitability between the aim followed and the means implemented, and gave an opinion on the protection of the test subjects. The study began after the approval of the survey committee.

2.5. Antioxidant Activity

DPPH assay: Antioxidant activity was evaluated by Bio-EC labs using the DPPH method, compared to L-ascorbic acid (vitamin C). The test measures the ability of an antioxidant to reduce the DPPH radical, with absorbance read at 517 nm using a UV-MC2 spectrophotometer (SAFAS, Monaco). Results were expressed as vitamin C equivalent antioxidant capacity (VCEAC) in mmol/L [29,48].

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is widely used to evaluate the antioxidant activity of various substances. It is based on the ability of antioxidants to donate hydrogen atoms or electrons to neutralize DPPH radicals, resulting in a color change that can be quantitatively measured. The underlying principle of the DPPH assay supports its use in assessing antioxidant activity because the capacity to scavenge free radicals is a hallmark of antioxidant behavior [19–21].

Concentration of SVX: A 1% SVX (*w*/*w*) dispersion in water was used.

Dilutions prepared: 1/500, 1/1000, 1/1500, 1/2000, 1/2500, 1/3000, and 1/4000.

EC50 Values: The concentration required to achieve a 50% antioxidant effect (EC50) for L-ascorbic acid is 0.3 mM. For SVX, the dilution necessary to achieve a 50% antioxidant effect (EC50) is 1/2500.

2.6. Environmental Pollution Modeling

Pollution modeling: Carbon black particles (PM2.5–10) were used to model environmental pollution. These particles were procured from Sigma-Aldrich (St. Louis, MI, USA) and applied to a skin model made from polyurethane. The polyurethane served as a skin model, with a 30 mm diameter circle marked on its surface.

Application procedure:

1. Formula application: 100 mg of the tested formula was applied within the marked circle using circular motions, allowing the solvents to evaporate.
2. Pollution application: Carbon particles were applied to achieve a concentration of 3 mg/cm^2 on the surface.
3. Washing process: The sample was then washed under running water for 20 s.
4. Scrubbing process: The polyurethane model was scrubbed with a brush for 20 s and washed again for an additional 20 s under running water.

Analysis: Each step was monitored using ImageJ analysis (version 1.53k) and delta L measurements, with five repetitions conducted for accuracy.

2.7. Hair Experiments

Experiments involving hair used European blond virgin hair:

Hair denaturation temperature: Measured using differential scanning calorimetry (DSC) with heating steps of 20 °C per minute from 100 °C to 300 °C under a nitrogen flow of 60 mL/min. The DSC-1 instrument is by Mettler Toledo (Greifensee, Switzerland). The crucibles are standard aluminum with a volume of 40 µL.

2.8. Slow-Release Mechanism and Protection Studies

Materials: Pure retinol was purchased from Sigma-Aldrich. Retinol 50 C Complex, a commercially available retinol complex, was obtained from BASF (Ludwigshafen, Germany). Hyaluronic acid (PrincipHYAL®) was provided by ROELMI Group (Lombardy, Italy).

Retinol stability measurement: Retinol stability was measured using a spectrophotometer.

FTIR analysis of hyaluronic acid (HA) release:

Complex creation: A complex of SVX:HA was created and monitored by FTIR techniques.

Measurement: An experiment to demonstrate the gradual release of hyaluronic acid was conducted using FTIR, comparing two peaks: the SVX peak at 1624 cm^{-1} and the HA peak at 1010 cm^{-1}.

2.9. Statistical Analysis

All experimental data were analyzed using a two-sample t-test to assess the significance of differences between test groups. A two-tailed test with equal variance was applied, where the first two arguments correspond to the two datasets being compared, the third argument (2) represents a two-tailed test, and the fourth argument (2) indicates equal variance (homoscedastic). A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. SVX—General Information

SVX is a novel raw material in the cosmetics industry, characterized by its unique properties and production methodology. SVX is synthesized through a fermentation process utilizing bacteria, resulting in the formation of particles with dimensions akin to bacteria themselves, approximately 0.7 microns (Figure 1). The significance of this size lies in its non-penetrative nature, ensuring that SVX remains on the skin's surface without causing irritation or adverse reactions. Due to its relatively large size, high surface area, and porous structure, SVX is potentially an ideal encapsulation and carrier for various active and sensitive ingredients commonly employed in cosmetics [39–45].

The benefits of SVX extend beyond its physical characteristics. As a sustainable and vegan biopolymer, SVX embodies the principles of green chemistry, aligning with the industry's shift towards eco-friendly formulations [49–56].

SVX further distinguishes itself by being readily biodegradable (Figure 2), aligning with the increasing emphasis on sustainability within the cosmetics field. This feature underscores SVX's commitment to environmental responsibility, offering a green alternative in cosmetic formulations without compromising efficacy or safety [57].

SVX represents an advancement in cosmetic raw materials, distinguished by its properties and safety profile. Testing has affirmed SVX's suitability for cosmetic applications, ensuring both efficacy and safety for consumers.

SVX has successfully undergone safety evaluations, including patch testing and human repeat insult patch testing (HRIPT). In patch studies, SVX demonstrated non-irritant properties after 48 consecutive hours of application on 22 volunteers' external arm surfaces. Similarly, in HRIPT studies involving 50 healthy subjects, both SVX raw material and final product SVX-based anti-wrinkle serum exhibited no dermal irritation or sensitization, indicating their safety for human use. Furthermore, evaluations of SVX's ocular irritation potential through in vitro methods, specifically utilizing the hen's egg test on the chorioallantoic membrane (HET-CAM), revealed a moderately irritant classification for SVX. However, it is noteworthy that the irritation observed was within acceptable limits,

ensuring minimal risk to ocular tissues. Additionally, assessments of SVX's phototoxic potential through in vitro tests yielded favorable results, with SVX categorized as "non-phototoxic". This signifies SVX's compatibility with prolonged exposure to light, ensuring its safety for use in cosmetic formulations intended for daytime application.

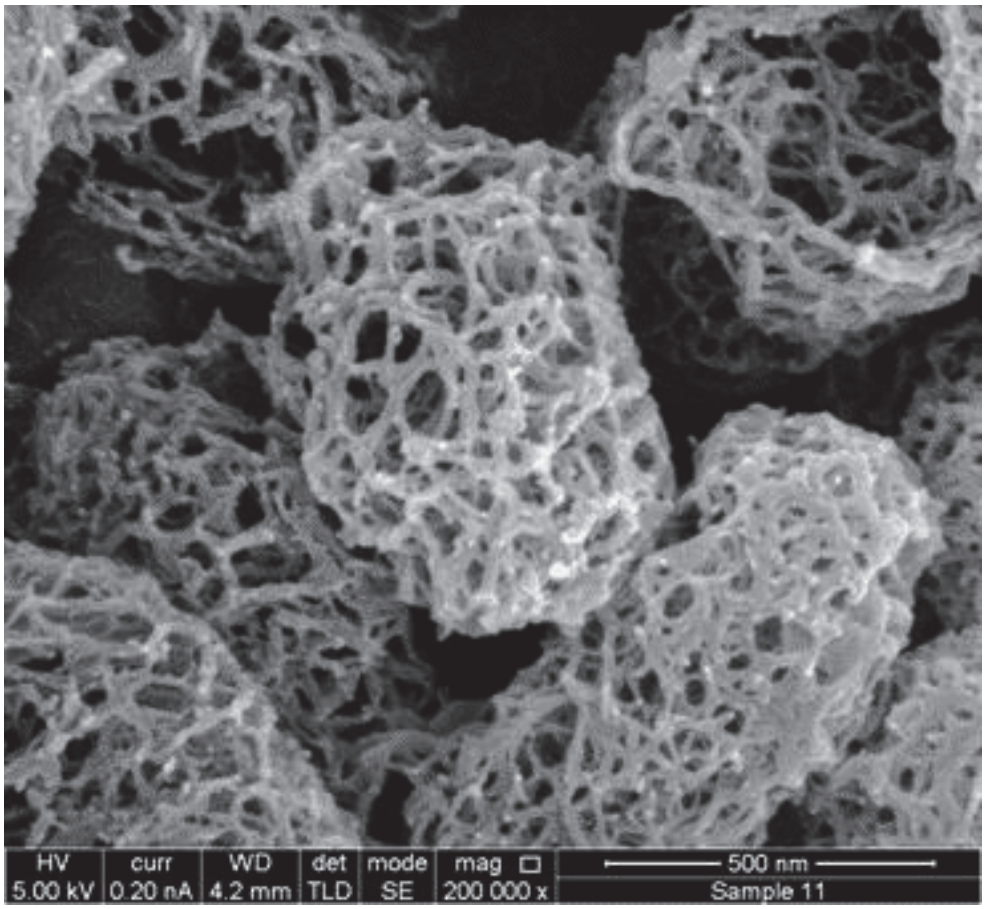


Figure 1. SEM analysis of SVX composed of 470,000 proteins.

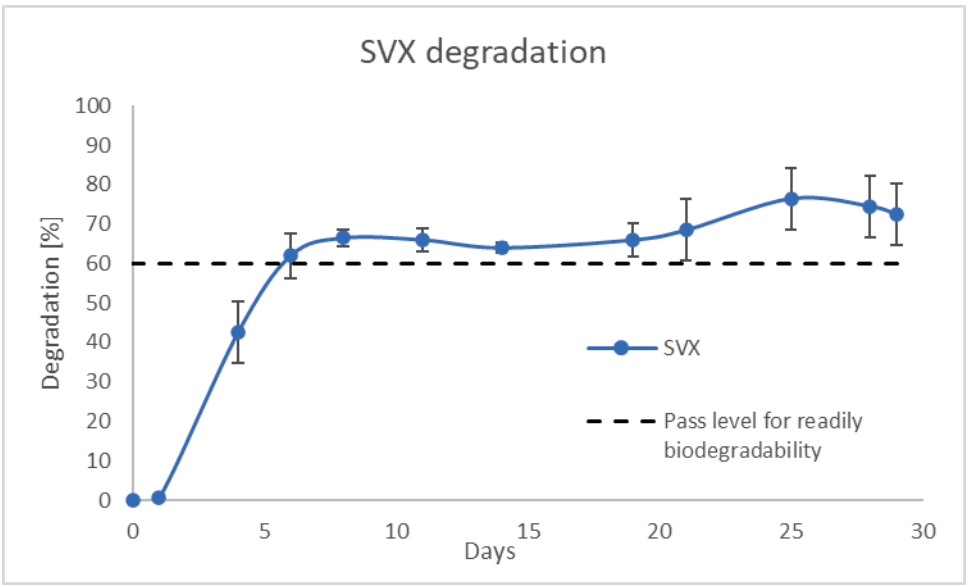


Figure 2. SVX is readily biodegradable, SVX biodegradable at 73%.

With its proven safety profile and multifaceted potential benefits, SVX emerges as a candidate for innovative and sustainable cosmetic formulations, intended to meet the evolving needs of consumers seeking effective, eco-friendly skincare solutions.

3.2. SVX—Antioxidant

Building upon SVX's antioxidant properties, this section presents findings from the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, a widely recognized method for evaluating antioxidant activity [58]. By quantifying SVX's ability to neutralize free radicals and inhibit oxidative damage, this section underscores the antioxidant potential of SVX and its implications for skincare formulations (Figure 3).

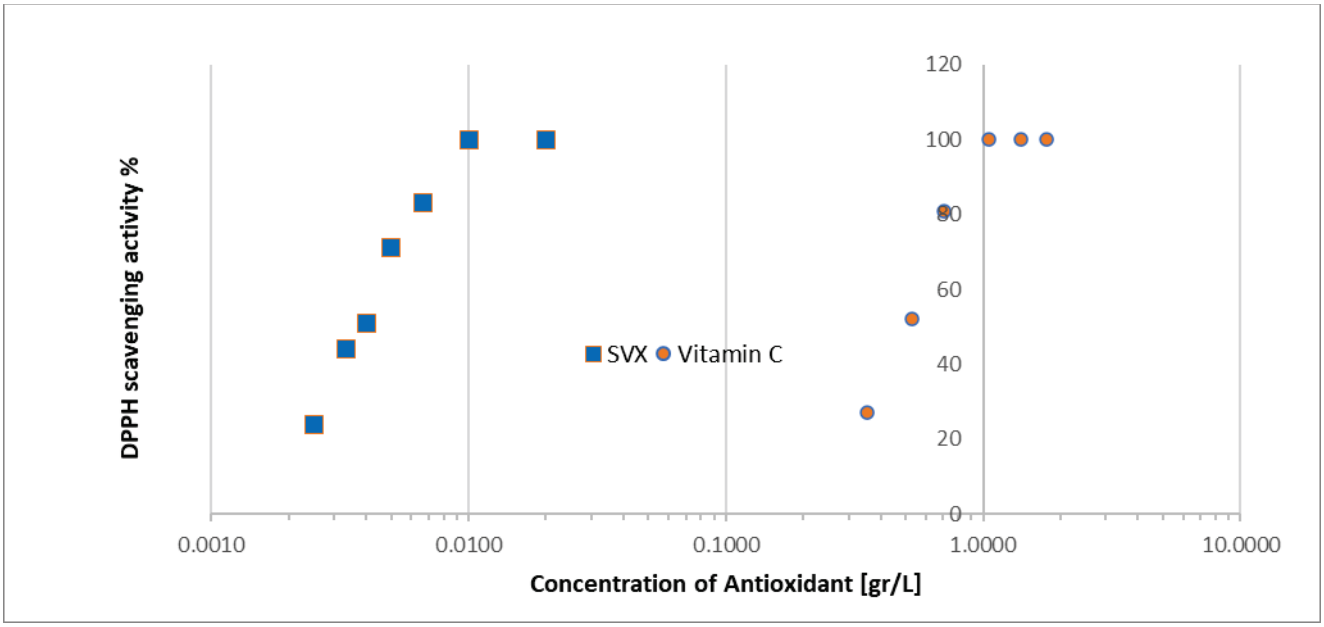


Figure 3. The graph represents the antioxidant activity of SVX compared to L-ascorbic acid, using the DPPH radical scavenging assay (logarithmic scale). The scavenging activity is measured by the percentage of DPPH radicals neutralized at varying concentrations of SVX and L-ascorbic acid. SVX demonstrates approximately 130 times more potent radical scavenging activity at equivalent concentrations compared to L-ascorbic acid.

To evaluate the antioxidant activity of SVX in comparison to L-ascorbic acid, a DPPH assay was conducted. This assay is based on the capacity of antioxidant compounds to reduce DPPH by hydrogen transfer, resulting in a measurable discoloration of the solution. The extent of this reaction is proportional to the antioxidant capacity of the tested substances. In this experiment, both SVX and L-ascorbic acid were exposed to DPPH radicals in parallel, with their scavenging activities being monitored.

The DPPH scavenging activity of L-ascorbic acid indicates that the concentration required to achieve a 50% antioxidant effect (EC50) is 0.3 mM, establishing it as a potent antioxidant. Figure 3 illustrates the DPPH scavenging activity of SVX, where the dilution necessary to achieve a 50% antioxidant effect (EC50) is 1/2500. This dilution highlights the potency of SVX as an antioxidant.

Further analysis revealed that the antioxidant activity of the SVX 1% in water at 1/2500 dilution corresponds to a vitamin C equivalent antioxidant capacity (VCEAC) of 0.3 mM. When converting the concentration of SVX and vitamin C to [g/mol], the concentration of vitamin C is 0.53 g/L and the concentration of SVX is 0.04 g/L, where both items achieve a 50% antioxidant effect (EC50). The ratio between the concentrations is 130, meaning that SVX is approximately 130 times more powerful as an antioxidant than L-ascorbic acid.

SVX showcases antioxidant properties that extend beyond skin protection, offering comprehensive potential benefits for cosmetic formulations. Its antioxidant ability plays a pivotal role in safeguarding against oxidative stress and preserving the integrity of various cosmetic products.

Studies have underscored SVX's efficacy in neutralizing free radicals, mitigating the detrimental effects of oxidative damage on the skin. Furthermore, SVX's antioxidant capacity extends to preserving color integrity, particularly in haircare formulations. By combatting oxidation, SVX serves to prevent color fading, ensuring long-lasting vibrancy and vitality of hair dyes.

The antioxidant properties of SVX position it as a potentially versatile ingredient in cosmetic formulations, offering not only skin protection but also color preservation benefits. As oxidative stress continues to be a significant concern in the cosmetics industry, SVX emerges as a possible solution for enhancing product stability and longevity, thereby meeting the evolving needs of consumers seeking effective and enduring cosmetic solutions.

3.3. SVX—Barrier and Protection

Through its unique properties, SVX creates a protective layer [59–63] on both skin and hair, guarding against various environmental stressors. Studies have demonstrated SVX's ability to fortify the skin's barrier function, enhancing its resilience against external aggressors such as pollutants [64–69] and free radicals (Figure 4, Table 1).

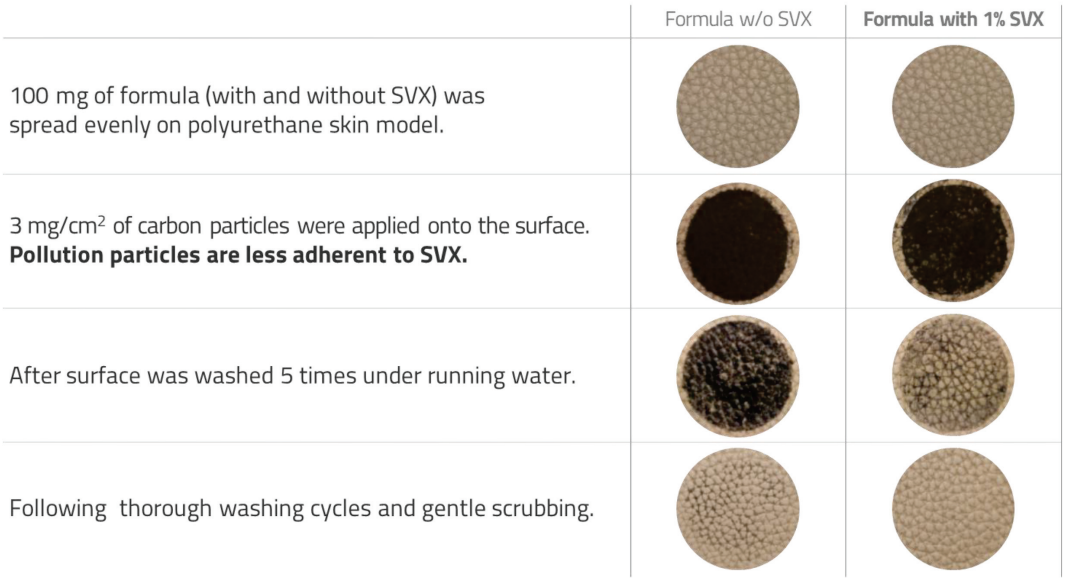


Figure 4. SVX acts as a protective barrier against environmental damage.

Table 1. Antipollution measurements.

Skin Model Tone	Formula w/o SVX (n = 7), Mean (SEM) [1]	Formula with 1% SVX (n = 7), Mean (SEM) [1]	*p* Value
Before application of pollution particles	142.1 (0.6)	141.7 (0.9)	0.5172
Immediately after application of pollution particles	35.6 (1.2)	40.5 (3.4)	0.0194
After 5 washes under running water	59.2 (3.7)	97.1 (3.9)	3×10^{-7}
After gentle scrubbing	117.1 (9.8)	138.2 (4.6)	2×10^{-3}

[1] Measured by ImageJ (version 1.53k).

SVX not only serves as a shield for the skin but also extends its protective benefits to hair strands. Through its properties, SVX creates a formidable protective layer on both skin and hair, guarding against various environmental stressors.

SVX imparts a protective shield on hair fibers, safeguarding against extreme temperatures and preventing color fading. This protective layer acts as a barrier, shielding the hair from the damaging effects of heat styling tools and environmental stressors, thereby promoting hair health and vitality (Table 2).

Table 2. Heat damage. Temperature denaturation measurement.

Hair Type	Temperature of Denaturation [°C] [1], n = 3
Hair before heat damage	256.7 (1.3)
Hair treated with formula with SVX after heat damage	246.8 (0.9)
Hair treated with formula without SVX after heat damage	233.9 (1.0)
Unprotected hair after heat damage	227.6 (1.5)

[1] Measured by DSC.

Table 2 demonstrates the efficacy of SVX in protecting hair fibers from heat damage, as evidenced by temperature denaturation measurements. Hair treated with a formula containing SVX after heat damage shows a denaturation temperature of 246.8 ± 0.9 °C, which is significantly higher than the 233.9 ± 1.0 °C observed in hair treated with a formula lacking SVX. This 12.9 °C difference ($p < 0.0001$) underscores the enhanced protective effect of SVX against heat. Furthermore, untreated hair exposed to heat damage denatures at a much lower temperature of 227.6 ± 1.5 °C, highlighting the substantial protection afforded by SVX.

In the color fading experiment (results shown in Figure 5, Table 3), fresh-colored hair was dipped into chlorinated water to demonstrate the damaging effects of pool water. The unprotected hair almost lost all its pigment, reverting to its natural color and becoming dry and brittle. Hair treated with a formula without SVX lost a significant amount of pigment and became very dry. However, the hair treated with the formula containing SVX retained almost the same color as freshly colored hair and maintained a smooth, soft texture. The difference in ΔE values of hair between the treatment with the formula containing SVX and the formula without SVX is 13.2 ($p < 0.0001$), underscoring the significance of SVX as a critical component for effective color protection. This experiment underscores the protective qualities of SVX, highlighting its effectiveness in preserving hair color and texture against harsh environmental conditions. SVX's ability to shield hair from oxidative damage and moisture loss underscores its potential as a valuable ingredient in haircare formulations aimed at prolonging color longevity and enhancing hair health.



Figure 5. Color fading following exposure to chlorinated and salt water for 3 days. SVX-coated hair samples showed only minimal fading.

Table 3. Total color change under the extreme oxidative conditions of pool water.

Hair Treatment	Delta E Calculation (n = 7), Mean (SEM) [1]
Unprotected hair	23.2 (0.9)
Hair treated with formula w/o SVX	18.2 (0.5)
Hair treated with formula with SVX	5.0 (0.6)

[1] Measured by ImageJ. $\Delta E = \sqrt{\left\{ (\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2 \right\}}$. ΔE is composed of the three-color units, and represents the interval between 2 points in the tri-dimensional color space—thus the difference of colors of 2 sets of L*a*b values. A high positive value indicates color is fading and/or losing the intensity.

Through its dual functionality in skincare and haircare, SVX emerges as a versatile ingredient with potential in cosmetic formulations. Its ability to create protective barriers on both skin and hair opens up new avenues for product development, catering to the diverse needs of consumers seeking effective, eco-friendly solutions for their skincare and haircare routines.

3.4. SVX—Smart Delivery System

The smart delivery system is an aspect of SVX's functionality, leveraging its properties to encapsulate and protect sensitive ingredients while enabling their controlled release [70,71]. SVX serves a dual role: It shields sensitive actives from environmental degradation and facilitates their gradual release onto the skin, enhancing efficacy and reducing potential irritation. This section presents experimental evidence supporting these capabilities.

Retinol: Retinol [72], a gold standard in skin rejuvenation, is highly sensitive to oxidation and can cause skin irritation. To evaluate the protective ability of SVX, we conducted a series of experiments exposing different retinol formulations to highly oxidative conditions (Figure 6a). Using materials including pure retinol, commercial retinol (Retinol 50C by BASF), and the SVX:retinol complex, we assessed stability in oxidative environments with hydrogen peroxide in an alkaline medium, monitored by FTIR techniques. The results clearly demonstrated that pure retinol degraded immediately, commercial retinol showed degradation over time, while the SVX:retinol complex maintained stability, preserving retinol in a fresh form. A similar experiment using a DPPH solution to expose the formulations to free radicals showed that the SVX:retinol complex had better stability compared to both pure and commercial retinol, highlighting SVX's protective capability (Figure 6b).

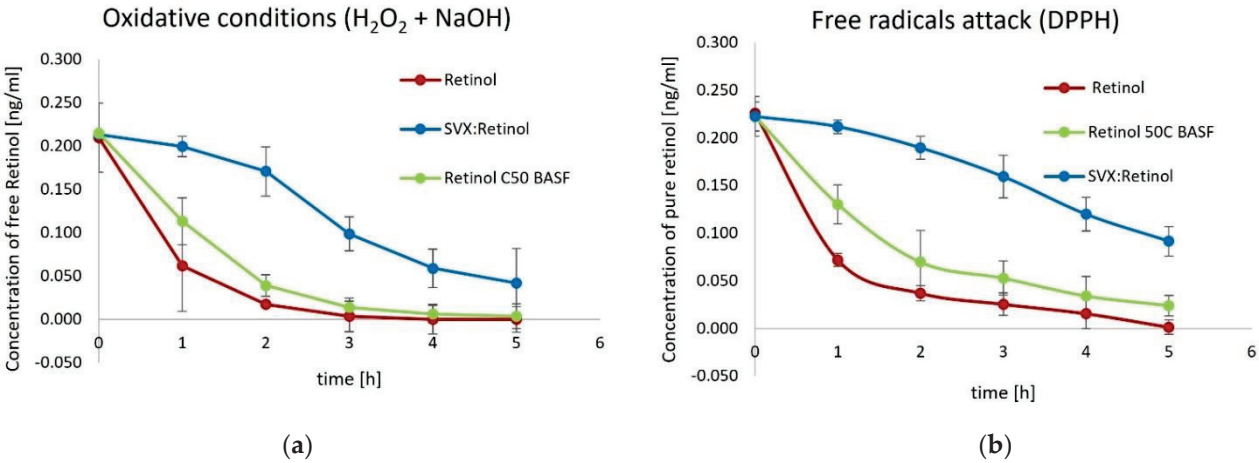


Figure 6. SVX protection of sensitive retinol: (**a**) under highly oxidative conditions; (**b**) under free radical attack.

Hyaluronic acid: In another set of experiments, we demonstrated the controlled release of hyaluronic acid (HA) using the SVX complex. SVX's porous structure, rich in various functional groups, facilitates the formation of hydrogen bonds, allowing it to absorb HA

efficiently and then release it gradually. Figure 7 shows the HA release rate from SVX in comparison to non-porous particles like silk and cellulose fibers, which can only interact with HA on the surface area. In contrast, SVX's large porous structure allows for significant absorption of HA, followed by a gradual release. The same experiments were conducted with glycolic and lactic acids, showing a similar trend. The benefit of gradual HA release is prolonged moisturization.

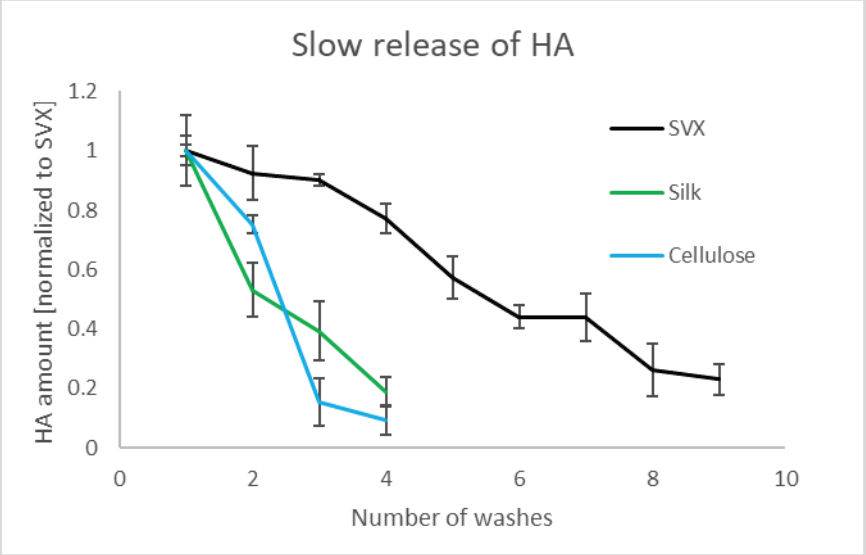


Figure 7. Slower release of hyaluronic acid (HA) measured by FTIR in comparison to non-porous silk and cellulose fibers.

This slow-release mechanism is crucial for reducing irritation while maintaining effective moisturization, addressing an unmet need in the cosmetic industry. High concentrations of HA provide prolonged hydration, but the SVX complex allows for a gentler, sustained moisturizing process.

Unlike traditional encapsulation systems, which are often sensitive to high temperatures and mechanical damage, SVX offers an alternative, making it a promising innovation for the encapsulation and delivery of sensitive cosmetic ingredients. In summary, the smart delivery capabilities of SVX enhance the stability and effectiveness of active ingredients like retinol and hyaluronic acid, offering advantages in cosmetic formulations. The dual role of protection and controlled release positions SVX as a potentially valuable addition to the field of cosmetic science.

4. Discussion

The development and application of SVX as a multifunctional biopolymer in skin and hair care formulations represent an advancement in the field. This study has demonstrated the benefits of SVX through various experiments, emphasizing its potential to enhance product performance and safety.

SVX's ability to form a skin barrier and protective layer has been substantiated through experiments using a polyurethane skin model. The application of SVX significantly enhanced the removal of PM2.5–10 pollution particles compared to control samples, illustrating its capacity to protect the skin from environmental pollutants. This protective barrier was also effective for hair, where SVX-treated hair maintained its color integrity better than untreated hair when exposed to seawater and pool water, highlighting its antioxidant properties that prevent color fading and structural damage.

The antioxidant capabilities of SVX were rigorously compared to vitamin C, the gold standard in skincare antioxidants. While vitamin C is known for its potency, its instability in the presence of air and water limits its efficacy. In contrast, SVX exhibited robust antioxidant

activity, maintaining its effectiveness over time and under various conditions. This stability is crucial for protecting skin and hair from oxidative stress and environmental damage.

Safety tests conducted by Bio-EC labs, including patch tests, HRIPT, phototoxicity, and ocular irritation potential, confirmed that SVX is non-irritant and non-sensitizing, making it suitable for cosmetic use. Additionally, SVX was shown to be readily biodegradable, addressing environmental concerns associated with many synthetic polymers used in cosmetics.

The smart delivery system of SVX further underscores its innovative potential. Through encapsulation experiments, SVX demonstrated superior protection and controlled release of sensitive ingredients like retinol and hyaluronic acid. Retinol, known for its skin rejuvenation benefits but prone to oxidation and irritation, was stabilized effectively within the SVX matrix. The SVX:retinol complex maintained its integrity under oxidative stress and free radical exposure, outperforming both pure retinol and commercial retinol complexes. Similarly, the SVX:HA complex provided a gradual release of HA, enabling prolonged moisturization.

These findings collectively indicate that SVX offers a multifaceted potential solution for enhancing the stability, efficacy, and safety of cosmetic formulations. Its ability to form a protective barrier, act as a potent antioxidant, and function as a smart delivery system could make it a valuable addition to the cosmetic industry's toolkit. The stability of active ingredients like retinol and HA within the SVX matrix not only improves their performance but also extends their shelf life, offering practical benefits for product development and consumer use.

The incorporation of SVX into skin and hair care products presents a promising approach to addressing common challenges in cosmetic formulations. Its multifunctional properties—ranging from environmental protection and antioxidation to smart delivery and biocompatibility—position SVX as a material with the potential to drive innovation and improve the overall effectiveness of cosmetic products. Future research should explore the long-term benefits of SVX in various formulations and its performance in broader environmental conditions to fully harness its potential in the cosmetics industry.

5. Conclusions

In conclusion, SVX emerges as a multifaceted potential solution addressing critical needs in the cosmetics industry. Through its unique properties, sustainable production process, and antioxidant activity, SVX offers a promising avenue for the development of eco-friendly, effective, and safe cosmetic formulations. By encapsulating and delivering sensitive ingredients while fortifying the skin's barrier and protecting against environmental stressors, SVX represents an advancement in cosmetic innovation, paving the way for a more sustainable and conscientious approach to skin and hair care without compromising its performance, positively changing consumers' experience.

6. Patents

This work has led to the filing of two patents that describe the cosmetic applications of the SVX biopolymer and its modifications, highlighting its multifunctional benefits in skincare and haircare formulations [12,13].

Author Contributions: Conceptualization, K.P., S.S. and N.H.; methodology, E.S.; software, T.K.; validation, A.M. and G.I.; formal analysis, K.P., N.H. and S.S.; investigation, N.H., M.G. and A.A.Z.; resources, A.M.; data curation, T.K.; writing—original draft preparation, K.P.; writing—review and editing, K.P., N.H. and S.S.; visualization, M.G.; supervision, K.P. and S.S.; project administration, K.P. and N.H.; funding acquisition, S.S. All authors have read and agreed to the published version of the manuscript.

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Bulk RNA Barcode Sequencing Reveals Role of RNA Splicing in Aging Dermal Stem Cell Modulation by a Botanical Extract

Julia Baumann [1,*], Valentine Vocat [2], Kathrin Nowak [1], Fred Zülli [1], Chennakesava Cuddapah [2] and Franziska Wandrey [1]

[1] Mibelle Group Biochemistry, 5033 Buchs, Aargau, Switzerland
[2] Curio Biotech Ltd., 3930 Visp, Bern, Switzerland
* Correspondence: julia.baumann@mibellegroup.com

Abstract: Skin aging is a complex, multifaceted process influenced by both intrinsic and extrinsic factors. Understanding the molecular mechanisms underlying skin aging is crucial for developing effective anti-aging strategies. Dermal stem cells play a pivotal role in maintaining skin homeostasis, but their functionality is compromised with aging. This study investigated the impact of aging on dermal stem cells and explored the potential of natural extracts in modulating their biological characteristics. Using bulk RNA barcoding and sequencing (BRB-seq), we identified differentially expressed genes (DEGs) between young and aged dermal stem cells, revealing alterations in cellular processes, including cell proliferation, ECM synthesis, and RNA splicing. We also demonstrated that a natural extract, comprising callus cells and Alpine rose leaf extracts, influenced RNA splicing in aged dermal stem cells, leading to improved dermal structure and integrity in vitro. Our findings suggest that natural extracts may exert their effects through senolytic activity and the modulation of RNA splicing, a process crucial to gene expression and cellular function. This study underscores the potential of integrating high-throughput transcriptomics in understanding skin aging, presenting new avenues for the development of innovative, sustainable, and effective anti-aging strategies.

Keywords: xeno-free; primary human 3D culture; PhytoCellTec^{TM}; emerging hallmark of aging; skin longevity; callus cells

1. Introduction

Skin aging is a multifaceted process influenced by both intrinsic and extrinsic factors, leading to changes in skin elasticity, texture, and resilience [1]. Understanding the molecular mechanisms of skin aging is notably critical for the development of effective anti-aging strategies. Stem cells, particularly dermal stem cells, play a pivotal role in maintaining skin homeostasis and regeneration [2]. However, these cells are not unaffected by the aging process. Aging-related changes in stem cell functionality, including diminished proliferative capacity and altered differentiation potential, can significantly contribute to tissue aging [3]. The cellular mechanisms underlying stem cell aging are of great interest, as enhancing stem cell regenerative capacity is a favorable strategy to promote healthy aging. Several molecular pathways affect skin stem cells at the cellular level, including alterations in DNA repair and stability, telomere shortening, oxidative stress, mitochondrial dysfunction, and altered hormonal signaling [4–6]. As such, these pathways may further be interdependent, delivering cues that affect the skin stem cell niche in a complex manner. For instance, estrogen signaling is reported to help maintain telomere length [7], while oxidative stress and the resulting reactive oxygen species result in DNA damage and telomere shortening [8]. Several reports have found that maintaining adult stem cells required for skin maintenance can prevent premature skin aging [9–11]. Therefore, exploring the complex impact of aging on skin stem cells is essential to comprehending and combating skin aging at a cellular level.

Plant extracts have shown significant potential in stem cell modulation, attributed in part to their high content of beneficial molecules such as flavonoids, polyphenols, and isoflavones [12]. For example, flavonoids identified in an Alpine rose leaf extract (*Rhododendron ferrugineum*) have been identified as probable senolytic molecules [13]. A limitation of natural plant-derived materials is the sustainable cultivation and sourcing [14]. In this context, plant callus cells offer a sustainable alternative to obtain bio-active material from whole plant extracts. Grown under controlled conditions, a consistent profile of bioactive compounds can be achieved, further eliminating the risk of contamination by environmental pollutants and pathogens [15]. Callus cell extracts have further demonstrated promising potential for skin cell treatments, as they have been found to promote antioxidant activity, enhance collagen formation, and stimulate cell migration and proliferation [16,17]. However, the effects of plant callus extracts on dermal stem cells remain largely unexplored.

RNA sequencing (RNA-seq) offers numerous benefits in the field of molecular biology and clinical diagnostics [18]. Particularly bulk RNA-seq, which is performed on pooled cell populations, has been proven useful in gaining novel insights into the biological mechanisms underlying pathologies, such as very early onset inflammatory bowel disease [19], glioblastoma [20], and systemic sclerosis [21]. Despite being a powerful tool, the limitations, including the extensive time and cost of the technology, have driven researchers to find viable alternatives to address these constraints. One such approach is bulk RNA barcoding and sequencing (BRB-seq), a cost-effective method for high-throughput transcriptomics, which combines cost-effective single-cell RNA-seq efficiency with the performance of the bulk RNA-seq procedure [22]. Employing this technology facilitates the generation of libraries that parallel the benchmark TruSeq in terms of gene expression quantification and quality, even applied to low-quality RNA samples [22,23]. As such, BRB-seq represents a valuable tool to explore the molecular mechanisms present in both physiology and pathology.

Our study primarily aims to investigate the impact of aging on dermal stem cells by exploring the gene expression changes in both young and aged cells. Given the reported potential of plant extracts, particularly plant callus extracts, in skin cell treatments and the detailed insight gained from BRB-seq analysis, we identify a significant research opportunity. Our study, therefore, will further investigate the impact of a natural extract, comprised of grape (*Vitis vinifera Linné*) and apple (*Malus Domestica Borkh*) callus cells, and Alpine rose leaf extract (*Rhododendron ferrugineum*) on the biological and molecular attributes of aged dermal stem cells. By employing BRB-seq, we aim to generate a comprehensive gene expression profile of young and aged dermal stem cells, thereby elucidating the cellular mechanisms occurring during aging, and explore whether they can be influenced by the callus cell extract.

2. Materials and Methods

2.1. Preparation of Extract

The extract evaluated in this study was prepared with a mixture of callus cells obtained from grape callus cells (*Vitis vinifera Linné*) and a specific apple variety of Malus Domestica (*Malus Domestica Borkh*), the Uttwiler Spätlauber, according to an established and patented in-house protocol following the principle of wound-induced callus formation [24]. The mixture was further enriched with an Alpine rose leaf extract (*Rhododendron ferrugineum*). The final extract, SenoCellTec™ (hereafter referred to as "SCT extract"), was provided by Mibelle Biochemistry, Buchs, Switzerland.

2.2. Validation of Xeno-Free Culture Medium

To validate the use of a xeno-free media for the primary culture of human dermal stem cells (DSCs), human dermal fibroblasts were cultured in DSC media supplemented with 2% human serum (Curio Biotech, Visp, Switzerland, reference CB-DSC-GM) for a total of 7 days. Control cells were cultured in CB-DSC-GM supplemented with 2% fetal bovine

serum (FBS). Brightfield images of the cultured cells, were taken after 7 days to compare cell morphology (Nikon ECLIPSE Ts2-FL, Tokyo, Japan).

2.3. CCK-8 Cell Viability Assay

For the determination of cell viability, cell proliferation and cytotoxicity assays using the Cell Counting kit-8 (CCK-8) (Cat. N.: HY-K0301, MCE (Med Chem Express, Monmouth Junction, NJ, USA)) were performed according to the kit's instructions. Briefly, 8000 cells/cm^2 were seeded into 96-well plates and preincubated for 24 h in a humidified incubator (37 °C, 5% CO_2). Then, various concentrations of the SCT extract were added into the appropriate wells, and plates were incubated for 48 h (37 °C, 5% CO_2). After this period, cell media were replaced with standard media, and 10 µL of CCK-8 solution was added into each well of the plates. After a further 2 h of incubation (37 °C, 5% CO_2), the absorbance was measured at 450 nm using a microplate reader.

2.4. Dermal Stem Cell (DSC) Isolation and Treatment

DSCs were isolated from human skin explants by a standard methodology. Human skin samples from the abdomen of Caucasian females from two age groups ("young" group, 18–30 years old and "aged" group, >70 years old, n = 3 per group) were acquired from a tissue collection site in France with license number AC-2017 3030 from French Ministry of Higher Education and Research according to French laws including ethics regulations. The tissues had been collected during plastic surgery after informed consent had been obtained. Tissues were washed and treated with dispase II enzyme (Sigma Aldrich, Taufkirchen, Germany), overnight at 40 °C. After, the epidermis was peeled, the microvessels from the upper part of dermis were extruded mechanically, and the remaining tissue was washed several times. The tissue was then chopped into tiny pieces with a scalpel blade and digested with collagenase I (Thermo Fischer Scientific, Waltham, MA, USA) till fully digested by shaking every hour. Isolated cells were filtered using a cell strainer (Milian, Switzerland) and cultured in validated (supplementary data) xeno-free dermal stem cell growth medium (CB-DSC-GM, Curio Biotech, Visp, Switzerland) containing 2% human serum (Type AB—heat inactivated, P30-2501-HI, PAN-Biotech, Aidenbach, Germany). Cells were grown to a confluency of 60% in growth medium (CB-DSC-GM), and then the media were exchanged with fresh medium containing 0.5% human serum and the cells treated for 2 days either in presence or absence of 0.4% SCT extract. After 2 days of treatment, cells were detached, lysed in RNA lysis buffer (Bio-Rad Laboratories, Hercules, CA, USA), and RNA extraction was performed according to the manufacturer's protocol. Untreated DSCs of young donors and aged donors were cultured as a control until confluency, without extract treatment, for 2 days according to the above-described procedure.

2.5. Flow Cytometry Analysis

The populations of stem-cell marker-expressing cells were determined on a Cytoflex S flow cytometer (Beckman Coulter, Brea, CA, USA). Cells were fluorescently labeled with anti-human monoclonal antibodies (BioLegend, San Diego, CA, USA) against CD29 (Cat. No. # 303015), CD49f (# 313607), CD133 (# 397905), CD34 (# 343503), CD31 (# NB100-65336AF488), CD73 (# 344003), CD90 (# 328107), and CD105 (# 323203). Unstained cells and appropriate IgG controls were used in parallel as controls. The protocol used was as follows: Cell suspensions were first fixed in 4% formaldehyde for 10 min, washed in a wash buffer (WB: 0.25% Tween-20 in DPBS + 5% FBS), and spun down. The cell pellets were resuspended in the presence of the labeled monoclonal antibody diluted in WB and incubated for 1 h on ice in the dark. Then the samples were washed twice with cold WB, and the cell pellets were resuspended in cold PBS and subjected to flow cytometry analysis. A total of 10^5 cells were measured at medium speed and gated to exclude doublets. Data acquisition s was performed with CytExpert Acquisition Software (version 2.5, Beckman Coulter, Brea, CA, USA) and analysis was performed using FloJo™ software (version 10.8.1, FlowJo, Ashland, OR, USA).

2.6. RNA Extraction and Quantification

Total RNA was extracted from the samples using the Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Briefly, adherent cells were lysed in lysis buffer, then one volume of 70% ethanol was added, and the resulting mix was directly added into each spin column and further processed following several sequential washes with high and low stringency buffers included in the kit. DNase I treatment was performed to eliminate eventual residual genomic DNA. Finally, RNA was eluted in 10 mM Tris-Cl pH 8.0. The resulting total RNA was quantified via NanoDrop 2000 apparatus (Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Bulk RNA Barcoding (BRB) and Sequencing

For BRB mRNA sequencing, library preparation was performed using MERCURIUS BRB Sequencing Library preparation Kit (Alithea Genomics, Epalinges, Switzerland) and were subjected to Illumina sequencing with 5 million reads/sample, as previously described [22]. Briefly, NanoDrop quantified RNA was further quality controlled for RNA integrity on BioAnalyzer (Agilent technologies, Santa Clara, CA, USA). RNA was reverse transcribed directly in the reverse transcription (RT) reaction with barcoded oligo-dT primers. The resulting cDNA was pooled in a single tube prior to cDNA second strand synthesis. The Illumina-compatible Unique Dual Indexed (UDI) library preparation was performed, and the libraries were sequenced on the Illumina NovaSeq (Illumina, San Diego, CA, USA) instrument. The FASTQ files were sample-demultiplexed and aligned to the reference genome using STARsolo version 2.7.9a [25]. The resulting count matrices were used for downstream gene expression analysis. For the identification of differentially expressed genes (DEGs), the data were analyzed using the DESeq2 R package, and functional enrichment tests were performed for finding associated biological pathways. First, the raw count data obtained from BRB-seq experiments were pre-processed to remove low-quality reads and filter out lowly expressed genes. The data were then normalized using DESeq2 to account for differences in library size and composition. The variance of the data was estimated using a negative binomial distribution to model the read counts. LRT hypothesis testing was performed to identify DEGs based on adjusted *p*-values and logFC (fold change). The detected DEGs were further analyzed to identify Gene Ontology terms and KEGG pathways associated with them using functional enrichment tests. Significant enrichment was indicated by a minimum gene overlap of 3, *p*-value cut-off of 0.01, and minimum enrichment factor of 1.5.

2.8. Dermis Culture and Treatment

DSCs from young and aged donors were seeded in 3D cell culture inserts with a seeding density of 200,000 cells/insert in growth medium (CB-DSC-GM), serum-free, overnight. The following day, the culture medium was exchanged with fresh medium containing 0.5% human serum and treated in the presence or absence of 0.4% SCT extract for 10 days, with media change and treatment renewal every alternate day. After exposure, 3D models were harvested at day 10, fixed and processed for histological staining.

2.9. Histology and Immunohistochemistry

Inserts were fixed in 4% PFA for 15 min, then PFA was changed for 70% ethanol. The inserts were then processed for paraffin embedding. Paraffin-embedded sections (5 mm) were stained with H&E (Hematoxylin and Eosin) for gross morphology. Bright field histological microscopy (Nikon TS2-FL, Tokio, Japan, magnification 20×) images were acquired, and the thickness of 3D dermis formed in the presence and absence of SCT extract was quantified using an open-source software, ImageJ (Version 20, NIH, Bethesda, MD, USA).

2.10. Statistical Analysis

Statistical comparisons were performed using GraphPad Prism 8 software (GraphPad, Boston, MA, USA), and all data are expressed as mean ± SD of at least 3 independent

experiments. Comparisons among different groups were made using one-way ANOVA or Student's *t*-test with homoscedasticity. p values < 0.05 were considered significant.

3. Results

3.1. Characterization of Dermal Stem Cells by Fluorescence-Activated Cell Sorting (FACS)

To ensure optimal culture conditions for human stem cells, including DSCs, ideally defined culture conditions are applied. This includes the removal of non-human animal-derived components, which helps to eliminate issues associated with batch variation and pathogen transmission [26]. Therefore, isolated DSCs were cultured, and the experiments performed in xeno-free culture media supplemented with human serum. The primary cell culture in the defined xeno-free media was validated prior to the experiment's start by assessing cell viability and morphology (Supplemental Figure S1) and provides, to the best of our knowledge, the first evidence of successful xeno-free human DSC culture. The isolated cells were characterized by FACS analysis to confirm their identity, with CD29, CD73, and CD90 as positive markers, whereas CD31 and CD34 were chosen as negative markers [27,28]. CD29 is a commonly used skin stem cell marker [29,30], which identifies dermal mesenchymal stem cells. Further, CD73 and CD90 are a favorable combination to label dermal stem cells [31,32], as they can distinguish different subsets of human dermal stem cells, including dermal stromal cells [33]. After cell isolation, FACS analysis of three independent donors demonstrated clearly negative signals for both CD31 and CD34, confirming the cells are not of a hematopoietic or endothelial origin (Supplemental Figure S2). In contrast, the isolated cell populations from all three donors had clear positive signals for the dermal stem cell markers CD29, CD73, and CD90. The combination of these three markers confirms that the cells, as isolated by the protocol used for this study, are indeed a dermal stem cell population.

3.2. Differential Gene Expression in Young and Aged Human DSCs

The BRB sequencing of DSCs isolated from young and aged donor tissues identified a total of 7409 genes, of which 75 were differentially expressed genes (DEGs). Compared to young DSCs, 20 genes were upregulated, and 55 genes were significantly downregulated in the aged DSC samples (Figure 1a). The volcano plot highlights the most significant up- and downregulated genes in aged donors, compared to young DSCs (Figure 1b). The full names and log2 fold changes of the top 10 up- and downregulated genes in aged cells are further depicted in Table 1. Compared to young DSCs, old cells had significantly upregulated genes associated with the inhibition of cell proliferation, such as PHLDA2 and SFRP1 [34,35]. Furthermore, protective factors such as glutathione peroxidase 3 (GPX3) and clusterin (CLU) were identified to be significantly downregulated [36,37]. Further downregulated genes include DKK1, CCN5, PTGIS, and SCARA5, which are involved in processes affecting cellular aging, such as cell proliferation [38], immune cell infiltration [39], and protein clearance [40].

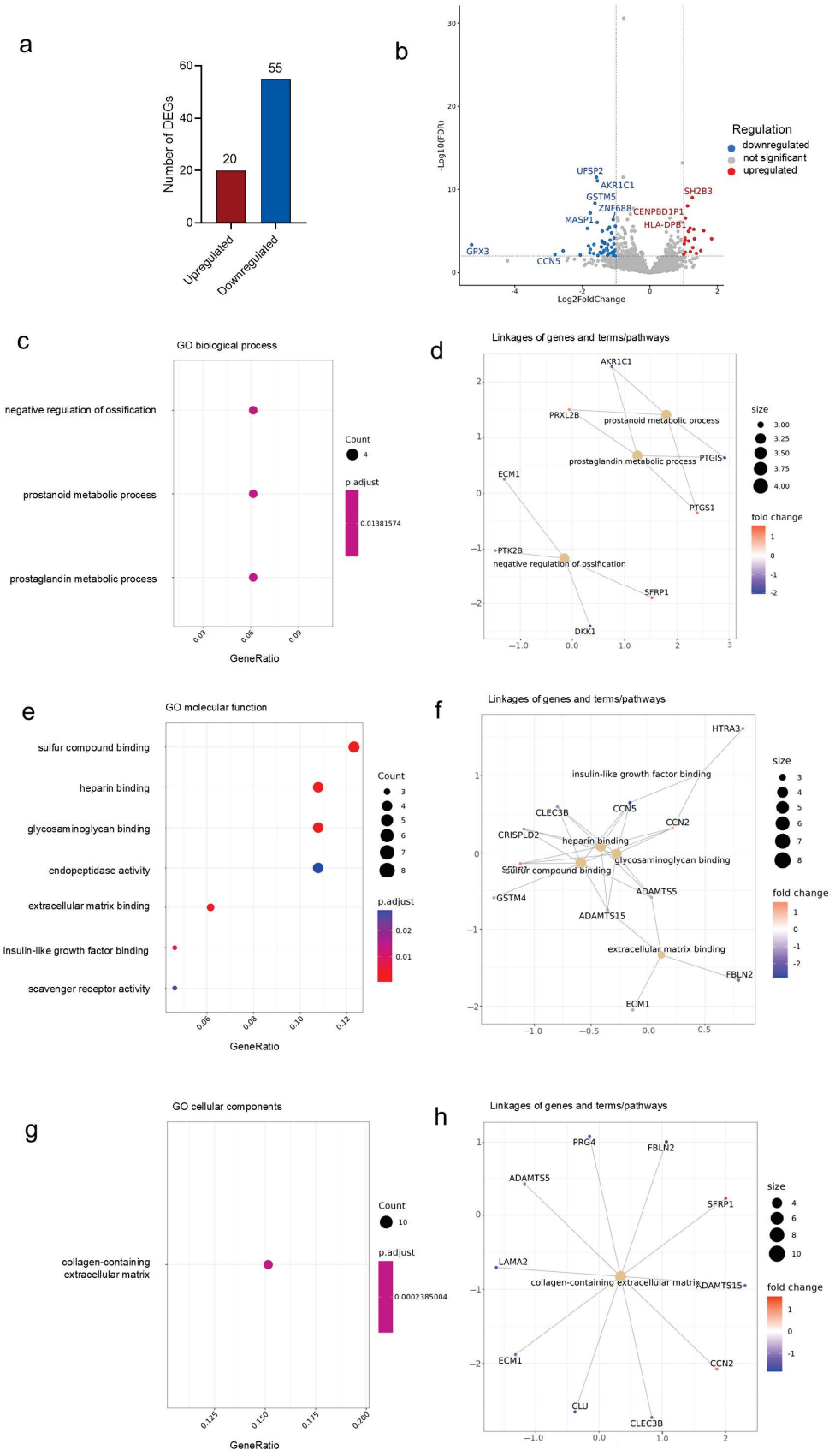


Figure 1. Differential gene expression in young and aged human DSCs. BRB sequencing of young and aged DSCs identified 75 differentially expressed genes (DEGs), with 20 upregulated, and 55 significantly downregulated in the aged DSCs compared to young DSCs (**a**). A volcano plot (**b**) was used to visualize

the DEGs identified. Gene Ontology analysis performed reveals changes in biological process (**c**), molecular function (**e**), and cellular components (**g**). Linkages of genes, terms, and pathways for the respective GO categories are graphed in (**d**,**f**,**h**), respectively. Colors correspond to significant fold change expression. Red, high expression; blue, low expression.

Table 1. Top 10 DEGs up- and downregulated in aged DSCs.

Upregulated				Downregulated			
Gene ID	**Gene Name**	**Log2 Fold Change**	***p* Value**	**Gene ID**	**Gene Name**	**Log2 Fold Change**	***p* Value**
PHLDA2	Pleckstrin homology-like domain family A member 2	1.83	6.02×10^{-3}	GPX3	Glutathione peroxidase 3	−5.28	4.90×10^{-6}
SFRP1	Secreted frizzled-related protein 1	1.59	3.83×10^{-8}	CCN5	Cellular communication network factor 5	−2.81	1.67×10^{-4}
IGF2BP3	Insulin-like growth factor 2 mRNA-binding protein 3	1.51	3.70×10^{-5}	SCARA5	Scavenger receptor class A member 5	−2.57	3.91×10^{-5}
FOSL1	Fos-related antigen 1	1.37	1.11×10^{-4}	PTGIS	Prostaglandin I2 synthase	−2.06	2.10×10^{-4}
PTGS1	Prostaglandin-endoperoxide synthase 1	1.32	6.28×10^{-7}	SPATC1L	Spermatogenesis and centriole associated 1 like	−1.85	1.84×10^{-8}
STAMBPL1	STAM-binding protein like 1	1.30	2.37×10^{-8}	FBLN2	Fibulin 2	−1.82	7.20×10^{-6}
PLK3	Polo-like kinase 3	1.27	1.27×10^{-5}	MEDAG	Mesenteric estrogen-dependent adipogenesis	−1.78	8.99×10^{-5}
SH2B3	SH2B adaptor protein 3	1.26	7.92×10^{-13}	MASP1	MBL-associated serine protease 1	−1.76	9.42×10^{-11}
SLC20A1	Solute carrier family 20 member 1	1.20	5.37×10^{-5}	DKK1	Dickkopf-related protein 1	−1.76	2.34×10^{-5}
SPRY2	Sprouty RTK signaling antagonist 2	1.19	1.63×10^{-8}	CLU	Clusterin	−1.66	1.16×10^{-4}

Gene Ontology (GO) functional enrichment analysis was further performed for the DEGs identified by RNA-seq and grouped according to biological process (Figure 1c), molecular function (Figure 1e), and cellular components (Figure 1g). The analysis revealed altered processes (and linked genes) in the aged DSCs, such as the negative regulation of ossification (downregulated ECM1, DKK1, and PTK2B and upregulated SFRP), and prostanoid and prostaglandin metabolic processes (downregulated AKR1C1 and PTGIS and upregulated PTGS1 and PRXL2B) (Figure 1d). When analyzing the molecular functions, an altered regulation of various cell binding processes (sulfur compound, heparin, glycosaminoglycan, IGF, and ECM) was identified, which was linked to cell-communication genes including CCN2 (upregulated), CCN5 (downregulated), and the extracellular matrix (ECM)-modulating factors ADAMTS5, ADAMTS15, FBLN2, and ECM1 (downregulated) (Figure 1f). The GO further identified the collagen-containing ECM to be a cellular component significantly altered in aged DSCs (Figure 1h). In line with this, the DEGs associated with the ECM include LAMA2, ECM1, ADAMTS5, ADAMTS15, FBLN2, CLU, CLEC3B, and PRG4 (downregulated) and CCN2 and SFRP1 (upregulated).

3.3. Natural Extract Affects RNA Splicing in Aged Cells

RNA-seq can yield detailed insights to the cellular transcriptome and has been frequently used to identify effects of botanical actives on biological processes in vitro [41,42]. To identify possible effects of the SCT extract on aged DSCs, cells treated with the extract were compared to their untreated aged and cultured controls. In total, 8006 genes were identified, wherein, interestingly, only seven DEGs were found to be downregulated with the treatment in comparison to untreated aged controls (Figure 2a). Further, no significantly upregulated genes were detected in this analysis. The heat map of the RNA-seq analysis shows the expression pattern of the downregulated DEG in both technical and biological replicates, highlighting the strong differential expression between untreated and SCT-treated aged DSCs (Figure 2b). The seven DEGs identified were PNISR, RSRP1, MEG3, PNN, LUC7L3, AHSA2P, and GOLGA4 (Figure 2b). The full names and log2 fold changes are highlighted in Table 2.

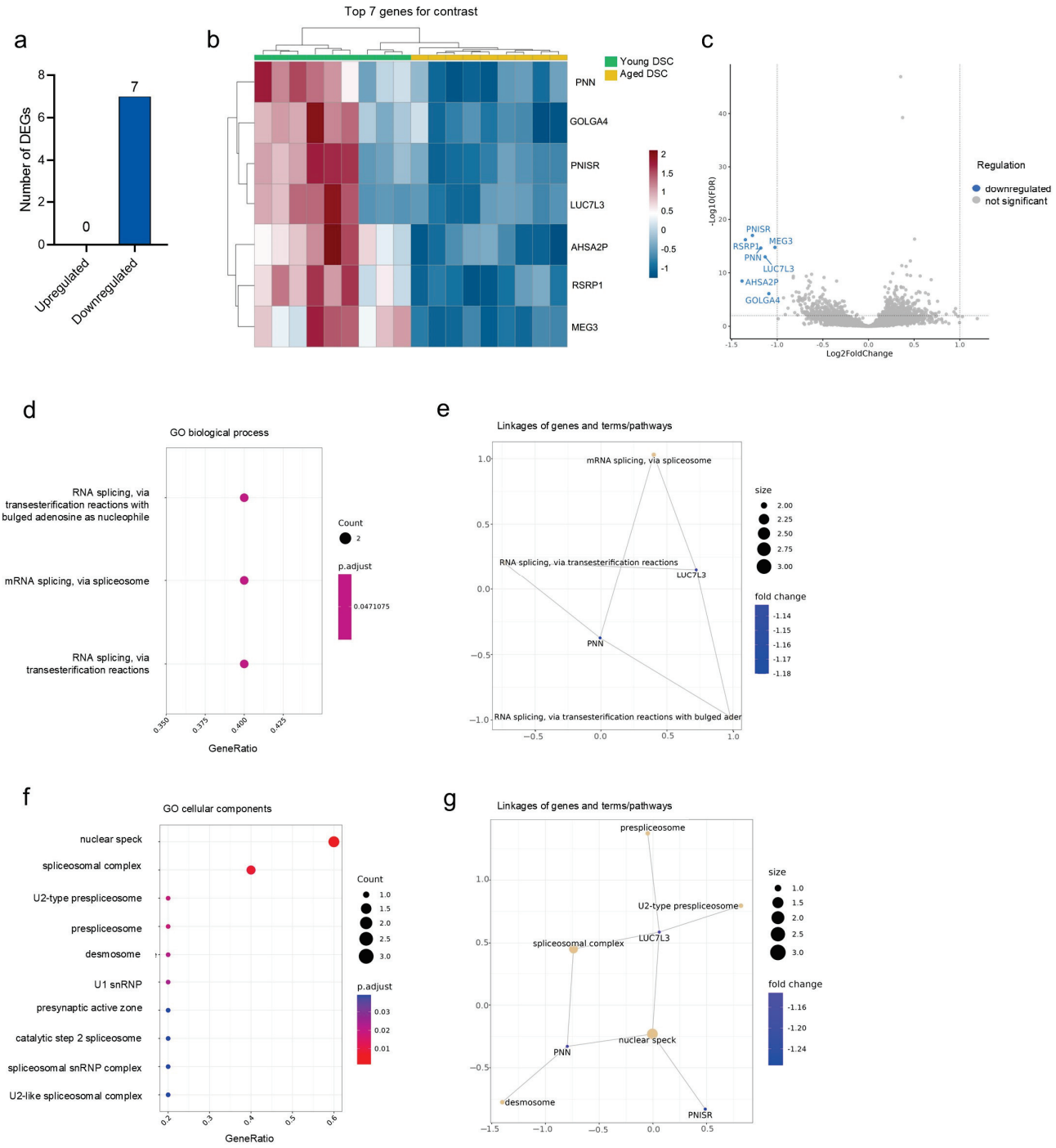


Figure 2. Altered gene expression in aged DSCs treated with SCT Extract. BRB sequencing of aged DSCs treated with or without SCT identified 7 significantly downregulated genes in the treated DSCs (**a**). The heat map highlights the expression pattern of the downregulated DEGs in the different groups (**b**), and the volcano plot (**c**) visualizes the DEGs identified. Gene Ontology analysis performed reveals changes in biological process (**d**) and cellular components (**f**). Linkages of genes, terms, and pathways for the respective GO categories are graphed in (**e**,**g**) respectively. Colors correspond to significant fold change expression. Red, high expression; blue, low expression.

Table 2. DEGs downregulated in aged DSCs treated with SCT extract.

Downregulated			
Gene ID	**Gene Name**	**Log2 Fold Change**	***p* Value**
AHSA2P	Activator of HSP90 ATPase Homolog2, Pseudogene	−2.81	1.67×10^{-4}
RSRP1	Arginine and Serine Rich Protein 1	−2.57	3.91×10^{-5}
PNISR	PNN Interacting Serine and Arginine-Rich Protein	−2.06	2.10×10^{-4}
PNN	Pinin, Desmosome-Associated Protein	−1.85	1.84×10^{-8}
LUC7L3	LUC7-like 2 Pre-MRNA Splicing Factor	−1.82	7.20×10^{-6}
GOLGA4	Golgin Subfamily A Member 4	−1.78	8.99×10^{-5}
MEG3	Maternally Expressed 3	−1.76	9.42×10^{-11}

Gene Ontology functional enrichment analysis identified molecular targets and pathways, which suggest that RNA splicing could underlie the biological activity of the SCT extract. Four of the seven DEGs identified, namely, PNN, PNISR, RSRP1, and LUC7L3, are associated with RNA binding activity and RNA splicing via spliceosome or transesterification reactions (Figure 2d) [43]. This was also reflected in the biological processes (Figure 2e) and cellular components identified to be involved, which included the nuclear speck, prespliceosome, and spliceosomal complex (Figure 2f). Further, GO analysis identified desmosomes as critical cell components modulated with the SCT treatment. This cellular component was in turn linked to PNN, which was originally identified as a desmosome-associated protein [44].

3.4. Improved Dermal Structure with Extract Treatment

To assess the functional outcome of the treatment, primary DSCs of aged tissues were cultured in the presence of SCT extract to form a stratified 3D structure and compared to untreated young and aged DSC controls. By adjusting the culture conditions and scaffold, the formation of organoids could be avoided [45], and the cells formed a stratified dermal structure. Compared to young DSCs, which formed a dense stratified multilayer (Figure 3a), the aged DSCs failed to form a comparable structure (Figure 3b). Morphologically, the structures appeared less dense and, upon the visualization and quantification of the dermis thickness (Figure 3d), it was observed that the dermal structures formed by aged DSCs were significantly thinner compared to those formed by young DSCs. Intriguingly, when aged DSCs were cultured in the presence of SCT extract, the structural integrity was rescued, and the DSC formed a dermal structure almost equivalent to the young DSCs (Figure 3c). This highlights the potential of the extract to stimulate aged cells, presumably by modifying RNA splicing machinery. As a result, dermal stem cell function was enhanced, which subsequently led to improved dermal integrity in vitro.

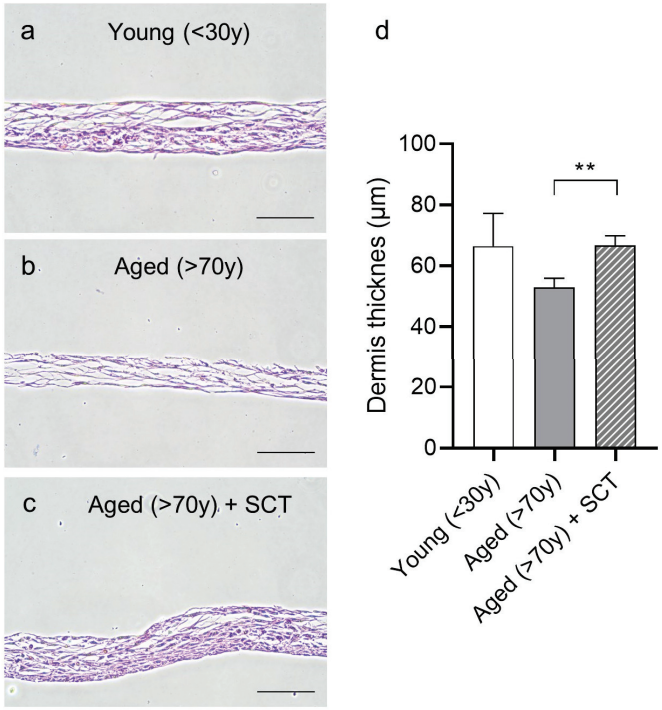


Figure 3. Effects of SCT treatment on 3D dermis reconstruction. Representative images of 3D reconstructed dermis of cells isolated from young (<30 y) donors (**a**), aged donors (>70 y), (**b**) and aged donors treated with SCT extract (**c**) after 10 days of culture. Quantification and graphical representation of dermis thickness (µm) (**d**). Scale bar = 100 µm. Students *t*-test, mean ± SD, n = 3, ** $p < 0.01$ compared to aged (>70 y) untreated DSCs.

4. Discussion

The process of skin aging is a complex and inevitable phenomenon that has garnered increasing interest in recent years. Understanding the molecular mechanisms underlying skin aging is crucial for the development of effective anti-aging therapies and tissue rejuvenation strategies. The skin's ECM plays a fundamental role in the aging process, both intrinsic and extrinsic [46]. Among the known ECM modulators, the family of CCN matricellular proteins plays a critical role in various physiological processes in the skin, such as cell proliferation, migration, adhesion, and ECM synthesis [47]. However, some may also contribute to pathological conditions such as fibrotic disease [48]. In this study, a differential regulation of two CCNs was identified in aged DSCs, with an upregulation of CCN2 and a downregulation of CCN5, respectively. The expression of different CCN proteins is indeed spatially and temporally regulated [49] and can further be modulated by environmental factors such as UV irradiation. In healthy human volunteers, the UV irradiation of sun-protected buttock skin demonstrated a significant increase in CCN2, whilst CCN5 was significantly reduced 24 h post irradiation [50]. Furthermore, in human fibroblasts, TGF-β-induced fibrosis led to the upregulated expression of CCN2 whilst CCN5, in contrast, was downregulated [48]. In regard to skin aging, studies report both the increased [51] and reduced [52,53] expression of CCN2 in human dermal fibroblasts. The increased expression of CCN2 was found to induce cellular senescence in human foreskin fibroblasts via p53 and $P16^{INK4a}$ [51]. In contrast, an age-related downregulation of CCN2 was observed in aged human skin, which was found to mediate collagen loss [52,53]. These differing observations may in part be allocated to the different culture conditions and exposures used. Elevated levels of CCN2 are further commonly observed in inflammatory and fibrotic skin conditions [54,55]. In contrast, CCN5 has been described as an antifibrotic molecule [56], and its expression may be induced by estrogen [57]. Considering that the DSCs of this study were obtained from female donors, it can be speculated that the

age-related decline of estrogen contributes to the observed decrease in CCN5. Moreover, the characteristic expression of CCN2 and CCN5 observed in the aged DSCs could be associated with age-related fibrotic processes. Interestingly, plant-derived molecules such as fisetin and curcumin have been found to benefit the skin via the regulation of the CCN pathways [58]. As such, the flavonoids present in the natural extract of this study could potentially modulate these pathways, leading to positive effects on aging skin.

GO functional enrichment analysis further identified changes in prostanoid and prostaglandin metabolic processes in the aged DSCs. Prostaglandins, particularly prostaglandin E2 (PGE2), play a significant role in skin aging [59]. In human dermal fibroblasts (HDFs), PGE2 inhibits collagen production and induces matrix metallopeptidase 1 (MMP1) expression, contributing to dermal matrix degradation and skin aging [60]. Additionally, PGE2 induces cellular senescence in HDFs, leading to decreased cell proliferation and increased senescence-associated β-galactosidase staining [61]. Furthermore, senescent cells synthesize prostaglandins, which further promote the senescence status and reinforce the proliferative arrest [62]. As such, blocking PGE2 is a favorable approach to combat skin aging. In line with this, several natural compounds have been reported to prevent PGE2 synthesis [63–65].

When evaluating the extract treatment in this study, a substantial effect of the SCT treatment was observed on functional outcome, despite only a small number of DEGs associated with RNA splicing having been identified. Alternative RNA splicing is a post-transcriptional regulatory mechanism by which a single pre-mRNA transcript can be differentially processed into diverse mRNA isoforms, thereby expanding proteomic complexity and functional diversity from a limited set of genes [66]. The dysregulation of this mechanism is described as an emerging hallmark of aging [67] and may serve as a predictor of biological age and life expectancy, offering potential for developing therapeutics to extend healthy lifespan in humans [66]. Indeed, a study evaluating gene expression changes in aging murine skin observed an increase in alternatively spliced genes [68]. These alternatively spliced genes were consistently allocated to functions, including RNA processing and splicing, and found to be enriched within cellular components such as the nucleus and the spliceosome. Targeting RNA splicing has, therefore, emerged as a promising approach for anti-aging interventions. One of the first studies addressing such a modulation found that treating human dermal fibroblasts (NHDFs) with novel small resveratrol analogs was associated with altered splicing factor levels and a reversal of cellular senescence [69]. Further, in models of accelerated aging including Cockayne syndrome, Hutchinson–Gilford Progeroid syndrome (HGPS), and Werner syndrome, all three progeroid dermal fibroblast types showed dysregulated splicing factor gene expression [70]. Intriguingly, treatment with the kinase inhibitor trametinib led to altered gene expression of a panel of splicing factors in the HGPS and Cockayne syndrome cells, which was accompanied by a reduced senescence burden. Our study found that the SCT treatment of aged DSCs affected genes associated with the spliceosomal complex, the prespliceosome, and the nuclear speckles. Nuclear speckles, also known as splicing factor compartments, are located in the interchromatin regions of the cell nucleoplasm and serve as reservoirs for pre-mRNA splicing factors [71]. Based on the modulations observed, it can be speculated that the SCT extract exerts its restorative effects by modulating splicing factors, which in turn leads to improved cell function in the aged DSCs. When evaluating the extended duration of treatment, from 2 days in 2D models to 10 days for the 3D dermis functional assay, it can be speculated that further downstream mechanisms are initiated after prolonged treatment with the SCT extract. Further investigations, for example, with the targeted transcriptomics and evaluation of senescence burden in appropriate cell models, will certainly provide more insight into this proposed mechanism.

Dermal stem cells play a crucial role in skin maintenance, for example, growth factors derived and secreted from human dermal stem/progenitor cells can ameliorate UV-A-induced damage in NHDF [2]. Preserving dermal stem cell function is critical for skin homeostasis, which could be achieved by regenerative treatment with natural sources

such as callus extracts. Indeed, it was previously observed that the plant-callus-derived shikimic acid effectively regenerates human skin by converting human dermal fibroblasts into multipotent skin-derived precursor cells, facilitating wound healing and dermal reconstruction [72]. Furthermore, the *Rhododendrum ferrugineum* extract was shown to have a senolytic effect in senescent fibroblasts [13]. Building on this understanding, the SCT extract examined in this study presents a promising strategy for addressing skin aging, as it potentially modulates RNA splicing, thereby influencing the expression and function of proteins critical to the maintenance of skin health and vitality. The successful cultivation of human DSCs under xeno-free conditions and the identification of DEGs between young and aged DSCs further offer a foundation for the exploration into the mechanisms of skin aging and potential therapeutic interventions.

5. Conclusions

Our findings highlight the potential of natural extracts in modulating biological characteristics of aged dermal stem cells. Importantly, our data suggest that these extracts may exert their effects through possible senolytic activity and the modulation of RNA splicing, a process crucial to gene expression and cellular function and described as an emerging hallmark of aging. In conclusion, this study underscores the potential of integrating high-throughput transcriptomics and natural extracts in understanding and combating skin aging. The findings present new avenues for the development of innovative, sustainable, and potentially more effective anti-aging strategies.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/cosmetics11050167/s1. Figure S1: Validation of xeno-free media; Figure S2: Fluorescence-activated cell sorting (FACS) characterization of human DSC.

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Revealing the Potential of Chios Mastic Gum and Its Constituents for Cosmetic Applications through Chemical Profiling and Biological Evaluation

Panagiota Stamou [1,†], Eleni V. Mikropoulou [2,†], Maria Chalkiadaki [2], Aikaterini Basdeki [2], Lemonia Antoniadi [3], Stéphane Poigny [4] and Maria Halabalaki [2,*]

[1] Division of Pharmaceutical Chemistry, Department of Pharmacy, National and Kapodistrian University of Athens, Panepistimiopolis Zografou, 15771 Athens, Greece
[2] Division of Pharmacognosy and Natural Products Chemistry, Department of Pharmacy, National and Kapodistrian University of Athens, Panepistimiopolis Zografou, 15771 Athens, Greece
[3] PharmaGnose S.A., 57th km Athens-Lamia National Road, 32011 Oinofyta, Greece
[4] Mibelle Group Biochemistry, Mibelle AG, 5033 Buchs, Switzerland
* Correspondence: mariahal@pharm.uoa.gr
† These authors contributed equally to this work.

Abstract: Chios mastic gum (CMG), the resin of *Pistacia lentiscus* var. *Chia*, is a product with great ethnopharmacological and economic significance. This study attempts to investigate, for the first time, the activity of CMG, its fractions and isolated compounds against specific enzymes, which play pivotal roles in the degradation of proteins contained in skin connective tissue. Initially, crude CMG was subjected to extraction, fractionation and isolation through different chromatographic techniques to obtain the acidic and neutral fraction of terpenes. Additionally, the characteristic and major active triterpene acids of CMG, masticadienonic and isomasticadienonic acids (MNA, IMNA) were isolated in pure form. All samples were analysed by means of High-Performance Thin-Layer Chromatography (HPTLC) with four distinct development systems to obtain their constituents' profile. Finally, samples were tested for their ability to inhibit the elastase and collagenase enzymes. According to our findings, for collagenase, a mixture of MNA and IMNA demonstrated the most potent activity with an IC_{50} value of 31.07 μg/mL, while for elastase CMG's acidic fraction provided the most promising results with an IC_{50} value of 17.30 μg/mL. Overall, these results attempt to fill the gap in scientific knowledge about the use of CMG and its constituents in skincare and cosmetic products.

Keywords: Chios mastic gum; collagenase; HPTLC; elastase; masticadienonic acid; isomasticadienonic acid; natural polymer; poly-beta-myrcene; skincare; triterpenes

1. Introduction

Skin aging is a complex process influenced by intrinsic and extrinsic factors, impacting the structure and function of the epidermis and dermis. Typically, alterations in skin structure, function, and appearance are more pronounced in photoaged skin compared to chronologically aged skin [1]. Skin exposure to UV radiation significantly contributes to the overproduction of reactive oxygen species (ROS), resulting in elevated levels of oxidative stress in the epidermis and the degradation of the extracellular matrix (ECM) by triggering the production of dermal enzymes such as collagenase (matrix metalloproteinase-1, MMP-1) and elastase. These enzymes break down collagen and elastin, which are essential components of ECM and maintain the skin's structural integrity and elasticity, helping to maintain a youthful appearance. This degradation manifests in various skin issues, including wrinkles, rough texture, dryness, reduced elasticity, and uneven pigmentation. Consequently, the increased activity of collagenase and elastase accelerates premature skin aging, making them primary targets for testing the efficacy of novel anti-aging treatments [2].

Chios mastic gum (CMG), the resin of *Pistacia lentiscus* var. *Chia*, is a product with great ethnopharmacological and economic significance. Both the resin and its essential oil are PDO products of Greece, while cultivation of the mastic tree on the island of Chios has been registered as an intangible heritage of humanity by UNESCO [3]. Based on mastic's EMA monograph issued in 2015, the resin can be used as a traditional herbal medicinal product with two indications: namely, the treatment of mild dyspeptic disorders and for the treatment of minor skin inflammations and minor wounds [4]. CMG's application in skin conditions has been known since antiquity. Among the most influential and esteemed physicians of the ancient world, Dioscorides, in the 1st century AD, described mastic as a skincare agent, among its other properties [5]. The Egyptians included the resin in their embalming rituals [6–8], while traces of mastic found in an Etruscan ointment of the 1st century BC testify to the widespread use of mastic across the Mediterranean [9]. Nowadays CMG is traded exclusively by the Chios Mastiha Growers Association (CMGA) and a plethora of products containing mastic can be found on the market, including foods and beverages, food supplements, oral hygiene products as well as cosmetics [3].

CMG comprises the natural polymer *cis*-1,4-poly-β-myrcene, the essential oil (Chios mastic oil—CMO), as well as the terpene fraction which can be further divided into acidic and neutral terpenes [3]. Over the last few decades, several studies have been published concerning the biological activity of the resin in diverse therapeutic targets such as inflammation [10], cardiovascular diseases [11] and metabolic syndrome [12], to name a few. Nevertheless, mastic's individual constituents have seldom been the focal point of research despite the promising data hinting in this direction [13,14]. This phenomenon can be largely attributed to the resin's chemical complexity and the presence of the insoluble polymer which often hinders sample handling [3]. Furthermore, the commercial unavailability of these bioactives further complicates the evaluation of their biological and pharmacological properties. However, mastic's extensive use in diverse applications highlights the need to overcome the challenges in the further exploitation of its constituents in order to unlock their full potential.

Taking the above into account, the current study involves an in vitro evaluation of the biological activity of CMG and its individual components against the elastase and collagenase enzymes, in order to highlight the benefits of their application in cosmetics and skincare products. In this framework, a multi-step extraction, fractionation and purification process was carried out and sample profiling was performed by means of HPTLC, a fast and versatile analytical technique, widely employed in the field of natural products.

2. Materials and Methods

2.1. Materials and Chemicals

Sulfuric acid (puriss. meets analytical specification of Ph. Eur., BP, 95–97%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide and isopropyl alcohol were acquired from Merck (Kenilworth, NJ, USA). Methanol (MeOH) for analysis [≥99.8% (HPLC)] and ethanol absolute (99.8% HPLC grade) were purchased from Fischer Chemical (Pittsburgh, PA, USA). Petroleum ether (RPE, for analysis) and ethanol 96° were supplied by Carlo Erba Reagents (Milan, Italy). Dichloromethane (DCM, reagent grade) was from Scharlau, Barcelona, Spain. Vanillin used for spot visualization (Acros Organics, Fair Lawn, NJ, USA) was 99% pure. Ethyl acetate (EtOAc), *n*-Hexane, cyclo-hexane, toluene, diisopropyl ether and MeOH used for extraction and HPTLC analysis were of analytical grade (Fisher Scientific, Loughborough, Belgium), while water (H_2O) was distilled. For the pH adjustment sodium hydroxide pellets (NaOH- penta CHEMICALS UNITED) and hydrochloric acid (HCl- analytical grade; Fisher Scientific) for extraction and enzymatic assays were used. Acetonitrile (ACN, Avantor Performance Materials, Poland), and H_2O (Fisher Scientific) used for preparative high-performance liquid chromatography (HPLC) analysis were of HPLC grade. Food grade CO_2 was obtained from Revival A. E. Ursolic acid was purchased from Sigma-Aldrich.

All the required reagents for the enzymatic assays, such as elastase type IV from porcine pancreas, N-succinyl-Ala-Ala-Ala-*p*-nitroanilide substrate, Trizma-base, elastatinal, collagenase from *Clostridium histolyticum* released from physiologically active rat pancreatic islets Type V, ≥1 FALGPA units/mg solid, >125 CDU/mg solid, Tris-HCl, MMP-2 substrate (MCA-Pro-Leu-Ala-Nva-DNP-Dap-Ala-Arg-NH2) and phosphoramidon were purchased from Sigma Aldrich. H2O was obtained from a Milli-Q purification system (Merck Millipore, Darmstadt, Germany).

2.2. Chios Mastic Gum Fractionation and Isolation of Terpenes

CMG was kindly provided by the Chios Mastiha Growers Association (CMGA). Firstly, a decantation process as described by Paraschos et al., was followed resulting in the Total Mastic Extract Without Polymer (TMEWP) [15]. A total of 500 g of CMG was dissolved in 500 mL of EtOAc and then 1500 mL of MeOH was added. After being left at room temperature for 2 days, the insoluble layer of the polymer, *cis*-1,4-poly-β-myrcene (150 g), was removed. After filtration and solvent evaporation, 350 g of TMEWP was obtained as white powder. Subsequently, the TMEWP was separated into the acidic (AF) and neutral terpene fractions (NF). In brief, 10 g of TMEWP was partitioned in a separatory funnel between a polar phase (20% NaOH in distilled water/MeOH 1:1 v/v, pH = 11) and a non-polar phase (*n*-Hexane/EtOAc 8:2 v/v), using 200 mL of each. The non-polar phase was then thrice re-extracted with 100 mL of the polar phase (100 mL each time), and following solvent evaporation, the non-polar extract produced a total of 4.5 g of mastic's NF. The combined aqueous phase (500 mL in total) was subsequently acidified with 1N HCl until pH = 3 and extracted with 500 mL of EtOAc, which, once evaporated, produced 5.5 g of AF. All solvent evaporations were performed using a rotary evaporator (Buchi) with a water bath at 40 °C.

The isolation of the mixture of MNA and IMNA was performed using preparative Reverse Phase High-Performance Liquid Chromatography (RP-HPLC-PDA) (Laballiance, State College, PA, USA). The system consisted of two Lab Alliance preparative 36 Pumps and an injection valve Rheodyne 7755–027 equipped with a 1 mL loop (Target Analysis, Thessaloniki, Greece) hyphenated to a photo diode array detector (ECOM SN 7007587, Czech Republic). 50 mg of the AF were injected in a reversed-phase column (Fortis, C18, 5 μm, 250 × 21.2 mm) (Fortis Technologies, Cheshire, UK). The prep HPLC system was controlled by ClarityTM ver. 8.2 (DataApex, Prague, Czech Republic). The UV detection wavelength was performed at 210 nm and the desired fraction was collected manually based on UV chromatograms. Elution was carried out with a solvent system consisting of H2O (A) and ACN (B) using a gradient elution with a flow rate of 14 mL/min, which produced 15 mg of MNA/IMNA mixture. The elution method started at 20% B, which increased to 70% B in 3 min, reaching 100% B in 12 min, and was maintained for 13 min before returning to the initial conditions in 4 min for an 8-min re-equilibration (40 min in total).

Pure MNA and IMNA were isolated using Supercritical Fluid Chromatography (SFC) as previously described, with some modifications [14]. Briefly, the instrumentation consisted of a Waters Prep 15 SFC Purification System coupled with a SQ Detector 2 mass spectrometer. The mass analyzer was equipped with a Z-spray ion source operated in ESI negative. For the purification of MNA and IMNA, a ChiralPak IC column (5 μm, 4.6 × 250 mm) was employed. The mobile phase consisted of supercritical CO_2 with MeOH/ACN 1:1 v/v as an organic modifier. Flow rate was set at 5 mL/min. Gradient elution started at 15% co-solvent and increased to 20% in 20 min before going back to the initial conditions in 3 min for a 3-min equilibration step. For the MS detection, a SIR function monitoring m/z = 453.40 as well as a full scan function with a mass range of 200 to 800 amu were used simultaneously. Isolated pure compounds were characterized using HRMS and 1D NMR (^{1}H and ^{13}C) and compared with the literature. ^{1}H NMR, ^{13}C NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer (Bruker Biospin AG, Faellanden, Switzerland) and on a Bruker aV400. For calibration, the solvent's peak

($CDCl_3$), set at 7.26 ppm for ^1H and at 77.23 ppm for ^{13}C, was used for reference. HRMS spectra were obtained on a hybrid Velos Pro Orbitrap Elite Mass Spectrometer (Thermo Scientific, Bremen, Germany). All spectrometric and spectroscopic data are provided in the Supporting Information.

2.3. HPTLC Profiling

HPTLC analyses were performed on a CAMAG (Muttenz, Switzerland) system consisting of an automatic TLC sampler (ATS4), an automatic development chamber (ADC2), a Visualizer 2 Documentation System and a Derivatizer, all controlled by the software platform VisionCats 2.5 (all from CAMAG). Extracts and fractions were prepared at 10 mg/mL in MeOH, while MNA, IMNA and their mixture were prepared at 0.5 mg/mL in the same solvent. Ursolic acid was prepared at three concentrations (0.25, 0.5 and 1.0 mg/mL) in MeOH and used as an intensity marker. For sample application, 20 × 10 cm HPTLC Silica gel 60 F_{254} glass plates (Merck) were employed. Sample solutions were applied bandwise with the autosampler ATS4 using a syringe of 25 µL (Hamilton, Reno, NV, USA) and a nitrogen aspirator with the following standard settings: tracks with 10 mm bands, 8 mm distance from the lower edge, 20 mm from the left and right edges, 14 mm between the different tracks, and 200 nL/s delivery speed. Four different mobile phases were tested: Development System 1 (DS1) consisted of DCM/MeOH (98:2 *v*/*v*), DS2 of cyclohexane/diisopropyl ether/acetic acid (60:40:10 *v*/*v*/*v*); DS3 of cyclohexane/EtOAc (80:20 *v*/*v*) and DS4 of toluene/EtOAc (90:10 *v*/*v*). In all cases, the application volume was set to 5 µL. The standard settings used in the development chamber were the following: 20 min chamber saturation, 10 min of plate activation (conditioning) at 33% relative humidity using $MgCl_2$ as a desiccant, and 5 min of plate drying. Solvent front was set to 8.5 cm. Plate images were recorded at 254 nm and 366 nm before spraying and at white light after spraying. For spot visualization, the plates were sprayed with *vanillin reagent R* [i.e., 2 mL of sulfuric acid added to 100 mL of a 10 g/L solution of vanillin in ethanol (96°)] and heated at 100–105 °C for 5 min in the oven.

2.4. Enzymatic Assays

2.4.1. Collagenase Assay

The inhibition against collagenase enzyme was determined regarding a previously described spectrofluorimetric protocol with slight modifications [16]. Firstly, 50 µL of Tris-HCl buffer solution (50 mM, pH = 7.3), 25 µL of the tested sample (dissolved in Tris-HCl buffer containing < 5% organic solvent), and 25 µL of 30 µg/mL collagenase solution in Tris-HCl buffer were preincubated in a black 96-well microplate for 10 min at 37 °C. Then, 25 µL of 50 µM MMP-2 substrate in the abovementioned buffer was added, and the plate was incubated for an additional 30 min under the same experimental conditions. The fluorescence intensity was measured at an excitation maximum of 320 nm and an emission maximum of 405 nm, at the same temperature, using an Infinite 200 PRO series reader (Tecan, Männedorf, Switzerland). During the incubation periods, light exposure was avoided. Phosphoramidon is a strong metallo-endopeptidase inhibitor, with an IC_{50} = 6.9 µM, and was used as the positive control, while Tris-HCl buffer containing less than 5% of organic solvent (DMSO or isopropyl alcohol) served as the negative control. All experiments were performed three times.

2.4.2. Elastase Assay

Elastase inhibition was determined by the presence of the UV-Vis absorbing product *p*-nitroaniline, after the reaction of the elastase enzyme with the substrate N-succinyl-Ala-Ala-Ala-*p*-nitroanilide, based on a previously described protocol with minimum modifications [16]. In a 96-well microplate, 70 µL Trizma base buffer (50 mM, pH= 7.4), 10 µL of tested sample (dissolved in Trizma base buffer containing <5% organic solvent) and 5 µL of elastase solution (initial concentration 0.90 U/mL, dissolved in Trizma base buffer) were incubated for 10 min at room temperature, avoiding light exposure. Next, 15 µL

of the substrate (initial concentration 2 mM, dissolved in Trizma base buffer) was added in every well and the mixtures were incubated for 30 min at 37 °C. The presence of *p*-nitroaniline was measured spectrophotometrically at 405 nm, at a constant temperature of 37 °C, using the abovementioned reader. Trizma base buffer containing less than 5% of the organic solvent used to dissolve the tested extracts (DMSO or isopropyl alcohol) served as the negative control, while elastatinal, a strong irreversible competitive elastase inhibitor (IC_{100} = 5 μg/mL, and IC_{50} = 0.5 μg/mL) served as the positive control. All experiments were performed in triplicate.

2.5. Statistical Analysis

The inhibition percentage of collagenase was calculated with the following formula:

$$\text{Inhibition (\%)} = (\text{FA} - \text{FB})/(\text{FA}) \times 100$$

where: FA = FControl − FControl Blank and FB = FSample − F_{Sample}Blank. FControl is the measured fluorescence intensity of the negative control and FSample is the measured fluorescence intensity of the tested sample. Blanks consisted of all the abovementioned reagents except for the enzyme.

Similarly, the inhibition percentage of elastase was calculated using the following formula:

$$\text{Inhibition (\%)} = (\text{Abs}_A - \text{Abs}_B)/(\text{Abs}_A) \times 100$$

where: $Abs_A = Abs_{Control} - Abs_{ControlBlank}$ and $Abs_B = Abs_{Sample} - Abs_{SampleBlank}$. $Abs_{Control}$ is the measured absorbance of the negative control and Abs_{Sample} is the measured absorbance of the tested sample. Blanks consisted of all the abovementioned components except for the corresponding enzyme.

GraphPad Prism software 8.0.1 was utilized for all the analyses. The means are represented as mean ± SD (n = 3) for all the biological assays and the occurrence of the statistical differences among the data was evaluated using one-way ANOVA. The IC_{50} calculation was performed using the four-parameter dose−response curve model.

3. Results and Discussion

3.1. Extraction, Fractionation and Isolation of Triterpenes from CMG

CMG is a particularly challenging substrate due to its resinous nature, which is largely due to its natural polymer *cis*-1,4-poly-β-myrcene [3]. Thus, most extraction efforts for the recovery of mastic's compounds often begin with the removal of the insoluble polymer, usually by decantation with an appropriate solvent system. In our case the method proposed by Paraschos et al. was employed with slight modifications [15], whereby the polymer is removed after a two-day decantation with a mixture of MeOH and EtOAc. Subsequently, the TMEWP was further separated into the acidic (AF) and neutral terpene fractions (NF) via a series of liquid/liquid extractions with different polarities and pH conditions. Through this process, the acidic terpenes are first transformed to sodium salts with the addition of NaOH—thus staying in the aqueous/polar phase—while neutral terpenes are partitioned in the organic/non-polar phase. Next, the aqueous solution is re-acidified with HCl and extracted with a non-polar solvent system to obtain the non-ionized forms of the acidic terpenes.

Afterwards, the AF can be utilized for the isolation of mastic's most characteristic compounds, namely masticadienonic (MNA) and isomasticadienonic acids (IMNA) which have been found to comprise approximately 25–30% of the resin's total weight [17]. In the current work, the AF was further fractionated by prep-HPLC-PDA in order to obtain a fraction rich in MNA and IMNA. Due to their structural similarity (they differ only by the position of one double bond), the two isomers are often observed to co-elute in reverse phase chromatographic separations, particularly when low resolution (preparative) columns are employed. It is important to note that the two triterpenic acids of CMG are not commercially available, a fact that hinders their further investigation and, most importantly,

a thorough assessment of their properties. However, in this case, in order to acquire the two triterpenes in pure form, a chiral chromatography semi-preparative SFC-PDA/MS method developed by our research group was employed, which has proven able to isolate the two compounds in high purity and yield [14]. Compound identification was based on the interpretation of 1D NMR spectra, as well as the compounds' HRMS and HRMS/MS spectra (Supplementary Information, Figures S1–S6) and comparison with the available literature [14,15]. A schematic workflow of CMG's extraction and fractionation is presented in Figure 1.

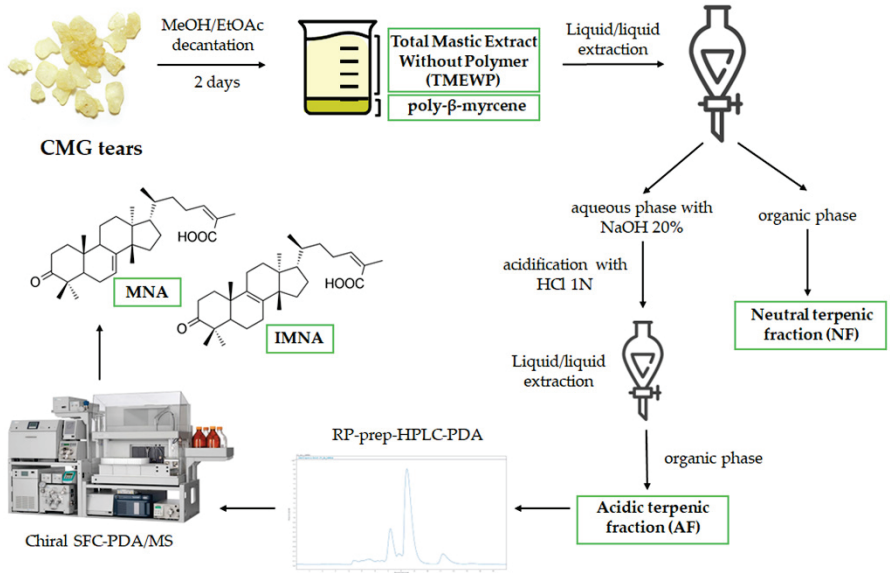


Figure 1. CMG extraction and fractionation workflow to obtain the different samples used in this study.

3.2. HPTLC Profiling of CMG Constituents

HPTLC is an excellent method for the rapid qualitative screening of plant extracts, fractions and isolated compounds, since it requires minimum solvent consumption and low sample quantities, as well as offering immense versatility in terms of chromatographical and derivatization conditions [18,19]. In one previous publication [20], we compared the method proposed by the European Pharmacopoeia [21] for the quality control of mastic with an in-house method for improved compound resolution. In this study, the in-house method consisting of DCM/MeOH 98:2 (*v*/*v*) (DS1) was compared against three additional development systems (DS2-4) that have previously been proposed for samples containing triterpenoids and steroidal compounds [22]. As shown in Figure 2, these were the CMG extract, fractions and isolated compounds, along with three concentrations of ursolic acid reference standard, which was used as an intensity marker [23]. All samples were profiled by means of HPTLC, offering a wide view of the samples' chemical composition. With the exception of DS2, whose increased polarity appears not to suit the analysis of our samples' constituents, the other three systems may contribute meaningful information to the analysis of CMG's products.

First, as a general remark, we observe that none of the proposed systems is able to fully distinguish the isomers MNA and IMNA, which in most cases elute as a single dark purple-blue broad band that appears to largely dominate the profiles of both the pure CMG and the TMEWP. Moreover, DS1 offers a significant advantage for the analysis of CMG's acidic terpenes since it is able to separate several bands that cluster tightly together with the other systems. Among these, we note two light blue-gray bands (DS1, *Rf* 0.35 and 0.42 respectively) that, according to our previous studies, correspond to the pentacyclic triterpenes and isomers of MNA and IMNA, oleanonic and moronic acids [20].

Additionally, DS1 allows us to observe the successful and satisfactory separation of mastic's AF and NF that seem to possess quite distinctive profiles.

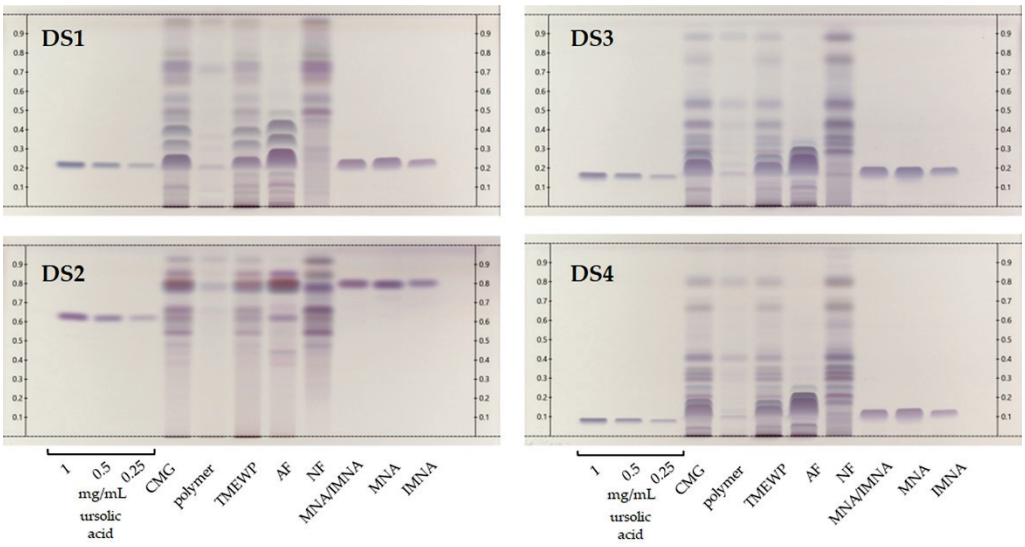


Figure 2. HPTLC chromatograms of CMG extracts, fractions and isolated compounds. Ursolic acid at three levels of concentration (0.25, 0.5 and 1 mg/mL) was used as intensity marker. DS1: DCM/MeOH (98:2 v/v), DS2: c-Hex/diisopropyl ether/acetic acid (60:40:10 $v/v/v$); DS3: c-Hex/EtOAc (80:20 v/v) and DS4: toluene/EtOAc (90:10 v/v). Detection at visible light after spraying with sulfuric vanillin reagent.

Moreover, even though DS1 is highly suited for the somewhat more polar, acidic terpenes, DS3 and DS4 offer a detailed view into the NF's complex chemical composition. Evidently, and despite the fact that mastic's NF is more diverse in nature compared to the AF, it has not been extensively studied, since to the best of our knowledge, so far only a handful of publications have reported data on its constituents [15,24–27] and even less on its biological activity [28–30]. In fact, NF has been found to contain mainly tirucallol, dammaradienone, 28-norolean-12-en-3-one, oleanonic and oleanolic aldehydes [15]. These compounds present a distinct biological interest since a study by Andreadou et al. demonstrated that NF, along with TMEWP, were able to reduce the infarct size in normal rabbits and reduced total cholesterol levels by 88% and 47% respectively in cholesterol fed animals [13]. Finally, special reference should be made to CMG's insoluble polymer, *cis*-1,4-poly-β-myrcene, which was first chemically characterized by Van Den Berg et al. [31] and whose medicinal properties have remained largely unexplored with only a few exceptions [32]. In our HPTLC analysis, the polymer appears as a very dark brown, almost black band at the base of the chromatogram and does not seem prone to elute even with relatively polar development systems (DS2). Nevertheless, in the authors' opinion, mastic's insoluble polymer presents promising therapeutic and chemical properties that merit further investigation.

Finally, special reference should be made to the adulteration attempts suffered by CMG and its products, which are commonly observed. These mainly concern blending or replacing authentic mastic resin with inferior quality products such as *Pistacia atlantica* resin, or other resins from *Boswellia* and *Pinus* species, along with other inorganic substances. Unfortunately, these phenomena lead to consumer fraud and/or low quality products. So far, there is no simple method for quality control purposes given the fact that terpenes are difficult to analyze with conventional HPLC methods due to their low absorbance under UV [17]. Thus, an HPTLC methodology like the one proposed herein could be easily employed for the quality assessment not only of crude CMG resin, but also its products.

3.3. Collagenase and Elastase Inhibition

Elastase and collagenase are important enzymes involved in the degradation of proteins contained in skin connective tissue [33,34]. More specifically, elastases are serine proteases that degrade elastin fibers and regulate, along with collagen, the mechanical properties of the skin including strength, tissue remodeling elasticity, and wound healing capacity. Elastin is a major protein in healthy human skin, comprising over 90% of the elastic fibers that provide skin with flexibility. The breakdown of elastin, primarily caused by the enzyme elastase, leads to sagging skin and the formation of fine wrinkles [35].

On the other hand, collagenases are transmembrane zinc endopeptidases that degrade the peptide bonds of collagen, the most abundant component of the extracellular matrix [36]. Collagen is a crucial component for human skin health, providing structural support, elasticity, firmness, and flexibility. Collagenases are enzymes that break down skin collagen, leading to the formation of wrinkles as we age. Under normal physiological conditions, these enzymes accurately regulate the maintenance of skin tissue homeostasis; nonetheless, under oxidative stress or UV radiation, these matrix metalloproteinases (MMPs) are overexpressed, leading to skin disorders such as premature skin aging, inflammation, or, more seriously, degenerative diseases [37,38]. Therefore, compounds capable of inhibiting this enzyme can potentially delay wrinkle formation and the photoaging process [35].

Although research for natural extracts or isolated compounds that could inhibit these enzymes has been extensive and thus of great interest for the cosmetic industry [39], there are limited scientific references regarding the potential dermo-cosmetic applications of *Pistacia lentiscus* resin and its main constituents, despite the fact that cosmetic products containing mastic can be found on the market. This literature gap may be attributed to mastic's challenging chemical composition, and particularly to the fact that it is highly insoluble in water, which serves as the base for most enzymatic assays [3]. Consequently, in the current study, following a series of extraction and fractionation steps, we investigated for the first time the elastase and collagenase inhibitory properties of not only the pure Chios mastic resin, but also its individual constituents. To counteract issues with solubility, other authors have proposed the use of alcohols for sample dilution when necessary [40,41]. In our case, particularly for the CMG extract, the polymer fraction and NF, isopropyl alcohol was employed for the initial sample dissolution, which was further diluted with buffer to a concentration below 5% (*v*/*v*) in the final sample solution.

The initial screening results displayed significant collagenase inhibitory effects at concentrations of 75 μg/mL, 150 μg/mL and 300 μg/mL for CMG, TMEWP, AF and the MNA/IMNA mixture, and at 150 μM, 300 μM and 500 μM for the pure compounds. In contrast, the polymer and NF exhibited minimal to no inhibitory effects (Figure 3a). For the CMG products with the most potent activity, further experiments were carried out to calculate dose−response curves ($R^2 > 0.97$) and the half-maximal inhibitory concentration (IC_{50}). The AF exhibited strong anti-collagenase activity (IC_{50} = 38.52 μg/mL), while the mixture of pure compounds MNA/IMNA was the most potent with an IC_{50} value of 31.07 μg/mL (Figure 3b); interestingly, the TMEWP extract demonstrated enhanced activity compared to the crude CMG resin. Finally, it is worth mentioning that the MNA/IMNA mixture was more effective than the isolated pure compounds, indicating a possible synergistic effect. What is more, when comparing the two isomers, IMNA showed stronger anti-collagenase activity; a fact which could be attributed to the position of the double bond on the B-ring of the triterpenic scaffold, which greatly influences the compounds' stereochemistry and therefore their capacity to bond to active sites of enzymes.

Regarding their capacity to inhibit elastase, all screened samples, except for the polymer fraction, exhibited significant activity, further encouraging their potential usage in the cosmetics industry. During the initial screening, the total CMG extract and its fractions were tested at concentrations of 50 μg/mL, 100 μg/mL, and 300 μg/mL, while the pure compounds were tested at 50 μM, 100 μM, and 200 μM (Figure 4a). The dose−response curves ($R^2 > 0.96$) and the IC_{50} values were calculated for all samples that demonstrated

anti-elastase activity at the initial screening. Further in vitro evaluation revealed the AF as the most potent again, with an IC_{50} value of 17.30 μg/mL (Figure 4b) while, similar to the collagenase results, CMG and all the fractions (except for the NF) exhibited remarkable inhibitory activity on elastase enzyme with IC_{50} values < 31 μg/mL. In this case, however, the MNA/IMNA mixture as well as the pure isolated compounds MNA and IMNA showed lower anti-elastase activity. Similarly to the collagenase results, IMNA demonstrated stronger anti-elastase activity compared to MNA.

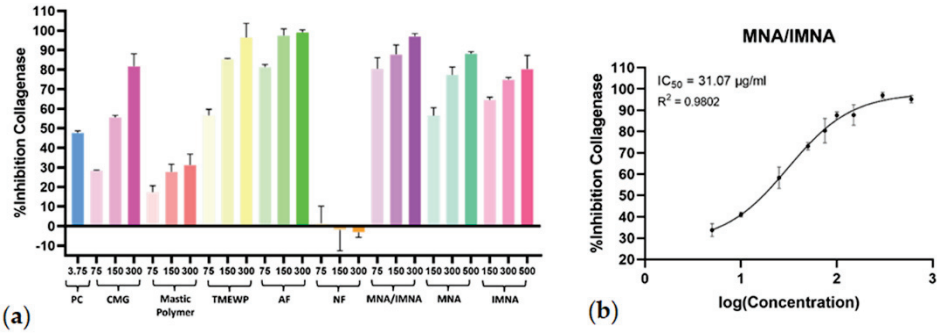


Figure 3. (**a**) Collagenase inhibitory activity of CMG, CMG fractions, MNA/IMNA mixture and isolated pure compounds. Bars represent mean ± S.D., *n* = 3. (**b**) Dose−response curve of the most potent anti-collagenase sample (MNA/IMNA mixture). PC = positive control.

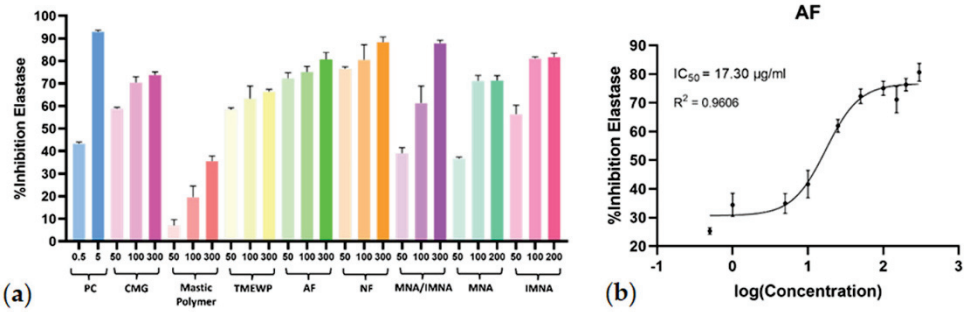


Figure 4. (**a**) Elastase inhibitory activity of CMG, CMG fractions, MNA/IMNA mixture and isolated pure compounds. Bars represent mean ± S.D., *n* = 3. (**b**) Dose−response curve of the most potent anti-elastase sample (AF). PC = positive control.

Representatives of the *Pistacia* genus have been employed since antiquity in wound healing and overall skin health, with many modern studies verifying the traditional use of these products through in vitro and in vivo assays [42]. Regarding *P. lentiscus* resin in particular, as previously mentioned, despite the widespread application of CMG in skincare and cosmetic products, information concerning scientific data of such use remains rather scarce. To the best of our knowledge, there are no reports regarding the effects of CMG products and CMG terpenes on elastase and collagenase enzymes. As demonstrated by the IC_{50} calculated values (Table 1) and the dose−response curves for the tested samples (Supplementary Information, Figures S7 and S8), this study shows that certain compounds in CMG can inhibit collagenase and elastase, highlighting their potential in cosmeceutical applications for preventing skin photoaging.

So far, only the anti-elastase and anti-tyrosinase activity of *P. lentiscus*' distilled leaves has been investigated, with the extracts standing out as potential cosmeceutical ingredients with promising activity [43]. In a following study, the same group attributed the observed results of a wound healing effect on an animal model to the presence of quercetin-3-*O*-rhamnoside and myricetin-3-*O*-rhamnoside [44]. In another interesting study, Chiocchio et al. highlighted the anti-elastase and anti-tyrosinase activity of both leaves and fruits of *P. lentiscus*, as part of an extensive screening of a hundred plant samples for potential cosmetic applications; in fact both extracts were among the most potent candidates in

both bioassays [45]. Furthermore, formulations containing pure CMG resin have shown promising activity using animal models, in skin conditions such as allergic dermatitis [46]. Additionally, other products of CMG such as Chios mastic oil (CMO), have stood out as remarkable cosmetic agents; in fact, CMO and pure compounds of the resinous extract, such as α-pinene and myrcene, have been shown to confer cytoprotective properties under oxidative stress conditions by upregulating a set of genes associated with antioxidant cellular response [47].

Table 1. IC_{50} values and standard error (SE) of screened samples for collagenase and elastase enzymes. (ND = Not Determined).

Screened Samples	Collagenase IC_{50}	Elastase IC_{50}
CMG	187.30 ± 0.11 μg/mL	20.50 ± 0.11 μg/mL
TMEWP	64.13 ± 0.06 μg/mL	30.18 ± 0.06 μg/mL
AF	38.52 ± 0.05 μg/mL	17.30 ± 0.08 μg/mL
NF	ND	27.89 ± 0.04 μg/mL
MNA/IMNA	31.07 ± 0.07 μg/mL	63.95 ± 0.09 μg/mL
MNA	145.9 ± 0.15 μM	94.59 ± 0.08 μM
IMNA	71.54 ± 0.07 μM	56.18 ± 0.05 μM

4. Conclusions

To sum up, the current study described a detailed extraction and fractionation process for CMG, which resulted in different extracts, fractions as well as isolated compounds, including mastic's major triterpenic acids, namely MNA and IMNA. All samples were profiled by means of HPTLC with different development systems, and to the best of our knowledge, this is the first published record of the application of HPTLC for the analysis of mastic's fractions (polymer, AF, NF) and isolated constituents (MNA, IMNA). Evidently, most tested systems offered diverse insight into the samples' chemical composition; nevertheless, the one containing DCM and MeOH at an analogy of 98:2 (v/v) was most suited for triterpene analysis. Additionally, as far as the authors are aware, this is the first time that CMG and its products are investigated for their anti-elastase and anti-collagenase activities. Based on the current study's findings, for collagenase, a mixture of MNA and IMNA demonstrated the most potent activity with an IC_{50} value of 31.07 μg/mL, while for elastase CMG's acidic fraction provided the most promising results with an IC_{50} value of 17.30 μg/mL. This is of particular importance for the cosmetics industry, given that it provides a scientific base for the inclusion of mastic in cosmetic applications and justifies the traditional use of this valuable natural product, whose skincare properties have been appreciated by Mediterranean populations ever since antiquity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cosmetics11050155/s1, Figure S1: ^{1}H (600 MHz, $CDCl_3$) spectrum of IMNA; Figure S2. ^{13}C (151 MHz, $CDCl_3$) spectrum of IMNA; Figure S3. ^{1}H (600 MHz, $CDCl_3$) spectrum of MNA; Figure S4. ^{13}C (151 MHz, $CDCl_3$) spectrum of MNA; Figure S5. HRMS spectrum of IMNA.; Figure S6. HRMS spectrum of MNA; Figure S7. Dose-response curves for anti-collagenase activity.; Figure S8. Dose-response curves for anti-elastase activity.

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Review

Medicalized Aesthetic Uses of Exosomes and Cell Culture-Conditioned Media: Opening an Advanced Care Era for Biologically Inspired Cutaneous Prejuvenation and Rejuvenation

Clara Rodriguez [1,†], Alexandre Porcello [1,*,†], Michèle Chemali [2], Wassim Raffoul [2], Cíntia Marques [1], Corinne Scaletta [3], Kelly Lourenço [1], Philippe Abdel-Sayed [3,4], Lee Ann Applegate [3,5,6], Fanny Pelissier Vatter [1,‡] and Alexis Laurent [3,7,8,*,‡]

[1] Development Department, LOUNA REGENERATIVE SA, CH-1207 Geneva, Switzerland; c.rodriguez@louna-aesthetics.com (C.R.); c.marques@louna-aesthetics.com (C.M.); k.lourenco@louna-aesthetics.com (K.L.); vatter.fanny@gmail.com (F.P.V.)
[2] Plastic and Reconstructive Surgery, Ensemble Hospitalier de la Côte, CH-1110 Morges, Switzerland; michele.chemali@ehc.vd.ch (M.C.); wassim.raffoul@ehc.vd.ch (W.R.)
[3] Regenerative Therapy Unit, Lausanne University Hospital, University of Lausanne, CH-1066 Epalinges, Switzerland; corinne.scaletta@chuv.ch (C.S.); philippe.abdel-sayed@chuv.ch (P.A.-S.); lee.laurent-applegate@chuv.ch (L.A.A.)
[4] STI School of Engineering, Federal Polytechnic School of Lausanne, CH-1015 Lausanne, Switzerland
[5] Center for Applied Biotechnology and Molecular Medicine, University of Zurich, CH-8057 Zurich, Switzerland
[6] Oxford OSCAR Suzhou Center, Oxford University, Suzhou 215123, China
[7] Manufacturing Department, LAM Biotechnologies SA, CH-1066 Epalinges, Switzerland
[8] Manufacturing Department, TEC-PHARMA SA, CH-1038 Bercher, Switzerland
* Correspondence: a.porcello@louna-aesthetics.com (A.P.); alexis.laurent@lambiotechnologies.com (A.L.); Tel.: +41-79-575-34-02 (A.P.); +41-79-778-48-58 (A.L.)
† These authors contributed equally to this work.
‡ These authors contributed equally to this work.

Abstract: Recent advancements in aesthetic medicine offer innovative cosmetic solutions to enhance patient skin quality and appearance. Advanced treatment options enable practitioners to effectively address skin aging signs, pigmentation imbalance, and loss of elasticity in ambulatory and home-based care regimens. Exosomes (nanoscale cell-derived vesicles) transport a variety of biomolecules and are pivotal in physiological intercellular communication. Importantly, exosomes have recently emerged as key endogenous players in tissular regeneration. More broadly (from an active ingredient purity standpoint), exosomes, stem cell secretomes, and cell culture-conditioned media have been clinically proven to exert multifaceted beneficial topical effects (anti-inflammatory, antioxidant, anti-aging, skin rejuvenation). Therefore, human, animal, and plant-derived exosomes or other refined sub-cellular biological fractions are gaining substantial interest within the aesthetic and cosmetic industries. Notably, such approaches are thought to be among the most promising novel contenders for advanced, biologically inspired skin prejuvenation and rejuvenation care. The present narrative review summarizes the latest clinically oriented research on exosomes and cell culture-conditioned media, highlighting their mechanisms of action in various topical applications. Furthermore, it explores the innovation landscape and currently commercially available products on the global cosmetic market and discusses the potential future applications of advanced, biologically inspired ingredients in the medical aesthetic industry.

Keywords: anti-aging treatments; cell culture-conditioned media; cosmetic products; dermatology; exosomes; extracellular vesicles; mesenchymal stem cells; secretome; skin rejuvenation; stem cell biology

1. Introduction

Within a dense commercial market, many innovative cosmetic solutions are currently being developed and proposed for the enhancement of consumer/patient skin quality attributes. Notably, several modern aesthetic medicine approaches enable practitioners to effectively manage hallmark signs of cutaneous aging, such as wrinkling, elasticity loss, and pigmentation unbalance [1–4]. Therein, recent surging interest has brought exosome-based technologies to the forefront of scientific and industrial innovation [5–10]. Specifically, there has been a growing use of cell secretomes and exosomes as skin boosters, aimed at locally delivering growth factors, proteins, and other substances to support tissue repair and regeneration [11]. These compounds and advanced ingredients are often used after physical/chemical treatments such as fractional laser, microneedling, radiofrequency, and microdermabrasion treatments to support the cutaneous healing processes [1,3,4].

Exosomes are sub-cellular vesicular bodies that are produced and derived from human, animal, and plant tissues/fluids or cell cultures. Their use as active ingredients is notably bioinspired, as the main physiological functions of exosomes comprise intercellular communication and small cargo transport [1,2,12]. In therapeutic topical indications, various cell-free ingredients (e.g., exosomes, stem cell secretomes, cell culture-conditioned media) have been reported to exert beneficial actions, such as anti-inflammation, antioxidant, or anti-aging effects [1–6]. From a physiological standpoint, exosomes have been mechanistically described as key endogenous effectors of tissular regeneration [2]. In medicalized aesthetic settings, exosome products are available as facials, which involve applying exosome-rich serums or creams to the skin. Such protocols have been reported to support the skin's natural healing abilities, improve texture, reduce inflammation, and promote a more youthful appearance [1,6,8].

While exosomes and cell secretome ingredients may be derived from an array of exogenous sources, they may also be obtained from autologous biological samples. However, due to important processing technical limitations, autologous exosomes are considered to be similar to platelet-rich plasma (PRP) preparations [13]. Indeed, existing point-of-care processes cannot guarantee that only exosomes are isolated specifically and selectively. This observation was widely noted at the last Aesthetic and Anti-Aging Medicine World Congress (AMWC2024, March 2024, Monte-Carlo, Monaco). As an example, the company Meta Cell Technology (Barcelona, Spain) is commercializing a CE-certified machine that is claimed to produce autologous exosomes from patients' cells and tissues [10]. However, no specific exosome isolation method is used in this technology, which leverages biological sample photo-biostimulation for exosome release [14]. The obtention and use of autologous exosomes were thus set outside of the scope of this review, based on technical and analytical limitations that currently characterize this specific sub-field.

The present narrative review aims to comprehensively analyze the latest preclinical and clinical scientific research on exosomes, secretomes, and cell culture-conditioned media for topical medicalized aesthetic uses. Therefore, following brief descriptions of the known mechanisms of action (MoAs) of exosomes in topical applications, the available data on therapeutic and aesthetic cutaneous indications are set forth and discussed. This review notably highlights the current and rapidly evolving innovation landscape of exosomes in the global cosmetic industry, providing in-depth descriptions of commercially available products. Parallelly, this work underscores key challenges (i.e., regulatory and technical) around the market implementation of exosomes and related ingredients for medical aesthetic uses. To conclude, several critical assessments and perspectives are provided on the potential of such biologically inspired ingredients in the medical aesthetic industry for enhanced skin prejuvenation and rejuvenation care.

2. General Biological Characteristics of Exosomes

Exosomes constitute a type of extracellular vesicle (EV) that also includes microvesicles and apoptotic bodies. They are released by almost all cell types, including prokaryotes and eukaryotes [15–17]. Structurally, exosomes are lipid bilayer-enveloped EVs with a size

ranging from 30 to 200 nm. They originate through the fusion of multivesicular endosomes with the plasma membrane within the endocytic pathway [15,16]. Therefore, the term "exosome" should only be used when the endosomal subcellular origin of the EVs can be demonstrated [18]. This distinction is highly important to note, as many research groups erroneously report scientific work on alternative sub-cellular fractions (e.g., cell secretome and its extracts) under the term "exosome".

Due to their secretion by diverse cell types, exosomes can be detected in biological tissues and fluids such as breast milk, blood, serum, urine, saliva, amniotic fluid, and synovial fluid [19]. As they circulate in the bloodstream, they undergo multiple cycles of absorption and release, reaching different tissue layers [20]. Of note, transcytosis is thought to be the most probable mechanism for transporting exosomes across the endothelium [21]. Originally considered as physiological byproducts or cellular waste, recent studies have revealed the highly organized structure, composition, and significant function of exosomes in facilitating intercellular communication between donor and recipient cells [22]. Notably, exosomes achieve horizontal communication by homing to specific targets through surface proteins known as "zip codes" and delivering their cargo [23–26]. Importantly, the preserved tropism in released exosomes serves as recognition signals for uptake by similar recipient cell types [27]. Furthermore, exosome-mediated signal transduction is crucial for coordinating various biological reactions, such as immune regulation, stem cell functions, neural signaling, metabolic control, heart health, and tissular regeneration [28,29].

Biologically, exosomes contain a diverse range of cellular components, such as functional proteins, metabolites, and nucleic acids, which can impact the function of recipient cells [15–17]. Notable lipid component examples include cholesterol, ceramides, sphingomyelin, phosphatidylinosol, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and gangliosides [21]. In their lumen, exosomes contain nucleic acids such as DNA, mRNA, miRNA, and noncoding RNA. These molecules can thus be transferred between cells and potentially influence the behavior of recipient cells while being shielded from RNAse-mediated degradation [30].

Exosomes also transport growth factors and cytokines, which regulate cellular signaling, stimulate cell proliferation, and support tissue regeneration. Moreover, they contain a variety of transmembrane proteins and antigen-presenting molecules (e.g., MHC class I and MHC class II), indicating their potential role in immune responses [31]. Importantly, tetraspanin transmembrane proteins such as CD9, CD37, CD53, CD63, CD81, and CD82 play a crucial part in exosome biogenesis and the targeting of their recipient cells. In particular, CD9, CD63, and CD81 are often used as exosome biomarkers, as they are widely distributed across exosomes from various tissues [32]. Adhesion proteins (e.g., integrin, p-selectin) facilitate the binding of exosomes to recipient cells, whereas glycoproteins such as the galectin-3-binding protein serve as recognition molecules contributing to their immune tolerance [25,33,34].

Exosomes also transport additional signaling receptors, such as Fas ligand and TNF receptors. They notably shuttle heat shock proteins, which play a major role in cellular stress responses and immune regulation, or enzymes involved in various metabolic functions. Furthermore, exosomes contain cytoskeletal proteins (e.g., actin, tubulin, and filamin), indicating their potential involvement in regulating both cellular structure and dynamics. As they originate from the endocytic pathway, exosomes contain the endosomal sorting complex required for transport (ESCRT) protein machinery for membrane transport and fusion proteins such as GTPases and Rabs (i.e., GTPases family) [35]. Overall, the specific composition of exosomes can drastically vary depending on the type and physiological state of the parent cells, which leads to a heterogeneous and large family of sub-cellular vesicles (Figure 1) [34,36,37].

Notably, exosomes, secretomes, and conditioned media are biologically distinct, but these terms are often used interchangeably in aesthetic medicine (Figure 1). Specifically, conditioned medium is a broad term for the liquid containing all substances secreted by cultured cells. Secretomes include a wide array of extracellular vesicles (i.e., including

exosomes) and proteins. Exosomes are a specific type of extracellular vesicle with a defined size and content. While the scientific distinctions are clear, the terminology in aesthetic treatments can be imprecise (Figure 1).

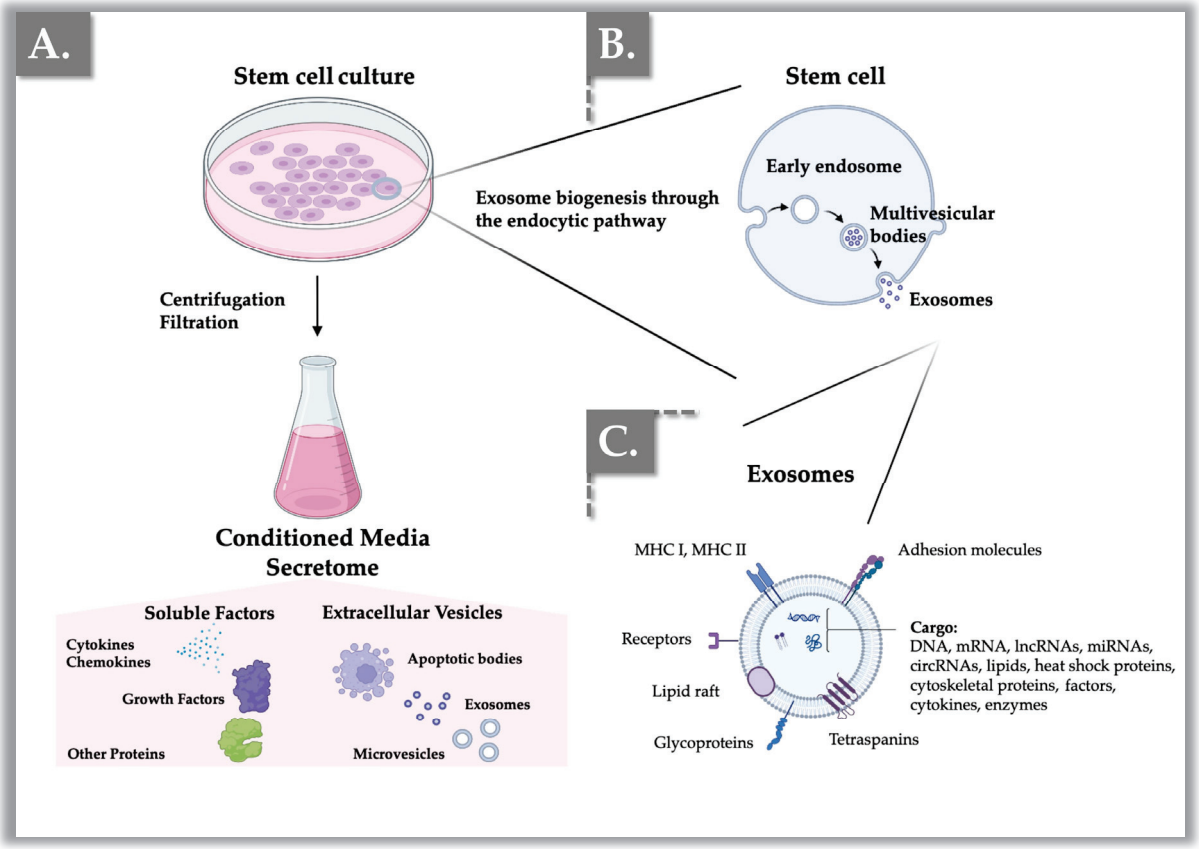


Figure 1. Exosome sourcing and characteristics. (**A**) Exosomes are obtained from stem cell cultures by centrifugation and filtration methods for primary isolation. (**B**) Exosomes are lipid bilayer-enveloped extracellular vesicles originating from the endocytic pathway. (**C**) Exosomes contain a diverse range of cellular components on their surface, such as adhesion proteins, antigen-presenting molecules, tetraspanins, receptors, glycoproteins, and lipids. They also transport cargo in their lumen, which may be shuttled to recipient cells. DNA, deoxyribonucleic acid; MHC, major histocompatibility complex; RNA, ribonucleic acid.

Of note, exosomes cannot be simply and specifically isolated from cell secretomes or conditioned media. Secretomes include all the molecules and biological factors released by cells into the extracellular space. The secretome mainly contains soluble factors (e.g., cytokines, chemokines, growth factors) and other soluble proteins and extracellular vesicles (e.g., apoptotic bodies, microvesicles, exosomes; Figure 1A). Therefore, the use of the term "exosome" requires that advanced separation processing and specific analytical methods are applied to the investigated biological extracts. Notwithstanding, few technical processes enable the obtention of exosome fractions with high degrees of purity, as soluble factors and alternative EVs are consistently present in trace amounts (Figure 1).

Importantly, exosomes are highly biocompatible and have enhanced stability, maintaining their integrity in circulation and even surviving gastrointestinal digestion [38–40]. Thus, exosomes offer several advantages in medical aesthetics, including low immunogenicity, good biocompatibility, targeting specificity, and strong tissue permeability. These properties also make them highly suitable as drug delivery vehicles. Their ability to transport bioactive molecules efficiently to targeted cells and tissues enhances the effectiveness of therapeutic applications in medical aesthetics.

3. Modes of Action of Exosomes in Cutaneous Applications

In the field of cosmetic and regenerative dermatology, several in vivo studies using EVs (i.e., sometimes incorrectly, inaccurately, or incompletely labeled as secretome or conditioned media) principally derived from stem cells have demonstrated promising potential in addressing various issues. Therein, the main indications of use include those for wound healing, scars, skin pigmentation, dermatoses, skin rejuvenation, and hair loss (Figure 2).

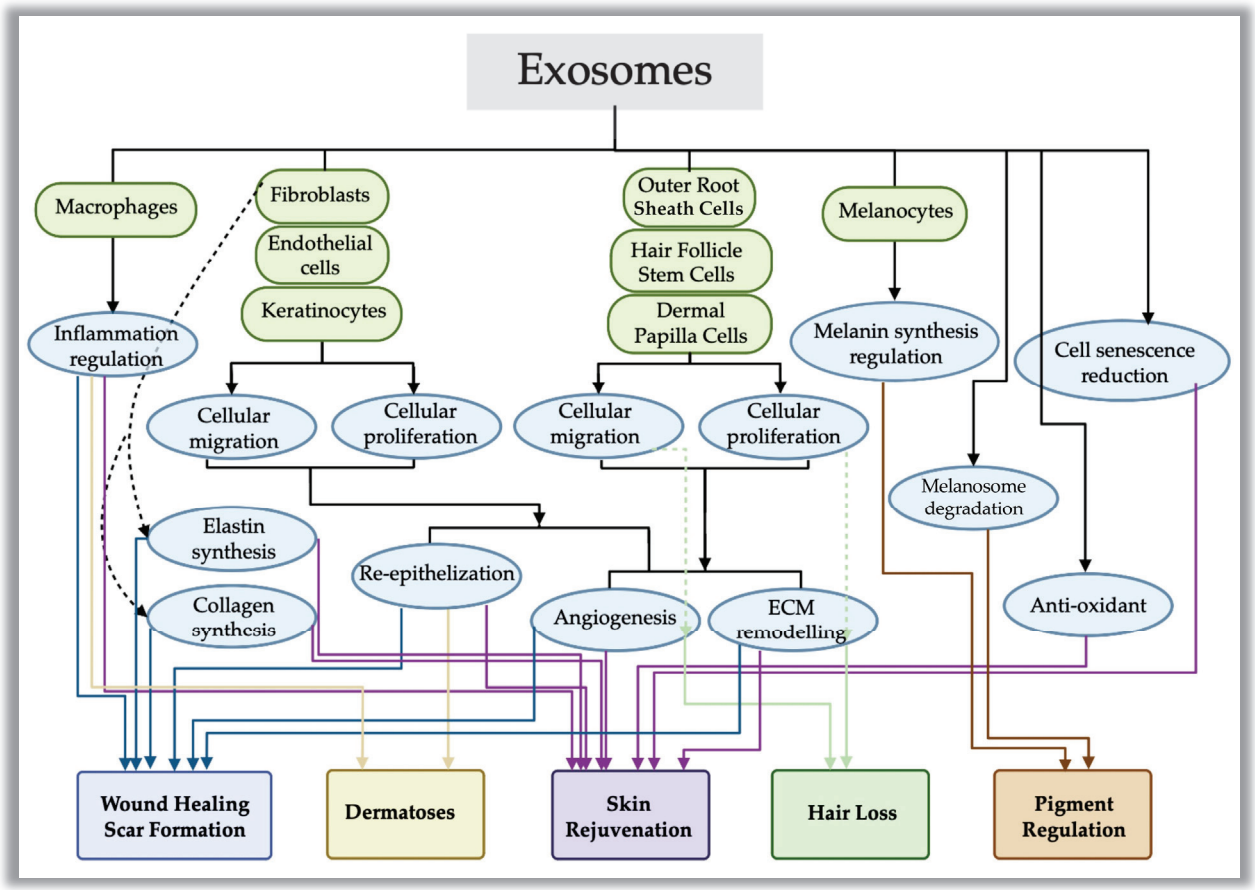


Figure 2. Mechanistic breakdown of the main documented effects of exosomes in skin-related applications. The listed biological functions were compiled from the available preclinical and clinical studies. ECM, extracellular matrix.

Importantly, exosomes' composition significantly influences their effects and in vivo applications. The presence of specific proteins and factors within exosomes dictates their regenerative properties. For example, adipose-derived exosomes, fibroblast exosomes, and those from umbilical cord cells are particularly noted for their regenerative potential [7–9]. To harness regenerative effects, exosomes must contain relevant proteins and factors associated with tissue repair and regeneration (Figure 1). Generally, the therapeutic potential and applications of exosomes in medicine are significantly influenced by the variability in their composition, which is determined by the cell type of origin and the physiological state of these cells. Exosomes reflect the molecular content of their parent cells, including proteins, lipids, RNAs, and other bioactive molecules, which play crucial roles in their therapeutic effects (Figures 1 and 2).

Of note, cells with regenerative properties, such as stem cells, are particularly valuable for exosome-based therapies. Among these, adipose-derived mesenchymal stem cells (AD-MSCs) are the most extensively studied. AD-MSCs possess robust regenerative and immunomodulatory capabilities, and their exosomes are rich in factors that promote tissue

repair, reduce inflammation, and modulate immune responses [5,9]. These properties make exosomes derived from AD-MSCs highly effective in therapeutic applications, particularly in regenerative medicine.

In contrast, exosomes derived from cells in pathological states or from less regenerative cell types may contain different cargo, which could limit their therapeutic efficacy or even pose risks. Thus, selecting the appropriate cell type, particularly those with regenerative capacities like mesenchymal stem cells, is critical for optimizing the therapeutic potential of exosomes in clinical applications. Due to the implication of exosomes in many biological pathways and their multimodal effects, their known MoAs are presented hereafter in Section 3.1, Section 3.2, Section 3.3, Section 3.4, Section 3.5 by therapeutic or cosmetic indications (Figure 2).

3.1. Roles of Exosomes in Cutaneous Wound Healing

Wound healing is facilitated and accelerated by a range of EV bioactive components, which influence inflammation regulation, increase skin re-epithelization and tissue regeneration through cellular proliferation and migration, or promote angiogenesis and extracellular matrix (ECM) remodeling through collagen synthesis [41–44]. The main effector cells implicated in wound healing are macrophages, endothelial cells, fibroblasts, and keratinocytes (Figure 2) [42,45,46]. In detail, the most frequently observed signaling molecules are miRNA, lncRNA, and circRNA, followed by growth factors and other specific proteins [42,45,47].

Interestingly, various sources of exosomes have been found to impact wound healing, comprising adipose stem cells (ADSCs), bone marrow stem cells (BMSCs), human umbilical cord mesenchymal stem cells (hUC-MSCs), induced pluripotent stem cells (iPSCs), expanded potential stem cells (EPSCs), fetal dermal mesenchymal stem cells (FD-MSCs), urine-derived stem cells (USCs), oral mucosal lamina propria-progenitor cells (OMLP-PCs), menstrual blood-derived stem cells (MenSCs), Wharton-jelly mesenchymal stem cells (WJ-MSCs), human amniotic mesenchymal stem cells (hAMSCs), human amniotic fluid stem cells (hAFSCs), and human embryonic stem cells (hESCs) [42,45].

Specifically, EVs derived from umbilical cord mesenchymal stromal cells (UCMSCs) were shown to reduce inflammation through the action of cytokines and nucleic acids, promote angiogenesis, and stimulate the proliferation and migration of fibroblasts through TGF-β pathway activation [48–50]. EVs from human cord mesenchymal stem cells (hCM-SCs) were shown to influence the Wnt4 pathway, which is crucial for wound healing processes [51]. Wharton's jelly MSC EVs exhibited potential in activating endogenous VEGF-A to enhance angiogenesis through miRNAs [52]. Adipose stem cell (ADSC) EVs also exhibited therapeutic benefits for wound healing by activating the PI3K/Akt signaling pathway [53,54].

Generally, MSC EVs inhibit inflammatory cells and promote the polarization of M1 macrophages [48]. Moreover, they enhance angiogenesis by stimulating the proliferation and migration of endothelial cells. They also support the remodeling of the skin ECM and inhibit scar formation by increasing the proliferation and migration of fibroblasts. In some cases, EVs containing MALAT1 contribute to improved wound healing through miR-124 activation of the Wnt/β-catenin pathway [55]. Furthermore, exosomes containing miR-29a reduced scar formation by inhibiting the TGF-β2/Smad3 pathway [56].

Of note, blood MSC EVs have been shown to facilitate wound healing by regulating the polarization of macrophages and inhibiting inflammation [48]. Additionally, they increase angiogenesis and the proliferation and migration of fibroblasts. Overall, two meta-analyses including a total of 47 in vivo studies (i.e., involving diabetic and non-diabetic animals) showed a significant enhancement of wound closure rates for wounds treated with EVs compared to the control groups [57,58]. Additionally, the treatment with EVs was associated with superior neovascular density, re-epithelization rates, collagen deposition, scar width reduction, and the downregulation of inflammatory factors.

3.2. Roles of Exosomes in Scar Formation and Cutaneous Pigment Regulation

Adipose MSC (ADMSC) and umbilical cord MSC (UCMSC) EVs have been found to reduce scar formation and fibrosis by regulating ECM reconstruction, inhibiting myofibroblast differentiation, and reducing excessive fibrotic tissue formation [59,60]. Most often, such EVs are observed to limit myofibroblast formation and modulate collagen deposition towards physiological balance [61]. Human ADMSC EVs are also involved in cutaneous pigment regulation, as they modulate melanocyte melanin synthesis through miRNA- and MITF-dependent pathways, as well as independent signaling pathways [46,62,63]. Exosomes are also involved in pigment regulation via enhanced melanosome-based melanin degradation [44,61].

3.3. Use of Exosomes for Managing Dermatoses

Potential therapeutic options for the treatment of dermatoses include topical exosomes, which regulate cutaneous inflammation and re-epithelization (Figure 2). Specifically, the use of EVs has been thoroughly investigated for various dermatological conditions such as psoriasis and atopic dermatitis [64–72]. Notable improvements were clinically recorded and supported the further investigation of EV-based treatment approaches for alternative inflammatory skin diseases.

3.4. Use of Exosomes for Skin Rejuvenation

From a medical cosmetic standpoint, EVs were shown to play a significant role in skin rejuvenation and photoaging by increasing fibroblast and keratinocyte proliferation, resulting in increased levels of collagen, elastin, hyaluronic acid, and localized dermal fat production [44,46,63,73–76]. Therein, the regulation of inflammation and the promotion of cellular migration contributed to enhanced re-epithelization and angiogenesis. As concerns photoaging, ADSCs, iPSCs, and HUCMSCs demonstrated preclinical proofs of their ability to reduce cutaneous damages [73]. In an in vitro model of UVB-irradiated human dermal fibroblast photoaging, it was demonstrated that UVBs inhibited miR-29b-3p levels compared with controls, but bone marrow mesenchymal stem cell-derived exosomes restored miR-29b-3p levels and reversed the inhibition of cell migration, the oxidative stress caused by UVB irradiation, and the production of MMPs [74]. Moreover, in vivo experiments on photoaged mice exposed to UV radiation showed that ADSC-EV treatment decreased wrinkles, increased collagen expression and production, and decreased UV-induced MMP expression [75–78].

Moreover, it has been demonstrated that exosomes deploy significant antioxidant and cell senescence reduction effects, both positively supporting skin rejuvenation [44]. Optimized ECM remodeling, enhanced autophagy, and reduced oxidative stress in EV-treated skin have also been set forth as key drivers of skin repair and regeneration [44,61]. Mechanistically, exosomes are known to play a role in various regenerative pathways in the skin, such as NFκb, AP-1, MAPK, P-AKT, NRF2, and SIRT-1 [79].

3.5. Use of Exosomes for Managing Hair Loss

Interestingly, EVs from various cell sources have been considered and investigated for managing hair loss concerns. These EVs, which carry essential proteins and miRNA, play a role in promoting the proliferation and migration of dermal papilla cells, outer root sheath cells, and hair follicle stem cells by activating or inhibiting different pathways (e.g., Wnt/β-catenin or LEF1 signaling) [46,80–85]. Generally, the topical use of exosome-based products for hair loss management is considered a promising option for mild to moderate cases, or as an adjuvant approach in post-graft care.

4. Clinical Studies on the Topical Cutaneous Use of Exosomes

Several clinical studies have investigated the use of exosomes as a topical treatment option for various cutaneous applications [86–98]. Some studies employed microneedling or electroporation to enhance product absorption, while other studies combined topical

exosome use with additional treatments such as laser conditioning. The reported outcomes included the recorded effects on pigmentation, scars, post-treatment recovery, erythema, hair growth, female sensitive skin, wrinkles, biostimulation, skin hydration, and skin elasticity (Table 1).

Table 1. Listing of notable clinical studies for the assessment of the cutaneous effects of topical applications of exosome-based products. ADSCs, adipose stem cells; CaHA, calcium hydroxylapatite; CM, conditioned medium; EVs, extracellular vesicles; HA, hyaluronic acid; hUCMSCs, human umbilical cord mesenchymal stem cells; MSCs, mesenchymal stem cells; SCs, stem cells.

Study Reference	Clinical Indication	Active Ingredients/ Concomitant Treatment	Clinical Results	Product/Protocol Details
Cho et al. [86]	Skin brightening	Exosomes from human adipose tissue-derived SC CM	Significant reduction in melanin levels; improvement in skin brightness	Topical formulation [1] with glycerin, 1,2-hexanediol, L-arginine, xanthan gum, carbopol, water for injection
Jang et al. [93]	Skin brightening	EVs from *Codium fragile* and *Sargassum fusiforme*	Improvement in skin brightness	Cream containing *Codium fragile* EVs, 5 μg/mL
Wang et al. [97]	Skin rejuvenation	Protein extracts from ADSCs/Microneedles	Improvement in melanin index, luminosity, brightness, elasticity, and wrinkles	Protein extracts from ADSCs
Proffer et al. [90]	Skin rejuvenation	Topical platelet exosomes for skin rejuvenation	Improvement in skin health; reduction in redness, wrinkles, and melanin production; improvement in luminosity and color evenness	Intensive Repair Serum from Rion containing human leukocyte-reduced apheresed platelet extracts
Lee et al. [94]	Skin rejuvenation	Human embryonic SC CM/0.25 mm microneedle roller	Significant improvement in pigmentation and wrinkles	CM secretory factors of endothelial precursor cells from human embryonic SCs
Chernoff et al. [88]	Tissue biostimulation	Exosomes from placental MSCs/Injected with CaHA/Cavitating ultrasound/LED therapy	Enhanced tissue biostimulation	Exosomes from Kimera Labs, 1 mL containing 10^6 exosomes, botulinum toxin, HA, and CaHA
Prakoeswa et al. [98]	Photoaging	Amniotic membrane SC CM/Microneedling	Significant improvement in photoaging [2]	Amniotic membrane SC CM
Lueangarun et al. [85]	Androgenetic alopecia, hair repigmentation	Exosomes from human adipose-derived MSCs/Fractional picosecond laser	Hair regrowth; repigmentation of gray hair and poliosis circumscripta	ASCE + HRLV-S [1]: 20 mg of lyophilized exosomes with 10^{10} exosome particles
Tak et al. [95]	Androgenetic alopecia	Adipose-derived SC extract for androgenetic alopecia	Increase in hair count and hair diameter	T-Stem product: 1% ADSCE-CE in distilled water/Gentle massage
Han et al. [71]	Dupilumab-related facial redness in patients with atopic dermatitis	Exosomes from human adipose-derived MSCs	Decreased erythema; reduced expression of inflammatory molecules; increased expression of angiogenesis proteins	ASCE SRLV-S [1]: 20 mg of lyophilized exosomes applied with prism sonophoresis

Table 1. *Cont.*

Study Reference	Clinical Indication	Active Ingredients/ Concomitant Treatment	Clinical Results	Product/Protocol Details
Park et al. [91]	Dupilumab-related facial redness in patients with atopic dermatitis	Human adipose-derived MSC exosomes for dupilumab-related facial redness	Improvement in erythematous facial lesions	ExoCoBio technology [1], 2×10^9 particles/mL/Electroporation
Ye et al. [92]	Female sensitive skin	Human MSC exosomes for female sensitive skin	Improved roughness, scaling, erythema, tension, burning, and itching symptoms	Exosomes from human MSCs
Kwon et al. [87]	Acne scars	Exosomes from human adipose tissue-derived SC CM/Fractional CO_2 laser	Significant improvement in acne scars; less erythema; reduced post-treatment downtime	ASCE gel [1], 1,2-hexanediol, glycerin, ammonium acryloyldimethyltaurate/VP copolymer, L-arginine, water for injection
Zhou et al. [96]	Atrophic acne scars and skin rejuvenation	ADSC-CM/Fractional CO_2 laser resurfacing	Improvement in skin hydration, elasticity, collagen, and elastin density	ADSC-CM
Wang et al. [89]	Melasma	hUCMSC-exosomes/Non-ablative fractional laser	Improvement in melasma symptoms	hUCMSC-exosomes with various non-ablative treatments

[1] Exosomes isolated through the ExoSCRT technology from ExoCoBio (Seoul, South Korea). [2] A clinical study demonstrated skin parameter improvements related to photoaging, such as reduced pore appearance, wrinkles, and UV spots in the group treated with AMSC-CM [98].

Exosome-based treatments have shown promising results in clinical studies for conditions like acne scars and melasma, often demonstrating faster healing and improved skin texture compared to traditional treatments like laser therapy or chemical peels (Table 1). However, the outcomes can vary due to the lack of standardization in exosome preparation, which can lead to inconsistent results. While exosomes offer the potential for more targeted and less invasive treatments, their efficacy relative to traditional therapies requires further investigation through controlled trials.

Interestingly, clinical studies have demonstrated that combining exosome-based treatments with microneedling or laser therapy is more effective than using microneedling or laser therapy alone (Table 1). The synergistic effects of exosomes with traditional methods lead to improved outcomes in photoaging treatment, with improved melanin indexes, skin brightness, skin roughness, wrinkles, skin elasticity, and subject satisfaction [96–98]. This combination approach leverages the strengths of both exosomes and established techniques, offering a superior therapeutic option compared to traditional methods used on their own [1–3].

Of note, among the 15 clinical studies listed in Table 1, two studies clearly mention the ASCE SRLV-S and ASCE HRLV-S products from ExoCoBio (Seoul, South Korea) [71,85]. Additionally, other clinical studies, albeit not directly mentioning the ExoCoBio products, were conducted by ExoCoBio and utilized the same exosome isolation technique that the company has patented (i.e., ExoSCRT technology) [86,87,91]. One clinical study mentioned the Kimera Labs product, and it should be noted that Kimera Labs (Miramar Beach, FL, USA) received a US Food and Drug Administration (FDA) warning and should not be currently commercializing their products for aesthetic indications [88]. Finally, one clinical study mentioned the use of the Rion cream product (Rion Aesthetics, Rochester, MN, USA) [90].

Importantly, no FDA or EMA EV-based products are approved at the moment. However, clinical trials related to wound healing (i.e., NCT05078385, NCT02565264, NCT04134676,

NCT06253975, NCT05475419, NCT04235296, NCT03686449, NCT04664738, NCT05475418), keloid formation (i.e., NCT04326959), androgenetic alopecia and hair loss (i.e., NCT06066827, NCT06239207, NCT05658094), skin rejuvenation (i.e., NCT05508191, NCT06217627, NCT05813379), psoriasis (i.e., NCT05523011, NCT05523011), atopic dermatitis (i.e., NCT05969717), and melasma (i.e., NCT06221787) are registered according to www.ClinicalTrials.gov (accessed on 25 June 2024).

5. Commercialized Exosome Products

5.1. Commercial Exosome Product Formula Analyses

For commercialized exosome products, the ingredients list is sometimes provided, but not consistently. The components may not be comprehensively listed, and the information may not be easily accessible or clearly presented. The ingredients list was reported for some products of ExoCoBio/Benev, Medipost, Rion Aesthetics, Elevai, hMSC Skincare, and DP Derm, which were retained for further analyses (Table 2).

Table 2. Listing of market-leading commercial exosome/secretome product information. Data were compiled from publicly accessible repositories [1] or product packaging and labeling materials. Detailed product ingredient listings are provided in Table S1. CM, conditioned medium; HA, hyaluronic acid; MSCs, mesenchymal stem cells; SCs, stem cells.

Company (Headquarters)	Product	Formulation Presentation	Active Ingredient Source	Uses/Applications	Storage Information [2]
ExoCoBio/Benev (Seoul, South Korea)	ASCEplus SRLV/HRLV/IRLV	20 mg lyophilizate + 5.0 mL solution	Plant/*Rosa damascena*	Topical/Skin rejuvenation	2–8 °C
ExoCoBio/Benev (Seoul, South Korea)	ExoBalm	20 mg lyophilizate capsule + 20 mL cream	Plant/*Rosa damascena*	Topical/Skin rejuvenation	4–8 °C during 28 days after reconstitution
ExoCoBio/Benev (Seoul, South Korea)	Soothing Gel Mask	Gel mask	Plant/*Rosa damascena*	Topical/Calming, cooling, recovery, hydration	Ambient
Croma Pharma (Leobendorf, Austria)	EXO/E Serum	Solution	Plant/*Ustilago cynodontis*, *Piper nigrum* L SC, *Withania somnifera* root SCs	Topical/Skin revitalizing complex	Ambient
Stemica Labs (Beirut, Lebanon)	Secretome from UCMSCs	Solution	UCMSCs	Mesotherapy/Skin rejuvenation and hair restoration	Ambient
Medipost (Seongnam City, South Korea)	NGF-574H Hair Serum	Solution	CM of hUCBMSCs	Topical/Hair growth in androgenic alopecia	Ambient
Medipost (Seongnam City, South Korea)	NGF-574H Solution	Solution (mesotherapy; 3 to 6 sessions every 2 weeks)	CM of hUCBMSCs	Mesotherapy	Ambient
Rion Aesthetics (Rochester, MN, USA)	Intense Serum	Serum	Human platelet extract	Topical/Anti-aging skin care, skin rejuvenation	Ambient
Exocel Bio (San Diego, CA, USA)	Exovex Revive	5.0 mL cryopreserved solution	Placental MSCs	Topical, microinfusion after microneedling	−80 °C to −20 °C

Table 2. *Cont.*

Company (Headquarters)	Product	Formulation Presentation	Active Ingredient Source	Uses/Applications	Storage Information [2]
Exoqure, Resilielle (Los Angeles, CA, USA)	Resilielle Age Zero Exosomes	5.0 mL cryopreserved solution	WJ-MSC	Topical with microneedling, laser	−80 °C, 15-month shelf life; −20 °C, 6-month shelf life; refrigerator, 3-month shelf life
JuveXo (Miami, FL, USA)	JuveXO Skin	5.0 mL solution	Umbilical MSCs	Topical with microneedling, dermabrasion, laser therapy	Ambient
PrimaCure (Incheon, South Korea)	E-50 Skin: Dry Ampoule	Lyophilizate	Salmon-tested cells cultivated in salmon embryonic SC media	Topical/Skin rejuvenation, skin inflammation, hair loss, hair growth	Ambient
AnteAge MDX (Irvine, CA, USA)	Exosome Solution	Lyophilizate + 6 mL HA solution	hBMSCs and hUCSCs	Topical with microneedling, radiofrequency, laser, and other ablative treatments	2–8 °C
DP Derm (North Miami Beach, FL, USA)	MG-Exo-skin Serum	5 mL solution (5×)	MSCs	Topical with microneedling	Ambient

[1] Reviews of accessible information (i.e., in English, French, or Spanish) were performed for products with a clear mention of exosomes, secretomes, and/or conditioned media. [2] When the storage temperature was not specified, it was typically assumed that the products are stored at ambient temperature.

It should be noted that only a fraction of the listed ingredients in the analyzed products are considered active, with the other components serving as excipients (Table S1). Specifically, as concerns the Benev ASCE plus SRLV product from ExoCoBio, leucine and isoleucine are considered the active ingredients, while all other ingredients (i.e., including exosomes) are considered inactive ingredients, according to the National Institute of Health (Tables 1 and S1) [99]. As concerns the Benev ASCEplus HRLV from ExoCoBio, the only active ingredient is biotin, according to an FDA report [100]. For the NGF-574H injectable solution, the distributor website cites panthenol as the only active ingredient [101]. In the Medipost shampoo, zinc pyrithione, panthenol, niacinamide, and biotin are considered active ingredients [102].

Generally, the available evidence suggests that exosome/secretome-based products contain a wide range of additional inactive constituents (Tables 2 and S1). Moreover, some companies claim to have growth factors, collagen, peptides, and other molecules in their products, but they do not specify whether these molecules are included in the exosomes, which, by definition, contain such cargo, or if they are molecules that were added during the product formulation process. Furthermore, one reasonable technical assumption is that all lyophilized products contain cryoprotectants and lyoprotectants such as sugars, amino acids, or glycerol to ensure the stability of the delicate ingredients throughout the lyophilization process.

Overall, it may be extremely difficult to precisely determine the exact composition of a given commercial product [103]. Specifically, a 2023 review by Asadpour et al. reported that there are 114 companies and private clinics in 28 different countries providing allogenic SC secretome-based treatments to consumers for skincare and hair loss. Therein, almost 50% of the companies were identified as marketing secretome-based therapies, and the authors noted that the cell sources for such treatments are frequently undisclosed [103].

However, due to the complex nature of finished product analysis from a quantitative standpoint, manufacturer-provided information currently represents the only tangible source for determining exact product compositions and contents.

From a general safety standpoint, it should be noted that long-term safety and efficacy data for exosome-based treatments in dermatology are currently limited. Nevertheless, in topical uses, and to the best of our knowledge, all the available studies highlighted an absence of adverse effects (Table 1).

5.2. Exosome Ingredient and Product Sourcing Considerations

As previously mentioned, exosomes can be derived from multiple biological sources, such as human donors, animal cells, or plant cells. Therein, critical importance is set on the selection of the starting donor material, which needs to be tested for communicable diseases (e.g., viruses, prions). Such basic qualification steps are necessary to ensure the safety and quality levels of the sub-cellular products that are derived from the retained starting materials. Moreover, age, sex, health status, and weight of donors are factors that have a potential impact on the regenerative potential or the proliferative capacity of the established cell source [104]. In detail, some companies (e.g., ExoCoBio) leverage the donor characteristics to support the quality attributes of their products.

In addition to the starting material sourcing and qualification phase, the specific in vitro manufacturing processes applied to the cells are directly related to the quality and functionality attributes of the isolated exosomes/secretomes. Indeed, the methods used to isolate and expand the cell source influence the basic characteristics and functionalities of the derived exosomes/secretomes [104,105].

5.3. Secretome Products for Potential Technical Rationalization

Generally, the isolation of exosomes from cultured cells presents significant biological and technical obstacles, making it a challenging task. Importantly, the act of proving the process of endocytic biogenesis for exosomes is crucial to ensure the unique presence of exosomes. Furthermore, methods including ultracentrifugation and ultrafiltration, commonly employed by companies commercializing exosomes, present technical challenges in isolating only exosomes without also co-isolating other sub-cellular and soluble particles.

As concerns most products that exist on the commercial aesthetic market, it is more appropriate to use the term "secretome" instead of "exosome" (Figure 1) [106,107]. The contents of the cell secretome are constantly dynamic and influenced by the specific type of cell that secretes it, as well as microenvironmental factors, such as cell culture conditions [108,109]. Another term for secretome is conditioned medium, which contains bioactive components responsible for tissue repair and regeneration through autocrine and paracrine effects in intercellular signaling [110–113]. As an example, an in vitro study demonstrated that human dermal fibroblasts pre-treated with ADSC-conditioned medium (CM) exhibited significantly increased proliferative activity and reduced cellular senescence compared to normal human dermal fibroblasts [114].

Topical applications of CM also demonstrated important effects related to skin conditions. A clinical study showed that the topical application of SC CM can significantly reduce wrinkles, pigmentation, and skin pores [111,112]. A topical gel containing hypoxically cultured human neonatal cell CM was assessed in a split-face clinical trial and showed significant improvement in wound healing following laser treatment, exhibiting a dose-dependent response. Additionally, it demonstrated a significant reduction in transepidermal water loss and decreased levels of inflammation by reducing macrophage and neutrophil infiltration, increasing regulatory T cell infiltration, and downregulating the expression of inflammation-related genes (i.e., interleukin-1β, interleukin-6, chemokine ligand 1, and chemokine ligand 2) [112,113]. Another topical gel, which contained hypoxically cultured amnion MSC CM, was demonstrated to enhance angiogenesis and re-epithelization as well as decrease inflammation in an in vivo model of wound healing [113].

One of the major challenges in successful exosome-based therapeutics is the inefficient productivity of exosomes. The yield of exosomes is typically less than 1 μg of exosomal protein (i.e., representing 1 billion EVs per 1 mL of culture medium), whereas the effective dose of exosomes is approximately 10–100 μg of exosomal protein per mouse in most studies [115,116]. Commercial EV products differ in their dosage, which typically range from 5 to 25 billion EVs/particles per application. Therefore, effective, large-scale exosome production methods are required to meet the therapeutic demands. The use of bioreactors offers a promising solution for the large-scale production of EVs. Depending on the cell types, bioreactor compatibility needs to be assessed to ensure the integrity and preservation of the biological activity of the exosomes [117].

5.4. Current Best Practices in Commercial Exosome Isolation

As previously mentioned, the standardization and consistency of exosome isolation are complex and unresolved issues in exosome-based research and applications. Currently, there is no accepted standard for ensuring the purity and consistency of exosomes, which poses a significant challenge across various fields, including aesthetic medicine and therapeutic applications.

In aesthetic medicine, manufacturers commonly rely on a combination of centrifugation, ultracentrifugation, and filtration techniques to isolate exosomes. These methods are widely used due to their relative simplicity and ability to yield exosomes in sufficient quantities for commercial use. However, these techniques often result in preparations that may contain a heterogeneous mix of vesicles and other contaminants, leading to variability in the final product [18,19].

In therapeutic applications, more precise and sophisticated methods, such as size-exclusion chromatography, immunoaffinity capture, and microfluidic techniques, are being explored to enhance the purity and consistency of exosome preparations. These approaches aim to isolate exosomes with greater specificity, but they also introduce additional complexity and are not yet universally adopted or standardized [18,19].

The lack of standardized protocols across different applications remains a critical issue, as it hampers the reproducibility and reliability of exosome-based therapies. Establishing rigorous standards for exosome isolation, characterization, and quality control is essential for advancing their clinical and commercial potential [18].

6. Analysis of EU and Global Regulatory Frameworks for Exosome-Based Products

6.1. Technical Hurdles in the Registration and Market Implementation of Exosome Products

Multiparametric factors contribute to the slow registration of exosome-based products. Notably, there are currently no injectable FDA-approved products, and available topical preparations are highly heterogeneous [118]. Furthermore, there is an absence of widespread consensus on standardized protocols for isolating, storing, and identifying exosomes [46]. It is important to underscore that treatments involving exosomes, secretomes, or EVs have not yet been approved (i.e., FDA or EMA) for application in aesthetic medicine. This fact is attributed to several factors, including the lack of standardization in isolation, purification, and characterization methodologies for exosomes [46,119,120]. The lack of standardization currently poses challenges for regulatory approval and consistency in clinical outcomes. Variability in exosome isolation methods, such as ultracentrifugation, size-exclusion chromatography, and precipitation, can lead to differences in the purity and functionality of the final product. Standardization would involve developing universally accepted guidelines for exosome purity, potency, and characterization, including the specific markers that define exosomes versus other extracellular vesicles. As an example, studies of toxicity profiles, biodistribution, and pharmacokinetics may be required. These standards would not only facilitate regulatory approvals but also ensure that exosome-based products are safe, effective, and of consistent quality across different clinical settings.

Of note, the FDA currently classifies exosomes as biological products. However, exosomes may be classified as cosmeceutical ingredients and therefore must respect the fact

that their effects must be restricted to the skin [120]. Overall, from a regulatory standpoint, developing a standardized quality control workflow for exosome products would be necessary [105]. Importantly, regulatory pathways for exosome-based products are likely to differ between therapeutic and cosmetic applications. In the United States, the FDA categorizes products based on their intended use. Therapeutic exosomes might be classified as biologics, requiring approval under the Biologics License Application pathway, which involves rigorous clinical trials and manufacturing oversight. Cosmetic exosome products, if not intended to treat, cure, or prevent disease, might be regulated as cosmetics or devices, but they still must meet safety and labeling requirements. In the European Union, the EMA may also classify exosome therapies as advanced therapy medicinal products (ATMPs) for therapeutic uses, necessitating a centralized marketing authorization process. For cosmetic applications, exosome products need to comply with the EU Cosmetics Regulation, which demands evidence of safety and efficacy but has less stringent requirements than ATMPs.

To potentially harmonize the classification and approval processes for exosome-based products, regulatory bodies could establish clear guidelines that differentiate between their use as cosmetics and therapeutic agents. This might involve creating specific regulatory pathways that consider the unique properties of exosomes, such as their cellular origin and intended use. Collaborative efforts between international regulatory agencies could also lead to unified standards, reducing the complexity and cost of obtaining approvals across different regions.

Overall, obtaining regulatory approval for exosome-based products is challenging due to the lack of clear guidelines and standardized manufacturing processes. The FDA and EMA have yet to approve any exosome-based products, largely because of concerns about product consistency, safety, and efficacy. These challenges could slow the adoption of exosome therapies in clinical practice, as companies may need to invest heavily in research and development to meet regulatory requirements. This delay could also affect the availability and affordability of these treatments.

6.2. Off-Label Uses and Illicit Commercialization of Exosome Products

Notwithstanding the described limitations of exosome product registration, several companies have taken the risk of marketing their products for aesthetic medicine indications. Most notably, in September 2023, Kimera Labs (Miramar Beach, FL, USA) received a warning letter from the FDA for commercializing MSC exosome products for various uses, including those concerning skin applications [121]. The warning letter explained that exosome products fall under the classification of biological products in the Public Health Service Act and are categorized as drugs. Moreover, Kimera Labs also demonstrated a negative response to the sterility test and exhibited notable deviations from the requirements of current good manufacturing practices (cGMPs). It is important to note that in May 2023, the company obtained an investigational new drug (IND) application for the intravenous product XoGloPro to address COVID-19 symptoms [122]. However, they have also gone beyond this framework and commercialized the product for skin repair, regrowth, and rejuvenation purposes.

As previously set forth, there are currently no exosome-based treatments or medical devices approved for clinical use by the FDA or the EMA, despite several ongoing clinical trials. However, exosomes may be defined as cosmetic ingredients. The FDA defines "cosmetics" as "articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body or any part thereof for cleansing, beautifying, promoting attractiveness, or altering the appearance" [123]. Similarly, the European Commission defines "cosmetics" in EU Regulation 1223/2009, Article 2.1.a) as "any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours" [124]. Notwithstanding, it is important to

stress again that exosomes have not yet been officially categorized for cosmetic use by the FDA; thus, the specific regulatory requirements remain uncertain [125].

Practically, within the EU, exosomes are included in the CosIng database, which lists all cosmetic ingredients. They are listed under the names "human adipose derived mesenchymal cell exosomes", "human cord blood progenitor cell exosomes", "human umbilical mesenchymal stem cell exosomes", "milk exosomes", "human adipose stromal cell exosomes", "human amniotic fluid induced pluripotent cell exosomes", "human dermal fibroblast induced multipotent cell exosomes", "human adipose derived mesenchymal stem cell exosomes", "human umbilical endothelial cell conditioned media", "human tooth pulp stem cell conditioned media", "human adipose derived stem cell conditioned media", "human neonatal fibroblast/keratinocyte conditioned media", "plant conditioned media", etc. Such ingredient nomenclature may be found on most marketed cosmetic products in the exosome field under the INCI component listing.

6.3. Dangers of Off-Label Uses for Commercial Exosome Products

Three main potential safety concerns are applicable to exosome-based products. Firstly, there is a risk of transmitting adventitious viruses or other pathogens from donor cells to recipients. Secondly, non-autologous exosomes may trigger immune responses, leading to inflammation, allergic reactions, or even rejection by the recipient's immune system. Thirdly, exosomes may carry oncogenic factors like specific microRNAs or proteins that could promote tumor growth or metastasis in recipients. Thus, the off-label use and illicit commercialization of exosome products carry significant risks, particularly when these products are not subjected to the rigorous testing (e.g., assays for viruses, mycoplasma, endotoxins, and sterility) and regulation required for safe medical use.

Furthermore, the long-term safety and efficacy of exosome-based products remain largely untested [126]. Moreover, toxicological evaluations have shown that some exosome products, such as those derived from human adipose tissue-derived mesenchymal stem cells (i.e., ASC-exosomes), are generally considered safe, with no adverse effects in various toxicity tests (e.g., skin sensitization, photosensitization, and acute oral toxicity) [126]. Lastly, the lack of standardized manufacturing processes and quality control measures in the commercialization of exosomes can result in products with varying potency and purity. This variability can compromise expected outcomes and safety. Therefore, a cautious and evidence-based approach is essential to ensure the safety and effectiveness of exosome treatments.

6.4. Harmonization of Regulations for Exosomes and EVs

Notwithstanding current industrial practices, the available evidence points towards global regulatory harmonization, under which, exosomes derived from cultured cell sources will be classified as biological products and drugs. Therein, there could be separation into various drug categories, depending on the product doses and intended application sites (e.g., topical use), similar to existing practices for over-the-counter painkillers and anti-allergy medications. Due to the dosing scale that is considered in exosome and EV products, the quantitative aspects of the preparations may even be considered close to those of homeopathic preparations. Notwithstanding, the use of cell culture methods requires the application of cGMPs for product manufacturing, including all QA and QC measures for release. Additionally, the availability of biological assays for the assessment of product activity is of high importance for the validation of product quality, potency, and stability.

7. Exosome Use in Cosmetic Products and Aesthetic Medicine Protocols

Exosomes have a significant scientific background, supported by in vitro, in vivo, and clinical studies. Their therapeutic effects have been demonstrated in this context along with their MoAs [1–4,20,31]. However, the use of exosomes in cosmetics differs from their therapeutic use due to several factors. These include dosages, application methods, formulations (i.e., including excipients), and other ingredients involved in the

finished product. Furthermore, EV function may be affected by various storage conditions, presenting considerable difficulties in maintaining their integrity within a formulation. Importantly, exosomes used as cosmetic ingredients may not produce identical effects to those examined in the aforementioned clinical studies [1,2].

Indeed, a clinical study conducted to evaluate the efficacy of ASCE plus exosomes (i.e., obtained from ExoCoBio) in treating dupilumab-related facial redness in patients with atopic dermatitis revealed significant changes not only in clinical scores but also in the expression of molecules within the stratum corneum [71]. This indicated that the product's clinical effects are produced through specific MoAs that alter the production of inflammatory and angiogenesis proteins.

Furthermore, the regulation and study of exosome MoAs are continuously evolving due to the youthful nature of the field. This necessitates further regulatory consensus and scientific research work. Over the past year, many websites and companies have updated their marketing strategies for exosomes. In detail, companies that previously marketed exosomes for aesthetic purposes have shifted towards developing drug-containing exosomes to treat diseases. Furthermore, several companies continue to sell products containing exosomes, but they have stopped explicitly mentioning "exosomes" in their marketing messages. With numerous changes occurring in this field, some previously available websites are no longer accessible.

From a safety standpoint, while it may appear that topically applied exosomes are less effective, they may in fact decrease immune reactivity. Indeed, the stratum corneum is highly reactive to immune responses, and topically applied exosomes effectively reduce immune reactivity in this layer, leading to a global decrease in inflammatory signals [67,68]. Moreover, an in vivo experiment in a mouse model demonstrated that topically applied exosomes are mostly confined to the stratum corneum [67]. Following topical application, mesenchymal stem cell exosomes exhibited minimal penetration beyond this outermost layer of the skin. Specifically, less than 1% of the exosomal fluorescence was detected in the culture medium after 24 h, indicating that the exosomes were largely retained within the stratum corneum rather than being excreted or systemically absorbed [67]. Locally, exosomes were utilized by the cells within the stratum corneum to inhibit complement activation, particularly the formation of the terminal complement complex (C5b-9). This inhibition led to a reduction in the release of IL-17, a pro-inflammatory cytokine primarily produced by neutrophils through the process of NETosis [67]. Overall, the data suggested that the exosomes exerted their therapeutic effects locally within the skin's stratum corneum by modulating the immune response, rather than being excreted from the body or entering the systemic circulation. Interestingly, despite this limited penetration, topically applied exosomes significantly reduced psoriasis biomarkers [67].

One effective method for achieving this outcome is micro-channeling (e.g., microneedling), which explains its wide application in clinics. In detail, microneedling allows molecules to bypass the stratum corneum barrier by creating numerous micro-holes, enabling transdermal molecule delivery to reach the epidermal/dermal tissue [3,75].

As regards clinical protocols, the topical application of exosomes could be regulated under cosmetic jurisdictions. However, many clinics offer exosomes combined with microchanneling to maximize the function of exosomes and enhance skin rejuvenation and regeneration effects. Therein, it remains unclear if substances applied with the aid of microchanneling are still classified as cosmetics. Notwithstanding, it is crucial to improve the penetration of exosomes applied topically into deeper layers of the skin [73,75,76]. Indeed, topically applied exosomes are unable to penetrate the skin barrier and therefore mainly act within the stratum corneum.

8. Galenic Form and Storage of Exosome-Based Products

8.1. Topical Formulation Possibilities for Exosome Products

Due to the biological nature of exosome/secretome ingredients, the finished product formulation and stability attributes are of critical importance for the quality of the provided

care. Specifically, the biological activity of secretome ingredients, including exosomes, is highly sensitive to storage conditions. Factors such as temperature, pH, and the use of cryoprotectants play critical roles in preserving the stability of these products. Improper storage, such as exposure to fluctuating temperatures or incorrect pH levels, can lead to the degradation of the exosomes' cargo and membrane integrity, reducing their effectiveness. Therefore, it is crucial to adhere to specific storage protocols, including the use of appropriate cryoprotectants (e.g., in lyophilized form), and maintain storage at ultra-low temperatures (e.g., if the product is in liquid form) to ensure the consistency and safety of exosome-based products. Additionally, no incompatibilities should exist between the components of the final formula (i.e., including primary packaging materials), and the finished product should maintain the integrity of the ingredient up until application. Importantly, due to the highly heat- and time-sensitive nature of exosome ingredients, specific aseptic formulation measures are necessary and terminal sterilization is excluded. Of note, most commercially available products are manufactured as semi-solid creams, serums, or lyophilizates (Figure 3).

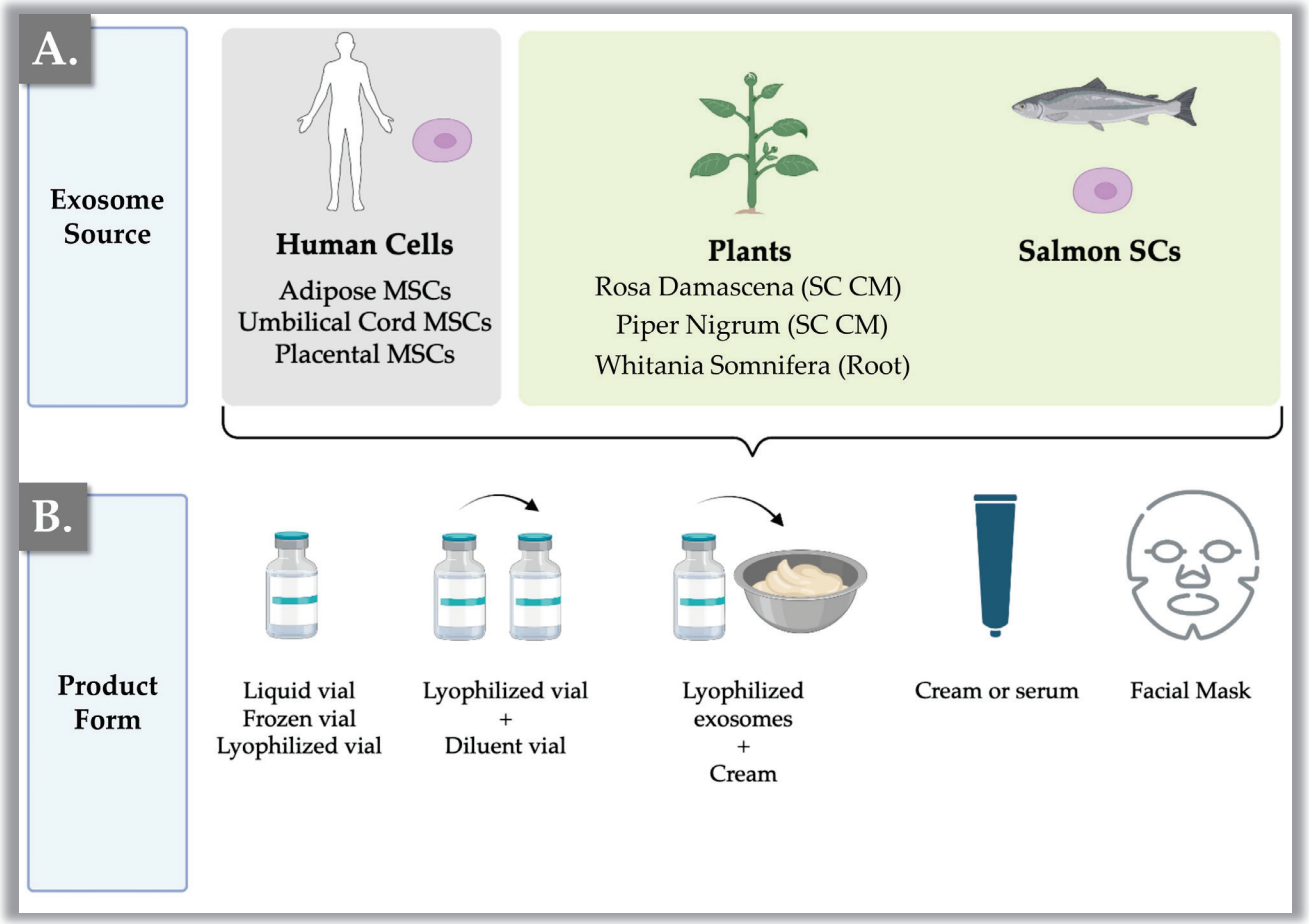


Figure 3. Current commercial landscape of exosome products. (**A**) In the commercial landscape, exosome products are primarily derived from human MSCs, but can also be derived from plant cells (e.g., *Rosa damascena*, *Piper nigrum*, *Whitania somnifera* root) and salmon stem cell CM. (**B**) The commercial products available on the market exhibit a diverse range of galenic forms. There are 3–5 mL vials that can be liquid (i.e., stored at room temperature), frozen (i.e., stored between −20 °C and −80 °C), or lyophilized. Lyophilized exosome vials usually need to be reconstituted with a diluent vial before application. Creams containing exosomes (i.e., ready to use or be reconstituted) can also be found. Exosome-containing facial masks and eye patches are also available. CM, conditioned medium; MSCs, mesenchymal stem cells; SCs, stem cells.

As concerns cream formulations, a preparation containing CM from 3D-cultured human adipose MSCs demonstrated a considerable skin whitening effect following a two-week application period [127]. The creme formulation involved an oil-in-water emulsion, with the CM being incorporated at 1% into the cream mixture [127]. Another formulation example by Dong Wha Pharmaceutical (Seoul, South Korea) offers a serum and cream that include lyophilized hUCBMSC CM and are combined with humectants and emollients before use [128]. In clinical studies, these products were shown to increase wound healing and decrease post-laser redness [128]. As concerns lyophilized commercial preparations, ExoCoBio commercialized an exosome-containing cream that consists of a 20 mL cream base formulation incorporating 20 mg of lyophilized plant exosomes, which the user needs to mix before application. The prepared cream should be stored at temperatures between 4 °C and 8 °C for up to 21 days. Additionally, ExoCoBio provides a calming gel mask that contains pre-solubilized plant exosomes (Table 2).

8.2. Storage Conditions and Stability of Exosome/Secretome Products

Storage conditions, particularly temperature, have a significant impact on the biological activity of exosome-based products. For example, storing exosomes at −80 °C helps preserve their integrity and functionality over time, while storage at higher temperatures, such as 4 °C or −20 °C, can lead to the degradation of key components like miRNAs, reducing their therapeutic potential. Freeze–thaw cycles and pH shifts during storage can also affect exosome stability, leading to diminished efficacy or altered biological effects.

In order to preserve the biological activities of secretome ingredients, it is important to store them appropriately. The secretome is highly unstable and requires specific storage conditions. Indeed, the molecules present in the secretome are notably prone to enzymatic degradation, protein oxidation, or protein aggregation [129,130]. It is crucial to remember that each study of storage conditions applies specifically to the type of cell being studied. Thus, it remains uncertain whether these results can be applied to EVs from different cell sources that vary in surface lipids or proteins and cargo content [131,132]. However, exosomes offer greater stability compared to other soluble factors in the secretome. One thermodynamic study of liposome stability showed that the specific surface composition plays a crucial role in maintaining the liposome's stability [133]. Various factors, such as temperature, pH, and preservation techniques, can influence the physiological and biological characteristics of the considered EVs [134].

For ready-made cosmetic products, long-term storage and sustained effectiveness at ambient temperature are important factors to consider [44]. At the ingredient level, a study demonstrated that storing EVs in PBS over time leads to drastically reduced recovery, particularly for pure EV samples at all tested temperatures, starting within days [135]. Alternative buffers may facilitate EV preservation at −80 °C, maintain stability throughout several freeze–thaw cycles, and drastically improve EV recovery in downstream applications [135]. To date, there is still a lack of consensus concerning the overall impact of storage conditions on EVs, yet it appears that process, formulation, and temporal parameters play key roles in frozen ingredient stability [135,136]. As concerns the use of lyophilization processing for ingredient stabilization, it was reported that a preparation supplementation with sugars (e.g., sucrose, trehalose, mannitol) significantly contributed to augmenting the maintenance of the quality attributes/therapeutic potency of biomolecules [137,138]. Furthermore, lyophilization offers the possibility of concentrating samples, which is especially important for CM-based preparations [137].

8.2.1. Frozen Storage for Exosome Products

In a patent describing EV formulation, it was demonstrated that the NTA-determined EV concentration and size remained stable after one week of storage at 4 °C, −20 °C, and −80 °C [132]. However, the levels of miRNA decreased to less than 50% after 30 days at temperatures of 4 °C and −20 °C. At a temperature of −80 °C, there was minimal alteration in the levels of miRNA [131]. Another study used flow cytometry analysis to investigate

the impact of the storage temperature on the physical and functional characteristics of antibacterial EVs derived from human neutrophilic granulocytes [139]. Storing the EVs at 4 °C for one day caused a reduction in EV quantity and antibacterial efficacy. Storing them at −20 °C for 28 days preserved the number of EVs but nearly eliminated their antibacterial effectiveness. Contrastingly, storage at −80 °C for 28 days maintained both the physical and functional characteristics of the EVs. While storage at −80 °C is optimal, it drastically increases costs and limits transportation and logistical options [139].

The clinical use of secretome products necessitates a minimum shelf-life extension of 6 months. According to MISEV2023 recommendations, storage should be at −80 °C in PBS [20]. However, it is underscored that freezing and thawing can cause alterations in the shape, activity, size, and concentration of EVs. Freezing may result in ice formation, exposure to ice–liquid interfaces, buffer salt precipitation causing pH shifts, and particle-rich phases due to the cryo-concentration of the vesicles. Furthermore, PBS use can cause an acidic shift during freezing, which, in turn, destabilizes the membrane proteins of the EVs [140]. Some authors reported that the concentration of EV fractions remained stable when preserved at either 4 °C or −80 °C for a period of one month [141].

8.2.2. Lyophilized Storage for Exosome Products

The lyophilization of exosome-based products presents several challenges, including the potential for aggregation and loss of bioactivity during the freezing and drying processes. The choice of cryoprotectants, such as trehalose, is critical to maintaining the structural integrity of exosomes. However, lyophilization can also introduce stresses that may cause changes in exosome size or surface properties, impacting their effectiveness. These challenges complicate the development of stable, commercially viable exosome formulations and require careful optimization of the lyophilization process to ensure product consistency and efficacy.

Lyophilization is commonly used to condense and preserve active ingredients during storage and transportation. Additionally, it offers an optimal ingredient format for subsequent cosmetic product formulation [44]. A notable in vitro and in vivo experiment demonstrated that lyophilized secretome had similar effects on wound closure rates. Importantly, this suggested that the process of lyophilization did not diminish the potency of the secretome [142]. Furthermore, polyacrylamide gel electrophoresis (PAGE) analysis indicated that the proteins and RNA in the secretome were preserved after lyophilization [143]. Of note, lyophilization and subsequent rehydration did not result in a significant decrease in the bioactivity of EVs either in vitro or in vivo when compared to EVs that were not subjected to lyophilization [131,132]. Notwithstanding, it was set forth that lyophilization can induce stress during the freezing, dehydration, and rehydration stages, necessitating the addition of suitable stabilizers. Indeed, rehydration can notably cause the swelling of amphiphilic molecules present in the vesicle bilayer and potentially detrimental osmotic effects [140].

Laboratory studies on the lyophilization of EVs from different cell sources, such as MSCs and HUVECs, have shown that the total particle amount decreases with lyophilization compared to storage at 4 °C or −80 °C. This decrease may be attributed to the formation of aggregates. Notwithstanding, it was found that the qualitative properties of EVs were not significantly influenced by lyophilization. Furthermore, trehalose was shown to be a superior cryoprotective agent compared to mannitol and polyethylene glycol 400 [144]. Overall, exosomes offer greater stability compared to other soluble factors and ingredients, and lyophilization processing appears to be an optimal technical solution for the preparation and storage of active exosome-based ingredients.

8.3. Stabilization Processing of Exosome Products

8.3.1. Formulation with Cryoprotectants/Lyoprotectants

Frozen storage as well as lyophilization require cryoprotectants. Cryoprotectants are commonly employed in preservation processes, with options such as penetrating

cryoprotectants (e.g., dimethyl sulfoxide [DMSO] or glycerol) and non-penetrating agents like trehalose and sucrose [134]. Frozen storage of the secretome at −80 °C with DMSO optimally maintains the stability of the sample, in contrast to frozen storage without cryoprotectants. This indicates that DMSO can be used in reasonable proportions (i.e., subtoxic amounts) as a cryoprotectant for the long-term storage of secretome ingredients, even though it may not preserve all vesicle morphologies in the sample [145]. Lyophilization also requires the use of cryoprotectants, such as trehalose, to prevent aggregation of the secretome [143]. It was demonstrated that EVs are more stable in the presence of sucrose or potassium phosphate buffer than in sodium phosphate buffer or phosphate-buffered saline during freeze–thaw cycles. A neutral pH also leads to less aggregation [140]. Generally, the use of sugars (e.g., mannitol, trehalose) as combined cryoprotectants and lyoprotectants is optimal for simple and effective biological ingredient stabilization.

Of note, the cryoprotectant used in the sourcing of cells for EV production is considered to be the most important. The overall cell survival and recovery should indeed be optimal (i.e., >80% cellular viability upon initiation). Specifically, if the retained cell preservation system does not allow high cell stability, it should be reconsidered before establishing the downstream processing of EVs from the cell source. Several studies have demonstrated that exosomes lyophilized with stabilizers such as trehalose maintain their bioactivity after rehydration, with minimal changes in size, structure, or functional properties [140–144]. However, the lyophilization process must be carefully controlled to avoid damage to the exosome membrane, which could occur during the freezing, dehydration, or rehydration stages. The use of appropriate cryoprotectants and careful optimization of the lyophilization cycle are crucial to ensuring that the exosome product remains potent and effective. We showed in a recent study the importance of assessing combinations of cryoprotectants with a grading table to compare the results after the process of lyophilization (i.e., including various quality controls, e.g., presence of a cake, residual water, uniformity, resuspension time, or meltback) [146].

8.3.2. Quality Considerations: Ingredient Resuspension after Lyophilization

An important parameter linked to the lyophilizate form is the resuspension time and quality, which can be limiting factors for clinicians and consumers. A study demonstrated that human red blood cells that had been lyophilized and subsequently rehydrated exhibited analogous levels of molecular components and cellular characteristics than the control group [147,148]. The parallel between red blood cells or liposomes and exosomes is interesting, as they share similar biological formats/structures. A study found that the reconstitution of highly concentrated lyophilized proteins was complete within one minute when the protein-to-sugar ratio was less than 1. However, when the ratio exceeded 1, the reconstitution times increased in a nonlinear manner [149]. Another study revealed that the reconstitution time was primarily determined by the formulation viscosity, which was dependent on the protein-to-sugar ratio [150]. The study determined that disaccharides do not offer a protective effect on lyophilized cells when they are present only in the extracellular environment and that extensive vitrification may not provide significant benefits [150]. Overall, high importance is set on the careful design of lyophilization formulas and cycle recipes in order to obtain an appropriately stabilized product with acceptable resuspension characteristics [151].

9. Conclusions and Perspectives

The growing use of secretomes, exosomes, and all potential formulations of EVs in the field of cosmetic and aesthetic medicine highlights their promising role in supporting tissue repair and regeneration. Although exosome- and secretome-derived products have demonstrated beneficial effects on skin texture, inflammation reduction, and overall skin quality attribute enhancement, important challenges remain regarding their isolation, characterization, MoAs, widespread cosmetic application, and regulation. Despite these challenges, in vitro, in vivo, and clinical studies confirmed their potential to address various important

cutaneous issues. Moreover, clinical trials using exosomes or secretome ingredients have notably confirmed their tolerability and efficacy, providing tangible scientific support for their future use. Differences in cosmetic applications, such as sourcing, dosage, formulation, and product stability, necessitate prospective research to optimize the use of exosomes and alternative EVs and facilitate their regulation. In detail, there are limitations due to the lack of reliable manufacturing methods, limited understanding of the mechanisms of action of the product owing to its complexity and involvement in multiple pathways, regulatory challenges, and scalability issues. Establishing standardized guidelines for manufacturing, isolation, storage, stability, dosage, sourcing, and safety is necessary. Addressing these factors and obtaining more clinical data will enable the homogeneous utilization and more straightforward regulation of secretome products and exosomes. The molecular profiling (i.e., of proteins and lipids) of secretomes could be an option to better characterize the products and help in establishing more precise mechanisms of action. Importantly, the commercial market for these products is constantly evolving, with frequent manufacturer website changes and applications shifting between aesthetic and therapeutic uses. Indeed, the market is rapidly expanding, with significant growth expected. Additionally, a substantial number of clinics are offering this treatment directly to consumers. Since 2024, numerous articles in cosmetic and beauty magazines have been highlighting these emerging beauty treatments, making exosomes a trending topic. However, the increasing interest in exosome therapies suggests that regulatory agencies will eventually develop specific guidelines. The implications for future market entry are significant. As the regulatory landscape evolves, exosome-based products may face stringent requirements for demonstrating safety, efficacy, and consistency in manufacturing processes. Companies entering this market will need to navigate these regulations carefully, which may involve extensive clinical testing and post-market surveillance activities. Generally, the promise of exosome and secretome products in the cosmetic industry holds substantial potential, requiring a rigorous approach to fully exploit their capabilities in improving skin health and appearance.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/cosmetics11050154/s1: Table S1: Listing of ingredients for the commercially available products described in Table 2.

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Abbreviations

ADSCs	adipose stem cells
ATMP	advanced therapy medicinal product
BMSCs	bone marrow stem cells
CD	cluster of differentiation
circRNA	circular RNA
CM	conditioned medium
CaHA	calcium hydroxylapatite
cGMPs	current good manufacturing practices
CHUV	Lausanne University Hospital
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EV	extracellular vesicle
EMA	European Medicines Agency
EPSCs	expanded potential stem cells
ESCRT	endosomal sorting complex required for transport
EU	European Union
FD-MSCs	fetal dermal mesenchymal stem cells
FDA	US Food and Drug Administration
HA	hyaluronic acid
hAFSCs	human amniotic fluid stem cells
hAMSCs	human amniotic mesenchymal stem cells
hCMSCs	human cord mesenchymal stem cells
hESCs	human embryonic stem cells
hUC-MSCs	human umbilical cord mesenchymal stem cells
HUVECs	human umbilical vein endothelial cells
INCI	international nomenclature of cosmetic ingredients
IND	investigational new drug
iPSCs	induced pluripotent stem cells
LED	light-emitting diode
lncRNA	long non-coding RNA
MenSCs	menstrual blood-derived stem cells
MHC	major histocompatibility complex
miRNA	microRNA
MoA	mechanism of action
mRNA	messenger ribonucleic acid
MSCs	mesenchymal stem cells
NLF	nuclear localization factor
NTA	nanoparticle tracking analysis
OMLP-PCs	oral mucosa lamina propria-progenitor cells
PRP	platelet-rich plasma
PBS	phosphate-buffered saline
PAGE	polyacrylamide gel electrophoresis
QA	quality assurance
QC	quality control
Rabs	GTPases families
RNA	ribonucleic acid
SCs	stem cells
TNF	tumor necrosis factor
WJ-MSCs	Wharton-jelly mesenchymal stem cells
UCMSCs	umbilical cord mesenchymal stromal cells
USA	United States of America
USCs	urine-derived stem cells
UV	ultraviolet

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Bioactive Triple-Helical Recombinant Collagen Gels for Improved Healing of Sunburned Skin

Yi Yang [1,2,3], Bei Tian [1,2,3], Yi Xie [1,2,3], Peng Liu [2,3], Linyan Yao [1,2,3,*] and Jianxi Xiao [1,2,3,*]

[1] State Key Laboratory of Applied Organic Chemistry, College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou 730000, China
[2] Gansu Engineering Research Center of Medical Collagen, Lanzhou 730000, China
[3] Joint Research Center of Collagen of Lanzhou University, China National Biotec Group, Lanzhou Biotechnology Development Co., Lanzhou 730000, China
* Correspondence: lyyao@lzu.edu.cn (L.Y.); xiaojx@lzu.edu.cn (J.X.)

Abstract: Excessive ultraviolet (UV) exposure can lead to sunburn, characterized by skin barrier damage, inflammation, pain, and an increased risk of skin cancer. Recombinant collagens have gradually attracted attention due to their high purity, low immunogenicity, batch-to-batch consistency, and excellent solubility. Additionally, the type of dressing significantly affects wound repair. Gels are ideal for sunburn treatment because they maintain a moist environment, adhere firmly, and do not need to be removed. Herein, we have created bioactive triple-helical recombinant collagen (THRC) gels for improved healing of sunburned skin. The THRC gels remained stable after a three-month stability test, displaying a rheological behavior characteristic of non-Newtonian pseudoplastic fluids. In vivo skin irritation tests conducted on New Zealand rabbits demonstrated that THRC gels were safe for use. A sunburned mice model was established to study the biological effects of THRC gels. Non-invasive combo evaluations indicated that THRC gels exhibited an exceptional capability of recovering dermis density, erythema index (EI), hydration, and transepidermal water loss (TEWL) of sunburned skin to a healthy state. Histological observations revealed that THRC gels significantly enhanced the repair of damaged skin by accelerating the recovery process, promoting collagen deposition and regeneration. Molecular biological characterizations further demonstrated their remarkable antioxidant properties, including the inhibition of lipoperoxidation and the enhancement in superoxide dismutase (SOD) and glutathione (GSH) activities. These safe and bioactive recombinant collagen gels provide a novel approach for sunburn repair and show potential for long-term cosmetic benefits.

Keywords: recombinant collagen gel; sunburn treatment; gel dressing

1. Introduction

Exposure to UV radiation is a common threat to skin health, causing acute sunburn symptoms such as erythema, edema, and dryness [1,2]. Within the UV spectrum, UVA (wavelength: 320–400 nm) penetrates deeper into the skin, reaching the dermis, while UVB (wavelength: 280–320 nm) primarily affects the epidermis [3,4]. About 50% of the UVA can penetrate to the dermis, eliciting apoptosis in cells [5]. UVB is a minor component of UV radiation; however, it is more detrimental to the skin than UVA [6]. UVB not only directly causes DNA damage, but also triggers a cascade of pro-inflammatory and intracellular signals, resulting in inflammatory cell infiltration, epidermal thickening, and flattening of the dermo-epidermal junction [7]. Excessive UV exposure can lead to acute photodamage and, with long-term accumulation, significantly increases the risk of skin cancer, particularly in individuals residing in high-altitude and coastal areas [8–10]. Therefore, it will be of great significance to develop effective strategies for the treatment of sunburned skin.

Natural polymers with high bioactivity and biocompatibility have been extensively developed to treat sunburned skin [11–13]. Hyaluronic acid (HA), a macromolecule of

the extracellular matrix, has been utilized to repair UV-damaged skin due to its efficacy in tissue remodeling, anti-inflammation, and antioxidant properties [14,15]. As the main dermal component in human skin, the damage of collagen has been widely known as a key feature of acute sunburn [16]. Recent research indicated that yak collagen, with its triple-helical structure, offers effective treatment for sunburn [17]. However, the animal-derived collagen suffers from allergic reactions and risks of disease transfer. Our previous study developed a type of triple-helical recombinant collagen that features high purity, low immunogenicity, consistent batch-to-batch quality, and excellent solubility [18]. This recombinant collagen has shown great promise in wound healing due to its high bioactivity and stability, attributes closely related to its triple-helical structure [19]. Consequently, THRC-based wound healing biocomposites remain a significant area of research.

Dressings have been designed to address skin damage promptly, providing effective care and promoting faster healing [20]. Based on the physical form, dressings are available in the types of ointment, film, foam, and gel, which can be applied to wounds with different characteristics [21]. A suitable type of wound dressing can provide a desirable environment for wound healing [22]. Gels offer several advantages over other dressings for treating sunburn, including protection from environmental contaminants, enhanced air exchange, antibacterial properties to combat microorganisms and infections, reduction in dead cells at the wound site, ease of replacement, and non-toxic and non-allergenic characteristics [21,23]. Additionally, gels are characterized by excellent sensory and rheological properties, such as a pleasing sensation when applied to the skin, and a low degree of viscosity [24]. Overall, gels are preferred by people for repairing sunburn wounds.

In this study, we developed a novel THRC gel and evaluated its stability, rheological properties, safety, efficacy, and antioxidant capabilities for repairing sunburned skin. The THRC gels, demonstrating favorable stability, excellent rheological behavior, and high safety, provide strong evidence supporting their potential for commercialization. We further established a sunburn animal model via UV irradiation and compared the healing effects of daily treatment over a 7-day period across different treatment groups. A non-invasive combo was applied to quantify changes in skin condition. A histological analysis and molecular biological methods were utilized to compare the effects of THRC gels and commercialized collagen dressings on sunburned skin. The THRC gels demonstrated superior safety and efficacy, highlighting their potential for applications in dermatology and skincare.

2. Materials and Methods

2.1. Materials

Glycerol, phenoxyethanol, methylparaben, and hydroxyphenylpropyl were purchased form Guangzhou Zhongguang Biotechnology Co., Ltd. (Guangzhou, China); HA with 120–160 w was purchased from Bloomage Biotechnology Co., Ltd. (Beijing, China); H&E, Masson, MDA, SOD, and GSH kits were all purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China); Hydroxyproline (HYP) (CRM), Chloramine-T (AR), citric acid (AR), isopropyl alcohol (AR), para dimethylaminobenzaldehyde (PDAB) (AR), and 70% perchloric acid (AR) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China).

2.2. Preparation of THRC Gel

THRC was expressed through the *Escherichia coli* (*E. coli*) BL21 strain, employing plasmid pColdIII-THRC in accordance with previous protocols [18,25]. The cells were cultured in 50 mL of a Luria–Bertani (LB) medium supplemented with 100 mg/L ampicillin overnight at 37 °C. Subsequently, the cells were transferred to 1 L of the LB medium and incubated at 37 °C. When the optical density at 600 nm (OD600) reached a range of 0.5–2.0, the temperature was reduced from 37 °C to 25 °C. Then, 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was introduced to induce protein expression. After 8–36 h of incubation, the cells were harvested and resuspended in a binding buffer (20 mM sodium

phosphate buffer, pH 7.4; 500 mM NaCl; and 20 mM imidazole). The cells were lysed using a homogenizer, and the resulting supernatant fraction was collected. Raw proteins were purified through a Ni-NTA Sepharose column using an elution buffer (20 mM sodium phosphate buffer, pH 7.4; 500 mM NaCl; 500 mM imidazole). THRC was obtained through the trypsin digestion of the purified protein, enabling the removal of the folding domain as outlined in previous descriptions. The degraded fragments were eliminated through dialysis using 50 mM phosphate-buffered saline (PBS) at pH 7.4. The purified THRC was lyophilized for future applications and confirmed by SDS-PAGE.

The formula of the THRC gel was as follows: 0.35% THRC, 1.45% HA, 8% glycerol, and a few medical preservatives (phenoxyethanol, etc.). The phase A mixture (water, glycerol, HA, and preservatives) was heated to 85 °C and stirred until complete dissolution. The phase A mixture was then homogenized for 3 min and incubated for 15 min. When the temperature decreased to 30 °C, phase B (THRC solution) was added. Finally, the mixture kept being stirred to reach a transparent solution. The THRC gels were packaged into plastic bottles that had been pre-sterilized with Co-60 irradiation. Each plastic bottle of a 30 mL gel mixture was sealed and stored at 4 °C.

2.3. Stability Assessment of THRC Gel

The formulations had to pass the centrifugation test, the heat test, and the freeze test to evaluate stability [26]. The centrifugation test was performed with 5 mL of the formulation in a plastic tube and subjected to centrifugation at 3000 rpm for 3 cycles of 30 min each. The formulations were stored in glass containers with lids (20 mL for each formulation) at temperatures of 60 °C and −4 °C for 12 h. The parameters evaluated were pH values and organoleptic characteristics (color, odor, and appearance). The determination of the pH values was performed with the formulations diluted in distilled water in a 1:10 ratio, according to the methods in QB/T 2872-2017 [27] and GB/T 13531.1-2008 [28]. The evaluations were performed at the initial time (0 weeks, 0 w) and subsequently at 28 days (4 weeks, 4 w), 56 days (8 weeks, 8 w), and 84 days (12 weeks, 12 w).

2.4. Rheological Behavior of THRC Gel

The rheological behaviors of the THRC gel were evaluated. Rheological experiments were conducted at a constant temperature of 25 °C using an Anton Paar rheometer (MCR 302, Anton Paar, Germany). The fresh gel samples were positioned on the sample stage of a rheometer, and changes in viscosity were evaluated across shear rates ranging from 1 to 100 s^{-1}. Then, we plotted a double logarithmic curve of shear stress versus shear rate. The flow and consistency index were obtained using the Ostwald–deWaele model, represented using the following equation where τ = shear stress (Pa); κ = consistency index ($Pa.s^n$); γ = shear rate (s^{-1}); and n = flow index (dimensionless) [29].

$$\tau = \kappa\gamma^n \quad (1)$$

2.5. Skin Irritation Test of THRC Gels on Rabbits

All animal experiments were conducted according to protocols approved by the ethics committee of the College of Chemistry and Chemical Engineering at Lanzhou University (No. G09, 20220711). All animals were fed with standard laboratory diets referring to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. All animal research complied with the commonly accepted "3Rs".

A skin irritation test was performed to evaluate the tolerance of rabbit skin to the THRC gels based on GB/T 16886.10-2017 (China) [30]. All rabbits weighed 2.0 ± 0.2 kg and were between 3 and 4 months of age. Three New Zealand white male rabbits with healthy skin were used. Three rabbits were fed in a single cage and adapted for 3 days. Then, 24 hrs before the experiment, fur from the backs of the three rabbits was removed and clipped into 10 cm × 15 cm areas with no damage to the epidermis. The shaved area was divided into four sections: control, normal (normal skin plastered with THRC gel), damage

(damaged skin plastered with THRC gel), and positive control (normal skin plastered with methanol solution). First, the dressing is applied for 4 h, which constitutes the contact phase. Then, the dressing is removed, and any residual test material is gently cleaned off with warm water, followed by careful drying of the area. Finally, at different time intervals (1 h, 24 h, 48 h, and 72 h), the skin of the test and control areas was observed under natural light and quantitatively scored for erythema and edema according to the scoring standards for erythema and edema formation. According to the GB/T 16886.10-2017, the primary dermal irritation indices (PDIIs) were calculated according to the following formula. Mean scores of 0–0.4 were recorded as very mildly irritating, 0.5–1.9 as mildly irritating, 2.0–4.9 as moderately irritating, and 5.0–8.0 as severely irritating.

$$\text{PDII} = \frac{\text{Sum erythema (all time points)} + \text{Sum edema (all time points)}}{\text{number of intervals} \times \text{number of animals}} \quad (2)$$

2.6. Animal Experiment of THRC Gels in Sunburn Skin Healing

The sunburned skin model was established by excessive UV exposure to study the repair efficiency of THRC gel for sunburn wounds. The 96 female Kunming mice were purchased from Lanzhou Animal Experiment Center. Before the test, all mice were depilated on the back in a 2 cm × 4 cm area. Firstly, 96 mice were assigned randomly into four groups with 24 mice in each group, as shown in Table 1. All mice except the blank group were irradiated with UVA (320–440 nm) and UVB (280–320 nm) lamps (Nanjing Huaqiang Electronics Co., Ltd., Nanjing, China) at 200 mJ cm^{-2} to induce acute skin inflammation [31]. One day after UV radiation, the THRC gel and product (medical recombinant collagen functional dressings (Gel Type), 30 g/bottle) were applied to repair the wound skin, respectively. Daily treatment of 15 min was applied at 24 h intervals. Six mice from each group were euthanized on days 1, 3, 5, and 7, respectively. Skin samples were then collected from the sunburned sites.

Table 1. The main information of experimental groups.

Group	UV Radiation	Treatment
blank	-	-
model	+	-
product	+	0.1 g/day
THRC gel	+	0.1 g/day

2.7. Non-Invasive Evaluation by Combo to Assess the Skin Condition

The DermaLab Combo analysis system was applied to evaluate the skin condition non-invasively. The five main parameters representing skin conditions, namely a dermatoscope, ultrasound, EI, hydration, and TEWL, were measured on day 1, day 3, day 5, and day 7 after UV irradiation. A dermatoscope uses positive or non-positive white light as a light source to magnify and image the surface of the skin. Meanwhile, Combo ultrasonic imaging relies on the properties of reflected sound waves through the tissue, which are highly correlated to the differences of collagen [32]. The increase in EI can be caused by sunburn, resulting in skin redness [32,33]. The hydration level of the skin surface can be accurately determined using Combo by measuring electrical capacity as the alternating voltage of SC. TEWL was particularly designed according to Nilsson's Vapor Pressure Gradient theory, with an open chamber method that provides minimal impact on the skin being examined with low statistical bias. Three measure points evenly distributed on dorsal skin were selected and measured three times at each point to reduce the measurement error. The average values were calculated according to the measured data.

2.8. Histological Analysis

Skin from euthanized mice models of UV-mediated acute skin injury was harvested. The skin tissues were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. The

tissues were sectioned to a 3.5 μm thickness on polylysine-treated glass slides. Sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome to evaluate epidermization, inflammation, cellular infiltration, dermal thickness, and collagen density/structure. The stained tissue sections were imaged on a metallurgical upright microscope.

2.9. Quantitative Analysis of Collagen Volume Fraction and Hydroxyproline

The collagen volume fraction was defined as the percent area fraction (%) = (area of collagen fibers/area of skin tissue) × 100%, and derived from Masson trichrome-stained histologic sections using Image J software (Fiji for macOS).

The concentration of hydroxyproline was determined in order to evaluate the gel's efficacy in promoting collagen regeneration following previously described procedures [34,35]. Briefly, 20 mg of fat-removed skin tissues was minced, and incubated with 2 mL of 6 M HCl for 12 h at 85 °C. pH of the solution was then adjusted to 7.0 by adding 10 mol/L NaOH at room temperature. Standard solutions of hydroxyproline were prepared at concentrations of 5.0, 7.5, 10, 15, and 20 μg/mL. Chloramine-T was added to the solution, and incubated at room temperature for 20 min. Ehrlich's reagent was then added, and incubated at 85 °C for 10 min. The mixture was cooled to room temperature and incubated for 30 min. The UV absorption at 560 nm was measured, and the concentrations of hydroxyproline in the hydrolytic samples were determined according to the standard curve. The concentrations of hydroxyproline in tissue homogenates were determined per milligram of dry tissue.

2.10. Effect of THRC Gels on MDA, SOD, and GSH Levels

Malondialdehyde (MDA), SOD, and GSH contents were determined using colorimetric assay kits. Skin tissues were initially weighed and placed into the extraction solution at a ratio of 0.1 g/mL, followed by homogenization in an ice bath. The homogenate was then centrifuged at $8 \times 10^3 \times g$ for 10 min at 4 °C, and the resulting supernatant was collected. The supernatant was taken and measured using a microplate reader to determine MDA, SOD, and GSH content, respectively.

2.11. Statistics

All data were expressed as means ± standard deviation. The multiple independent groups were compared with a one-way analysis of variance (ANOVA) in the software SPSS ver.24. Statistical significance was defined as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. GraphPad Prism8.0.2 software (San Diego, CA, USA) was used for charts.

3. Results

3.1. Stability Determination of THRC Gels

The stability of the THRC gels was assessed at the initial time (0 w) and subsequently at 28 days (4 w), 56 days (8 w), and 84 days (12 w). THRC gels were uniform, transparent, and not delaminated, and no water came out after centrifugation, heating, and freezing at different times (Table 2). In addition, the pH of THRC gels remained within the physiological skin range of QB/T 2872-2017. These results demonstrated the good stability of THRC gel. A longer-term storage test may need to be conducted to ensure the stability of the THRC gel before it is marketed [26].

Table 2. The stability of THRC gels at different times (n = 3).

Time	pH (25 °C)	Centrifugation Test	Heat Test	Freeze Test
0 w	6.43 ± 0.03	no significant difference	no significant difference	no significant difference
4 w	6.57 ± 0.05	no significant difference	no significant difference	no significant difference

Table 2. *Cont.*

Time	pH (25 °C)	Centrifugation Test	Heat Test	Freeze Test
8 w	6.44 ± 0.08	no significant difference	no significant difference	no significant difference
12 w	6.50 ± 0.04	no significant difference	no significant difference	no significant difference

3.2. The Rheological Behavior of THRC Gels

The rheological behaviors of THRC gels were investigated using an Anton Paar rheometer. Within the range of 1 to 100 s^{-1}, the viscosity of the THRC gels gradually decreased with an increasing shear rate, indicating the presence of shear-thinning behavior (Figure 1a). A double logarithmic plot of the shear rate versus viscosity in the range of 1 s^{-1} to 100 s^{-1} approximated a straight line with the equation y = 0.2864x + 0.7033 (Figure 1b). According to Equation (1), we obtain n = 0.2846, which is less than 1. These results demonstrated that the THRC gel exhibited non-Newtonian pseudoplastic behavior.

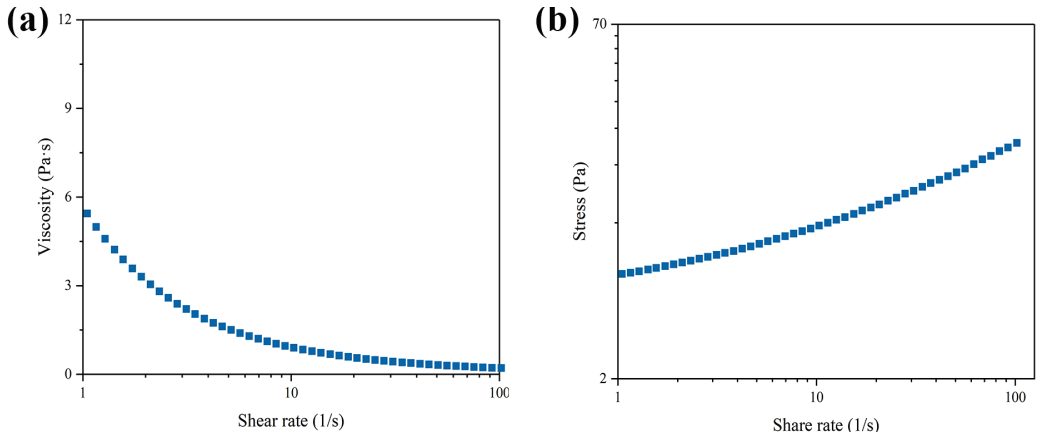


Figure 1. The rheological behavior of the THRC gel. (**a**) The viscosity measurement; (**b**) a double logarithmic plot of the shear rate versus viscosity.

3.3. Skin Irritation Evaluation of THRC Gels on Rabbits

The skin irritation by a single administration assessment was performed by an in vivo rabbit test to evaluate the safety of THRC gels applied to the skin. The shaved rabbit skin was first divided into four parts. The scores of skin edema and erythema for the normal/broken area where the gel was applied were both "0" in each rabbit at any time point. PDII was calculated as 0 according to the scores, indicating that the skin irritation caused by the THRC gels was negligible (Table 3). The results indicated that the THRC gels exhibited no skin irritation for either normal or damaged skin.

Table 3. Results of the skin irritation test to the THRC gels (n = 3).

Group	Score	PDII	The Intensity of Irritation
control	0	0	no irritating
normal	0	0	no irritating
damage	0	0	no irritating
positive control	1.66	0.55	mildly irritating

3.4. DermaLab Combo Evaluation of the Performance of THRC Gels on the Repair of Sunburned Skin

A UV-mediated acute sunburned skin model was established according to the method described in Section 2.6. The THRC gels and commercialized collagen dressings were

applied to the severe sunburned sites, respectively. Meanwhile, the untreated group was the model group.

A Combo dermatoscope was utilized to evaluate the representative macroscopic appearance of sunburn at different points (Figure 2). At the first day after model preparation, all groups except the blank group exhibited typical sunburn reactions, including redness, swelling, scaling, and epidermal peeling, with no significant differences between these experimental groups. On day 3, the THRC gels group showed complete scabs on the sunburned sites, which began to fall off. The commercially available products group had scabs with slight epidermal damage. The redness and swelling of the treated group basically subsided. The model group skin cracked due to inflammation and dryness. On day 5, the scabs of the THRC gels group were completely removed, and skin essentially returned to normal but there was slight dryness. The skin of the commercially available products group crusted and quickly fell off. The model group skin was crusted completely with slight redness. On day 7, the skin with THRC gels returned to normal. The scabs of the commercially available product group were removed completely but slight dryness remained, while the scabs of the model group were not completely removed. The results demonstrated that the sunburn model was successfully constructed. Compared to the untreated group, the treated groups indicated much accelerated repair of the skin wounds. Notably, THRC gels showed superior efficacy in repairing sun-damaged skin compared to commercialized collagen dressings.

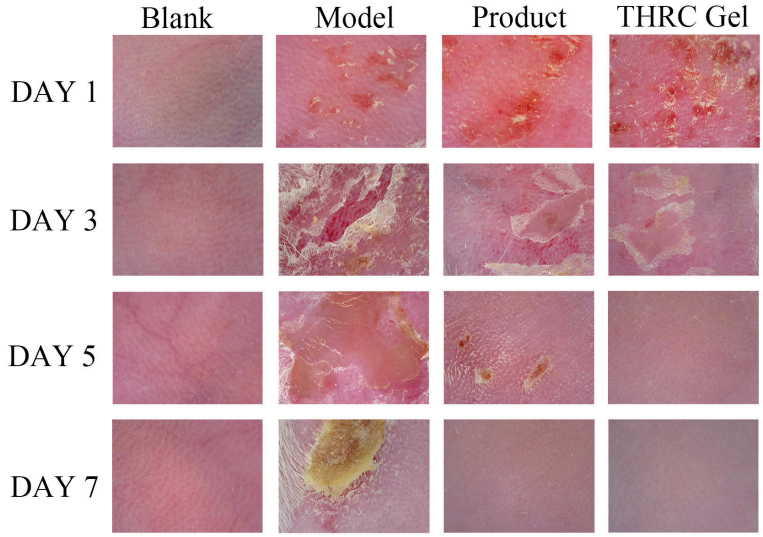


Figure 2. Combo dermatoscope images of the skin in blank, model, product, and THRC gel groups on days 1, 3, 5, and 7 at the magnification of 50×.

Using the DermaLab Combo skin analysis system, the dermis density, EI, hydration, and TEWL of the skin were measured. One day after UV radiation, no significant difference was observed among the different groups expect the blank group, no matter the dermis density, EI, hydration, or TEWL (Figure 3b–e). The representative ultrasound scanning images as well as dermis density were obtained by skin ultrasonic inspection at different time points (Figure 3a,b). In the treated group with THRC gels, the dermis density increased along with the extending of experiment time, increasing to 32.70 ± 3.39, 44.53 ± 4.19, and 57.81 ± 6.11, respectively, at 3, 5, and 7 days, while the dermis density in the commercially available product group increased to 30.63 ± 3.92, 38.91 ± 5.70, and 48.33 ± 3.29. The differences between these two groups were significant ($p < 0.01$) at 7 days. In the model group, the dermis density decreased to 22.36 ± 4.53, 23.99 ± 2.46, and 24.45 ± 3.49, respectively, at 3, 5, and 7 days. The result was consistent with the photodamaging descriptions reported in the literature, which indicated the successful establishment of the sunburned animal model [11,36]. Along with the extending of experiment time, the EI in

the THRC gels decreased to 7.91 ± 0.97, 4.46 ± 0.60, and 3.34 ± 0.46, respectively, at 3, 5, and 7 days. Meanwhile, in the commercially available product group, the reduction in EI was 9.62 ± 0.56, 7.16 ± 0.33, and 3.95 ± 0.82 at 3, 5, and 7 days (Figure 3c). The differences between these two groups were significant at 3 days. The hydration had a similar trend to that of dermis density (Figure 3b). At 3, 5, and 7 days, the increment of hydration in the THRC gel group was 136.61 ± 14.34 μS, 198.81 ± 18.42 μS, and 290.07 ± 13.36 μS, respectively. The hydration of the commercially available product group decreased to 91.95 ± 16.43 μS because of cracking skin at 3 days, but increased to 156.37 ± 20.67 μS and 221.33 ± 23.95 μS at 5 and 7 days. The hydration of skin showed significance along with the experiment time between these two groups. The TEWL paralleled EI (Figure 3e). At 5 days postoperatively, the TEWL of the THRC gels group showed no significant difference from the blank group. The data attained by the DermaLab Combo skin analysis system indicated that UV radiation impacts skin parameters including the dermis density, EI, hydration, and TEWL. Additionally, the THRC gels enhanced the skin conditions, while the efficiency was better than the commercialized collagen dressings.

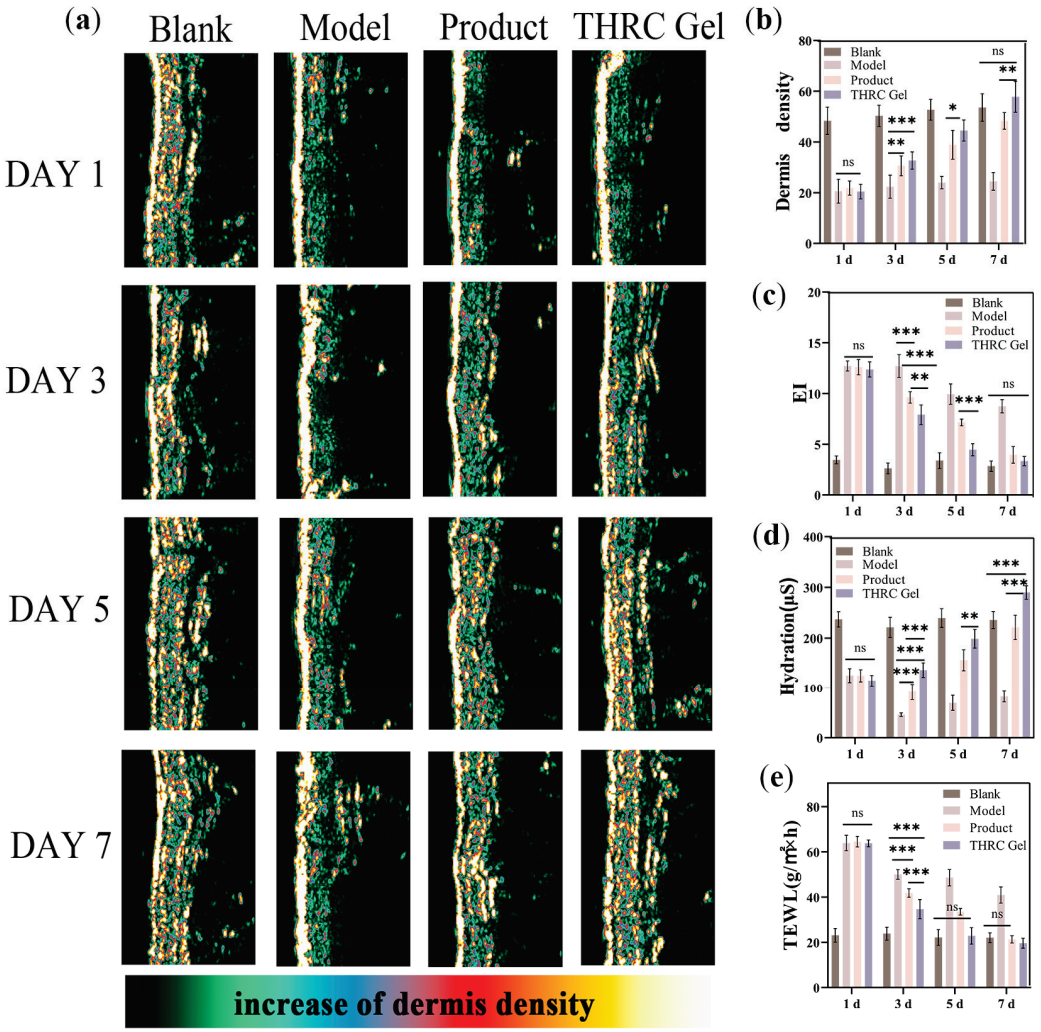


Figure 3. The Combo non-invasive evaluation of the performance of THRC gels on the repair of sunburned skin. (**a**) Ultrasonic image; (**b**) dermis density; (**c**) EI; (**d**) hydration; and (**e**) TEWL of mice skin in different groups including blank, model (untreated), and two treatment groups (product and THRC gels) on days 1, 3, 5, and 7. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns > 0.05, $n = 6$.

3.5. Histological Analysis to Investigate the Repair Ability of THRC Gels

H&E staining indicated the biocompatibility of the THRC gels, revealing no obvious inflammation or granuloma observed (Figure 4). Throughout the experiment, the blank group's skin remained healthy, showing no inflammatory reactions. The epidermal layer remained continuous and intact, with cells arranged in an orderly fashion, and there were no spines at the epidermal–dermal junction. On the first day, the histopathological analysis revealed sunburn symptoms in all groups except the blank group. Keratinocytes were vacuolated, numerous sun-damaged cells were present in the stratum spinosum, spines were evident at the epidermal–dermal junction, and dermal edema along with inflammatory cell infiltration led to a blurred epidermal–dermal boundary. By day 3, notable epidermal changes occurred in the UV radiation groups. Increased melanocytes and inflammatory cells were evident in the epidermis, with the highest counts observed in the model group, followed by the commercially available product group, and then the THRC gels group. Keratinization was incomplete in the model group, whereas both the commercially available product and the THRC gels groups exhibited crusted keratinization. By day 5, minimal change was observed in the model group compared to day 3, except for the presence of scabs. The scabs of the commercially available product group began to fall off, accompanied by epidermis thinning, reduction in inflammatory cells, and subsiding dermal edema. The THRC gels group basically returned to a normal state. By day 7, the epidermis of the commercially available product group was slightly thickened compared to the blank group. The THRC gels group returned to normal. These results demonstrated that THRC gels displayed enhanced epithelization capability, accelerated wound healing, and improved healing efficacy, effectively reducing the risk of infection in sunburned skin.

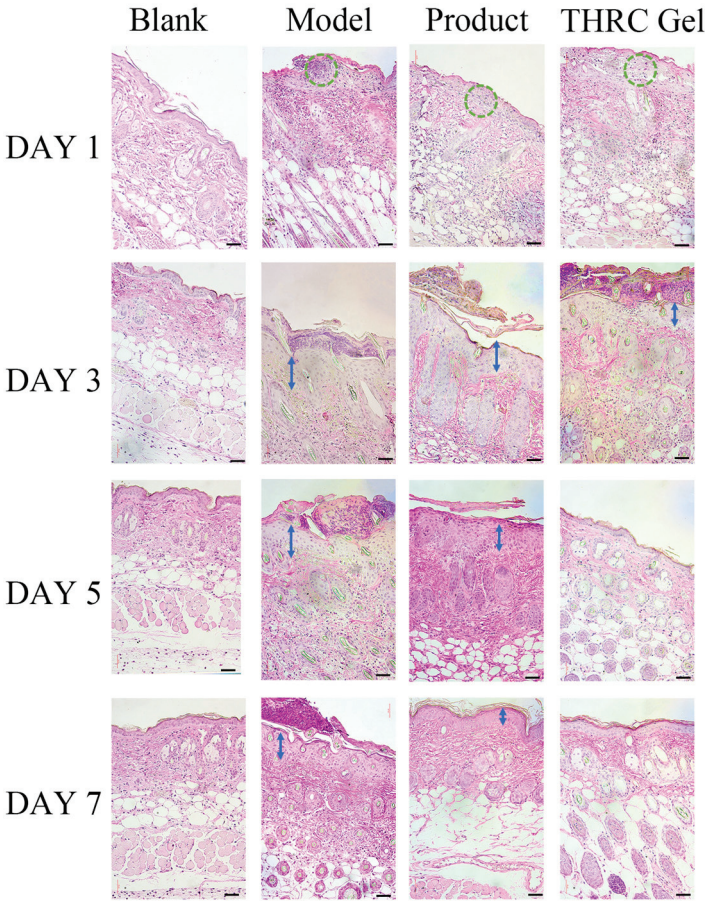


Figure 4. H&E staining to evaluate the repair ability of THRC gels on days 1, 3, 5, and 7. Green circles (apoptotic keratinocytes or sunburned cells); blue double arrows (the thickness of epidermis). Scale bar: 70 μm.

Masson staining images of the untreated group showed abundant collagen debris and short aggregates, which could result from collagen polypeptide chain breakage and the degradation of collagen fibers due to severe UV irradiation (Figure 5a). In contrast, the treated groups both showed a significant increase in the amount of well-ordered dense collagen fibers with interspersing fibroblasts, which well resembled the normal dermis. Collagen volume fraction results further illustrated remarkable collagen deposition in the treated groups (Figure 5b). The results indicated that THRC gel promoted collagen deposition to accelerate sunburn wound healing.

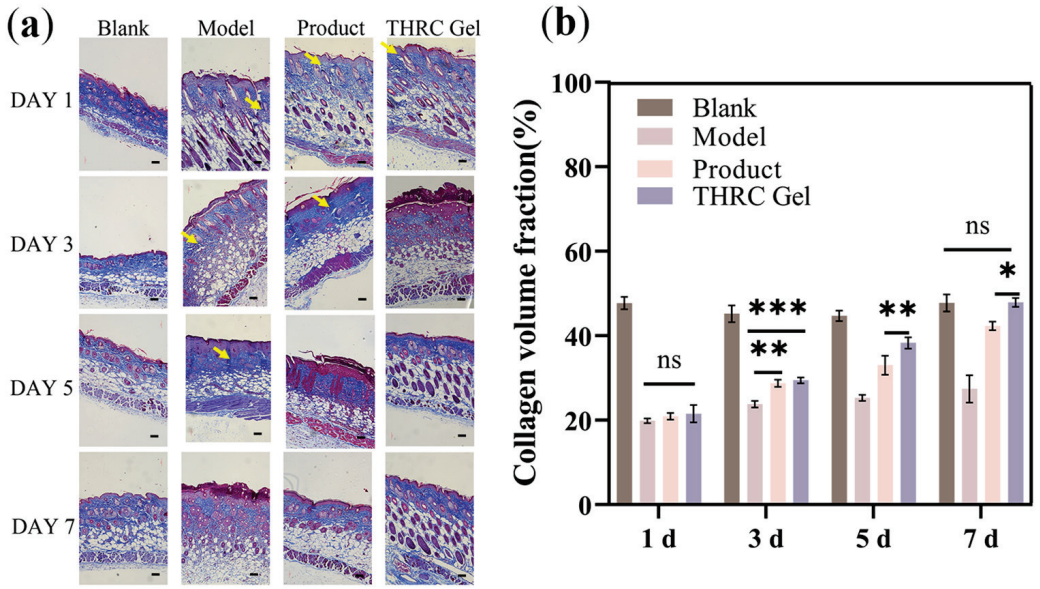


Figure 5. Masson staining evaluation of the ability of THRC gel to promote collagen regeneration. Masson staining images (**a**) and collagen fraction volume (**b**) of the blank, model, product, and THRC gel groups on days 1, 3, 5, and 7. Yellow arrows (collagen fiber breakage). Scale bar: 40 μm. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns > 0.05, $n = 3$.

3.6. Effect of THRC Gels on Hyp, MDA, SOD, and GSH Levels

Hyp, a characteristic amino acid in the collagen sequence, was measured to quantify collagen content. The content of Hyp in the blank group served as the standard. Compared to the blank group, the untreated group exhibited a significant reduction in Hyp content due to UV damage (Figure 6a). Following treatment with THRC gels, the Hyp content was increased, indicating collagen enrichment within 7 days. Similarly, treatment with the commercially available products also increased Hyp content. These results suggested that THRC gels possessed the power to accelerate the healing of severe UV-mediated skin injury by promoting the regeneration of dermal collagen. Additionally, the result was superior to those obtained with commercialized collagen dressings.

Exposure to UV radiation would generate large amounts of reactive oxygen species (ROS) and causes DNA damage in skin tissue, leading to oxidative stress [37,38]. MDA, a major product of lipid peroxidation, significantly increases due to UV radiation, resulting in the oxidation of macromolecules [39]. Thus, the MDA content was evaluated to discuss the degree of sunburn in skin tissue. Compared to the blank group, the model group showed a significant increase in MDA content (1.72 ± 0.14 nmol/g), suggesting that UV radiation induces lipid peroxidation in the skin (Figure 6b). Meanwhile, both of the two treated groups showed a significant decrease in MDA content, with the values of 1.22 ± 0.11 nmol/g (THRC gel) and 1.40 ± 0.05 nmol/g (product group). Notably, the MDA content in the THRC gel group was not significantly different from the blank group.

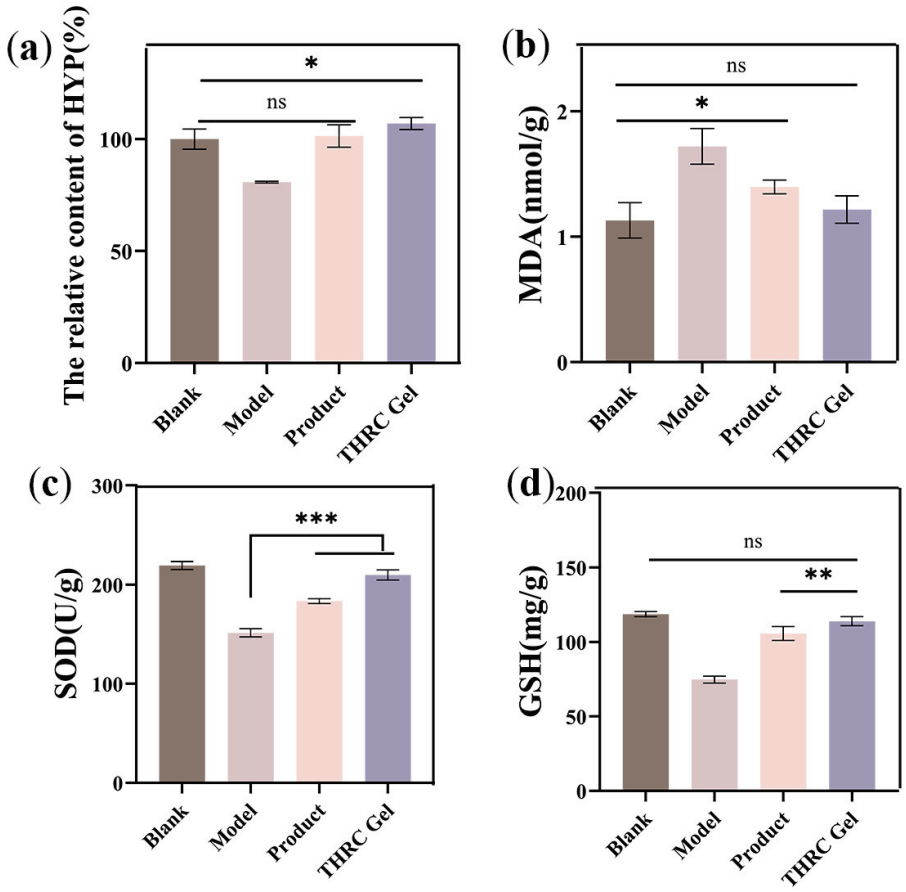


Figure 6. The quantitative analysis of the effect of THRC gels on the repair of sunburned skin. The content of Hyp (**a**), MDA (**b**), SOD (**c**), and GSH (**d**) of the blank, model, product, and THRC gel groups on day 7. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns > 0.05, $n = 3$.

The activities of SOD and GSH were further investigated to study the effect of THRC gels on the repair of sunburned skin. Excessive UV radiation has also been reported to induce a decrease in SOD and GSH activity, disrupting the physiological equilibrium of the skin [11,39]. The activities of SOD and GSH decreased to 151.35 U/g and 74.67 ± 2.37 mg/g in the model group, respectively, displaying significant decreases compared to the blank group (Figure 6c,d). The SOD activity in the treated groups showed a significant difference compared to the model group. Specifically, the THRC gel group exhibited a SOD activity of 209.77 ± 5.07 U/g, while the product group showed an activity of 183.38 ± 2.39 U/g. The GSH activity significantly increased to 113.86 ± 3.02 (THRC gel) and 105.67 ± 4.67 (product). These findings suggest that THRC gels are more effective in revitalizing sunburned skin than the commercial product, likely by significantly increasing SOD and GSH levels while inhibiting MDA overexpression.

4. Discussion

Sunburn is arguably the most prevalent skin disease induced by UV irradiation particularly in high-latitude regions, and it has been considered as a crucial risk factor for skin cancer [10,40]. As the main component of the dermis, collagen degradation has been identified as a critical feature of acute sunburn. Consequently, THRC gels with high biocompatibility and bioactivity exhibit notable protective effects against UV-induced sunburn. Compared to traditional liquid dressings, gels treating sunburn wounds may offer enhanced efficiency. Initially, the gel forms a protective film on the wound surface, isolating it from bacterial contamination, preventing fluid loss, and fostering an optimal environment for cell proliferation, thereby accelerating tissue healing. Additionally, it

adheres to the wound longer than a liquid dressing, facilitating healing. It maintains a moist environment on the wound surface and can be repeatedly administered in situ, avoiding new injuries due to dressing adhesion to the wound surface during dressing changes, and effectively alleviating patient discomfort [23]. Our results revealed that THRC gels represented a promising and more effective strategy for sunburn treatment.

We have reported the development of biocompatible and nonirritating gels composed of triple-helical recombinant collagen, HA, and glycerol for the accelerated healing of photodamaged acute skin wounds and use in cosmetics. The physicochemical evaluations of THRC gels over different time periods revealed their remarkable stability, attributed to the triple-helix conformation [19]. The skin irritation test of New Zealand rabbits further demonstrated that the THRC gels are highly safe and non-irritating.

The sunburn healing efficacy of THRC gels was examined using a UV-induced acute skin injury mice model, characterized by exfoliation, erythema, dryness, and epidermis damage. DermaLab Combo results demonstrated that the THRC gels may improve wound healing by accelerating inflammation [41]. Skin barrier damage is characterized by the diminishment of hydration and increment of TEWL, while the moisture content in the skin typically determines the health of skin [39]. THRC, HA, and glycerol are efficient moisturizers since the THRC gels can enhance the hydration of the stratum corneum, and film-forming properties contribute to mitigate TEWL. The histological analysis achieved a consistent result. H&E and Masson staining images indicated that the untreated group showed epidermal damage, cellular infiltration, and dermal inflammation. In contrast, 5 days of treatment with THRC gels resulted in the robust revitalization of sunburned mice skin, evidenced by the prominent acceleration of epithelization and collagen deposition, whereas the reported fish scale extract natural hydrogel showed persistent redness on day 5 [42]. Furthermore, THRC gels remarkably promoted the arrangement and deposition of collagen fibers, which could be accomplished by supplementing contact collagen, reducing denatured collagen, and stimulating collagen regeneration according to our previous research [31].

UV radiation causes the overexpression of ROS, which is a reduction product of oxygen in the body and could induce the activation of signaling molecules and pathways such as the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathway [3]. As one of the main causes that could clear ROS generated by UV radiation, SOD is important in cellular overall defense mechanisms. GSH, a master antioxidant inside the cells, is essential for preventing oxidative cell damage, and could be detected and considered as an antioxidant activity index [39]. Additionally, long-term exposure to UV accelerates lipid peroxidation, resulting in increased MDA content. The THRC gels showed higher levels of SOD and GSH as well as lower MDA content, which meant the most potent antioxidant effect. Therefore, it could be deduced that THRC gels possessed a remarkable antioxidant performance, which was beneficial to prevent the photodamaging caused by UV radiation.

5. Conclusions

In conclusion, we have demonstrated a novel type of THRC gel for the enhanced healing of sunburned skin. The THRC gels displayed great stability and rheological behavior, showing no aggregation over a wide range of temperatures and time periods, with a pH that met the required standards. Non-invasive combo evaluations revealed that the THRC gels contributed to recovery of dermis density, EI, hydration, and TEWL values of sunburned skin to normal levels. The histological analysis further indicated that THRC gels significantly enhanced the repair of sunburned skin by preventing inflammatory reactions and promoting collagen regeneration. In addition, THRC gel demonstrated excellent resistance to oxidative stress, including the inhibition of lipid peroxidation and increased SOD and GSH activity. Notably, the THRC gels demonstrated a faster recovery rate compared to commercial collagen dressings, highlighting their superior efficacy in treating sunburn wounds. These highly biocompatible and bioactive THRC gels offer an

effective treatment strategy for sunburn and daily skincare, with broad applications in cosmetics and dermatology.

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Institutional Review Board Statement: All animal experiments were performed with protocols approved by the ethics committee of the College of Chemistry and Chemical Engineering at Lanzhou University (No. G09, 20220711).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Review

Cosmetology in the Era of Artificial Intelligence

Vasiliki Sofia Grech *, Vasiliki Kefala and Efstathios Rallis

Department of Biomedical Sciences, School of Health and Care Sciences, University of West Attica, Campus 1, 12243 Athens, Greece; valiakef@uniwa.gr (V.K.); erallis@uniwa.gr (E.R.)
* Correspondence: vgkrek@uniwa.gr

Abstract: The integration of artificial intelligence (AI) in cosmetology is transforming the industry in numerous ways, including the introduction of advanced tools such as at-home skin analysis devices that can evaluate skin quality and augmented reality applications that allow users to virtually try on various makeup products. These innovations empower individuals to make well-informed decisions about their cosmetic care and enable cosmetologists to predict treatment outcomes with higher accuracy. In this way, AI enhances patient satisfaction by better aligning expectations with achievable results. A computerized database search was performed to identify articles relevant to this topic. A comprehensive search was applied to the following electronic databases: IEEE Xplore, PubMed, Google Scholar, and Research Gate. This review explores four key areas in the current literature where AI contributes to cosmetic procedures. Firstly, AI democratizes skincare by making products and services more accessible to everyone. Secondly, it bridges the gap between physicians and cosmetic suppliers by enlightening collaboration and innovation. Thirdly, it improves the assessment of cosmetic ingredients by ensuring better safety and efficacy, and lastly, AI provides an ethical alternative to animal testing by replacing the Local Lymph Node Assay (LLNA) and the Guinea Pig Maximization Test (GPMT) with in silico models. While AI offers significant benefits, it also raises concerns about data privacy, informed consent, and the potential for promoting unrealistic beauty standards. Addressing these challenges involves implementing measures such as anonymization and de-identification techniques to protect sensitive data and safeguard informed consent for data collection and processing. This article aims to highlight the responsible and ethical use of AI in cosmetology, emphasizing the importance of accuracy and customization in cosmetic care, which represents a significant advancement in the industry.

Keywords: artificial intelligence; machine learning; cosmetology; custom cosmetics; acne; dermatological diversity; safety; data privacy; ethical AI

1. Introduction

Cosmetology is a unique field focused on otherwise healthy patients, often involving local treatments such as creams for addressing acne, aging, and other skin issues. Although these beauty-related dermatological conditions do not pose a direct threat to physical health, they can significantly impact the quality of life [1]. In recent years, AI and machine learning (ML) techniques have been integrated into esthetics to improve cosmetic outcomes. Nonetheless, there has been limited research on the applications of ML and AI in this field.

The literature review conducted by Vatiwutipong et al. in 2023 [2] is one of the first to compile recent and relevant research on employing AI technologies to address challenging questions in cosmetology [2]. Choosing the right cosmetic product can be challenging for customers due to the vast array of ingredients and product availability. AI's predictive capabilities can align expectations with achievable cosmetic results and resolve unrealistic expectations, allowing patients to make informed decisions [3]. Additionally, AI is increasing access to cosmetologists and personalized cosmetic products for diverse and underrepresented populations through tele-esthetics [4], home-based applications, and personalized skincare, which is an important step in democratizing cosmetology [5].

Moreover, in silico models utilize AI to calculate the sensitizing potential of chemicals contacting the skin. They are less expensive, faster, and not restricted to laboratories compared to in vitro models, which are characteristics that make them more attractive tools for hazard evaluation, particularly in a screening context [6]. Today, in silico models are completely replacing animal testing in the European Union [7]. Our paper aims to highlight the contributions of AI in cosmetology, particularly in bridging the gap between cosmetologists and physicians in managing acne [8]. AI is creating more sophisticated and accessible diagnostic tools, combining machine learning expertise with domain-specific knowledge to address real-world healthcare challenges, offering advanced diagnostic capabilities, personalized product recommendations, and the possibility of remote surveillance by dermatologists. Additionally, this paper underlines the importance of personal data safety in the cosmetic industry, highlights the safety of custom cosmetic products, and addresses the limited representation of dermatological diversity in cosmetology through AI.

2. AI in Cosmetic Outcome Prediction: Aligning Expectations with Achievable Results

AI in cosmetology has advanced significantly, offering predictive capabilities that enrich patient satisfaction by aligning expectations with achievable results. This technology allows patients to make informed decisions about cosmetic procedures by providing a tangible preview of expected outcomes. Accurate outcome prediction is crucial as it helps cosmetologists and patients focus on feasible and satisfactory plans. Unlike medically compulsory procedures, cosmetic treatments offer various options influenced by patients' preferences and financial considerations. By effectively forecasting responses, practitioners can associate patient expectations with achievable results, thereby enhancing overall satisfaction and trust in the process [2,3].

Customer satisfaction is a critical factor in the cosmetic industry as it influences product purchases. Recent studies have broadened and explored AI's potential in emotion recognition and utilized it for personalized cosmetic advice. More specifically, Kim et al. [9] developed Convolutional Neural Network (CNN) models for analyzing electroencephalography (EEG) data to objectively measure consumers' emotions during the application of cosmetic creams. The aim was to understand consumers' emotions using EEG as they applied four different creams with various textures. The EEG data, categorized based on the subject's preference score as "like (positive)" or "dislike (negative)", was analyzed using frequency bands—alpha, beta, low gamma, and high gamma. These data were then organized into a matrix that incorporates both frequency and spatial information, and seven CNN-based models were developed and evaluated, achieving a 75.4% accuracy rate in predicting user satisfaction. This approach provides valuable insights into user preferences, aiding cosmetologists and patients in making informed decisions about cosmetic products. This is the first study to apply a CNN-based deep learning method using EEG data to evaluate preferences for cosmetic creams, highlighting an under-researched area in AI applications within cosmetology [9]. However, this is not the first study to develop deep learning techniques by using EEG, since a year ago, EEG and galvanic skin response (GSR) data have been employed to measure emotional responses to different emollients and lip balms during consumer experiences [10]. This research could be expanded to include not only the texture of a product but also other sensory aspects, such as smell, and provide an even more comprehensive understanding of consumer preferences.

Cosmetic makeup is a global practice that enhances beauty and expresses emotions, continuously adapting to cultural and societal trends. Despite the wide array of publications detailing cosmetic techniques, the application of makeup has traditionally been a multidisciplinary process. In 2007, Tong et al. [11] were among the first to introduce a method for simulating virtual make-up applications. This method involved using a computer-based technique called "cosmetic transfer", which utilized the ratio of "before" and "after" makeup images to replicate makeup effects on a target face. The process computed these effects on a pixel-by-pixel basis from three aligned images sharing the same 2D geometry. While the approach was successful in achieving realistic cosmetic transfers, it

was acknowledged that the results were not flawless [11]. Since then, more sophisticated AI-based algorithms have been developed to assess facial features for makeup applications. By addressing the complexity introduced by factors such as face shape, eye color, skin tone, etc., an automatic system was developed by Flament et al. [12] to provide "blind" expertise advice. Before makeup application, an AI-based automatic descriptor performs a comprehensive analysis of 23 facial traits, hairs included, from customers' selfies as an aid for make-up practices. This system was validated by a panel of 12 makeup experts and included a diverse group of participants, demonstrating its ability to offer accurate, personalized cosmetic advice across a wide demographic range [12].

In the realm of skincare, Shi et al. [13] developed an AI tool called SkincareMirror, which predicts a user's appearance after long-term use of skincare products. This tool is particularly beneficial for customers as it provides a visual forecast of the anticipated results using a combination of product function labels, user images, and efficacy ratings. SkincareMirror has proven especially useful for individuals with varying levels of skincare knowledge, helping them make quicker and more informed decisions by visualizing the potential long-term effects. The tool's effectiveness is evidenced by increased satisfaction and efficiency among users, especially men with less skincare expertise, compared to traditional shopping methods [13].

Advancements in AI and deep learning models hold significant promise for the cosmetic industry, as seen in Figure 1. These technologies assist dermatologists and patients in selecting suitable treatment options, potentially leading to the development of sophisticated simulation tools. These tools could simulate multiple cosmetic procedures simultaneously and consider a broader range of patient characteristics and preferences. As research progresses, these innovations are expected to transform cosmetic practices, ensuring that patients receive treatments that meet their expectations and enhance their overall experience [2].

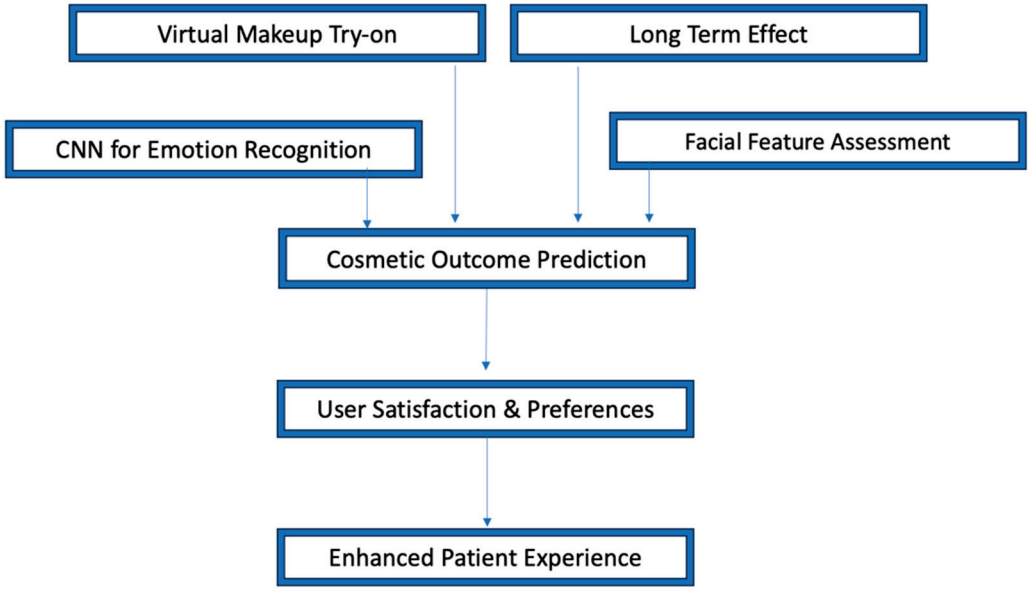


Figure 1. AI methods used for cosmetic outcome prediction in cosmetology.

3. The Role of AI in Democratizing Skincare: Transforming Accessibility and Personalization

AI democratizes cosmetology by making esthetic care more accessible, affordable, and convenient. Technologies like tele-esthetics enable remote consultations by analyzing skin conditions through photographs, benefiting individuals in remote or underserved areas. This reduces the costs associated with traditional in-person visits, making expert advice more affordable and accessible. The convenience of receiving personalized skincare recommendations from home saves time and effort, particularly for those with busy schedules or

mobility issues. Additionally, the scalability and decreasing cost of AI tools ensure that high-quality care is available to a broader population. These technologies also provide safe and effective remote services for basic esthetic concerns and facilitate appropriate referrals for more complex cases, thereby enhancing inclusivity and equity in access to cosmetic and skincare services [4].

Furthermore, AI is transforming skincare by expanding accessibility and improving the assessment of dermocosmetic ingredients across diverse populations. A fully remote study showcased an AI-based classification system that analyzed selfie images from a varied and representative group of 1041 US women of different ages and ancestries, including East Asian, Non-Hispanic Euro-American, African American, and Hispanic Euro-American, covering all Fitzpatrick phototypes. This study, which did not provide specific instructions for taking selfies, demonstrated that the AI system could accurately and clinically assess seven facial signs. The results, cross-verified by evaluations from 50 diverse US dermatologists, highlighted the system's precision, although it noted areas for improvement in skin tone analysis, particularly for pigmentary spots and in the younger, older, and darkest phototype groups. To enhance accuracy, new datasets from numerous countries have been obtained, and the automatic grading system has been upgraded. The study emphasized the importance of using well-distributed datasets across all demographics to ensure fair representation and reduce bias, as demographic factors can significantly affect biometric algorithms. This approach enables more inclusive and personalized skincare recommendations, thereby democratizing access to advanced skincare assessments and enhancing equity in skincare practices [14].

Despite advancements in AI, its application to the skin of color (SOC) faces significant limitations due to underrepresentation in datasets. Current AI programs often rely on the Fitzpatrick Skin Phototype (FST I-VI) scale to classify skin tones, but this scale is flawed for SOC classification. A 2024 review [15] found that only 30% of AI programs in dermatology reported data related to the SOC, highlighting the extent of this issue. The FST scale, originally designed to determine the risk of burning during phototherapy among Caucasians, is an unreliable indicator of skin pigmentation for diverse populations. Studies have shown that the FST does not effectively represent the Black community, often exaggerating the prevalence of type IV skin among them. This can lead to inadequate cancer risk assessments and a lack of accuracy in evaluating the SOC. Consequently, AI programs may perform poorly in identifying lesions in the SOC, and biases in algorithms can disproportionately impact individuals with darker skin [15].

An alternative classification tool, the Monk Scale [16], developed by Harvard Professor of Sociology Dr. Elias Monk, offers a more inclusive categorization by dividing individuals into 10 different skin type categories based on skin color and ethnic background. This scale aims to capture a broader spectrum of human skin pigmentation, providing a more accurate representation of diverse skin tones. Google has partnered with Dr. Monk to enhance the diversity of images displayed across its products, including image searches. Similarly, Facebook/Meta's Casual Conversations data suggest using the Monk Scale alongside the Fitzpatrick Skin Phototype (FST) scale to improve skin tone representation in AI algorithms, promoting fairness and diversity while minimizing bias. Adopting comprehensive scales like the Monk Scale can expand the categorization of skin tones and demonstrate a commitment to inclusive representation across industries. Addressing these challenges by utilizing well-distributed datasets and inclusive classification tools is crucial for overcoming bias and ensuring equitable and accurate dermatologic and cosmetic care [15].

In summary, AI technologies are revolutionizing skincare by making it more accessible and personalized, particularly for diverse populations. Table 1 below provides an overview of how AI democratizes skincare. These advancements highlight the importance of individuality in skincare, empowering people to make informed decisions about their hair and skin through home-based applications and personalized products. Companies are utilizing AI through skincare quizzes that gather data on demographics, skin characteristics,

and esthetic preferences, which machine learning algorithms then analyze to recommend suitable cosmetic products tailored to individual needs. Virtual try-ons further enhance this experience, allowing users to experiment with makeup styles from the comfort of their homes. Additionally, free applications and devices offer online skin analyses and personalized recommendations, using high-definition cameras and portable tools for objective assessments. By catering to the unique needs of diverse populations, AI is promoting greater accessibility and inclusivity in skincare, ensuring that everyone can benefit from customized and effective treatments [5].

Table 1. A summary of how AI democratizes skincare.

Key Area	Description
Remote Consultations	AI technologies enable remote skin consultations through photograph analysis.
Cost Reduction	AI lowers costs by facilitating remote consultations and assessments.
Convenience and Personalization	Provides personalized skincare recommendations accessible from home.
Scalability and Accessibility	Ensures high-quality skincare services are available to a broader population.
Diverse Population Representation	Improves skincare assessments' accuracy across diverse populations.
Enhanced User Engagement	Uses tools like virtual try-ons and personalized product recommendations.
Addressing Skin of Color (SOC) Limitations	Adopts inclusive classification tools to reduce bias in skincare assessments.
Objective Assessments	Uses high-definition cameras and portable tools for objective skin assessments.

4. AI Is Bridging the Gap between Physicians and Cosmetologists

Collaboration between dermatologists and cosmetologists plays a crucial role in managing and treating acne, a chronic multifactorial inflammatory disease of the pilosebaceous follicle that significantly impacts patients' quality of life. Acne affects over 85% of adolescents and often continues into adulthood, with an increasing incidence of adult acne being characterized by periodic flare-ups [1]. Cosmetologists often serve as the first point of contact for patients, offering preventive care through personalized skincare treatments and educating them on proper skincare routines. Depending on the severity of the acne, dermatologists and cosmetologists work together to select appropriate treatments, with dermatologists addressing inflammatory effects and cosmetologists helping to maintain clear skin. This teamwork enhances long-term treatment efficacy and patient satisfaction [8].

Understanding the unique characteristics of each type of acne is crucial for effective diagnosis and tailored treatment approaches. Cosmetologists must have adequate skills, and dermatologists should oversee the treatment of medical skin conditions [17]. The integration of AI has significantly advanced the diagnosis and treatment of acne by focusing on the unique characteristics of various acne types. Table 2 shows AI software applications for acne diagnosis. AI tools, such as CNN-based methods, have become essential in this field, offering advanced diagnostic capabilities. For instance, Shen et al. [18] introduced a CNN-based approach in 2018 that could automatically identify seven types of acne lesions (nodules, pustules, cysts, papules, blackheads, whiteheads, and normal skin) with an accuracy of 81%. This method utilized a binary classifier to distinguish skin areas from non-skin areas and a seven-class system for detailed acne classification by leveraging pre-trained models like VGG16 for feature extraction [18].

In 2019, Junayed et al. [19] developed AcneNet, a deep residual neural network that categorizes five types of acne lesions, namely closed comedo, open comedo, cystic, pustular, and keloidalis, achieving over 94% accuracy. This model employs convolutional layers, pooling layers, fully connected layers, and activation functions to enhance its classification capabilities [19]. In 2022, Kim et al. [20] introduced a method for detecting and counting facial acne lesions, offering a reliable means of classifying acne lesions into inflammatory and non-inflammatory categories, and it was validated on a dataset of 20,699 manually labeled lesions. This system also proved to be valuable for teledermatology by visually highlighting acne lesions on the screen, thereby assisting dermatologists in remote consultations [20].

Table 2. AI software applications for acne diagnosis.

AI Acne Classification Studies	Applications	Details
"An Automatic Diagnosis Method of Facial Acne Vulgaris Based on Convolutional Neural Network" (Shen et al., 2018) [18]	Classification of seven types of acne lesions	Identifies nodules, pustules, cysts, papules, blackheads, whiteheads, and normal skin with 81% accuracy. Uses VGG16 for feature extraction.
"AcneNet—A Deep CNN Based Classification Approach for Acne Classes" (Junayed et al., 2019) [19]	Categorization of five types of acne lesions	Classifies closed comedo, open comedo, cystic, pustular, and keloidalis acne with over 94% accuracy. Utilizes deep residual neural networks.
"Automated Facial Acne Lesion Detecting and Counting Algorithm for Acne Severity Evaluation and Its Utility in Assisting Dermatologists" (Kim et al., 2022) [20]	Detection and counting of acne lesions	Differentiates between inflammatory and non-inflammatory acne. Useful in tele-dermatology for remote consultations.
"Acne Detection Care System using Deep Learning" (Yadav et al., 2021) [21]	Deep learning-based acne detection and personalized care	Uses ResNet-18 to predict the number, location, and severity of acne lesions, providing personalized care with 90% accuracy.

Accurate assessment of acne severity is essential for patient care and clinical studies, yet traditional methods like global severity grading and lesion counting can be time-consuming and inconsistent. AI algorithms, such as PP-YOLO, have demonstrated clinically valid performance in detecting and counting facial acne lesions, improving the reliability of severity assessments [20]. Recently, Yadav et al. [21] introduced "Acne Care", a system using deep learning techniques and the ResNet-18 architecture for acne detection and personalized care. This system can accurately predict the number, location, and severity of acne lesions, providing a useful tool for both self-assessment by patients and clinical diagnosis by doctors. The ResNet-18 model, known for its depth and skip connections, classifies acne severity into four levels—normal, level 0, level 1, and level 2—with up to 90% accuracy. This research highlights the potential of AI to create sophisticated and accessible diagnostic tools, combining machine learning expertise with domain-specific knowledge to address real-world healthcare challenges in acne management [21].

The declining number of dermatologists in Europe [22] has made it challenging for patients to secure timely appointments, leading to irregular follow-ups and suboptimal treatment outcomes. This shortage exacerbates issues such as the inadequate management of skin conditions, increasing the risk of scarring and residual hyperpigmentation and causing a decline in patients' quality of life. Timely and appropriate management is crucial to prevent long-term complications. AI tools significantly enhance patient outcomes by assisting dermatologists and cosmetologists in various ways. These tools facilitate efficient diagnosis and screening by rapidly analyzing skin conditions like acne and recommending treatment plans, thus accelerating the diagnostic process and allowing dermatologists to focus on more complex cases. AI also supports remote consultations and tele-esthetics by providing accurate assessments through images or videos, ensuring that patients receive timely advice and treatment. The continuous monitoring and follow-up capabilities of AI tools help maintain patient adherence to treatment regimens and allows treatments to be adjusted as necessary. Moreover, AI provides educational support to cosmetologists, enhancing their ability to manage skin conditions and fostering effective collaboration with dermatologists. This integration of AI into dermatological care helps bridge the gap left by the declining number of specialists, ultimately improving patient care and quality of life [8,23].

In conclusion, the integration of AI in dermatology and cosmetology is crucial in bridging the gap left by the declining number of specialists. By providing tools for efficient diagnosis, remote consultations, and personalized treatment plans, AI ensures that patients

receive timely and accurate care. This advancement not only enhances collaboration between dermatologists and cosmetologists but also improves patient access to essential skincare services, ultimately leading to better health outcomes and quality of life.

5. Using AI in Ingredient Assessments for Cosmetic Recommendations

Choosing the right cosmetic product can be challenging for customers due to the vast array of ingredients and products available in the industry, ranging from natural to chemical formulations. Common ingredients like glycerin, hyaluronic acid, retinol, and ceramides contribute to the effectiveness of cosmetics, but evaluating all possible combinations is impractical as the cosmetic industry includes an estimated 20,000 to 200,000 ingredients and products [24]. Since the pandemic, there has been an increased interest in health and safety, leading users to scrutinize ingredients themselves before purchasing cosmetics. This trend indicates that an ingredient-based approach can provide more precise and reliable recommendations to today's users [24,25].

To assist in this process, algorithms and ML methods are increasingly being used. Researchers have applied various techniques, such as Natural Language Processing (NLP), content-based filtering, and neural collaborative filtering, to analyze customer reviews, product similarities, and user-related data for personalized recommendations. NLP enables computers to understand human language in a meaningful and useful way by extracting explicit information from customer reviews, and neural collaborative filtering provides personalized cosmetic recommendations based on users' past online behaviors and preferences [26,27]. Additionally, the Ingredient Frequency–Inverse Product Frequency (IF-IPF) method validates cosmetics according to skin type, age, and ingredient composition by analyzing cosmetic review sites and user evaluations to identify ingredients believed to have the best effects [28].

Some researchers have focused not only on analyzing cosmetic components but also on evaluating users' skin conditions to better meet users' demands. AI skin analysis, which includes image processing algorithms and deep learning models like YOLOv4, automatically removes noise, enhances face characteristics, and extracts features for skin condition classification [29]. Additionally, another CNN called U-Net [30], initially designed for biomedical image segmentation and uses three-dimensional data for image classification and object recognition, is now used for skin analysis and evaluation. This model achieves the simultaneous segmentation of wrinkles and pores by analyzing the morphological structures of the skin rather than relying on color-based analysis. When provided with an entire face image from various shooting positions, the U-Net architecture excels at localizing wrinkles and pores, outperforming both conventional image-processing approaches and recent deep-learning methods [31]. The prediction results from these machine-learning approaches can provide suitable product recommendations, offering more precise and reliable cosmetic suggestions tailored to individual skin concerns.

In 2024, Lee et al. [24] proposed a complete cosmetic recommendation system that combines ingredient analysis with AI skin analysis to provide personalized recommendations. Existing systems do not quantitatively analyze their performances using proper evaluation metrics, nor do they consider the order of ingredients in cosmetic analysis. In contrast, Lee et al.'s system features specialized AI models for each skin problem, and instead of providing a direct measure of a product's efficacy, it analyzes the order of ingredients in its composition. By using a deep neural network encoder to analyze sequential ingredient data and a deep neural network for skin analysis, the system offers precise and reliable cosmetic suggestions tailored to individual skin concerns. This advancement is expected to provide more objective results on cosmetic effects by offering a more tailored cosmetic prediction for each user's skin status [24].

6. Advancements of In Silico Models in Cosmetology

Cosmetics are likely the most common reason why patients undergo patch testing. Cosmetics can cause delayed type IV hypersensitivity responses, known as Allergic Contact

Dermatitis (ACD), which occurs within 48–72 hours after exposure and presents with symptoms such as rashes, blisters, and swelling, significantly impacting patients' quality of life. The eyelids, being highly sensitive due to their thin stratum corneum and occlusion when the eyes are open, are particularly vulnerable, often developing dermatitis from substances applied to the scalp, face, or hands, even when these primary sites remain unaffected. Small amounts of allergens transferred from the fingers can trigger eyelid dermatitis without visible signs elsewhere. While many cosmetics are free of common allergens, nonspecific irritation still occurs, leading to conditions like *status cosmeticus*, [32] where every cosmetic or soap applied to the face causes itching, burning, or stinging sensations. The face and hands are most commonly affected, but the neck, anogenital region, and other areas can also be impacted [33]. In a current retrospective analysis of Brazilian patients who underwent patch tests for suspected ACD between 2004 and 2017, cosmetics were identified as the fundamental cause of the condition in 16.5% of the patients, of which 89.7% were women [34].

Predicting the possibility of cosmetic components to trigger skin sensitization is crucial. Before 2004, cosmetics and their ingredients were primarily tested on animals using methods like the Guinea Pig Maximization Test (GPMT), the Bühler test, and the Local Lymph Node Assay (LLNA). However, the European Union's cosmetic directive mandated alternatives to animal tests, resulting in a complete ban on animal testing for cosmetic products and ingredients by 1 October 2004 and a total ban by 11 March 2013, regardless of the availability of alternative methods [35]. Consequently, non-animal test methods or New Approach Methodologies (NAMs) have become the preferred choice for risk documentation and descriptions of skin sensitizers, allowing for the development of a non-animal, Next-Generation Risk Assessment (NGRA) framework [36].

Current in vitro tests include the Human Cell Line Activation Test (h-CLAT), Direct Peptide Reactivity Assay (DPRA), LuSens, Myeloid U937 Skin Sensitization Test (MUSST), and the KeratinoSens method [35]. Despite their advantages, the accuracy and reliability of in vitro methods compared to traditional animal tests remain debated. In vitro tests per se cannot substitute animal and human tests since they emphasize only one Adverse Outcome Pathway (AOP). This limitation has led to the development of mathematical models called in silico models to calculate the sensitizing potential of cosmetic ingredients derived from historical epidemiological data [35].

Various skin sensitization data, including non-animal and animal data, along with chemical structure information, have been published. The Cosmetic Ingredient Database (CosIng), an online resource created by the European Commission, compiles data and structural alerts to construct predictive models. The quality of data is crucial in developing these models, and creating an in silico model without clean or pure data is challenging. Effective development strategies involve collecting extensive experimental data and understanding biological mechanisms to predict bioactivity and biochemical processes. Currently, diverse simulation approaches such as K-Step Yard Sampling (KY), Decision Trees (DTs), Artificial Neural Networks (ANNs), Support Vector Machines (SVMs), AdaBoost, Logistic Regression (LR), Iterative Least Squares Linear Discriminant (TILSQ), consensus methods, and Bayesian networks are used to develop skin sensitization predictive models [6].

In silico methods also offer significant advantages in the toxicology of cosmetic ingredients via safety assessments, enabling high-throughput screening of extensive chemical libraries without requiring physical experiments. This capability allows for potential candidates to be prioritized before the costly and time-consuming process of molecule synthesis. These approaches utilize existing data more efficiently and support the 3Rs principle (Replacement, Reduction, and Refinement), thereby reducing the reliance on animal testing [37].

Recognizing the complexity of skin sensitization, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) under the US Department of Health and Human Services employed a multi-faceted approach that includes machine learning techniques. Specifically, they used LR and SVM to train models on 72 substances

and tested these models on an additional 24 substances. The SVM models achieved the highest accuracy, reaching 92%, by integrating data from DPRA, h-CLAT, and read-across, with some models also incorporating KeratinoSens and log P. These machine learning models outperformed traditional methods like the LLNA and individual in vitro methods, highlighting the efficacy of computational approaches in predicting human skin sensitization hazards [38].

Another approach involved developing an ANN for in vitro risk assessment systems. This model uses descriptors like the maximum change in cell-surface thiols (MAC value), CV75 (indicating 75% cell viability), and threshold concentrations for CD54 and CD86 expressions (EC200 and EC150). The ANN model, named "iSENS ver. 1", showed a strong correlation with published LLNA thresholds, suggesting its effectiveness in calculating the skin sensitization potential of chemicals using in vitro data [39].

Additionally, in silico models can be applied to entire cosmetic compounds, and not just individual ingredients. Some commercial or cosmetic products contain sensitizers that do not induce adverse effects when administered in low doses. For example, products that are washed off, such as body lotions or shampoos, have different exposure levels compared to those in direct and prolonged contact with the skin, such as foundations or creams. The disparity in exposure between these groups can be as much as 30 times greater. As a result, qualitative in silico models, which often rely on binary classification, have limited application in the pharmaceutical or cosmeceutical markets. These models typically focus on a single biological pathway and fail to capture the full spectrum of sensitization responses, including weak or moderate effects. They also do not consider variations in product application and exposure levels, such as the difference between leave-on and wash-off products. Therefore, there is a need for more comprehensive and quantitative prediction models. These models provide detailed assessments of the sensitization potency, enabling precise risk evaluations and helping to meet regulatory requirements. Quantitative models improve product safety by identifying safe ingredient levels, aiding in formulation, and protecting consumers from skin reactions. Additionally, they facilitate the comparative analysis of different ingredients, ensuring the development of safer and more effective cosmetic products [6].

In the context of cosmetic ingredient assessments, hundreds of in silico models are available to predict various properties of interest for different endpoints, with some models being offered for free and others requiring a fee. To support this process, the SpheraCosmolife package was developed. This package provides tools to evaluate cosmetic ingredients and predict genotoxicity, mutagenicity, and skin sensitization based on factors such as concentration and product type (e.g., shower gel or lotion). The SpheraCosmolife package facilitates and standardizes the safety assessment of cosmetic products by providing a Margin of Safety (MoS) based on systemic exposure doses. It incorporates models for both exposure and hazard prediction, enhancing the accuracy and reliability of safety evaluations in the cosmetic industry [40].

In 2021, Golden et al. [7] compared eight in silico models against two human datasets, achieving accuracies ranging from 70% to 80%, similar to traditional animal tests like the LLNA. These models were evaluated based on parameters such as coverage, accuracy, sensitivity, and specificity, with additional consideration for optimization features like the probability of accuracy and applicability domain when available. The binary skin sensitization outcomes predicted by each model were compared to the actual sensitization status of the chemicals in the datasets. Interestingly, the models did not consistently mispredict the same compounds, suggesting that a combined approach might improve predictive accuracy. This study highlights the potential of these in silico models as cost-effective tools for predicting skin sensitization [7].

Existing human tests for skin sensitization, including the Human Maximization Test (HMT) and the Human Repeat Insult Patch Test (HRIPT), provide direct results but are hindered by the scarcity of consistent data, ethical concerns, and subjectivity. Conversely, animal tests offer predictive models but are fraught with ethical issues and limitations in

accuracy. The increasing adoption of non-animal assays, driven by ethical considerations and technological advancements, has elevated the role of in silico models in supporting preliminary evaluations and complementing experimental data. As seen in Figure 2, the evolution of testing methods is tremendous. Despite these advancements, animal tests, notably GMPT and LLNA, remain mandatory in several countries including Brazil, Canada, Japan, China, and the United States [6].

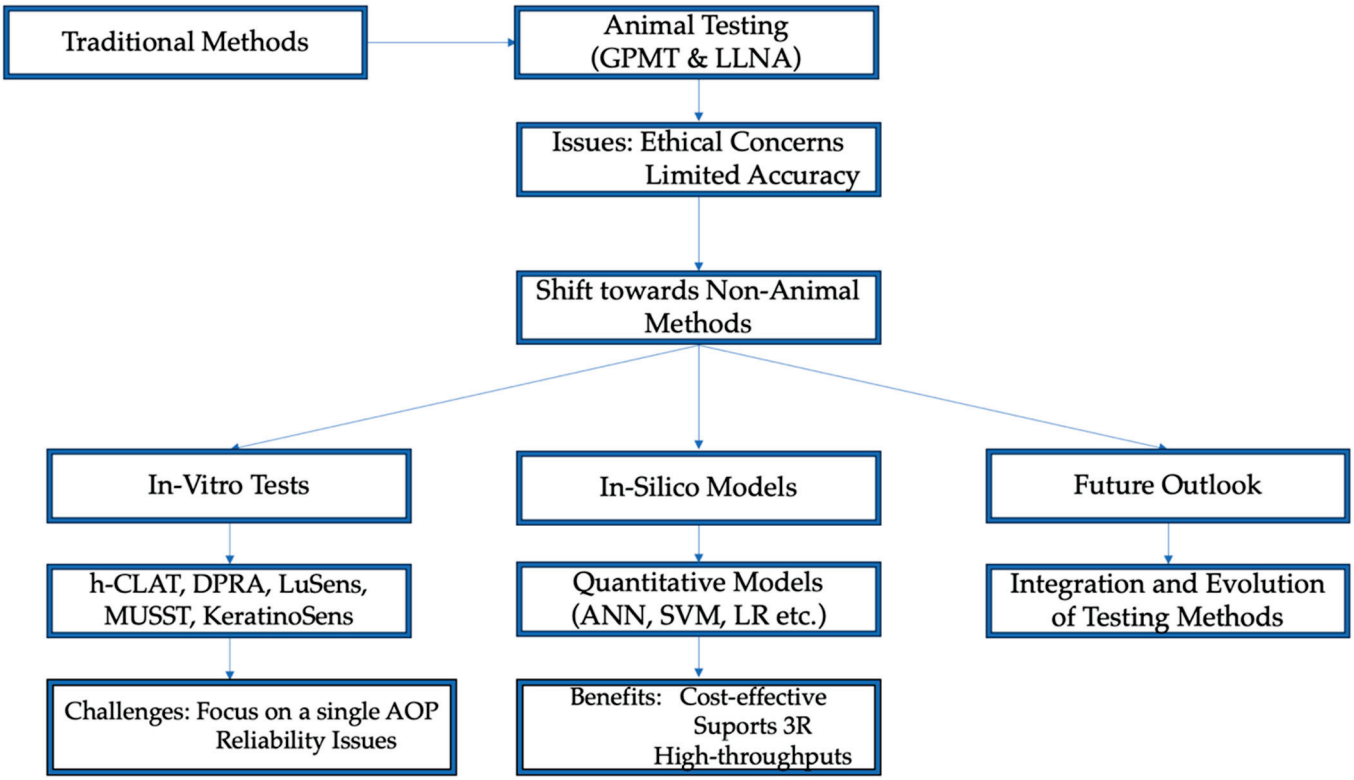


Figure 2. Evolution of testing methods in the cosmetic industry.

7. Ethical Considerations and Data Security in AI Applications for Cosmetology

AI and ML technologies have recently become central to the beauty and skincare industries, revolutionizing consumer services by using advanced techniques to analyze skin and hair characteristics. These digital tools offer personalized product recommendations and treatments based on complex data interpretation. However, as AI assumes tasks traditionally performed by humans, it can inherit biases that lead to unjust discrimination. For instance, in cosmetology, ethnicity-related biases often stem from the underrepresentation of certain skin tones, while gender biases may overlook hormonal influences on skin health. Age biases can also contribute to unrealistic expectations regarding youthful appearances. These biases can significantly impact product recommendations and treatment effectiveness. If the training data predominantly feature certain demographics, AI may disproportionately recommend products that are most effective for those groups, leading to inadequate suggestions for individuals with different skin characteristics. This can result in suboptimal treatment outcomes and perpetuate narrow beauty standards, marginalizing those who do not conform to these ideals. Therefore, while AI has the potential to enhance customer experiences with tailored solutions, addressing and correcting these biases is crucial to ensure fair and effective recommendations for all users [41].

The European Union's AI Act, established in 2021 under the title "Laying Down Harmonized Rules On Artificial Intelligence And Amending Certain Union Legislative Acts", serves as a crucial regulatory framework to address the challenges and safeguard fundamental rights in AI applications [42]. This act is essential for ensuring fairness and safety in AI-driven solutions by standardizing the use of AI technologies, promoting trans-

parency, and reducing bias. In the context of AI, fairness involves ethically and equitably treating individuals or groups during the creation and implementation of AI algorithms. Safety pertains to the responsible and dependable use of AI, ensuring that systems operate securely and ethically, thereby protecting users from harm. Data governance involves strategies, policies, and procedures to manage data responsibly, ensuring quality, privacy, and security throughout AI initiatives. These standards are crucial for ensuring that AI algorithms in cosmetology are fair and transparent, offering equal treatment to all individuals, regardless of skin type, ethnicity, or age. Addressing AI bias is both an ethical obligation and a matter of legal and corporate responsibility, essential for maintaining consumer trust as AI systems increasingly influence product recommendations and treatment plans. The AI Act mandates regulatory measures, such as human oversight, accurate training data, and meticulous recordkeeping, to enhance the safety and reliability of AI applications in cosmetology. This comprehensive framework aims to protect consumers' rights and ensure equitable access to beauty and skincare products and services for all users [43].

Non-conformity with global beauty standards presents a significant issue, as traditional cultural ideals have evolved into international norms that prioritize traits such as youthfulness, symmetry, clear skin, slimness, and "Western" features. Biases in AI technologies within the beauty industry can arise from various factors, including algorithmic design, inadequate training, or biased datasets [41]. For instance, the Beauty.AI contest in 2016 revealed a notable bias towards white faces, reflecting broader disparities in representation and fairness [44]. This globalization of beauty standards often disregards cultural diversity, fostering discrimination and promoting unrealistic beauty ideals, which can have health implications. For example, the side effects of whitening cosmetics have been documented, highlighting that cosmetic ingredients can be a serious health concern. Certain ingredients in brightening cosmetics, such as hydroquinone, corticosteroids, and mercury, can cause severe complications, including exogenous ochronosis (also known as "fish odor syndrome"), Allergic Contact Dermatitis (ACD), "steroid addiction syndrome", and acne [6].

Transparency remains a critical challenge in AI systems, which often function as black boxes as they obscure their decision-making processes [45]. Ensuring the reliability of data analysis requires efforts to eliminate performance gaps and to report algorithmic performance across different phenotypic and demographic subgroups. However, many AI service providers are pushing facial analysis technology into conventional use without ensuring the fairness and reliability of these systems [46]. In a study by Buolamwini et al. [47], two commercial facial analysis benchmarks were evaluated concerning phenotypic subgroups. The results show error rates as high as 34.7% for darker-skinned females, making them the most frequently misclassified group, compared to a maximum error rate of 0.8% for lighter-skinned males. These significant disparities, as described in Table 3, highlight the urgent need to develop transparent, fair, and accountable facial analysis algorithms. Today, AI is not only used in cosmetology and medicine but also plays a role in critical decisions like hiring, loan approvals, and even determining the length of prison sentences [47].

Data privacy in AI-driven cosmetology is crucial as the beauty industry often relies on sensitive information such as biometric data and facial analysis. These data, used for personalized beauty product recommendations, typically include background information like location and demographic details (gender and age), skin types and goals, allergies, and behavioral data such as browsing history, transaction records, search keywords, and consumed content [48]. During the 25th International Master Course in Aging Science (IMCAS) in 2024, a digital questionnaire was disseminated to attendees via a QR code scan and Google Forms. Out of 47 doctors from various specialties in cosmetic medicine who responded, nearly half (48.9%) expressed concerns about the data privacy and security implications of AI. This response underlines the necessity for strong protective measures to safeguard personal data in the application of AI technologies [49].

Table 3. Ethical considerations in AI cosmetology.

Ethical Consideration	Description
Bias and Discrimination	AI systems may inherit biases related to ethnicity, gender, and age, leading to unfair treatment and recommendations.
Transparency and Accountability	AI often functions as a "black box", making it difficult to understand its decision-making processes and identify biases.
Data Governance	The responsible management of data is crucial to ensure diversity, quality, and security in AI training and deployment.
Global Beauty Standards and Cultural Sensitivity	AI may perpetuate narrow beauty standards, prioritizing traits like youthfulness and "Western" features and ignoring cultural diversity.
Safety and Health Implications	AI-driven recommendations must consider the safety of cosmetic ingredients and potential health risks to users.
Regulatory and Ethical Oversight	Frameworks like the EU's AI Act emphasize the need for fairness, transparency, and safety, requiring human oversight and accurate data.

Data privacy challenges in AI arise from concerns about protecting personal information, where inadequate measures can lead to breaches, legal issues, and ethical dilemmas. Techniques such as anonymization and de-identification are essential for protecting sensitive data, while secure storage and transmission methods, like encryption, enhance privacy protection. Obtaining informed consent from individuals for data collection and processing is crucial as organizations must clearly communicate the reasons for which the data will be used. Additionally, organizations must ensure that data are used solely for their intended purposes and not repurposed without explicit consent. To address these challenges, organizations should establish robust data governance frameworks and implement stringent quality control processes. Transparency and explainability are vital for building user trust and ensuring accountability. Collaboration with data providers and stakeholders is necessary to facilitate knowledge sharing and address privacy concerns. Adhering to regulatory compliance and incorporating privacy-by-design principles are critical steps in developing ethical and responsible AI systems in cosmetology [50].

8. Future Directions of AI in Cosmetology

The beauty industry is undergoing a profound transformation, particularly within the metaverse, where the alpha generation—children born after 2010—are spearheading change. In the post-COVID-19 era, there has been a marked shift towards non-face-to-face interactions, driving the need for innovative platforms where consumers can test and purchase cosmetics virtually. Thus, the metaverse, combining "meta" (virtual) and "universe" (the universe), offers a rich landscape for these changes. Here, companies can engage in fandom marketing and enhance customer experiences in a way that traditional face-to-face methods cannot, expanding the technological adeptness of the alpha generation to create more personalized and engaging cosmetic experiences [51].

With the advent of Fourth Industrial Revolution technologies, the development of personalized beauty products has become virtually limitless, and the rise of custom cosmetics is a notable trend in the evolving beauty landscape. Consumers now favor cosmetics tailored to their specific needs, a shift that has been accelerated by the COVID-19 pandemic and the increase in mobile shopping. The Republic of Korea has been at the forefront of this trend, implementing a custom cosmetics system that caters to individual preferences through mobile shopping. According to a study by Lee et al. [52], the use of non-face-to-face mobile shopping for customized cosmetics is growing in the contactless era. This trend is closely linked to the increasing awareness of skincare and the recognition of the benefits of customized cosmetics, including the use of interactive apps. However, the burgeoning market for these personalized products also raises concerns about safety, quality control, and the sourcing of raw materials, underscoring the need for stringent safety management practices. As custom cosmetics become more prevalent, ensuring that these products meet

high standards for safety and efficacy will be critical to maintaining consumer trust and satisfaction [52].

The safety of custom-made cosmetics is a critical concern, as highlighted in a study by Kim et al. [53]. This study, from the Republic of Korea—the world's eighth-largest cosmetic market—points out safety issues with products made on-site by consumers and sellers. While the hygienic conditions of the environment, instruments, and handlers in customized cosmetic shops were generally good, the study revealed high microbial counts in the final products, indicating possible contamination during production. The key risk factors include semi-solid products, pH values above 10, the transfer of cosmetics to new vessels, and raw materials. Proper heat management during processing is crucial for controlling microbial levels. Although microbial contamination in cosmetics is currently rare, it can compromise product quality and pose health risks, leading to occasional global recalls. The preservation systems of custom-made cosmetics are often not as robust as those of mass-produced items, as these products are created and delivered directly on-site, increasing the risk of contamination during production [53].

Therefore, ensuring the microbiological stability of custom cosmetics requires stringent safety management. In 2021, members of the same scientific team suggested that distributors employ a government-authorized "dispensing chemist of customized cosmetics" for on-site mixing and shop management. According to a law that took effect in 2020 in the Republic of Korea, all substances used for making cosmetics—except forbidden materials, those with limited use, and some designated functional ingredients—are permitted in custom-made products. This approach promises a future where personalized beauty products are both effective and safe, aligning with consumer demands in an evolving market [54].

Moreover, bioprinted skin, a momentous advancement in the integration of AI and cosmetology, has been further enhanced through the use of 4D laser-assisted bioprinting and advanced skin culture protocols. These innovations have resulted in bioprinted skin that closely resembles natural human skin, offering a more ethical and precise alternative to animal testing for evaluating cosmetic ingredients [55]. A notable feature of bioprinted skin is its ability to mimic complex human skin characteristics, including sensory-like neurons that can replicate neuroinflammatory responses to harmful stimuli. For example, when capsaicin, a known neurosensitizing agent, was applied within a basic facial moisturizing formulation to these innervated skin models, there was a significant release of neuropeptides and pro-inflammatory cytokines such as substance P, indicating a response similar to what occurs in vivo [56].

This application resulted in a considerable increase in the concentration of CGRP, a neuroinflammatory marker, in the culture medium, demonstrating the model's ability to effectively mimic real skin reactions. This evidence suggests that such bioprinted skin models can be instrumental in screening cosmetic and therapeutic topical formulations for potential inflammatory responses, thereby ensuring product safety. As reliable and reproducible platforms, these Human Skin Equivalents (HSEs) are crucial for confirming the safety of new cosmetic products before they reach consumers. Future enhancements, such as the integration of human-derived neurons, are expected to further improve the accuracy and applicability of these models, reinforcing their importance in the safety assessment of cosmetic and therapeutic products [56].

The integration of robotics in cosmetology has led to significant advancements in various procedures, including laser hair removal and facial esthetic treatments. In laser hair removal, robot-assisted automatic systems have been designed to identify different shapes of treatment areas and ensure uniform laser irradiation across designated skin regions. The use of algorithms and robotics aims to achieve a consistent distribution of laser pulses, thereby minimizing potential side effects. Comparative studies between robot-assisted and physician-directed laser hair removal have shown superior hair removal rates with robotic assistance, highlighting its potential to enhance cosmetic outcomes. Ongoing advancements focus on refining algorithms to optimize performance on curved or slanted skin surfaces,

broadening the range of applications for robot-assisted laser hair removal. Research on these systems has demonstrated reliable beam reach and a consistent number of pulses emitted. Additionally, automated scanning devices offer benefits such as shorter pulse durations, precise energy placement, quicker treatment sessions, and reduced operator fatigue [5,57].

Similarly, the use of robotics in cosmetology, particularly in dermatology and plastic surgery for facial esthetic procedures, has brought significant advancements. Robotic systems enhance safety and accuracy by delivering injections with consistent distribution, minimizing human error. These systems operate at a controlled pace, using smaller needles and less pressure, which helps reduce patient discomfort. Moreover, robots standardize procedures by adhering to programmed protocols, ensuring injections are made at specific depths, angles, and locations. This precision helps avoid critical areas of the face, reducing the risk of complications and providing uniform results. Robotic systems can also expedite treatment processes and improve access to these procedures, particularly in remote areas where skilled practitioners may be scarce. These benefits mark significant steps forward in enhancing the safety and efficiency of cosmetic treatments [58].

However, the use of robotics in cosmetology also presents several challenges and risks. The high costs associated with purchasing, maintaining, and repairing robotic systems can make these treatments more expensive for patients. Robots' limited flexibility can restrict their ability to adapt to individual patient needs, leading to less personalized care. The absence of human interaction is another concern, as robots cannot provide the same level of empathy and rapport-building that human practitioners can, which is crucial for patient comfort and trust. Additionally, robots may struggle with making creative adjustments during procedures, potentially impacting the quality of esthetic outcomes. There are also risks of injury from robotic malfunctions or incorrect calibrations, and robots are less capable of effectively assessing and managing patient pain or discomfort. Ethical concerns include issues related to patient data privacy and the broader acceptance of robotic involvement in medical procedures. To address these issues, it is crucial that the use of robotic technology in cosmetology is transparent and patient-centered, ensuring that patients are well-informed about their treatment options and the role of robots in their procedures, allowing them to make decisions that align with their values and preferences [58]. In Table 4 below, key areas of transformation and innovation in AI cosmetology and their potential challenges have been listed, including metaverse integration, custom cosmetics, bioprinted skin, robotic assistance, and ethical and safety considerations.

Table 4. Key areas of transformation and innovation in AI cosmetology.

Category	Description	Potential Challenges
Metaverse Integration	- Virtual testing and purchasing of cosmetics. - Fandom marketing and personalized consumer experiences.	- Ensuring safety and authenticity in virtual environments.
Custom Cosmetics	- Personalized beauty products tailored to individual needs. - Increased demand for custom products via mobile shopping.	- Safety, quality control, and raw material sourcing. - Managing the microbiological stability of on-site mixed products.
Bioprinted Skin	- Ethical and precise alternative to animal testing for cosmetic ingredients. - Mimics human skin, including sensory neurons for accurate testing.	- Technical challenges in replicating complex skin functions and responses. - Cost and scalability of bioprinted skin models.
Robotic Assistance	- Enhances safety and accuracy in procedures like laser hair removal and facial injections. - Standardizes procedures, reducing human error and ensuring consistent results.	- High costs and limited flexibility in adapting to individual needs. - Lack of human interaction and empathy.
Ethical and Safety Considerations	- Patient data privacy and ethical use of AI and robotics. - Necessity for stringent safety management and adherence to regulatory standards.	- Transparency in treatment options and potential risks of robotic involvement in procedures. - Potential for injuries due to malfunctions or incorrect calibrations.

9. Discussion

AI and machine learning (ML) technologies in cosmetology are reshaping the industry, enhancing the personalization, accessibility, and efficiency of beauty products and services. In Figure 3 below, a schematic representation of the key points of the article is illustrated. This transformation is largely driven by specific AI technologies, such as CNNs, NLP, and in silico modeling, with each playing a pivotal role in advancing the field.

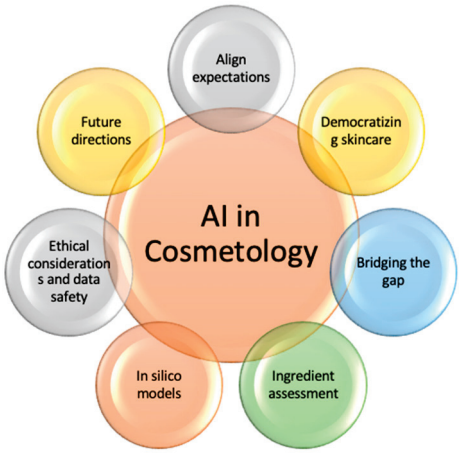


Figure 3. A schematic representation of the key points of this article.

CNNs are a cornerstone in image analysis applications within cosmetology. They have enabled the development of sophisticated diagnostic tools that analyze various skin conditions, such as acne, with remarkable accuracy. For example, CNN-based systems can classify different types of acne lesions (e.g., nodules, cysts, and blackheads) and assess their severity, providing valuable insights that support dermatologists and cosmetologists in treatment planning. This capability is crucial for the early detection and management of skin conditions, significantly improving treatment outcomes [16]. Beyond diagnostics, CNNs are integral to virtual try-on applications, allowing users to visualize the potential results of cosmetic procedures or products before committing to them. This functionality not only helps set realistic expectations but also enhances user engagement and satisfaction, as consumers ca n experiment with different looks in a virtual environment [13].

NLP technologies facilitate the analysis of textual data, such as customer reviews and product descriptions, to extract meaningful insights. In cosmetology, NLP interprets consumer feedback, identifying common concerns and preferences, which is invaluable for refining product recommendations to align closely with user needs [26,27]. NLP also plays a critical role in ingredient analysis, identifying potentially harmful components and suggesting safer alternatives. This capability is particularly relevant in personalized skincare, where understanding the nuanced language of ingredients and consumer feedback can significantly improve product formulation and recommendation accuracy [24]. NLP thus bridges the gap between complex scientific data and consumer-friendly communication, enhancing transparency and trust in cosmetic products.

In silico modeling refers to computational methods used to predict the biological effects of cosmetic ingredients. These models employ machine learning algorithms, such as SVMs and ANNs, to simulate human skin reactions to various compounds, aiding in the assessment of potential allergens and irritants [6]. This method offers a significant advantage over traditional testing, especially in regions where animal testing is restricted or banned [38]. However, in silico models often rely on simplified assumptions and may not fully capture the complex interactions between multiple ingredients in a cosmetic product. As such, they may fall short in predicting the full spectrum of possible skin reactions, especially in products with diverse active components [6]. Despite these challenges, in silico modeling represents a critical tool in the push towards more ethical and efficient cosmetic testing.

While these technologies offer substantial benefits, their application in cosmetology is not without challenges. One significant issue is the inherent bias in AI systems, often due to the underrepresentation of diverse skin tones in training datasets [15]. This bias can result in suboptimal outcomes for individuals with darker skin, as current models may not accurately predict the efficacy or safety of products for these users [47]. The reliance on classification systems like the Fitzpatrick Skin Type (FST) scale exacerbates these issues, as it inadequately represents the diversity of human skin tones. Ethical considerations surrounding data privacy are also critical. AI tools frequently handle sensitive personal and biometric data, raising concerns about transparency and the potential for misuse [50]. The "black box" nature of many AI algorithms—where the decision-making process is not easily understood—compounds these concerns, making it difficult for consumers to trust the technology [45].

Looking forward, the future of cosmetology in the age of AI is poised for continued growth and innovation. The integration of advanced AI technologies will likely lead to more personalized and safer beauty products, as well as more efficient and accessible cosmetic services [54]. Key to this future will be the development of more inclusive AI models that can accurately serve diverse populations, ensuring that all consumers benefit from these advancements. As the industry evolves, there will be a greater emphasis on balancing technological innovation with ethical considerations, particularly regarding data privacy and bias mitigation [14]. The ongoing collaboration between dermatologists, cosmetologists, and AI developers will be crucial in refining these technologies and enhancing their applicability in real-world settings [8].

10. Conclusions

In conclusion, the future of cosmetology in the era of AI holds great promise, offering new levels of personalization, efficiency, and inclusivity. Embracing these technologies will be essential for redefining the cosmetology landscape and improving the overall quality of life for individuals worldwide.

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Design, Development, and In Vivo Testing of the Hydrating Effect and pH Maintenance a Cosmetic Formulation Incorporating Oils and an Extract from Peruvian Biodiversity

Jorge Huaman [1], Lourdes Victoria-Tinoco [1,*], Jorge Rojas [1], Ana María Muñoz [2] and Patricia Lozada [1]

[1] Centre for Cosmetic Research, Technology and Innovation, San Ignacio de Loyola University (CITIC-USIL), Lima 15024, Peru; jhuaman@usil.edu.pe (J.H.); jrojasv@usil.edu.pe (J.R.); jlozada@usil.edu.pe (P.L.)
[2] Institute of Food Science and Nutrition, San Ignacio de Loyola University (ICAN-USIL), Pachacamac Campus, Lima 15823, Peru; amunoz@usil.edu.pe
* Correspondence: lvictoria@usil.edu.pe; Tel.: +51-987918896

Abstract: Vegetable oils and extracts have been used from ancient times for skin care. The aim of this study was to design and evaluate the physicochemical, organoleptic, and microbiological characteristics and the instrumental efficacy in vivo of a cosmetic formula named "ASC Cream", containing sangre de grado resin extract (*Croton lechleri*) and vegetable oils obtained from moriche palm (*Mauritia flexuosa* L.), goldenberry (*Physalis peruviana*), super sacha peanut (*Plukenetia huayllabambana* sp. nov.), and sacha peanut (*Plukenetia volubilis* L.). Instrumental efficacy, skin hydration and skin pH were tested in vivo in 24 healthy female volunteers between 40 and 65 years old, using non-invasive skin bioengineering equipment from Courage + Khazaka Electronics, both in the short term (30 min, 1 h and 3 h) and long term (14 and 28 days). The main findings were increased immediate hydration (132.4%) and long-term hydration (143.6%), showing a statistically significant average improvement ($p < 0.05$) without altering the skin pH. In conclusion, a balanced combination of the extract and oils significantly increases hydration levels while maintaining skin pH.

Keywords: natural oils; natural extracts; instrumental efficacy; skin hydration

1. Introduction

The skin is the first line of defense against external factors and threats and acts as an effective barrier like the endocrine and immune system, preserving homeostasis, excretion, body boundaries and metabolism. With optimal water content, the stratum corneum is fully hydrated to maintain the skin strong, elastic, luminous, soft, and smooth. When its water content is low, the skin looks rough, lacks elasticity and may develop peeling and dehydration lines [1].

Peru boasts an extraordinarily wide range of ecosystems and is home to approximately 20,375 plant species. These plants are the focus of basic research leading to the applied research of active ingredients with potential industrial, medicinal, and cosmetic applications [2]. The Peruvian Amazonia is one of the most diverse sources of natural resources; some of its endemic species are *Plukenetia volubilis* L. and *Plukenetia huayllabambana* sp. nov. [3].

The natural oils used in this research such as *Plukenetia volubilis* L. and *Plukenetia huayllabambana* sp. nov. have high contents of polyunsaturated fatty acids of the linolenic and linoleic type, 46.8% and 36.2% for the former and 52% and 28% for the latter [4]. Linolenic acid present in *Plukenetia huayllabambana* has been reported to be much higher than in *Plukenetia volubilis* L. [5]. This species from the Peruvian Amazon is considered a promising source of quality vegetable oil, in addition to its high protein and lipid content [6].

The pulp oil of *Mauritia flexuosa* L. contains large amounts of oleic acid (75%), which is the main component of its fatty acid profile, as well as linoleic and linolenic acids,

carotenoids, polyphenols and tocopherols [7,8]. Its effect as an antioxidant [7] and inflammation mediator has been studied and reported [8].

Linoleic acid is the most predominant fatty acid in *Physalis peruviana* seed oil (73%), which is even higher than in the oil obtained from the peels and berries [9]. It also contains other monounsaturated oils (MUFA), oleic acid and polyunsaturated acids (PUFA), palmitic and stearic acids, polyphenols and tocopherols [10,11]. Oleic acid activates lipid metabolism, restoring the barrier function of the epidermis and retaining moisture in the skin; linoleic acid has the function of maintaining the permeability of the skin barrier, and linolenic acid has an immunomodulatory activity, inhibiting the production of GE2 prostaglandins, which are responsible for inflammation, thus strengthening the immune system [12,13]. A high level of linoleic acid is associated with the prevention of cardiovascular disease [14].

The resin of blood-grade Croton lechleri, included in the cosmetic formula, has skin-conditioning effects [15]. In Peru, it is well known and used in folk medicine; studies show its antioxidant, healing, anti-inflammatory, analgesic, antitumor, antibacterial and immunomodulatory properties, attributed to the presence of catechins, proanthocyanidins and the tapsine alkaloid in its chemical composition [16].

Nowadays, consumers prefer natural ingredients, and vegetable oils have been incorporated into cosmetic formulations as emollients and occlusive agents, which prevent transepidermal water loss and increase skin hydration, mainly due to their unsaturated fatty acids such as oleic and linoleic acids [17]. Furthermore, such formulations containing natural oils are adjuvants in common skin conditions such as atopic dermatitis, contact dermatitis, psoriasis and acne [18]. Fatty acids are essential for the preservation of structure, emolliency and prevention of the development of skin eczema [17]. Cosmetic formulations using vegetable oils in which linoleic acid was found in higher proportions have shown increased hydration and decreased transepidermal water loss even after a single topical application, and it has been concluded that they promote an improvement of the epidermal barrier [19]. The Research, Technology and Cosmetic Innovation Center (CITIC) of the Universidad San Ignacio de Loyola in Lima, Peru Laboratory developed a cosmetic formula for ASC Cream (Andean Skin Care) that allowed mixing different bioactive compounds from plant species of Peruvian biodiversity that are stable and have synergistic effects.

There is extensive research on the chemical composition and properties of these plant oils, which has highlighted the importance of long-term skin bioengineering studies. Instrumental efficacy studies have been developed on 2-month skin; however, no cosmetic formulation has been found in which the percentage improvement in skin hydration is highly significant [20,21]. In addition, long-term studies on topical creams have also been conducted without adequate sample sizes to support the hydration or elasticity effect [22]. Therefore, this research supports the dermatological properties and long-term efficacy of ASC Cream.

The moisturizing effect and improvement of transepidermal water loss after a single application of raspberry, sesame and coconut seed oils on the skin of young women has also been studied, confirming the positive influence of these oils on the skin barrier function [23]. Therefore, it is important to highlight the evaluation of long-term moisturization after daily application of a cosmetic formula [21]. The evaluation of ASC Cream containing more than one vegetable oil shows a highly significant moisturizing effect after 4 weeks of daily use.

The evaluation of ASC Cream, containing four plant oils and one extract, shows a significant positive influence of the bioactive blend on skin hydration.

2. Materials and Methods

2.1. Elaboration of the Cosmetic Formula

This cosmetic product was made from natural oils of *Mauritia flexuosa, Physalis peruviana, Plukenetia huayllabambana, Plukenetia volubilis* and a *Croton lechleri* resin extract. The formulation of ASC Cream is shown in Table 1.

Table 1. ASC Cream final formulation and component proportion.

Phase	N°	Trade Name	INCI Name	%
A	001	Aqua	Aqua	64.52
	002	Propylene glycol	Propylene glycol	5.00
	003	Sangre de grado	Croton lechleri resin extract	5.00
	004	Triethanolamine	Triethanolamine	0.03
B	005	Sacha inchi	Plukenetia volubilis seed oil	5.00
	006	Super sacha inchi	Plukenetia huayllabambana seed oil	5.00
	007	Sepimax zen	Polyacrylate Crosspolymer-6	0.65
	008	Aguaymanto	Physalis peruviana seed oil	5.00
	009	Aguaje pulp oil	Mauritia flexuosa fruit oil	2.00
	010	Emulgade 1000 NI	Cetearyl alcohol	3.50
	011	Montanov 202	Arachidyl alcohol	3.00
	012	Vitamin E	Tocopheryl acetate	0.20
C	013	Saliguard EHGP	Ethylhexylglycerin phenoxyethanol	1.00
	014	Rosewood (*Aniba rosaeodora*)	Aniba rosaeodora wood oil	0.10

It is an emulsion in which the aqueous phase (Phase A) is mixed with the oily phase (Phase B) at a temperature between 65–75 °C under constant agitation; then, when the system reaches a temperature below 50 °C, phase C is added.

The Sacha inchi, Aguaymanto and Aguaje oils were purchased from Candela Peru (Lima, Peru); Super sacha inchi from Cloud Forest, (Lima, Peru); Sangre grade resin from Pebani (Lima, Peru). The raw materials Propylene glycol, Triethanolamine, Emulgade 1000 NI, and Vitamin E were purchased from Basf SE (Ludwigshafen, Germany); Montanov 202 from Seppic (Paris, France); Saliguard EHGP from Salicylates and Chemicals (Nagar, India); Rosewood from Essential Oils Peru (Lima, Peru) and water was purchased from the water treatment system of the Faculty of Engineering-USIL.

2.2. Accelerated Stability Studies

Two ASC Cream batches (1001 and 901) were formulated and subjected to accelerated stability studies at 40 ± 2 °C for 6 months [24].

2.2.1. Organoleptic Characteristics

Both batches of the ASC Cream cosmetic formula were observed for appearance, color and odor during the six-month accelerated stability studies.

2.2.2. Determination of pH

The pH of the cosmetic formulation was determined during accelerated stability studies using a potentiometer (Thermo Scientific, Orion Star A211, Waltham, MA, USA) to 25 °C [25].

2.2.3. Viscosity Determination

The viscosity of the cosmetic formulation was determined during accelerated stability studies using a viscometer (Brookfield AMETEK, DV-E, Middleboro, MA, USA) to 25 °C [26].

2.3. Antimicrobial Efficacy or Microbiological Challenge Tests

This test was performed against the microorganisms *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231 and *Aspergillus brasiliensis* ATCC 16404. The microbial load was monitored at 0, 7, 14 and 28 days; the logarithmic reduction was calculated each time. This test was carried out at SMASAC Laboratories, Lima, Peru.

2.4. In Vivo Instrumental Efficacy Study Protocol

2.4.1. Description of the Study Design

An experimental, analytical, longitudinal, and prospective study was conducted at the Research, Technology and Cosmetic Innovation Center (CITIC) of the Universidad San Ignacio de Loyola in Lima, Peru from October to December 2023.

All healthy volunteers signed an informed consent form before participating in the study. The study was performed in accordance with the Declaration of Helsinki and followed the national legislation of Peru's National Health Institute (INS). The clinical trials regulation was approved by Supreme Decree No. 021-2017-SA24 [27].

2.4.2. Study Settings and Locations

This research was carried out in two CITIC laboratories: the Cosmetic Formulations Laboratory conducted the formulation process and product stability studies, and the Bioengineering and Cosmetic Safety Laboratory measured biomechanical skin attributes. CITIC specializes in applied research and innovation for the generation of formulation patents, and its technological equipment allows the development of skin bioengineering studies. Additionally, it promotes the first postgraduate program in Lima, Peru that teaches cosmetic technology and innovation.

2.4.3. Study Population

The study included healthy volunteers from the database of the CITIC effective as of 2022. Personal data are protected in accordance with the provisions of the Directorate for Personal Data Protection pursuant to Article 34 of Law No. 29733 (Law on Personal Data Protection) and Articles 76, 77, 78, 79 and 81 of the Law on Personal Data Protection Regulation approved by Supreme Decree No. 003-2013-JUS. The personal data collected are used exclusively for the purposes set forth in this document.

2.4.4. Study Sample

The study sample consisted of 24 healthy volunteers aged 40–65 years who provided informed consent prior to participation.

2.4.5. Inclusion Criteria

- Being a healthy female volunteer aged 40–65 years with healthy skin, no wounds, and no tattoos on the front of the legs.
- Agreement to adhere to the procedures and requirements of the study and going to the laboratory on the day and time established for the examinations.
- Acceptance of participation in the study by signing the informed consent form.

2.4.6. Exclusion Criteria

- Presence of any skin disease, tattoos, or scars in the product application area.
- Pregnancy.
- Current use of topical or systemic drugs, such as corticosteroids, immunosuppressants, and antihistamines.
- History of episodes of allergic and irritant reactions to plant and cosmetic raw materials.
- Skin diseases, such as vitiligo, psoriasis, lupus, and atopic dermatitis and other diseases that may directly interfere with the study or put the subject's health at risk during the study process, such as diabetes mellitus, opportunistic infections, hypothyroidism, history of hypoglycemia, and immune insufficiency.

2.4.7. Skin Bioengineering Instruments

High-tech non-invasive skin bioengineering equipment from Courage + Khazaka Electronics, Cologne, Germany, was used. A Courage + Khazaka Corneometer® CM 825 and a Skin-pH-Meter® were used for skin hydration and pH assessments, respectively.

The Corneometer® CM 825 uses a cylindrical probe to indirectly measure the water content of the stratum corneum by means of capacitance. Any change in the dielectric constant due to changes in the water content of the epidermis alters the capacitance of the precision capacitor, which is recorded on the device [28].

The Skin-pH-Meter® measures skin pH.

2.4.8. Procedures and Techniques

The application and tests were performed on the front of the leg, in an area of 7 cm × 5 cm. For short-term measurements, this area was divided into three equal surfaces. The distribution of ASC Cream was randomly distributed between the right and left legs.

2.4.9. Working Procedure

- On day 1 of the visit, the volunteers were informed of the study objective, methodology, duration, expected benefits and limitations. They read the consent form and signed it. They were also instructed about the mode and frequency of product use. Measurements were carried out at two time points: immediate measurements at 30 min, 1 h and 3 h and long-term measurements at 14 days and 28 days, respectively.
- Prior to both immediate and long-term measurements, participants were acclimatized in a room with a temperature of 20 ± 2 °C and 50% ± 5% relative humidity for at least 30 min before baseline measurements.
- For short-term measurements, hydration and pH were measured on the front of the legs, recording the baseline condition for each indicator. Subsequently, the product was applied (first application), and measurements were taken 30 min, 1 h and 3 h after the first application.
- For long-term measurements, participants were instructed to apply the product twice a day, and they were instructed not to apply it the day before their visits (day 14 and day 28). In subsequent controls (visits on days 14 and 28), participants were acclimatized in the same way as in visit 1.

2.5. Statistical Analysis

Measurements were performed in triplicate for each participant. Corneometry and pH results were analyzed statistically with the IBM SPSS Statistics 29.0 software. Values are expressed as mean, standard error, percentage of improvement, and percentage of positive cases in the short and long term. Given the amount of data, Shapiro–Wilk normality tests were used, and Student's *t* test was used for parametric tests.

3. Results

3.1. Accelerated Stability

Satisfactory results in terms of emulsion stability and stability of its physicochemical characteristics, such as pH, viscosity, and organoleptic features.

The organoleptic characteristics of the ASC Cream batches are shown in Table 2; both batches had the same organoleptic characteristics during the 6 months of the study.

Table 3 shows the results obtained on the two laboratory batches of ASC Cream, which are within the recommended pH for cutaneous application [29]. The samples were stored for 6 months at 40 ± 2 °C as part of the accelerated stability study protocol, based on NTS No. 182-MINSA/DIGEMID-2022 [24]. According to this technical standard, significant changes in accelerated stability studies involve alterations in pH, change in organoleptic characteristics and phase separation; these changes in characteristics were not observed in ASC Cream during the accelerated stability study. The results in terms of physicochemical parameters are preliminary, as these are laboratory batches; these characteristics will be corroborated when industrial production or larger batches are manufactured as part of the technology transfer.

Table 2. Organoleptic characterization of the ASC Cream formula.

N° Formula/Code: CITIC-46-F007		Organoleptic Characterization		
Batch Number	**Duration (Weeks)**	**Aspect**	**Color**	**Odor**
Batch 1001	0 2 4 8 12 16 20 24	Homogenous	Lightly brownish	Characteristic
Batch 901	0 2 4 8 12 16 20 24	Homogenous	Lightly brownish	Characteristic

Table 3. Physical and chemical characterization of ASC Cream.

N° Formula/Code: CITIC-46-F007		Physicochemical Parameters	
Batch Number	**Duration (Weeks)**	**pH (25 °C)**	**Viscosity (25 °C)**
Batch 1001	0	5.72	21,000
	2	5.31	20,500
	4	5.1	20,100
	8	4.95	19,800
	12	4.85	19,100
	16	4.72	19,500
	20	4.56	17,420
	24	4.47	17,600
Batch 901	0	6.3	21,200
	2	6.15	20,900
	4	5.98	20,100
	8	5.81	19,800
	12	5.65	19,500
	16	5.41	19,200
	20	5.21	18,900
	24	5.37	16,200

3.2. Antimicrobial Efficacy

ASC Cream met the assessment criteria for:

Bacteria: A log reduction of no less than 2.0 from the baseline count was reported at 14 days (4.6×10^5 CFU/mL, 3.9×10^5 CFU/mL, and 8.2×10^4 CFU/mL for *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 9027, respectively). No increase was observed at 28 days regarding the value observed at day 14.

Yeasts and molds: No increase was observed at 14 and 28 days with respect to the baseline count of 7.6×10^4 CFU/mL for *Candida albicans* ATCC 10231 and 6.5×10^4 CFU/mL for *Aspergillus brasiliensis* ATCC 16404.

It can be concluded that the preservatives used result in a safe product from a microbiological point of view.

3.3. Instrumental Efficacy In Vivo

3.3.1. Evaluation of Skin Hydration

ASC Cream showed higher hydration values ($p < 0.05$) over the 4 weeks compared to its baseline control (Table 4, Figure 1).

Table 4. Hydration results by corneometry.

Control	Basal Control	Immediate 30 min	1 h	3 h	2 Weeks	4 Weeks
n	24	24	24	24	24	24
Average	17.10	39.74	34.75	33.26	37.45	41.65
Max	29.40	65.50	51.12	52.76	55.00	54.08
Min	7.94	23.86	17.84	21.64	23.50	26.68
SE	1.28	2.01	1.59	1.37	1.52	1.76
IC 95%	2.64	4.17	3.28	2.82	3.15	3.65
% Positive cases	-	100%	100%	100%	100%	100%
Positive cases number	-	24	24	24	24	24
p-Value	-	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Average improvement as a percentage	-	132.4%	103.2%	94.5%	119.0%	143.6%

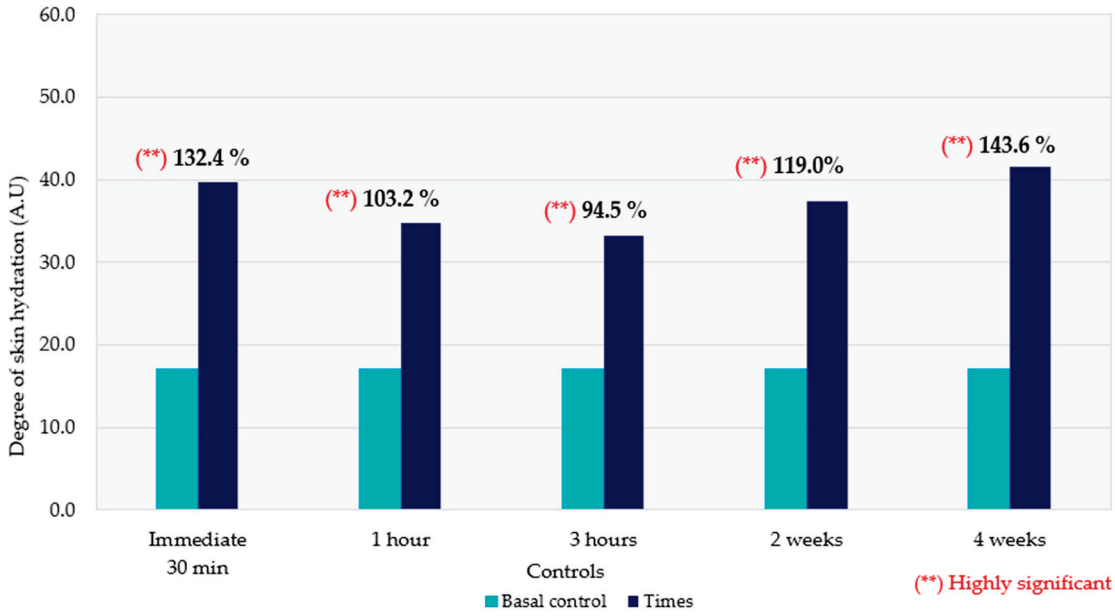


Figure 1. Comparison of skin hydration values measured with the Corneometer CM825 after application of ASC Cream up to 4 weeks.

3.3.2. Skin pH Assessment

After 4 weeks, ASC Cream exhibited statistically significant short- and long-term differences; however, this increase is within the normal range of lateral leg pH (4.5–6.5). (Table 5, Figure 2.)

Table 5. Comparison of skin values.

Control	Basal Control	Immediate 30 min	1 h	3 h	2 Weeks	4 Weeks
n	24	24	24	24	24	24
Average	5.71	5.92	5.85	5.93	5.29	5.34
Max	6.00	6.16	6.06	6.09	5.91	6.18
Min	5.23	5.74	5.46	5.66	4.49	4.80
SE	0.05	0.02	0.03	0.02	0.08	0.09
IC 95%	0.09	0.05	0.07	0.05	0.16	0.18
% Positive cases	-	79%	75%	88%	8%	29%
Positive cases number	-	19	18	21	2	7
p-Value	-	0.000	0.006	0.000	0.000	0.001

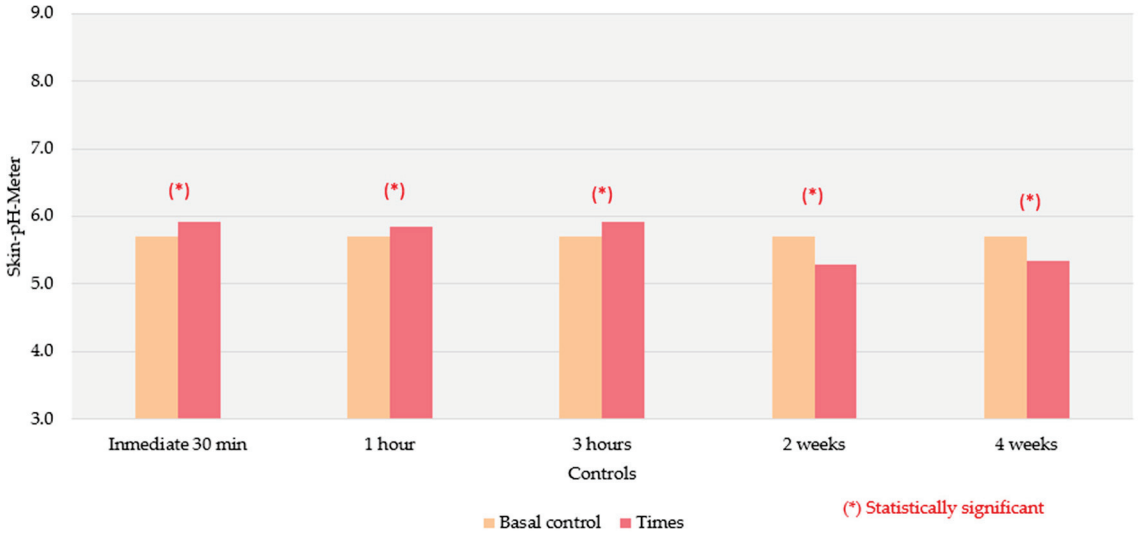


Figure 2. Comparison of skin pH values measured with the Skin-pH-Meter® after application of ASC Cream up to 4 weeks.

4. Discussion

Throughout the treatment, 100% positive results were obtained, which strongly supports the hydrating effect of ASC Cream. All external factors that could have influenced the results, such as probe pressure, relative humidity, relative temperature, and seasonal period, were controlled during measurements [30].

ASC Cream is an o/w emulsion-type formula, containing 0.65% Sepimax Zen, which is a gel that gives the cream a non-Newtonian behavior, meaning that it can vary its viscosity when subjected to shear forces [31]. Therefore, the decrease in viscosity does not represent instability; there is no phase separation, its organoleptic characteristics and cosmetic form are maintained, and it remains a cream that is easy to spread on the skin. Sepimax Zen, whose INCI name is Polyacrylate Crosspolymer-6, is a gel former and contributes to the stability of emulsions containing large amounts of oils [32]; this ingredient is what imparts the thinning behavior to our product, which is why, although the viscosity decreases as time passes at a high temperature of 40 °C, it is stable and there is no phase separation.

Some authors claim that the bioactive compounds of cosmetic formulations containing vegetable extracts or oils may have synergistic or antagonistic effects [19]. However, our study shows that the design and elaboration of cosmetic products containing several oils containing essential fatty acids and catechins improves the hydration effect and maintenance of skin pH, ensuring synergism between these components. The results obtained in this skin bioengineering study scientifically support these claims. The extracellular

matrix is characterized as hydrophilic due to the high presence of glycosaminoglycans produced by the fibroblast [33]. Based on these previous studies, it can be assumed that ASC Cream could be stimulating the synthesis of these polymers, so its daily application is recommended to ensure its moisturizing efficacy, as it is relevant for improving skin capacitance. The same occurs with the amide and polyol varieties included in commercial moisturizing and hydrating cosmetic products, which should also be applied daily [34]. The epidermal hydration caused by moisturizers demonstrates a favorable influence on the biomechanical properties of the skin [22], which may be a reason to extend the study of ASC Cream to examine its behavior in viscoelastic characteristics.

The epidermis is a semi-permeable barrier that maintains a physiological gradient in water evaporation [17]. This capacity is attributed to glycerol produced by the sebaceous glands. Hydrating creams help reduce transepidermal water loss, improving hydration [35]. ASC Cream would improve natural hydration factors by forming a more consistent hydrolipidic layer which retains water, keeping skin highly hydrated.

Due to the hydrophobicity of its acyl chain, oleic acid is known to spontaneously and easily penetrate the stratum corneum to the epidermal–dermal junction, contributing to skin permeability and the formation of separate domains enriched with oleic acid [36]. It also helps to restore the barrier function of the epidermis and allows a greater amount of moisture to be retained [12]. Linolenic acid (omega 3) strengthens the immune system and inhibits the production of prostaglandins GE2, which are responsible for inflammation. Linoleic acid as an acyl glycosylated ceramide component maintains the water permeability barrier of the skin [13]. These natural oils have penetration-enhancing effects due to their chain length and double bonds, which promote skin permeability through lipid fluidization in the stratum corneum, even more than saturated fatty acids [37]. This bioactive mixture benefits the hydration power of ASC Cream without altering normal skin pH.

Quantification of polyphenols such as flavonoids in hydroalcoholic extracts has evidenced the anti-enzymatic activity of the elastase and collagenase enzymes [38]. Several types of flavonoids have been identified in *Croton lechleri* [16,39], which may promote collagen increase. This may be supported by an additional study of the cream to identify its viscoelastic properties.

Long-term assessments of other vegetable oils such as canola have been conducted in a wider age range and evidenced a moisturizing increase; however, they did not show a significant difference with baseline controls [19]. The combination of the vegetable oils included in the ASC Cream formulation reports statistically significant results that strongly support its hydrating effect.

In other studies of skin hydration with cosmetic products where the active ingredients were catole coconut, licurí and almond oils, which also contained the fatty acids mentioned above, significant increases in hydration values were observed after 20 days [21]. However, the area of topical application should be clearly established, as well as the application instructions for volunteers to avoid confusion. In addition, considering that the time from stratum corneum formation to its removal is 20 to 30 days in young adults [40], long-term studies should last 4 weeks or longer.

The skin on the legs is more likely to be affected by dryness than the skin on the forearms [41]. ASC Cream may foreseeably be used on diabetics, who develop skin dehydration that leads to vulnerability to dermatological conditions, especially on the lower limbs. The results of this research were satisfactory in the population of healthy volunteers, which could be continued with populations of people with dry skin [42]. In addition, by containing plant species from the Peruvian biodiversity in their formulation, they can boost the economic activity of the inhabitants of the respective regions.

After 4 weeks, ASC Cream exhibited statistically significant differences in the short and long term. However, this increase is within the normal pH range of the side-of-the-leg skin (4.5–6.5) and to maintain the care of the acid mantle [43–47]. No volunteers showed any visible adverse reaction in the application area throughout the treatment, demonstrating that ASC Cream does not alter skin pH. Free fatty acids can maintain skin

pH between 4.0 and 6.5; dermatoses are mostly characterized by an impaired skin barrier, increasing the pH of the skin surface [48]. Skin pH helps control the physical properties and stability of the lipid membrane, preserving skin structure and health [49]. When pH increases, corneodesmolysis occurs and the synthesis of ceramides by glucosylceramides is retarded [50].

5. Conclusions

ASC Cream is an exclusive formulation that combines four oils and a natural extract, all with different chemical compositions, rich in omega 3, omega 6, omega 9 and polyphenols that combined synergistically potentiate their effectiveness for the maintenance and improvement of skin health. These five natural ingredients were correctly mixed in an o/w emulsion vehicle, allowing a better availability at the skin level, favoring, after a good dispersion, its efficacy at the application site. Because of the efficacy found, it could be recommended to help treat skin conditions such as dehydration and its associated problems effectively and safely.

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cosmetics

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Review

Unwanted Skin Reactions to Acrylates: An Update

Liborija Lugović-Mihić [1,2,*], Eva Filija [1,2], Vanja Varga [1,2], Lana Premuž [1,2], Ena Parać [1], Renata Tomašević [1,2], Ema Barac [1] and Bruno Špiljak [2,*]

[1] Department of Dermatovenereology, University Hospital Center Sestre Milosrdnice, 10000 Zagreb, Croatia; efilija@sfzg.hr (E.F.); vvarga@sfzg.hr (V.V.); lpremuz@sfzg.hr (L.P.); enaparac7@gmail.com (E.P.); renatavalo@gmail.com (R.T.); ema.barac@gmail.com (E.B.)

[2] School of Dental Medicine, University of Zagreb, 10000 Zagreb, Croatia

* Correspondence: liborija@sfzg.hr (L.L.-M.); bruno.spiljak@gmail.com (B.Š.)

Abstract: Acrylates and methacrylates, though common in a wide variety of products, especially in the dental industry, can cause adverse skin reactions. These compounds, including 2-hydroxyethyl methacrylate, triethylene glycol dimethacrylate, and bisphenol A-glycidyl methacrylate, are strong contact irritants or allergens. Found in dental prostheses, composite resins, dentin bonding materials, and glass ionomers, they pose a higher risk of exposure for dental personnel. Clinically, acrylate allergies manifest as facial rashes, eczema with cracked skin on fingers (pulpitis), nail dystrophy, and periungual dermatitis. Recently, however, the highest frequency of allergic reactions to acrylates has been observed in the beauty industry due to increased use in artificial nails, eyelashes, and hair extensions. This has led to greater sensitization. Acrylates are also used in medical applications such as bone cement for orthopedic endoprostheses, soft contact lenses, hearing aids, histological preparations, and wound dressings, which can also cause allergic reactions. For example, acrylates in surgical glue can cause severe dermatitis, and diabetic medical devices are also potential sources of allergic contact dermatitis. Given the extensive use and prolonged skin contact of products containing acrylates and methacrylates, this review aims to present current knowledge from the literature on reactions to these compounds across different industries.

Keywords: acrylates; methacrylates; contact reactions; skin; dentistry; cosmetics; allergy; contact dermatitis; nails; sensitization

1. Introduction

Acrylates are plastic materials obtained by polymerizing monomers derived from acrylic or methacrylic acid [1–4]. The type of acid utilized in the esterification process of these monomers influences their reactivity [5–9]. Acrylates serve as the basis for acrylic resins [10]. The double bonds of acrylates allow for polymerization to occur quickly at room temperature (spontaneously) or with the help of heat/ultraviolet (UV) light [11].

Acrylate monomers have many applications [11,12]. Thus, the presence of acrylates and methacrylates (MAs) is widespread, primarily in the dental industry. They are used in dental prostheses, composite resins, dentin bonding materials, and glass ionomers [9,10,13–15]. Historically, the most frequent human exposures to acrylates that were related to production processes were the most common forms of exposure to acrylates, especially printing, dyeing, coating, the metallurgical industry, and dentistry [11,12]. Familiar sources of acrylates include floor waxes, leather treatments, and textile and paper products. Today, many (meth)acrylates are mainly used in dental binders, printing inks, artificial nails, etc. By profession, dental personnel are particularly at risk for acrylate and MA allergies [16].

Contact dermatitis is an inflammatory dermatosis that can be triggered by various chemicals that cause irritant (toxic) effects or induce immune responses [4,6]. Allergic contact dermatitis (ACD) occurs only in persons who are sensitive to certain substances,

while the irritant type may occur in all people and depends on related factors like the amount and concentration of the substance, duration and frequency of exposure (short concentrated exposure or low repeated/prolonged exposure), previous skin damage, atopy constitution, etc. [7]. Contact dermatitis may manifest as an acute or chronic form (after repeated exposures) [8,9]. Contact dermatitis is a relatively frequent dermatosis, where the irritant type is more common than the allergic form [10]. By comparison, the irritant type is a non-specific skin reaction to direct chemical skin damage with the release of inflammatory mediators, while the allergic type is a delayed hypersensitivity reaction to allergens that includes immune responses [11]. Thus, irritant contact dermatitis (ICD) does not involve immune reactions, no sensitization (previous exposure to the substance) is required, and most individuals exposed to the same substance manifest a similar reaction [15]. Sometimes contact dermatitis (irritant or allergic) can be related to occupational activities and different workplace factors [17].

Acrylates and MAs can be irritants and allergens. Thus, acrylates and, more rarely, MAs such as 2-hydroxyethyl methacrylate (2-HEMA), triethylene glycol dimethacrylate (TEGDMA), and 2,2-bis[4-(2-hydroxy-3-methacryloxypropoxy)phenyl]propane (bis-GMA) are strong contact irritants or allergens. These compounds have been found in uncured dental adhesives and cement in 50 to 90% concentrations. It is also important to mention that methyl methacrylate (MMA) can penetrate through thin protective disposable gloves as a small molecular acrylate. While exposure to solid acrylic plastics is generally harmless, MA can evaporate even when solid, increasing the risk of exposure and irritation [8]. Thus, ACD caused by MA has been well documented. In ACD, skin reactions appear 24–48 h after repeated contact with an allergen/substance to which the skin was previously exposed and developed sensitization [17]. In addition, many papers on occupational ACD caused by (meth)acrylate monomers have also been published [11].

2. Chronological Development of the Application of Acrylates and the Appearance of Adverse Reactions

Starting in the 1930s, acrylates and methacrylates were developed for use in paints, glues, inks, and, most notably, plastic glass, trademarked as Plexiglas. Thus, their use became ubiquitous in a number of industries, including the medical and cosmetic industries [18–22].

Since the 1950s, many cases of ACD have been documented due to MMA exposure. Numerous papers have been published on the occupational origin of ACD caused by (meth)acrylate monomers since that time [11]. Occupational allergy to (meth)acrylates is also possible, which most often occurs among dental staff. In the 1990s, dental professionals observed a significant increase in sensitization to acrylates [23]. Dental personnel are exposed to many potential contact allergens, including untreated plastic (acrylate) resins such as acrylic monomers, MAs, urethane acrylates, and epoxy acrylates. These have been used in dentistry in dentures, dentin bonding materials, and glass ionomer materials [11,24]. Due to the frequent observation of these reactions, (meth)acrylates were named "allergen of the year" in 2012 by the American Society for Contact Dermatitis [8,10]. Additionally, isobornyl acrylate (IBOA) was 2020's contact allergen of the year [11,25].

In recent decades, allergies to (meth)acrylates have been on the rise. These allergies are prevalent not only in dentistry but also in the beauty and medical device industries. For example, with the popularity of permanent manicures, reactions to acrylates in permanent nail polish have contributed to the rise in incidence. Another example is reactions caused by medical devices for diabetics [11].

3. Acrylates in Cosmetics and Medicine

The first case of an acrylate allergy to artificial nail products was reported by Canizares in 1956 [11,26–29]. Now, (meth)acrylate allergies in the beauty industry outpace the incidence in dentistry [26]. Artificial eyelashes and nails, as well as hair extensions, are the most common sources of exposure [27,28]. The wide availability of nail techniques based on

acrylates, MAs, or cyanoacrylates and the increased use of artificial nails has led to greater sensitization in beauticians and their clients [11,16,30]. Also, with the use of commercial nail kits for home use, there is an even greater risk of allergy development, already reported in Australia, Sweden, and Spain [27,31]. Notably, the appearance of an allergy can occur even after a person has already used artificial nails for months or years [11].

Nowadays, new sources of acrylates have appeared [11]. For example, a case of acrylate ACD caused by a liquid adhesive for a fixed hair prosthesis (in a man with scarring alopecia) was described after 4 weeks of use (Ghostbond) [32]. The patient developed pruritic eczematous lesions on the scalp and, despite switching out the liquid adhesive for tape, no improvement was seen [32]. A patch test confirmed an allergy to hydroxypropyl methacrylate (2-HPMA), hydroxyethyl acrylate, butyl acrylate, adhesive tape, and Ghostbond glue (2+). However, IBOA, previously used mainly in coatings, inks, cosmetics, and paints, is also a potential allergen, though less frequent than other (meth)acrylates [33].

Acrylates are also widely used for various medical purposes; thus, contact with anything from bone cement and wound dressings to histological preparations can trigger allergic reactions. Surgical glue, for instance, has been known to cause severe dermatitis, and adhesives used on the skin are known to pose a high risk for primary sensitization [11].

Multiple reports also indicate that diabetic medical devices are an important cause of ACD. For example, new technologies for rapid glucose measurement have been implicated [34]. According to the results of one multicenter study, skin reactions to Freestyle Libre, a flash glucose monitoring (FGM) system (which involves fixing the device to the skin with adhesive for two weeks), may occur directly below the adhesive part of the sensor and most patients exhibited a proven allergy to isobornyl acrylate, a component of the device [11,33,34]. In another study, following 70 diabetics (type 1) with a suspected contact allergy to their glucose sensor (tested by patch test), 81% of Freestyle Libre users exhibited IBOA reactions [33]. Recently, reactions to IBOA in insulin pumps have also been identified (OmniPod) [11,35]. Therefore, based on current observations and recommendations, manufacturers need to eliminate this allergen from these devices due to the increased prevalence of acrylate allergies [34]. Other acrylates, such as N,N-dimethylacrylamide (DMAA) and 2-ethyl cyanoacrylate adhesive, are also potential sensitizers in insulin infusion and glucose monitoring devices [36]. As a preventive measure, in cases of ACD caused by acrylates, patients can use hydrocolloid plates as a barrier that limits skin exposure to device adhesives [11,37,38].

Other sources of sensitization to acrylates include electrodes for transcutaneous electrical nerve stimulation (TENS) and electrocardiogram electrode adhesives [38,39]. Allergic reactions to acrylates can also occur in plastic surgery. For example, a case was described where a patient developed ACD to polymethyl methacrylate (PMMA) after an intradermal filler was used for the tip of his nose. Subsequently, an allergy to MMA (3+) was proven [40].

4. Acrylates as a Cause of Allergic Contact Dermatitis and Other Disorders

Clinically, an allergy to acrylates most often appears as a rash on the face and/or eyelids, hand eczema with cracking of the finger skin (pulpitis), nail dystrophy, and periungual dermatitis (Figure 1) [5,19]. When a person is sensitized to acrylate, lesions can appear anywhere on the skin, though the site of direct contact is where they commonly appear, often the hands and face. The overall clinical picture of skin allergy can also manifest as acquired leukoderma on the fingertips or sometimes with simultaneous dermatitis of the face or neck [11,16,30,41]. Nail abnormalities similar to psoriasis (due to onycholysis and severe subungual hyperkeratosis) can occur. However, not all skin lesions at contact sites can be attributed to ACD alone; other manifestations, such as irritant (non-allergic) contact dermatitis, may also occur. In a differential diagnosis of skin lesions, "acne mechanica," which manifests as inflammatory papules and pustules caused by mechanical pressure, should also be considered [42]. In addition, acrylates and MAs can also cause asthma [43].



Figure 1. Skin lesions in the person who works as a manicurist (image from the archive of the main author, Prof. Dr. Lugović Mihić).

Different frequencies of allergies to certain acrylate haptens have been observed among dental personnel. For dentists and dental nurses, common allergens are 2-HEMA and MMA, while ethylene glycol dimethacrylate (EGDMA) is common for dental technicians [44]. According to the results of a study testing allergies in dental technicians and the connection with work-related ACD (patch tests conducted from 2001 to 2015 in Germany), the most common contact allergens were MAs and acrylates [24]. Eczema of the hand and fingertips were observed as typical clinical manifestations of allergies to (meth)acrylate in these dental professions, though generalized dermatitis is also possible [44–47]. The face and eyelids can also sometimes be affected due to acrylate particles in the air or the transmission of allergens via contaminated tools or hands [27]. Additionally, acrylates can cause respiratory hypersensitivity, such as difficulty breathing, asthma, or rhinoconjunctivitis.

Among other unwanted changes/lesions induced by acrylates, the term allergic contact stomatitis (ACS) is described in the literature. This condition has been seen in connection to dental prosthetic replacements where the acrylates used were not properly polymerized. [48]. Patients with ACS may experience oral symptoms like burning or pain in the mouth, loss of taste, numbness, inflammation of the mucous membrane, vesicles, erosions, and lichenoid reactions limited to the area of contact with dental materials [11]. Therefore, caution is recommended when working with sensitized patients who need dental procedures, as they may experience complications during dental work [49]. However, sometimes inadequately performed dental prosthetic work (which, by its impact, disrupts the biological width of the periodontium) can cause inflammation of the underlying mucosa (with hyperemia and erythema), or patients, after the creation of new prosthetic work, can report clinical problems (burning mouth syndrome), and so often wrongly/unfairly declare it an oral allergic reaction. Therefore, clinicians need to consider these possibilities when working with patients [50,51]. It is also important to note that lip and perioral skin inflammation can be triggered by certain metals or acrylates [52]. When cheilitis results from an allergic reaction, the lesions are usually localized to the site of contact.

Looking at adverse reactions when working in cosmetics, ACD caused by acrylates in artificial nail techniques is frequently overlooked due to the unobserved association of manicure product use with nail abnormalities [12,53]. In establishing a diagnosis, the clinical picture involvement of all the nails and the absence of psoriasis of the nails (e.g., "salmon patches") can help (nail biopsies often are not necessary) [54]. According to research, acrylate allergy occurs most often in young female beauticians who suffer from hand dermatitis and sometimes facial dermatitis [27]. According to an international retrospective study [11 European Environmental Contact Dermatitis Research Groups (EEC DRGs)], most cases of ACD were to acrylates in nail styling materials (67%): 43% of patients were users/consumers, and 56% were contractors/workers. Furthermore, most occupational ACD cases (65%) were detected during the first year on the job. Study results highlight the high sensitizing power of acrylates [8,11,28]. A positive reaction to two or more acrylates was found in most patients, 2-HEMA, 2-HPMA, EGDMA, and ethyl cyanoacrylate being the most common allergens [11,28].

Looking at the negative impact of acrylates on beauticians, the mucosa of the eye, nasal passages, and respiratory tract can also be involved. In beauticians, there is a possible direct connection between exposure to allergens at the workplace, including (meth)acrylates, with lung function and airway inflammation [55]. Their nasal and respiratory symptoms can be associated with the strong irritating properties of acrylates [11,55]. Conjunctivitis, asthma, and rhinitis are all possible [49,55].

The negative influence of acrylates when performing eyelash extensions (a modern cosmetic trend) should also be mentioned, with possible unwanted reactions resulting from the process of glueing on artificial eyelashes (eyelash by eyelash on the natural eyelashes of clients) with glue that often contains cyanoacrylates. Clinically, possible unwanted manifestations in clients include eye disorders such as blepharitis and hand eczema in the beautician [56]. Conjunctivitis and rhinitis may occur first during the application of acrylate glue for eyelashes (which stimulates the influx of eosinophils into the tears and then into the nasal mucosa). A case was described of a beautician who used eyelash glue with acrylate to extend artificial eyelashes for two years (containing ethyl-2-cyanoacrylate, alkoxy-2-cyanoacrylate, and PMMA), leading to eye and nasal symptoms (discontinuing the use of these adhesives led to the withdrawal of symptoms) [11].

5. Diagnosis and Proof of Allergy to Acrylates

Patch testing is the gold standard for confirming the diagnosis of allergy to acrylates and to metals and other contact allergies (Figures 2 and 3) [11]. Thus, patch testing is useful for the investigation and confirmation of allergies to various substances (including acrylates) that cause allergic contact dermatitis. The test involves applying allergens (appropriately diluted) to the skin of the back for 48 h (In some specific situations when it is not possible to apply the allergens to the back, it may be carried out on another part of the body.). After 48 h, the allergens are removed, (the first reading) and, at 96 h, the results are recorded a second time (sometimes, also after 7 or 10 days for some allergens). In the case of positive allergic reactions at the sites of application, vesicles, papules, or blisters, etc., may be seen. Standard allergen sets for patch tests vary by country, but additional allergens may also be tested. Generally, aside from the patch test, which confirms type IV contact allergic reactions, there is also the prick test, which confirms early type I allergic reactions to, e.g., inhalant allergens and food, but is not important in the case of allergy to acrylates [1,11,14].



Figure 2. Positive patch test for contact allergens/standard testing procedure (image from the archive of the main author, Prof. Dr. Lugović Mihić).



Figure 3. Positive patch test for acrylates and MAs in a dentist (image taken from the doctoral dissertation of Dr. Iva Japundžić [57], courtesy of Dr. Iva Japundžić Rapić).

Many commercial patch tests include acrylic monomers used in dentistry and the cosmetics industry [44]. A polyvalent allergy to acrylates is also common in test results (multiple positive reactions in the patch test), although individual patients likely would

not be exposed to each of these substances. In these cases, acrylic monomers may be cross-reacting due to their simultaneous presence in some products [8,44]. According to a study on acrylic compounds and cross-reactivity, sensitized patients were allergic to multiple compounds from the test (66.3%), which points to the need for preventive measures [10]. According to the results of a study conducted on 67 patients allergic to (meth)acrylates, reactions to one or two (meth)acrylates were proven in 42% of them, while allergies to $\geq$5 (meth)acrylates in 33% [24]. According to Raposo et al., positive patch tests for HEMA acrylates were observed in more than 90% of patients with an allergy to acrylates [21]. In comparison, 64.1% were positive for 2-HPMA, which could be a useful screening marker [21]. For this reason, 2-HEMA and HPMA should be included in the European basic patch test series [11,30]. Also, since acrylic monomers often cause cross-reactions, sensitized persons should not be exposed to any of these compounds [10,58].

In study results of allergy tests for nail acrylates and the results of their patch tests ("European Baseline Series and Acrylate Series Nails Artificial" and 10% ethyl cyanoacrylate), significant contact allergies to triethylene glycol diacrylate were observed [22]. In other studies, reactions were seen to dental and surgical products and medical devices. Therefore, clinicians and patients/clients who are in contact with acrylates should be adequately informed about possible adverse reactions, including how to recognize and confirm them [11,48].

According to research by Lyapina, more than two-thirds of all dentistry workers/students who reported skin symptoms (61.9%) were third- and fourth-year students and practitioners (most often without skin symptoms were dental professionals) [10]. According to these results, the general sensitization rates to MAs in the studied population were relatively high: from 25.9% (for MMA) to 31.7% (for TEGDMA). In addition, a significantly higher frequency of sensitization to MMA and TEGDMA in third- and fourth-year students compared with dental professionals was also observed. A significantly higher frequency of sensitization to EGDMA, BIS-GMA, 2-HEMA, and tetrahydrofuryl methacrylate was recorded in patients compared with dental professionals. Therefore, greater awareness of the possible adverse effects of exposure to MAs is needed [10].

Regarding the diagnostics of potential oral manifestations from acrylates, although the patch test is a standard method for confirmation of contact allergies, the correct interpretation of the results and their clinical importance for oral mucosal lesions (histologically different from the skin) can be complex. Differences in the concentration of haptens in the oral mucosa compared with standard substances and differences in pH values are essential for the patch test results, which can lead to false positive/negative results or non-specific irritant reactions [59]. Also, sometimes inadequately made prosthetic works can imitate allergic reactions, and assessing the clinical importance of haptens in dental materials is difficult.

6. Allergy to Acrylates and Methacrylates in Dental Workers and Students

Many acrylic monomers are used in dentistry, so dental workers (dentists, dental technicians, patients, or dental students) can become sensitized when it is necessary to examine the possibility of adverse reactions to dental preparations based on the acrylates to which they are exposed. Numerous studies confirm the high frequency of sensitization to (meth)acrylates in dental professionals and their patients (who are exposed to resin-based materials during dental treatments) [10]. In their daily activities, dental professionals and dental students are exposed to numerous chemical substances, primarily from various dental materials and drugs. Thus, dental products contain numerous allergens and irritants that can cause health problems for dental professionals (during their professional exposure), dental students (during their education), and patients to whom the products are applied.

According to the literature data, the most common positive allergens among dentists and dental nurses are 2-HEMA, ethyl acrylate (EA), TEGDMA, and bis-GMA [10]. At the same time, 2-HEMA is considered the most critical allergen for dentists and dental nurses, while MMA and EGDMA are significant for dental technicians. The relevance of

the positive reaction is also crucial, as positive patch-test reactions to bis-GMA, diethylene glycol diacrylate (DEGDA), TEGDMA, ethyl methacrylate (EMA), and EA are relevant in some patients [10,44,58,60]. In the study population, overall sensitization rates to MAs are relatively high, varying from 25.9% for MMA to 31.7% for TEGDMA. The lowest frequency of sensitization is in dental professionals, while the highest rates vary (depending on the allergens and examined groups). There was significantly more frequent sensitization to MMA and TEGDMA in third- and fourth-year students than in dental professionals, likely related to their recent and intensive exposure to these substances during the second year of training [10]. Due to the occupational nature of the exposure, a patch test for contact allergens is required for the complete diagnosis and evaluation of professional skin allergy to MA in dental staff [9].

Numerous studies confirm the high frequency of sensitization to (meth)acrylates in dental professionals, and in patients who undergo dental treatment and are exposed to resin-based materials [14,61,62]. Several other studies have been conducted to assess the frequency of sensitization to acrylates in various populations (Table 1) [1–7,13,14,19,63,64].

Table 1. Selected data on unwanted reactions to acrylates obtained by different studies.

Author, Year [Reference Number]	Examinees/Patients	Analyzed Factors/Methods	Results	Conclusions
Kanerva L et al., 1993 [64]	4 patients (an orthodontist, 2 dental technicians, and an in-house trained dental worker)	Patch testing for allergens in dental prostheses	All patients had positive allergic patch test reactions to MMA.	Dental personnel working with prostheses had higher risk of sensitization. Patients working with dental prostheses should be patch-tested with MMA, 2-HEMA, dimethacrylates, epoxy acrylates, and urethane acrylates to detect occupational ACD.
Kiec-Swierczynska MK, 1996 [63]	1619 patients suspected of occupational CD (examined between 1990 and 1994)	Patch testing to acrylates and methacrylates including EGDMA, MMA, 2-HEMA, and TEGDMA	The most frequent sensitizers were EGDMA (5 positive patch tests), MMA (4), 2-HEMA (4), and TEGDMA (4). Sensitivity to acrylates was diagnosed in 9 patients (4 dental technicians, 4 dentists, 1 textile printer).	Dentists were more sensitive to (meth)acrylate allergens and other allergens (metals and rubber additives). Dental technicians were mainly sensitive to methacrylates. The textile printer was only sensitive to acrylates.
Geukens S and Goossens A, 2001 [7]	3833 patients suspected of CD (from 1978 to 1999)	Patients were tested by patch test	The top three sensitizers were EGDMA (17 positive patch tests), 2-HEMA, (14), and TEGDMA (6). Almost half the examinees (14/31) were affected by (meth)acrylate-containing dental materials (including dentists and dental technology students).	An increasing trend in dermatological issues associated with the expanding use of (meth)acrylates, particularly in dental professions.

Table 1. *Cont.*

Author, Year [Reference Number]	Examinees/Patients	Analyzed Factors/Methods	Results	Conclusions
Wrangsjö K et al., 2001 [13]	174 dental personnel referred as patients to the Department of Occupational and Environmental Dermatology, Stockholm (1995–1998)	Patch testing according to the Swedish standard series and a dental screening series. Testing for IgE-mediated allergy to natural rubber latex (NRL).	Hand eczema affected 63% of participants: 67% ICD, and 33% ACD. 59% of participants had positive reactions to standard series substances and 40% to the dental series; 22% had positive reactions to (meth)acrylates, primarily to multiple test preparations, including HEMA, EGDMA, and MMA. Allergy to NRL was recorded in 10% of patients.	Irritant hand dermatitis was the predominant diagnosis among dental personnel. Contact allergy to (meth)acrylate in around 20% of tested patients, mostly to 3 test substances: HEMA, EGDMA, and MMA. (Meth)acrylate allergy often coexisted with atopy and/or other contact allergies.
Goon AT et al., 2006 [14]	1639 subjects were patch-tested at the Department of Occupational and Environmental Dermatology, Malmo, Sweden	Patch tests for either dental patient series or dental personnel series including (meth)acrylate allergens and identification of common allergens and their prevalence in each group.	Positive patch tests for (meth)acrylate allergens were seen in 2.3% (30/1322) of the dental patients and 5.8% (18/310) of the dental personnel. The most common allergens for both groups were 2-HEMA, EGDMA, and MMA.	2-HEMA is an important screening allergen to detect contact allergy to (meth)acrylates used in the dental profession.
Isaksson M et al., 2007 [4]	A case report (dental nurse with facial eczema allegedly caused by airborne methacrylates in the workplace)	Patch testing with serial dilutions of several methacrylates and work provocations in methacrylate environments.	High reactivity to patch testing. Repeated exposure to methacrylates at work led to facial eczema; resolved when away from work. Efforts to collect the sensitizers using air pumps and filters failed. 37/122 patients showed a positive patch test with an extended (meth)acrylate series. 25 cases (67.6%) were occupational.	Facial dermatitis may be associated with airborne methacrylate exposure, which may involve allergy to $\geq$1 allergens.
Ramos L et al., 2014 [5]	An observational and retrospective study (January 2006–April 2013)	Evaluation and correlation of epidemiological and clinical parameters and positive patch test results for (meth)acrylates.	Hand eczema with pulpitis in 32 patients: 28 related to artificial nails, 3 to dental materials, and 2 to industrial work. Oral lesions associated with dental prostheses in 4 patients. 31/37 positive to >1 (meth)acrylate. Beauty technicians with artificial nails accounted for 80% of occupational cases.	HEMA detected in 80.6% of cases; may serve as a reliable screening allergen. A broader range of allergens is advisable for accurate diagnosis.

Table 1. *Cont.*

Author, Year [Reference Number]	Examinees/Patients	Analyzed Factors/Methods	Results	Conclusions
Muttardi K et al., 2016 [19]	A retrospective study of 241 patients who were patch-tested with meth(acrylates) and cyanoacrylates (January 2012–February 2015)	Patch testing with the mini-acrylate or extended acrylate series.	16/241 patients had positive patch-test reactions to (meth)acrylate or cyanoacrylate. Female predominance (M/F ratio of 1:15).	(Meth)acrylate allergy is mainly occupational, but more common in younger women, especially beauticians and nail technicians.
Havmose M et al., 2020 [2]	1293 female patients were patch-tested with HEMA	Two groups of patients based on their positive/negative patch test reactions to HEMA. MOAHLFA characteristics analyzed for both groups.	31 (2.4%) of the tested examinees tested positive for HEMA.	Sensitization and elicitation of ACD to HEMA primarily from artificial nail modeling systems; a significant health issue for consumers and certain professions.
Gregoriou S et al., 2020 [6]	156 female patients with ACD-using/performing cosmetic nail procedures (January 2009–December 2018)	The incidence of positive sensitization to (meth)acrylates assessed using patch tests.	Contact allergy to $\geq$1 (meth)acrylates in 74.4%: 88.5% occupationally exposed, and 11.5% consumers. A statistically significant increase in (meth)acrylate ACD from 2014 to 2018 (79%) compared with 2009 to 2013 (55%). EGDMA was the most common sensitizer positive in 72.4%. Among acrylate-positive patients, the rate was 97.4%.	A global trend of increasing (meth)acrylate sensitization among nail technicians and users of nail products with ACD. Enhancing preventive measures is essential.
Opaliñska S et al., 2022 [3]	8 women with CD related to acrylates found in hybrid varnishes	Manicure using a home acrylic nail kit and a non-professional UV lamp. Clinical and dermoscopic features were assessed.	Allergen contact areas (skin and nails) were affected. Severity correlated with exposure duration. Common findings: subungual hyperkeratosis and onycholysis (8/8 patients), eczematous finger pulp fissuring (2/8 patients) (more specific).	Nail changes from hybrid manicures may resemble onychomycosis or nail psoriasis (patch tests in uncertain cases). ACD was suspected. Confirmed acrylate allergies require patient awareness and avoidance.

Table 1. *Cont.*

Author, Year [Reference Number]	Examinees/Patients	Analyzed Factors/Methods	Results	Conclusions
de Groot AC, Rustemeyer T, 2024 [1]	24 studies presenting case series and 168 case reports on patients with ACD attributed to HEMA	Review of cross- and co-sensitization, atypical contact allergy manifestations, HEMA versus other (meth)acrylates, HEMA's screening sensitivity, and its presence in commercial products.	Strong cross-allergy exists between HEMA, EGDMA, and HPMA. Reactions to EGDMA often from primary HEMA sensitization. Rare atypical manifestations of HEMA allergy include lichen planus, lymphomatoid papulosis, systemic CD, leukoderma post-positive patch tests, and systemic side effects (nausea, diarrhea, malaise, palpitations).	HEMA is the most common patch test-positive methacrylate; an effective screening agent for other (meth)acrylates allergies. Sensitization to HEMA 2% pet. in patch tests is exceedingly rare.

Abbreviations: ACD—allergic contact dermatitis; CD—contact dermatitis; EGDMA—ethylene glycol dimethacrylate; F—female; HEMA—hydroxyethyl methacrylate; HPMA—hydroxypropyl methacrylate; ICD—irritant contact dermatitis; M—male; MMA—methyl methacrylate; MOAHLFA—male, occupational relevance, a history of atopic dermatitis, hand eczema, leg dermatitis, and facial dermatitis, age > 40 years; TEGDMA—triethylene glycol dimethacrylate; 2-HEMA—2-hydroxyethyl methacrylate.

Due to the frequent use of acrylic monomers in dentistry, dental workers (including staff, patients, and students) sometimes develop sensitivities, as indicated by a positive patch test. It is essential to identify these individuals and assess the significance of their allergies. In addition to proving sensitization to allergens by patch testing, it is necessary to examine the relevance of these reactions [65]. It is also crucial for all acrylics in products, even those in low concentrations, to be labeled.

Safety precautions for dental patients regarding acrylates are crucial to prevent allergic reactions, minimize exposure, and ensure overall health. Before using acrylate-based materials, dental practitioners should review a patient's medical history for any known allergies to acrylates or related compounds. This helps to identify patients at risk for allergic reactions [66]. When possible, dental practitioners should use acrylate materials formulated to minimize allergenic potential. Some newer formulations have reduced levels of residual monomers that can cause sensitization [67]. Acrylate materials should be handled and stored according to manufacturer instructions to prevent premature polymerization and reduce the risk of exposure to volatile compounds [68]. Dental staff should wear appropriate personal protective equipment (PPE), such as gloves and masks, to prevent direct skin contact with uncured acrylate materials. Patients should also be protected from direct skin contact with these materials [69,70]. Dental clinics should be equipped with adequate ventilation systems and fume extraction units to reduce the concentration of airborne acrylate vapors, which can cause respiratory sensitization [71]. Also, ensuring that acrylate materials are properly cured can significantly reduce the amount of residual monomer, thereby minimizing the risk of sensitization and allergic reactions. Furthermore, proper curing protocols should be followed as per the manufacturer's guidelines [72]. Finally, patients should be monitored for any signs of allergic reaction or sensitization after procedures involving acrylates. Immediate steps should be taken if any adverse reactions are observed [66].

7. Preventive Procedures

Health professionals need to be aware that skin reactions in the workplace can be due to acrylates and that applying preventive measures can avoid contact dermatitis [9]. PPE, such as protective gloves, goggles, masks, and disposable aprons should be used. Non-contact techniques are also important ways to avoid exposure [73].

One good option is the use of nitrile rubber gloves, which offer good resistance to MA penetration and lower monomer penetration rates. Nitrile gloves, as opposed to latex gloves, are a more effective option when exposure is shorter (15–20 min), as demonstrated by a patch test on glove fragments [11]. Gloves should also be changed frequently. Morgado et al. concluded that nitrile gloves should be changed during procedures after 30 min [74].

In the beauty industry, in the case of methacrylate contact from nail polish gel 4H (ethylene vinyl/alcohol polyethylene) finger covers can be used under regular gloves when the use of 4H gloves is not preferred [11,28,40,48]. Applying basic preventive measures consistently ultimately helps patients avoid having to change professions due to an allergy [9]. It should not be overlooked that adequate personal protection among students is also essential [10]. In recent years, the number of dental staff allergic to MAs has somewhat decreased due to the increased awareness that acrylates can cause sensitization [11,75].

Looking at the prevention of reactions to acrylates in cosmetics, many irritating reactions of the mucous membranes and skin of beauticians could be prevented with proper ventilation and PPE use [11,55]. Also, the quality of devices, such as UV devices, and following appropriate curing times for nail gel, which is higher when applied at home by untrained individuals, reduce the risk of sensitization to acrylates. [11,31].

Career counseling for a person with an adverse reaction to acrylates, including a potential allergy to acrylates, is also an important issue. Allergy to acrylates can significantly influence a person's ability to work in a particular job or profession [28]. The literature includes the case of a manicurist who, after only three months of work, had an allergic reaction to acrylates. Without understanding that acrylates are also present in dentistry, she became a dental nurse [11]. Shortly thereafter, her skin manifestations recurred due to exposure to 2-HEMA, 2-HPMA, EGDMA, and other acrylates. Thus, she had to change jobs again. Education about re-exposure risks in other industries could prevent reoccurrence and professional hardships [11,76].

Considering this problem, there are various other measures to consider. To improve the quality of acrylates, several techniques and approaches can be employed. Adjusting the conditions under which polymerization occurs, for example, can significantly impact the quality of acrylates. Factors such as temperature, pressure, and the presence of initiators or catalysts can also be optimized [77]. Incorporating cross-linking agents can improve the mechanical properties and stability of acrylates. In other words, cross-linking enhances the structural integrity of the polymer network [78]. Also, stabilizers, such as UV absorbers and antioxidants, can protect acrylates from degradation due to environmental factors [79]. Another possible solution could be altering monomer composition, such as by copolymerizing with other monomers, which tailors the properties of acrylates to specific requirements [80]. Additionally, removing impurities from monomers and polymers can improve the clarity and performance of acrylates [81]. Incorporating nanoparticles into acrylates can enhance their mechanical properties, thermal stability, and other characteristics [82]. Managing molecular weight and its distribution during polymerization can also affect the physical properties of the final product. Techniques such as controlled radical polymerization could also be used [83].

It is important to note that the tolerance exposure limit for acrylates can vary depending on the specific type of acrylate and the regulatory guidelines of different countries. Generally, exposure limits are set to ensure that workers and the general population are not adversely affected by the chemical. Many countries have established occupational exposure limits (OELs) for acrylates. These limits are often expressed as time-weighted averages (TWA) over an 8 h workday. For example, the OEL for methyl methacrylate is set at 50 ppm (parts per million) by OSHA in the United States [84]. Some acrylates also have short-term exposure limits (STELs), where higher concentrations are permitted for short periods (usually 15 min). For instance, the STEL for ethyl acrylate is 25 ppm [85]. Studies on the toxicity of acrylates indicate that prolonged exposure can cause respiratory and skin sensitization. Methyl methacrylate, for example, has been shown to cause respiratory irritation and ACD [86]. Regulatory agencies like the Environmental Protection Agency

(EPA) and European Chemicals Agency (ECHA) provide guidelines on safe handling and exposure limits. For instance, the EPA has set a reference concentration (RfC) for methyl methacrylate at 1 mg/m^3 based on respiratory effects [87]. Continuous monitoring of acrylate levels in workplaces is crucial. Techniques such as gas chromatography can be used to ensure that the concentrations remain below the set limits [88].

Alternatives with similar properties to acrylates are sought after in various industries due to concerns about acrylate toxicity, environmental impact, and specific application requirements. Epoxy resins, for example, are known for their excellent adhesion, chemical resistance, and mechanical properties. They are widely used in coatings, adhesives, and composite materials [89]. Polyurethanes offer versatility in terms of flexibility, durability, and resistance to abrasion and chemicals. They are used in coatings, foams, and elastomers [90]. Silicone polymers exhibit excellent thermal stability, flexibility, and biocompatibility. They are often used in medical devices, sealants, and coatings [91]. Vinyl ester resins provide good chemical resistance and mechanical properties, similar to acrylates. They are commonly used in corrosion-resistant coatings and composite materials [92]. Polystyrene and styrene acrylic copolymers can be tailored to achieve similar properties to acrylates, such as clarity, hardness, and chemical resistance. They are used in various applications, including coatings and adhesives [93]. Lastly, polyester resins are known for their durability, chemical resistance, and ease of processing. They are used in coatings, adhesives, and composite materials [94].

In addition, additives can significantly impact the properties of acrylates and potentially reduce unwanted skin irritations. Adding photoinitiators that allow for more complete curing of acrylate resins can reduce the presence of residual monomers, which are often responsible for skin irritation. When properly selected and balanced, photoinitiators ensure better polymerization [95]. Also, inhibitors and stabilizers can be added to acrylate formulations to reduce the likelihood of premature polymerization during storage and handling, which in turn reduces the risk of exposure to reactive monomers that can cause irritation [96]. The method of incorporating plasticizers can make acrylate materials more flexible and less prone to cracking, reducing the risk of skin irritation from rough or brittle surfaces [97]. Antioxidants can be added to acrylate formulations to prevent oxidation and degradation of the polymer, which can produce irritant by-products [98]. Another possible measure could be adding biocompatible modifiers, such as polyethylene glycol (PEG), which improve the biocompatibility of acrylates and make them less likely to cause skin irritation [99]. Also, formulations designed with lower monomer content or using high-purity monomers can reduce the likelihood of skin sensitization and irritation [100].

Therefore, for the successful retraining of people allergic to acrylates, it is crucial to consider the various industries where acrylates are present and how to work with modified materials and products [11,28,40,48,101,102]. Clinicians should be appropriately educated and trained to provide suitable patient care. For example, if a medical procedure is planned, patients allergic to acrylates require cementless arthroplasty and dental materials that do not release acrylates.

8. Ways to Deal with the Unwanted Effects of Acrylate Reactions and Treatment

After confirmation of an acrylate allergy, further contact with acrylates must be avoided. However, when dermatitis (eczema) does appear, it is necessary to treat the lesions adequately. Treatment primarily includes topical corticosteroid preparations and emollients, while, in resistant cases, immune-modulating calcineurin inhibitors (tacrolimus and pimecrolimus preparations) may be helpful. In severe cases, oral steroids may be used but only for a short time. Sometimes, secondary infections may appear and topical or oral antibiotics may be used. Other therapy options include phototherapy or immunosuppressive drugs [103].

9. Conclusions

Acrylates have become integral to numerous industries from their initial applications to their widespread use in various medical and cosmetic products. While their usage offers numerous advantages, the risk of adverse reactions, particularly ACD, should not be overlooked. This review highlights that allergic responses to acrylates, especially ACD, pose a significant health problem across various professions. In the dental industry, acrylates are prevalent in dental prostheses, composite resins, dentin bonding materials, and glass ionomers. Dental personnel are particularly at risk, with common manifestations including facial rashes, eczema with cracked skin on fingers, nail dystrophy, and periungual dermatitis. The beauty industry has also seen a rise in allergic reactions due to the increased use of acrylates in artificial nails, eyelashes, and hair extensions. Additionally, acrylates are used in medical applications such as bone cement for orthopedic endoprostheses, soft contact lenses, hearing aids, histological preparations, and wound dressings, which can also cause allergic reactions. Clinically, ACD due to acrylates presents as skin lesions, often on the face and hands, and can lead to significant morbidity. Diagnosis is primarily confirmed through patch testing, which remains the gold standard for identifying specific acrylate allergens. The frequent cross-reactivity among different acrylates necessitates comprehensive testing and preventive measures to avoid exposure. Preventive strategies are essential to mitigate the risk of ACD among individuals exposed to acrylates. These strategies include the use of appropriate PPE, such as nitrile gloves and protective eyewear, and modifications to work practices to minimize skin contact with uncured acrylates. Increased awareness and education about the risks associated with acrylate exposure are crucial for both professionals and consumers. Given the growing prevalence of acrylate allergies in both occupational and non-occupational settings, there is a pressing need for manufacturers to develop safer alternatives and improve the formulation of products to reduce sensitization risks. Future research should focus on identifying less allergenic substitutes for acrylates and further elucidating the mechanisms of sensitization to enhance prevention and treatment strategies. In sum, while acrylates provide significant benefits across various industries, the potential for adverse skin reactions necessitates vigilant preventive measures, thorough diagnosis, and continued research to safeguard the health of users and professionals alike.

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cosmetics

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Review

Harnessing the Potential of *Helinus integrifolius* in Cosmeceutical Research: Toward Sustainable Natural Cosmetics

Hlalanathi Y. Gwanya [1], Afika-Amazizi N. Mbuyiswa [2], Ompelege J. Phokwe [1], Nomagugu Gxaba [1] and Madira C. Manganyi [3,*]

[1] Department of Biological and Environmental Sciences, Botany Section, Walter Sisulu University, Nelson Mandela Drive, Mthatha Campus, Mthatha 5117, Eastern Cape, South Africa; hgwanya@wsu.ac.za (H.Y.G.); ophokwe@wsu.ac.za (O.J.P.); ngxaba@wsu.ac.za (N.G.)

[2] Department of Nature Conservation, Mangosuthu University of Technology, 511 Griffiths Mxenge Highway, Umlazi 4031, Durban, South Africa; mbuyiswa.naledi@mut.ac.za

[3] Department of Biological and Environmental Science, Sefako Makgatho Health Sciences University, P.O. Box 139, Ga-Rankuwa 0204, Pretoria, South Africa

* Correspondence: madira.manganyi@smu.ac.za; Tel.: +27-12-521-5843

Abstract: The growing demand for natural and sustainable ingredients in cosmetic formulations has driven scientists to explore a wide range of botanical resources. *Helinus integrifolius*, a lesser-known botanical entity, has recently emerged as a formidable contender in this field. This review explores the possibility of incorporating *Helinus integrifolius* in the cosmetics industry, synthesizing data from the literature on pharmacological, botanical, and cosmetic research. Additionally, this highlights the versatility and multipurpose nature of this botanical resource in cosmetic applications. Furthermore, concerns regarding the safety of contemporary cosmetic products, formulation compatibility, and regulatory aspects are addressed to accelerate its integration into cosmetic products. Ultimately, this review highlights the untapped potential of *Helinus integrifolius* as a treasured resource for the cosmetics industry, emphasizing its ability to meet the growing consumer demand for eco-friendly, effective, and naturally inspired cosmetic solutions. Through thorough exploration and analysis, this paper aims to stimulate further research and development initiatives aimed at harnessing the cosmetic capabilities of *Helinus integrifolius*, thus contributing to the advancement of innovation in natural cosmetics.

Keywords: botanical resources; cosmeceutical; *Helinus integrifolius*; pharmacological properties; natural

1. Introduction

The concept of beauty is deeply ingrained in society, and a universal standard for facial beauty that is based on ideal facial proportions is being established [1]. This standard is often pursued by using cosmetics, which are subject to quality control measures that ensure products meet specific standards [2,3]. The use of cosmetics, particularly for enhancing appearance, has been prevalent throughout history and is linked to the desire for ideal beauty and social competition [4]. Cosmetics play a significant role in enhancing appearance, protecting from harmful rays, and boosting confidence, hence the medicalization of female beauty [5]. The increasing popularity of cosmetic procedures further reflects the influence of societal beauty standards [5]. The global beauty industry, which includes cosmetics, is currently valued at $532 billion [6]; however, it is anticipated to increase to $758.05 billion by the year 2032, revealing a CAGR of 9.8% over the forecast period [7]. These figures indicate substantial growth and increased revenue for the cosmetics industry. However, there is a global shift towards more eco-friendly, sustainable, and green products that is driven by a growing awareness of the environmental impact of synthetic chemicals. The shift has led to a recent surge in consumer demand for natural and sustainable ingredients in cosmetics [8–12]. The global green cosmetics market is anticipated to increase at a CAGR of 5.1% from 2024 to 2029 [13]. Consequently, the

focus is largely on botanical resources, with cosmetic scientists and formulators increasingly leveraging the potency of natural compounds in skincare formulations [14,15]. Despite this trend, concerns about their safety have led to calls for more stringent regulations [9]. To address some of the concerns, over 60 amendments have been revised by Regulation 1223/2009. The Regulation 1223/2009 is a European Union regulation that governs the safety and labeling of cosmetic products sold within the EU market. It establishes a comprehensive framework for ensuring the safety of cosmetic products to protect consumer health [16].

The potential effectiveness of many botanical ingredients in skincare products is still being researched, with a need for more clinical trials to assess their biological effects [17]. Hence, leveraging the inherent potency of natural compounds in skincare formulations will provide a competitive edge. The use of medicinal plants in skincare formulations is gaining popularity due to their natural and beneficial properties [18]. Plant extracts in skincare products have been reported to have antioxidant, antimicrobial, and other biological effects, further supporting their potential in skincare formulations [19–21]. Among these botanical resources, *Helinus integrifolius* (Lam.) Kuntze, also known as Soap Bush, is a valuable botanical resource with significant potential in skincare due to its rich repository of bioactive compounds [22]. This plant has been traditionally used by indigenous African communities for its medicinal properties, particularly in treating dermatological conditions and enhancing skin health [23].

H. integrifolius is identified as a medicinal plant that is used by Xhosa communities in South Africa to manage skin disorders [24]. This is consistent with the use of herbal oils and natural emulsifiers in topical applications [25]. The potential of *Helianthus tuberosus* in treating atopic dermatitis, which is a chronic inflammatory skin disease, has also been explored [26]. These studies collectively accentuate the plant's therapeutic value, which is further highlighted by its diverse skincare benefits [27]. The potential of *H. integrifolius* in skincare is a promising area for further research and development. The use of *H. integrifolius* in natural cosmetics is in line with the growing demand for botanical-based solutions that prioritize efficacy, safety, eco-friendliness, and sustainability. This review seeks to unveil the rich potential of *H. integrifolius* as a natural, eco-friendly, and sustainable ingredient or product in cosmetic formulation innovations, regulatory considerations, and future prospects.

2. Botanical Characteristics

Helinus integrifolius (Lam.) Kuntze is a perennial climbing shrub that belongs to the family Rhamnaceae [28]. It is native to the tropical and subtropical regions of Africa and Asia. It is commonly found in thickets (along riverbanks and gassy areas), forest margins, woodlands, bushlands, dry savannah, rocky places, and elevated areas [29]. The common names of this plant include Soap Bush, Soap Creeper in English; Seepbos or Seepbossie in Afrikaans; Ubulawu Obude, Ukumbuqwekwe or ithyolo in isiXhosa; and uPhuphuphu, Uxuphukwekwe, or uBhubhubhu in isiZulu [28]. The genus name *Helinus* means tendril and refers to the fact that the shrub has climbing tendrils [29]. It is known for its distinct botanical characteristics. It has a hairless stem with coiled tendrils that help it climb to other plants for support [29]. *H. integrifolius* has simple, alternate, large oval leaves. They are elliptical to lanceolate in shape, with entire margins that are smooth with no indentations or serrations pressed with silky pubescence. (Hence, the specific epithet "integrifolius", meaning "with entire leaves"). *H. integrifolius* produces small, white, yellowish star-shaped flowers with five petals [30] as shown in Figure 1. They are born in pendulate umbels with a long peduncle and a short, slender pedicel that lacks showy petals but is nonetheless important for the plant's reproductive cycle. The fruits are three-lobed, with the calyx remaining on the tips. The seeds are small, black, and triangular [31]. Understanding the botanical characteristics of *H. integrifolius* is essential for both botanical enthusiasts and those interested in utilizing this plant for various purposes, including landscaping, conservation, and botanical as well as cosmeceutical research.



Figure 1. The botanical morphology of *Helinus integrifolius* (Photo by Mike Bingham) [32].

3. Sustainability in Cosmetics

There is a growing global need for natural resources to produce cosmeceuticals [33]. In many cultures around the world, using natural resources, especially plant material, for skin conditions and cosmetic purposes is a long-standing custom [34]. Natural treatments are not just for cosmetic purposes but also for therapeutic purposes aimed at curing different skin conditions [35]. Statistics South Africa states that while seeking treatment for common ailments, such as skin conditions, South Africans turn to both traditional healers and public health institutions [36]. Despite the ongoing disregard for traditional cosmeceuticals in favor of synthetic ones, many rural communities still use natural resources for skin health [33,37–39]. Alshammari et al. [40] state that "the move away from synthetic flavors and fragrances towards plant extracts, particularly for the food, drink, and cosmetic industries", is one reason why the usage of natural product extraction has grown in popularity recently. The market is expected to rise at a compound annual growth rate of 6.0% from 2021 to 2026, where it is expected to reach $55.3 billion [41]. The cosmetics industry is placing a greater emphasis on sustainability due to the increase in the need for natural ingredients derived from medicinal plants. Sustainability has become a cornerstone of the cosmetic industry, driven by increasing consumer awareness of environmental issues and a desire for eco-friendly products. Natural ingredients, such as those derived from *H. integrifolius*, play a pivotal role in advancing sustainability initiatives within the industry [42]. Therefore, *H. integrifolius* sustainability in cosmetics is a crucial aspect that aligns with the global trend towards sustainable practices in the cosmetics industry [43]. However, some businesses might find it difficult to adopt sustainability practices because of a lack of long-term planning, hierarchies, and investor overvaluation [44,45], although other businesses in the industry are progressively embracing long-term sustainable strategies, integrating green chemistry principles, and concentrating on sustainable supplier selection [46–48]. Rapid changes in environmental and socioeconomic conditions may result in the extinction of targeted plant species and the indigenous knowledge that goes along with them [35]. In this context, evaluating the ecological footprint becomes paramount.

3.1. Ecological Footprint

The harvesting and subsequent depletion of natural resources for human consumption is a global conservation and sustainability issue [49,50]. The depletion of natural resources not only negatively impacts biodiversity and water availability but also exacerbates the effects of climate change on the environment [49,50]. The increased use of medicinal plants such as *H. integrifolius* in the cosmetics industry has been shown to have a significant ecological

footprint, spanning from the harvesting of the plant, the extraction of bioactive compounds, the production and manufacturing of the cosmetic to the consumer pre and post use [51–54]. The unregulated harvesting of natural resources depletes the resource from the environment, thereby reducing biodiversity as well as the amount of plant material available for the absorption of carbon dioxide from the atmosphere. Information on the commercial cultivation and harvesting of *H. integrifolious* is limited, although numerous studies report on the harvesting of the plant from its natural environment in the wild [35,55,56]. To produce and manufacture cosmetic products from harvested medicinal plants, saponins are extracted from plant parts and, in the case of *H. integrifolius*, the entire plant is utilized [51,55,56]. The extraction process requires extensive electricity and water usage, thereby contributing to an electricity indirect greenhouse gas emission, which increases the carbon and water footprint [51,54,57]. Greenhouse gas emissions are further exacerbated during the transportation of the finished product due to emissions from transportation vehicles [57]. In addition to greenhouse gas emissions, chemical spills during the production process, and effluents deposited in water during use by consumers, further pollute the environment. This reduces the quality of ground and river water, thereby affecting access to safe and clean drinking water for humans. Polluted water may also lead to eutrophication, thereby affecting aquatic life [51,54,57].

3.2. Sustainable Practices

Numerous studies make reference to sustainability practices being employed in the entire process of product formulation, that is, from harvesting, manufacture, and packaging, to distribution, as shown in the case of the L'Oreal group of companies [50,52,57,58]. Although this is the case, emphasis is placed largely on sustainable production, manufacturing, distribution, consumer use and after-use practices, while research on sustainable harvesting is limited [51,57]. In addressing the biodiversity conservation concern, which impacts the carbon footprint, emphasis needs to be placed on regulating the sourcing of natural resources. Although *H. intergrifolius* and most of the medicinal plants that are used in the cosmetics industry are placed in the Least Concern category of the threatened and endangered species red list by the International Union for Conservation (IUCN; [56]), the continued use of these plants needs to be monitored to prevent overexploitation, given their demand in the quest for "green cosmetics". The harvesting of a portion of the plant for tissue culture and propagation, rather than the whole plant as in the case of *H. integrifolius*, should be explored. This would ensure that wild populations of these plants continue to thrive.

To limit energy consumption and reduce the carbon and water footprint, reports in the literature include the use of renewable energy, a reduction in greenhouse gas emissions, the recycling and reuse of water in a loop, the use of biobased ingredients and packaging materials from sustainable sources, the use of biobased formula ingredients derived from minerals or circular processes, the recycling of packaging plastic, and the recycling and reuse of generated waste, such as almond shells, orange peel and used cooking oil [52,53,58]. However, Rocca et al. [50] argue that looking at these aspects does not consider the social and economic standing of consumers, nor does it highlight standardized and precise regulations that pertain to sustainability. Table 1 emphasizes the sustainable qualities of *Helinus integrifolius*, making it an ideal candidate for use in natural cosmetics formulations.

Table 1. Sustainability attributes of *Helinus integrifolius* for natural cosmetics.

Sustainable Sourcing	Indigenous to tropical and subtropical regions, promoting local biodiversity—cultivated by indigenous communities using sustainable practices
Organic Certification	Traditionally grown without synthetic pesticides or fertilizers, ensuring purity and naturalness
Biodegradable Ingredients	Plant-derived compounds naturally degrade, minimizing environmental impact and waste
Eco-Friendly Formulations	Abundant in antioxidants and bioactive compounds, ideal for natural skincare formulations Cultivated using environmentally friendly agricultural practices
Renewable Resources	Utilizes renewable plant resources, reducing reliance on finite resources and supporting ecosystem health
Packaging	Plant-derived extracts can be incorporated into biodegradable packaging materials, reducing plastic waste
Certifications and Standards	*Helinus integrifolius*-derived ingredients can meet organic and natural cosmetic standards, ensuring quality and authenticity
Environmental Impact Assessment	Low environmental footprint due to sustainable sourcing and minimal processing, minimizing ecological harm
Transparency and Communication	Ingredient transparency through clear labeling and communication fosters consumer trust and informed choices

4. Limitations and Drawbacks of Contemporary Cosmetic Products

Cosmetics contain organic, inorganic, or synthetic ingredients that enhance their effectiveness to meet certain social or peer-approved standards. However, some of the ingredients pose a threat to human health and the environment. The study conducted by Sheikh [59] highlighted 10,500 harmful synthetic ingredients used in cosmetics, whereas the study by Abed et al. [60] indicated 70,000 chemicals currently used in cosmetics, an extreme increase in the number of synthetic chemicals used in cosmetics over an 8-year period. In addition, an estimated 1000 new compounds are expected to be introduced every year. Surprisingly, it has been brought to our attention that about 7500 cosmetic products contain more than one ingredient that has never been evaluated or tested for safety, toxicity, or permissible allowance [59]. It is reported that in the United States, there are approximately 12,500 different compounds used in cosmetics. Among these, only 2500 are claimed to be safe for use. Still, a large number of the remaining compounds have been identified as unsafe, with only 11 of them currently being restricted [60]. Naveed [61] has mentioned that a large group of harmful ingredients persists in the cosmetics industry, which shockingly includes 100 carcinogens and 15 endocrine disruptors.

Furthermore, a number of these chemicals pose health complications, such as carcinogens, pesticides, reproductive toxins, endocrine disruptors, plasticizers, degreasers, and surfactants. While some chemical ingredients may only cause minor irritations or allergic reactions, others are associated with more severe health issues, such as cancer, hormonal disruptions, immunosuppressants, tumor promoters, and reproductive problems [60–62]. It is important to consider that in the process of manufacturing, in addition to the chemicals used, synthetic preservatives are also used, which can have negative effects on human health. These preservatives can cause chronic symptoms, such as cardiovascular disorders, hypersensitivity, an estrogenic effect, endocrine disruption, pulmonary irritation, carcinogenicity, neurotoxicity, cytotoxicity, antibiotic resistance, sensitization, and more [63].

The skin is the body's largest organ, protecting the entire body and interacting directly with cosmetics of various types. The interactions may result in the absorption of these active ingredients into the body, which may potentially cause harm and contribute to health complications. Certain cosmetics are associated with provoking negative effects on one's overall health over time or through continuous exposure. This is because the toxic chemical ingredients in these products can accumulate in the bloodstream, disrupt hormones and cell functioning structures, and increase the risk of developing chronic diseases, such as cancer [62]. The repeated application of certain cosmetic products may result in irritation or complications.

In the study conducted by Sullivan et al. [62], it was found that eye makeup can directly cause ocular surface and adnexal diseases, leading to various symptoms such as infections, inflammation, allergies, trauma, neoplasia, dysfunction, and degeneration. In a survey of 10 makeup cosmetics, prominent symptoms such as toxicity, cytotoxicity, allergens, irritants, immunosuppressants, carcinogens, endocrine disruptors, neurotoxins, meibomian gland dysfunction, and antibiotic resistance were identified. It is important to note that the above-mentioned acute disease symptoms were only collected from a survey

of 10 eye cosmetics. However, it is worth considering that there are many other cosmetic products that people use every day alongside eye makeup, such as toothpaste, body lotion, perfume, hair conditioner, aftershave, lipstick, and nail polish; people that have tattoos must also be considered. All of these products contain different synthetic chemicals that could potentially lead to adverse health effects and negatively affect the environment.

As reported in many studies, including those by Sullivan et al. [62], the current cosmetic products are produced with several chemicals that endanger humans and the environment. These compounds cause chronic conditions like neurotoxicity, endocrine disruptors, meibomian gland dysfunction, and toxicity to epithelial cells. Different heavy metal ingredients have been associated with the potential to cause definite or varying acute or chronic conditions; as such, exposure to heavy metals like cobalt, mercury, beryllium, and chromium is associated with chronic health (cancer, neurological, and reproductive complications) or other non-cancerous health disorders that include impaired neurological, immunological, respiratory, and reproductive functioning [60,64].

In the past few decades, cosmetic production has invested in heavy metal ingredients that ultimately contribute to symptomatic conditions, including toxicity of different kinds, depending on the types of metals involved and the fact that they easily penetrate the skin [60]. Heavy metals such as aluminum, lead, cadmium, mercury, chromium, cobalt, manganese, copper, titanium, nickel, zinc, iron, and arsenic are classified as harmful metals that are associated with causing acute toxicity and poisoning that leads to disorders in the nervous system, blood, lungs, kidneys, and liver [60,65]. It has been observed that cosmetic products contain significantly higher levels of certain metals compared to our diet. The accumulation of metals in the skin leads to several health issues, such as cancer, allergies and neurological problems, and can cause birth defects. This, therefore, raises concerns about the safe utilization of these cosmetic products with harmful substances [65].

Recently, in a study by Abed et al. [60], cosmetic products contained heavy metals at levels that exceeded the permissible limit set by the Food and Drug Administration. Some examples of these metals include mercury at 65 ppm, lead at 10 ppm, arsenic at 3 ppm, and chromium at 50 ppm. However, the permissible limits of these metals may vary depending on the specific cosmetic product. Inorganic compounds are other elements that have generated interest for use in cosmetic production due to their broad photoprotective or UV filters that offer dual protection against UVB and UVA radiation [66]. Users are increasingly opting for cosmetic products that contain inorganic compounds instead of organic ones, despite being unaware of the potential risks involved. This, therefore, means that humans and the environment will suffer as users prefer products that offer more activity, and producers will always manufacture and introduce new chemicals to enhance effectiveness or relevance to the demand or market. The application of skincare products containing heavy metals, synthetic chemicals, or inorganic compounds can cause the generation of reactive oxygen species, which can ultimately lead to skin damage and a loss of elasticity [59,66]. Hydroquinone is a depigmenting agent in skin-lightening cosmetics that causes irritations, redness, burning, and ultimately exogenous ochronosis that is triggered by extreme cytotoxicity to melanocytes and potentially linked with mutagenic mammalian cells [65]. Other compounds, like black henna, would cause symptoms like blisters, oozing, swelling, and rashes.

The improper disposal of cosmetic products can release heavy metals into waterways and soil, leading to severe environmental impacts [60]. Numerous studies have concluded that people frequently use cosmetic products that contain complex chemical compounds that directly interact with the skin, penetrate it, and leach into the blood, causing both carcinogenic and non-carcinogenic health disorders, as well as chronic or acute conditions. Harmful ingredients are present at high levels in the soil, drinking water, and the food we consume. The combined effect of these compounds increases exposure not only to humans but also to animals and the environment. Exposure to inorganic, heavy metal, and synthetic chemicals in cosmetic products can be minimized by adjusting manufacturing and preservation techniques or toxic chemical compounds. It is highly recommended to

consider using natural-based products that are safe, with chemical ingredients that are regulated and tested for safety.

5. Cosmetic Formulation Innovations (Natural Products)

H. integrifolius has been traditionally used in the treatment of stroke, leg pain, hysteria, nervous system ailments [34,67] and as a soap substitute for the treatment of hair loss and skin disorders [25,34,35,55,68]. This herb, like most soapy plants, foams when agitated in water [68]. The soapy properties are attributed to the presence of saponins [68]. Saponins are amphyillic glycosides characterized by hydrophobic aglycones attached to varying numbers of hydrophilic sugar chains [55,68]. Saponins from *H. integrifolius* are not only used as foaming agents, as they can also be utilized as natural washing and emulsifying compounds, and skin health-enhancing agents [68]. The hydrophilic sugar moieties are usually in the form of either hexose or pentoses [56]. The phytochemical studies revealed that the type of saponins in *H. integrifolius* were triterpenoid and steroidal saponin [55]. However, this depended on the type of aglycone they carried [67]. Most of these saponins accumulated in roots rather than in the other parts [55]. Both saponins have been reported to contribute to skin hydration [69,70]. They achieve this by reinforcing the skin's natural barrier and interacting with lipids in the stratum corneum or by using their hydrophilic sugar moieties to attract and retain water [69]. Steroidal and triterpenoid saponins have attracted significant attention as important anti-inflammatory compounds which inhibit pro-inflammatory mediators (e.g., IL-1β, TNF-α and COX-2) and the NF-kB pathway, thereby calming irritated skin [71,72]. Several plant-derived saponins have been reported to exhibit a collagen synthetic property, which improves skin firmness [73]. They accomplish this by stimulating fibroblasts, which are responsible for producing collagen and thereby enhancing skin firmness and elasticity [69,71]. The other way this is achieved is by inhibiting collagen-degrading enzymes [70]. These saponins also have antimicrobial activity [56,71], wound healing, and skin regeneration properties; hence, they can be used to heal eczema and psoriasis [73,74]. These saponins also have a skin lightening capability, wherein they inhibit tyrosinase and reduce melanin production, leading to a more even skin tone [69]. This is also achieved by applications such as those of antioxidants and cytotoxic activities [70]. The presence of saponins in *H. integrifolius* is a groundbreaking discovery that unequivocally demonstrates the plant's potential for cosmeceutical applications. Its multifunctionality, ranging from formulation benefits to skin health improvement, make it an attractive natural ingredient that people can use in skincare products.

This plant has other key innovations in traditional cosmetic formulation, although the literature on commercial cosmetic products derived from *H. integrifolius* is limited. Numerous chemical compounds with possible health benefits are found in the plant species *Helinus integrifolius*, which has been investigated for its medicinal qualities. According to studies conducted on *Helinus integrifolius*, the leaves contain triterpene 1, squalene, spinasterol, oleanolic acid, phytol, and lutein; the stems contain squalene and spergulagenin A, and the roots contain spinasterol [75]. The aforementioned compounds demonstrate the potential of *Helinus integrifolius* as a bioactive compound exhibiting a wide range of pharmacological activities.

Helinus integrifolius, particularly the stem bark, has been traditionally used for treating various skin infections [25,76]. In addition to stem bark, the leaves are cited as the most used plant part for traditional concoctions using *H. integrifolius*, due to ease of preparation for treatment. Leaves are crushed and mixed with water for a softer consistency [34,35], Shai et al., 2013 [35]. The entire plant, consisting of roots, stems, and leaves, is also used to form a poultice for treating acne [35]. In some instances, the whole plant is harvested, although only the leaves or roots are required [77]. Poultices are formed from mixing either roots, leaves or stem bark with water and applied as a topical [35]. In African folklore, roots and bark are used to form a spiritual connection with ancestors and to induce dreams [77]. The reason this plant is used to treat skin ailments is the fact that it is rich in antioxidants, such as phenolic compounds. The plant-derived phenolic antioxidants help neutralize free

radicals, protecting the skin from oxidative stress and premature aging [70]. *H. integrifolius* was among the plant species that have been used in the treatment of skin-related issues, including wounds [35]. The bark extract of *H. integrifolius* comprises bioactive compounds (friedelin and lupeol), which have shown significant wound healing properties by reducing the wound area and enhancing keratinocyte migration [78]. Additionally, *H. integrifolius* root and leaf extracts have shown significant activity against skin disorder, inducing bacteria such as Staphylococcus typhi [79] and Staphylococcus aureus, fungi that include Candida albicans [80] as well as diabetes-inducing enzymes [81].

By using consistent manufacturing procedures and logical formulation development, formulations containing *Helinus integrifolius* can demonstrate reproducibility. A few crucial actions must be taken in order to guarantee the reproducibility of *Helinus integrifolius* formulations and produce consistent outcomes throughout manufacturing. Initially, to ensure constant concentrations of active ingredients, standardized extraction techniques and strict quality control procedures must be put in place. Standardizing active ingredient concentrations and guaranteeing the consistency of raw material sources both contribute to uniformity. Consistency and control over all aspects of production are guaranteed when Good Manufacturing Practices (GMP) are followed [82]. It is confirmed that the active ingredients continue to work overtime by performing formulation stability testing. Comparing new batches with older ones to make sure the same quality is maintained requires batch-to-batch consistency checks. Tracking any variations and taking quick corrective action is also possible by keeping complete documentation and traceability [83]. Producing consistent, high-quality *Helinus integrifolius* formulations is ensured by manufacturers in adhering to these guidelines.

Such cosmetic formulations incorporating African olive extract can, therefore, contribute to maintaining skin health and vitality. *H. integrifolius* has been implicated in the treatment of hair loss [77]. Innovative cosmetic formulations leveraging the diverse benefits of *Helinus integrifolius* are poised to revolutionize skincare routines, offering consumers effective solutions for maintaining healthy, radiant skin [25]. Similar to other plants, *Helinus integrifolius* poses certain challenges when used in formulations. These challenges include the need to ensure the quality and purity of raw materials, manage plant composition variability due to environmental factors, and standardize extraction techniques to maintain constant concentrations of active compounds [84]. There are also a lot of obstacles to overcome in terms of these compounds' stability, formulation compatibility with other ingredients, and regulatory compliance [85]. Strict quality control must be maintained throughout production in order to achieve consistent efficacy and consumer acceptance. Notwithstanding these obstacles, if they are methodically resolved, the plant's potential for secure and efficient use in supplements and cosmetics can be realized.

6. Regulatory Considerations

A body of evidence has explored regulatory frameworks and guidelines related to sustainable sourcing and the use of botanical ingredients in cosmetics. The European Union (EU) regulatory framework is cited as governing the use of botanical food supplements (BFS) and herbal medicines (HM), ensuring their safety and quality while taking into account potential interactions between botanical ingredients, HM, and traditional medicines [71–73]. Furthermore, the development of the Cosmetics Sustainable Supplier Selection (C-SSS) model, which assesses suppliers in an unbiased manner, has been driven by the need for a long-term sustainable strategy in the cosmetics industry. Although specific guidelines for sustainable sourcing and use of botanical ingredients in cosmetics are not explicitly mentioned, the EU regulatory framework and the C-SSS model provide essential considerations for promoting sustainability in this area [47].

International standards, such as ISO 16128, developed by the International Organization for Standardization (ISO), provide directives for natural and organic cosmetic ingredients, incorporating sustainability criteria [74]. In addition, certification programs like COSMOS (Cosmetic Organic and Natural Standard) ensure compliance with rigorous

standards for organic and natural ingredients, as well as sustainable sourcing and manufacturing practices [86]. Regulatory bodies, such as the European Commission's Scientific Committee on Consumer Safety (SCCS) and the U.S. Food and Drug Administration (FDA), are responsible for evaluating the safety of cosmetic ingredients, including botanicals, while considering their environmental and sustainability aspects. Moreover, ethical sourcing and fair-trade practices are emphasized in certain regulatory frameworks, with certifications such as fair trade ensuring equitable remuneration for producers and adherence to environmental and social standards [87].

The regulatory landscape for saponins and other compounds in *Helinus integrifolius* involves a complex interplay of global regulatory considerations. Herbal preparations can be registered as botanical drugs or sold as dietary supplements in the United States. In contrast, the medicinal product laws of Europe have created a regulatory class known as "herbal medicinal products". Based on the therapeutic good/drug model, Australia and Canada have implemented a comprehensive approach that includes the majority of herbal and traditional medicines. Asian countries have created distinct legal frameworks to accept traditional medical practices such as traditional Chinese medicine (TCM) and Ayurveda. Certain products used in traditional medicine can be altered and sold in India thanks to hybrid categories [88].

Saponins, such as those found in *Helinus integrifolius*, have been identified as key phytotherapeutic principles with significant molecular bases for their antidiabetic, anti-obesity, and plant growth regulatory effects [89]. The global demand for herbal biomolecules underlines the importance of stringent regulatory policies to ensure quality, safety, and efficacy in the marketing of herbal drugs, including those derived from *Helinus integrifolius* [90]. The lack of standardization, the misidentification of plant species, contamination, adulteration, uneven dosages, interactions with medications, and adverse effects are among the safety issues with herbal medicines [91]. Strict quality control testing, the standardization of active ingredient concentrations, clinical trials and scientific testing to assess safety and efficacy, unambiguous labeling with details on ingredients, dosages, side effects, and drug interactions, and post-market surveillance to monitor safety after products are on the market are some of the regulatory measures used to address these problems. By following federal guidelines, these precautions help guarantee that herbal medicines are safe to use [92]. Furthermore, the need for improved methods to enhance the production and diversification of saponins in plants like *Helinus integrifolius* highlights the potential for metabolic engineering to address challenges related to limited yields and resource availability.

Furthermore, labeling requirements mandated by regulatory agencies necessitate the accurate disclosure of botanical ingredient sourcing and origins, enabling consumers to make informed decisions and support sustainable practices [93]. The importance of supply chain traceability is growing, as it is crucial for ensuring transparency and accountability in ingredient sourcing practices. This involves the comprehensive documentation of the entire supply chain, extending from procurement to production and distribution [86]. In summary, following regulations and guidelines is essential for promoting the responsible sourcing and utilization of botanical ingredients in cosmetics, which in turn supports environmental preservation, the protection of biodiversity, and ethical trade practices.

7. Comparative Analysis

As previously highlighted, cosmetic production and active ingredients include a variety of synthetic and organic chemicals, inorganic compounds, and heavy metals that negatively impact human health and the environment [62]. A body of evidence has highlighted the negative effects of using synthetic cosmetic products with various forms of reactions (internally or externally), and this raises major concerns [94,95], including alignment with the market requirements and recommendations, including the use of herbal cosmetics. Current research on *H. Integrifolius* is limited to indigenous knowledge systems in traditional cosmetics and treatments as well as spiritual significance to sangomas [35,55,67]. Although the plant has

been evaluated for the presence of bioactive compounds [79] that have the potential to reduce the incidence of acne-causing bacteria and the prevalence of diabetes [75], scientific evidence on the full potential of the plant is still lacking. Additionally, no commercial cosmetic products specifically formulated from *H. integrifolius* are readily available or stipulated in the literature. This highlights the need for further biochemical research on the plant to accentuate its traditional benefits scientifically. There is a growing demand for herbal cosmetics that is triggered by vital benefits that include product safety, effectiveness, no adverse effects, or multiple benefits due to a complex package of bioactive compounds [96]. Bioactive compounds in herbal products are hypoallergenic and compatible with sensitive skin. Additional benefits include an improvement in sleep, endurance, emotional and physical health, as well as boosting the immune system, aiding in digestion, and reduced weariness [97–99]. The literature sources demonstrate that synthetic cosmetics have ingredients that are not sustainable and pose a threat to cosmetic society or users, and their non-sustainability also poses a threat to the health environment [63,96,98]. Figure 2 highlights the various adverse effects of synthetic cosmetics and organizes them into distinct categories related to skin and overall health.



Figure 2. Negative effects of synthetic cosmetic products: categorized impacts on skin and health.

Alternatively, herbal cosmetics have been revealed to have beneficial contributions to developing countries and poor people. In essence, herbal cosmetics offer an eco-friendly, pure, stable, and renewable form compared to synthetic cosmetics [99]. However, there are limited studies conducted to test or evaluate the cosmetic efficiency and the application of the active compounds identified in *H. integrifolius*. Moreover, the plant's bioactive compounds are worth investigating, as there is a growing demand for herbal cosmetics. This, therefore, requires an evaluation of the bioactive compounds of *Helinus integrifolius*, as the cosmetics industry requires an increasing amount of new and evolving bioactive compounds to address safety concerns in the application of cosmetics, especially herbal cosmetics, as shown in Table 2.

Table 2. Summary of various skin treatments offered by *Helinus integrifolius*.

Active or Bioactive Ingredients	Skin Conditions or Disorders	References
Friedelin Lupeol	Treatment against hair loss	[25]
	Soap application (skin issues)	[35]
	Hair loss and skin infections	[83]
	Treat sandworm infections Used as an emetic and soap substitute	[100]
	Inflammation, antioxidants and pains	[101]
	Various skin conditions Canine demodicosis treatment	[102]

8. Future Prospects

Current research on medicinal plants in cosmeceutical research has made significant strides in highlighting the therapeutic benefits of plants in natural cosmetics [103]. However, gaps exist in the field, including a lack of standardized methods for quality control and consistency in active ingredients [104], an insufficient understanding of the biological mechanisms of action, and limited long-term safety and efficacy studies [105]. Additionally, research on the bioavailability and skin absorption rates of active compounds is inadequate, and interactions between medicinal plant extracts and other ingredients are not well studied [106,107].

In order to progress the application of *Helinus integrifolius* in cosmetics, it is imperative to support investigations that tackle open-ended issues and establish a stronger scientific basis. Given its enormous biological potential, *Helinus integrifolius* is an excellent option for eco-friendly cosmetics that are both sustainable and suitable. To guarantee that the active ingredient concentrations in *Helinus integrifolius* extracts are constant, research should concentrate on standardizing quality control procedures. In-depth research on the biological processes that this plant uses to improve skin health is required in order to create cosmetic formulas that are more precise and potent [108]. Long-term safety and efficacy research will also offer important insights into the long-term advantages and possible drawbacks of *Helinus integrifolius* in cosmetic products. Product performance can be improved by maximizing the bioavailability and absorption rates of its active ingredients through sophisticated formulation techniques [109]. Furthermore, developing safe and effective formulations will be made easier by knowing how *Helinus integrifolius* extracts interact with other cosmetic ingredients. To ensure environmental responsibility, a focus on sustainable cultivation, harvesting, and processing techniques is necessary [110]. Finally, carrying out carefully planned clinical trials will produce strong proof of *Helinus integrifolius*'s safety and effectiveness, encouraging regulatory approval and consumer confidence. Taking up these research topics will be important.

As sustainability becomes increasingly integral to the cosmetic industry, the exploration of botanical resources like *Helinus integrifolius* for eco-friendly formulations presents promising avenues for future development. It is crucial that, while using non-crop plant species, conservation is taken into consideration. Training on how to use fruits and leaves should be part of these methods [57]. The attainment of effective biodiversity protection necessitates the persistent efforts of all parties involved, particularly rural communities, whose livelihoods are directly dependent on the local biodiversity [111]. A study by Chigor [112] emphasizes the significance of protecting our native plant resources from extinction, since some of them may be pharmacologically active or have unique chemicals with biological activity against specific ailments. The intensive harvesting and overexploitation of these plants have resulted in a decline in some species and extinction in others, with a general decrease in biodiversity in high-use areas [113–115]. Some plant traders do not harvest sustainably; therefore, they need to be educated on responsible harvesting techniques, as heavy harvesting leads to the scarcity of popular medicinal plant species like *Helinus integrifolius* [113].

The preservation of *Helinus integrifolius*, a species of considerable conservation concern, is influenced by various ecological factors. Grazing intensity has been identified as a critical

determinant of its conservation status, as noted by Yang et al. [111]. Research by Edelstein and Shriberg [112] underscores the importance of heavy grazing in Sweden for enhancing germination rates and seedling survival of this species. However, habitat fragmentation and the reduction in natural disturbances like wildfires pose significant threats to its habitat, as highlighted by Olaokun and Ntini [75]. Chauhan and Mahajan's [113] findings emphasize the vulnerability of *Helinus integrifolius* to mortality during the growing season, particularly under drought conditions, necessitating tailored management practices. Conservation agriculture (CA), as advocated by Widén [114], offers a holistic approach to sustainable productivity enhancement and natural resource preservation. Kassam [115] further argues for the integration of such conservation strategies to support biodiversity and ecosystem health. Despite its endangered status, *Helinus integrifolius* has been assigned an automated status of Least Concern due to a limited detailed assessment and exclusion from screening processes, as noted by Foden and Potter [29].

For the large-scale conservation and propagation of medicinally significant and endangered plants, in vitro culture techniques provide incredibly effective instruments [116]. A study conducted by Ndawonde et al. [116] identified unthreatened and threatened plant species sold by plant traders in Zululand to select species for propagation in communal gardens. In this way, plants like *H. integrifolius* can be sustained. Most of the harvesting is carried out by the community; therefore, their knowledge and perception regarding plant availability are important because they would contribute to a better understanding of plant conservation at the local level [117]. Conservation used to be carried out using law enforcement, but it became futile; therefore, propagation has become necessary [109,118].

It is recommended that future research on *Helinus integrifolius* concentrates on standardizing extraction techniques, comprehending its biological mechanisms, and carrying out long-term safety and efficacy trials. Comprehensive clinical trials, ingredient interactions, bioavailability optimization, and the promotion of sustainable practices should all be the focus of future research. Through these initiatives, its use in sustainable, safe, and effective supplements and cosmetics will be improved. Closely addressing these gaps will improve its application in creative, potent, and environmentally friendly cosmetics.

9. Conclusions

In conclusion, the exploration of *Helinus integrifolius* as a botanical resource for the cosmetics industry reveals promising avenues for sustainable and effective formulations. By conducting a thorough analysis of its pharmacological, botanical, and cosmetic characteristics, this manuscript highlights the capacity of *Helinus integrifolius* to address the rising need for natural and environmentally friendly cosmetic remedies. The discussion around safety, compatibility in formulation, and adherence to regulations in this critique promotes the inclusion of *Helinus integrifolius* in cosmetic merchandise. The emphasis on its adaptability and versatility in this article draws attention to the unexplored possibilities of *Helinus integrifolius* in reshaping the cosmetics industry. As consumer preferences increasingly prioritize sustainability and natural ingredients, the utilization of *Helinus integrifolius* represents a significant step towards meeting these demands. Ultimately, this review seeks to inspire further research and development efforts aimed at unlocking the full cosmetic capabilities of *Helinus integrifolius*, thereby propelling innovation in natural cosmetics and contributing to a more sustainable future.

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cosmetics

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Review

Regenerative Cosmetics: Skin Tissue Engineering for Anti-Aging, Repair, and Hair Restoration

Paula Pleguezuelos-Beltrán [1,2,3,4], Sara Herráiz-Gil [5,6,7,8], Daniel Martínez-Moreno [1,2,3,4], Iria Medraño-Fernandez [9], Carlos León [5,6,7,8] and Sara Guerrero-Aspizua [5,6,7,8,*]

1 BioFab i3D Lab-Biofabrication and 3D (Bio)printing Laboratory, Department of Human Anatomy and Embryology, Faculty of Medicine, University of Granada, 18016 Granada, Spain; paulapb97@go.ugr.es (P.P.-B.); daniel0mmd@go.ugr.es (D.M.-M.)
2 Excellence Research Unit "Modeling Nature" (MNat), University of Granada, 18016 Granada, Spain
3 Instituto de Investigación Biosanitaria ibs.GRANADA, University of Granada, 18016 Granada, Spain
4 Biopathology and Regenerative Medicine Institute (IBIMER), Center for Biomedical Research (CIBM), University of Granada, 18016 Granada, Spain
5 Department of Bioengineering, Carlos III University, UC3M-IISFJD-CIEMAT-CIBERER, Av. de la Universidad 30, Leganés, 28911 Madrid, Spain; sherraiz@ing.uc3m.es (S.H.-G.); cleon@ing.uc3m.es (C.L.)
6 Network Research on Rare Diseases (CIBERER), U714, C/Melchor Fernández Almagro, 3, 28029 Madrid, Spain
7 Health Research Institute-Jiménez Díaz Foundation University Hospital (IIS-FJD), Av. de los Reyes Católicos, 2, 28040 Madrid, Spain
8 Centre for Energy, Environment and Technology Research (CIEMAT), Av. Complutense 40, 28040 Madrid, Spain
9 Department of Neurosciences and Biomedical Sciences, Faculty of Health Sciences, Carlos III University, Calle Madrid 126, Getafe, 28903 Madrid, Spain; imedrano@salud.uc3m.es
* Correspondence: sguerrer@ing.uc3m.es; Tel.: +34-914968206

Abstract: The quest for youthful, healthy skin and full, vibrant hair has long been a driving force in the dermocosmetics field. However, traditional approaches often struggle to address the underlying causes of aging, damage, and hair loss. Regenerative cosmetics powered by skin tissue engineering offer a transformative alternative. This review explores the emerging field of using engineered skin tissues for cosmetic purposes, focusing specifically on their potential for anti-aging, repair, and hair restoration applications. We discuss how these technologies aim to rejuvenate aging skin by promoting collagen production, reducing wrinkles, and improving overall skin function. Additionally, the use of engineered skin for wound healing and scar reduction is examined, highlighting their potential to improve the appearance and functionality of damaged skin. Finally, we advance the exciting prospects of utilizing skin tissue engineering techniques to regenerate hair follicles, potentially offering solutions for hair loss and promoting denser hair growth.

Keywords: regenerative cosmetics; skin; tissue engineering; hair restoration; hair follicles; anti-aging; regenerative medicine

1. Introduction

The pursuit of rejuvenating skin and hair to achieve a fresh, younger look has been a longstanding motivation for the cosmetics industry. However, conventional treatment strategies frequently target only the most apparent manifestations and not the core causes of deterioration and aging. This review delves into an exciting new wave of cosmetic solutions based on tissue regeneration. Regenerative cosmetics, powered by the cutting-edge field of skin tissue engineering (TE) and all related fields, offer a transformative alternative with the potential to revolutionize the way we care for our skin and hair.

1.1. Importance of Healthy Skin and Hair in Society

The skin, a complex organ with multiple layers, plays a crucial role as the body's first line of defense, protecting us from harmful environmental factors like UV radiation, pathogens, and allergens. It also plays a vital role in regulating body temperature and maintaining hydration [1]. It is a key target for dermocosmetic products aimed at enhancing skin health and appearance [2]. Additionally, healthy hair is a protective barrier for the scalp and helps regulate heat loss [3].

The condition of our skin and hair significantly impacts our psychological well-being and social interactions [4,5]. Radiant and healthy skin and hair can boost our confidence and self-esteem, contributing to a positive self-image [6]. Regardless of its cause or severity, hair loss is a distressing condition often underestimated. It encompasses various pathogeneses, including androgenetic alopecia (AGA, the most widespread type of hair loss), alopecia areata, diffuse alopecia, and therapy-induced hair loss. Despite treatment advancements, affected individuals commonly face stigmatization, which may exacerbate psychological conditions and diminish overall contentedness [7]. Concerns about blemishes, wrinkles, or hair loss can lead to insecurity and social anxiety, impacting our quality of life and interactions with others [8]. Therefore, maintaining healthy skin and hair is not only essential for physical health but also contributes significantly to our overall well-being and social integration.

1.2. Limitations of Traditional Cosmetic Approaches

Traditional cosmetic approaches primarily focus on superficial effects. They operate on the surface of the skin or hair, offering temporary improvements like hydration, masking imperfections, or providing color. These products often lack the ability to address the underlying biological processes that contribute to aging, damage, or hair loss [9,10].

Furthermore, the effectiveness of traditional cosmetics can vary significantly depending on individual factors like age, skin type, and the severity of the concern. Additionally, some individuals may experience adverse reactions to certain ingredients, limiting their suitability [11] or requiring continuous use to maintain the desired effects, leading to inconvenience and cost burdens for consumers [12].

Finally, traditional testing methods for these products, such as animal testing and two-dimensional (2D) cell cultures, have significant drawbacks. Animal testing raises ethical concerns and often fails to represent human skin physiology accurately. Similarly, 2D cell cultures lack the complexity of three-dimensional (3D) tissues, hindering the evaluation of product safety and efficacy [13,14].

1.3. The Promise of TE-Based Dermocosmetics

Dermocosmetics, formulated with ingredients that target specific biological pathways or are based on TE principles, offer a more directed approach than traditional cosmetics. TE is a rapidly evolving field that combines the three well-known key components (cells, scaffolds and signals). Some of them are included in Table 1, summarizing relevant research data from the literature for each of these key components.

Table 1. Key TE components that have an important role in dermocosmetics applications.

Component	Description	References
Cells	**Adult stem cells**: – Mesenchymal stem/stromal cells (MSCs) – Adipose-derived stem cells (ADSCs) **Differentiated skin cells**: – Fibroblasts – Keratinocytes	[15,16]

Table 1. *Cont.*

Component	Description	References
Scaffolds	**Natural scaffolds**: – Collagen – Gelatin – Silk – Chitosan – Hyaluronic acid (HA) **Synthetic scaffolds**: – Polyethylene glycol (PEG) – Polyurethane (PU) – Polyglycolic acid (PGA) – Polylactic acid (PLA) **Hybrid scaffolds**: – Combining natural and synthetic materials (e.g., collagen-PU)	[15,17]
Signals	**Bioactive molecules** **Growth factors**: – Epidermal growth factor (EGF) – Fibroblast growth factor (FGF) – Platelet-derived growth factor (PDGF) **Vitamins** **Antioxidants** **Mechanical or electrical stimulation**	[15,16]

By incorporating these components, TE-based dermocosmetics offer several advantages over traditional approaches:

- **Addressing the root cause:** TE-based dermocosmetics aim to address the underlying biological processes responsible for various skin and hair concerns. This can involve stimulating collagen production for anti-aging effects, promoting wound healing through the delivery of growth factors, or even facilitating hair follicle (HF) regeneration through the use of bioengineered scaffolds [18].
- **Enhanced efficacy:** By targeting specifically the dermal compartment, dermocosmetics derived from TE, including new delivery methods, improve the efficacy of the bioactive compounds or key proteins such as collagen. This can significantly improve areas like scar regeneration and wound healing [19,20].
- **Long-lasting results:** Some TE techniques, like the application of stem cells or their exosomes, show promise for promoting long-lasting results by stimulating cell proliferation and collagen production. This can significantly reduce the need for frequent product application and improve patient compliance [21,22].
- **Better testers:** Ex vivo skin models, such as 3D "skin-on-a-chip" (SoC) systems combined with microfluidics, offer a promising alternative to traditional testing methods. These models provide a more realistic recreation of human skin architecture and function, enabling more accurate dermocosmetic product testing [23,24].

While traditional cosmetics play a role in aesthetics, they often have limitations. In contrast, advancements in TE-based dermocosmetics hold significant promise for the future of personalized and effective cosmetic interventions (Figure 1).

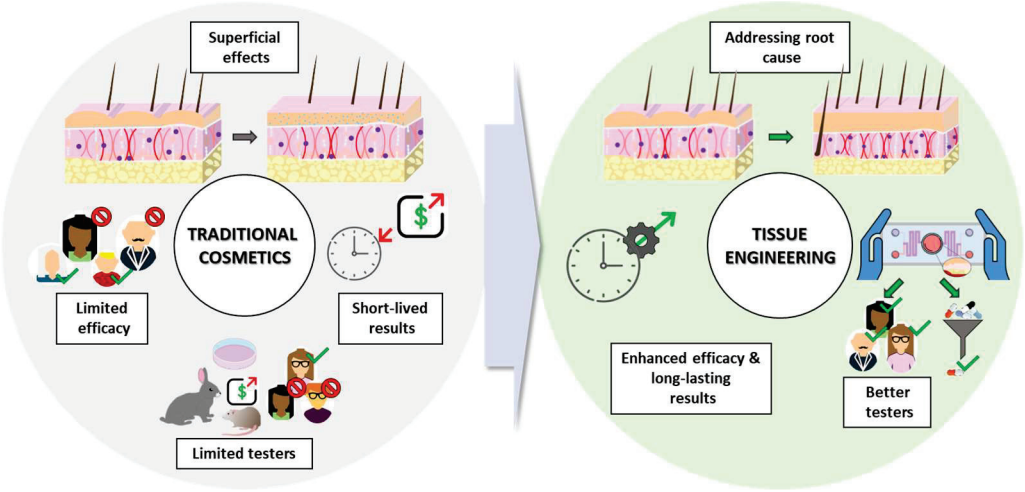


Figure 1. Limitations of traditional cosmetic approaches and the promise of tissue engineering. Source of some vectors: https://scidraw.io/ (accessed on 17 June 2024).

2. Mechanisms Involved in Regenerative Cosmetics

Regenerative cosmetics, situated at the intersection of dermatological science and innovative biotechnology, focus on mechanisms underlying skin aging. This multifactorial process involves oxidative stress, cellular damage, and senescence. Additionally, fibrosis affects skin architecture, reducing elasticity and resilience [25]. Moreover, the HF regeneration approach explores stem cell dynamics, signaling pathways, and microenvironmental cues, offering the potential for advanced anti-aging interventions (Figure 2).

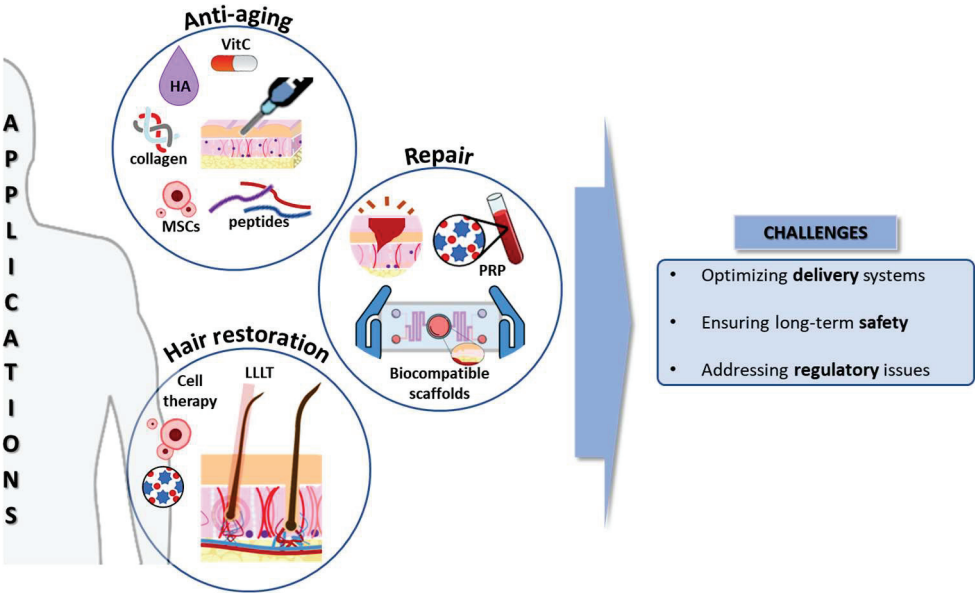


Figure 2. Applications and challenges of regenerative cosmetics. HA: Hyaluronic acid; LLLT: Low-level laser therapy; MSCs: mesenchymal stem/stromal cells; PRP: platelet-rich plasma; VitC: vitamin C. Source of some vectors: https://scidraw.io/ (accessed on 17 June 2024).

2.1. Skin Aging

- Skin aging is characterized by a decline in collagen production and a reduction of cell proliferation, together with a decrease in stemness from each tissue, among other factors [25]. Regenerative cosmetics offer solutions to combat these age-related changes, here is a summary of the key approaches in regenerative medicine applied to address age-related skin changes (Table 2).

Table 2. Summary of the key approaches to addressing age-related skin changes using regenerative cosmetics.

Approach	Components	Function	References
Stimulating collagen production	**Growth factors** (e.g., TGF-β, PDGF, IGF-1)	Promote collagen synthesis by skin cells	[26,27]
	Vitamins (e.g., vitamin C, vitamin A)	Enhance collagen production and skin cell proliferation	[26]
	Peptides (e.g., Palmitoyl pentapeptide-4)	Stimulate collagen and elastin synthesis	[27]
Wrinkle reduction	**HA fillers**	Plump up the skin and reduce the appearance of wrinkles	[28]
	Microneedling	Create controlled micro-injuries to stimulate collagen production and reduce wrinkle depth	[29]
	Exosome-based cosmetics	Promote collagen synthesis and improve skin elasticity through paracrine effects	[30]
Improving skin restoration	**Engineered skin substitutes**	Enhance barrier function, hydration, immune response, and wound healing	[31]

2.2. Oxidative Stress

Life in an oxygen-rich atmosphere comes with a toll to pay. While oxygen's reactivity supports cellular energetics, its unique properties also lead to partial reduction into potentially damaging molecular species known as reactive oxygen species (ROS).

Being the most exposed area of the body, environmental-derived ROS activity affects skin health and appearance. Oxidative stress, an imbalance between free radicals and endogenous antioxidants, plays a central role in aging [32] and plays a major role in the etiopathogenesis or aggravation of atopic [33], acne [34], rosacea [35], melasma [36] and alopecia areata [37], and rare skin disorders [38]. Thus, it comes as no surprise that the dermocosmetics industry has paid great attention to combating its effects.

Modern skincare routines frequently aim to mitigate oxidative stress, with vitamin C being a popular ingredient. In its natural form or modified in more stable derivatives, ascorbic acid acts as a potent hydrosoluble electron donor, neutralizing free radicals [39]. In cosmetology, this molecule possesses several relevant properties. It regenerates the endogenous antioxidant α-tocopherol, protecting against lipid peroxidation [40]. Additionally, it inhibits the tyrosinase enzyme responsible for hyperpigmentation collagen synthesis and processing [41]. However, the extensive list of prophylactic or therapeutic antioxidant agents in dermocosmetics includes both synthetic and naturally obtained bioactives [42].

Notably, some of these compounds may impact stem cell pluripotency and differentiation [43–45], warranting cautious application. While reactive oxygen species (ROS), particularly H_2O_2, play physiological roles at low concentrations, excessive antioxidant doses could disrupt endogenous regenerative processes, potentially worsening skin conditions [46]. In the context of skin, these include cell survival proliferation and tissue repair pathways [47–49]. Thus, excessive antioxidant doses could disrupt endogenous regenerative processes, worsening skin conditions. Indeed, many reactive oxygen species (ROS) mechanisms remain unclear and may be cell-specific. Interestingly, preconditioning with low H_2O_2 doses in regenerative medicine shows promise [50–52]. Guided by these efforts, in the near future, oxidants and antioxidants will be carefully and timely dosed to rescue pathology without challenging the function of the other. To this end, defining their respective action windows is of utmost importance and an extremely relevant objective to pursue the development of novel dermocosmeticals.

2.3. Repair vs. Regeneration

The shift from repair to regeneration in dermocosmetics is crucial for sustainable skin health. Regeneration aligns with the skin's natural healing, offering restoration of its structure and function. This approach not only preserves the skin's youthful properties but also ensures compatibility with its biological processes, minimizing long-term damage.

Emphasizing regeneration represents a significant leap in skin care, promising lasting benefits and alignment with natural dermal recovery mechanisms. Skin damage caused by wounds, burns, or scars can significantly impact both appearance and functionality. Regenerative cosmetics offer several aspects:

- **Promoting wound healing:** Engineered skin substitutes like biocompatible scaffolds provide a structure for cell migration and tissue regeneration, accelerating wound healing and minimizing scar formation [53]. Current skin substitutes have been tested for addressing regeneration in burn patients, chronic ulcers (diabetes), and rare genodermatoses (Epidermolysis bullosa) [54]. Novel technologies, including injectable cell suspensions and 3D scaffolds, are promising for improving wound healing and skin regeneration [55].
- **Scar reduction:** Microneedling and fractional laser therapy, combined with regenerative ingredients like growth factors, can stimulate collagen production and improve the appearance of existing scars [56,57]. Additionally, platelet-rich plasma (PRP) therapy is gaining traction as a potential scar reduction technique. Studies suggest that PRP injections may improve scar quality and reduce scar tissue formation [58], which is particularly interesting in relation to acne scars.

2.4. Fibrosis and Connective Tissue in Skin Rejuvenation

Fibrosis, the excessive deposition of collagen and other extracellular matrix (ECM) components, can occur following wound healing or in response to chronic inflammation. Proper management of connective tissue is essential for maintaining skin elasticity and preventing the stiffening associated with aging. While essential for wound closure, excessive fibrosis can lead to scar formation and impair skin function [59], which is particularly interesting in a pathological context in some genodermatoses such as Epidermolysis bullosa [60]. By understanding and controlling fibrotic pathways, we can enhance skin rejuvenation and promote a more youthful appearance. Regenerative cosmetics aim to:

- **Modulate the fibrotic process:** by understanding the molecular mechanisms underlying fibrosis, researchers can develop strategies to control collagen deposition and promote scarless wound healing, and also highlight the role of macrophages in the inflammatory phase [59].
- **Enhance the functionality of the connective tissue:** supporting the health and organization of the connective tissue, which provides structural support and elasticity to the skin, is crucial for maintaining a youthful appearance and function, and this is particularly interesting when the role of MSCs is studied in UV-associated skin aging [61].

Wound healing involves many cell types working together in a specific order. While some interactions are essential, others have redundancies to ensure successful repair [62].

2.5. Hair Follicle Regeneration

The HF is composed of epithelial and mesenchymal components, whose interaction regulates HF morphogenesis and regeneration. The epithelial elements comprise three concentric layers: Outer root sheath (ORS), inner root sheath (IRS), and hair shaft (HS). The bulge, an engrossed part of the ORS, is considered the largest reservoir of hair follicle stem cells (HFSCs). The HF mesenchyme includes the dermal papilla (DP), composed of dermal papilla cells (DPCs), and the dermal sheath (DS). During the anagen phase of the hair cycle, the quiescent HFSCs in the bulge are stimulated by the DP through signaling molecules and transcription factors to proliferate and initiate the hair growth process [63,64]. Therefore, the TE strategies to form bioengineered constructs capable of regenerating HFs when transplanted to the patient should focus on the epithelial–mesenchymal interactions (EMIs) [65].

Non-follicular cell sources for HF regeneration include either autologous mesenchymal (adult dermal fibroblasts, skin-derived precursors [SKPs]) and epithelial (epidermal stem cells [Epi-SCs], adult epidermal keratinocytes) precursors obtained from outside the HF; or non-autologous reprogrammed pluripotent stem cells, such as human embryonic

stem cells (hESCs) or human-induced pluripotent stem cells (hiPSCs) [66]. Conversely, follicular-based approaches are based on the combination of epithelial and mesenchymal cells from the HF. For that, DPCs and HFSCs need to be isolated from the patient and expanded in culture, but DPCs lose their innate trichogenic capacity in 2D in vitro culture [64]. To avoid this and better mimic the in vivo HF microenvironment, the current approaches are mainly focused on 3D cultures to preserve intercellular interactions. DPCs in spheroid and organoid cultures have been shown to preserve their characteristics even in high passages [64].

Furthermore, the cell–ECM interactions are also crucial in the HF cycle [67]. A variety of biomaterials have been used to create spheroids or hydrogel scaffolds to enhance HF regeneration by simulating the cell–ECM interactions, such as collagen [68], gelatin [69,70], PRP [70,71], chitosan [70,72], HA [73], Matrigel [74], or human placenta decellularized ECM [75,76].

Despite the advances, these in vitro TE approaches often lack the intricate physiological, structural organization and microenvironment of the native HF niche, which is essential for HF regeneration. To better mimic the native tissue, 3D bioprinting enables the automated, reproducible, and high-throughput biofabrication of advanced skin models incorporating several cell types and adnexal structures [77]. Regeneration of HF-like structures has been achieved by 3D bioprinting complex skin constructs incorporating DPCs, as well as Epi-SCs and SKPs [77–79] (Figure 3).

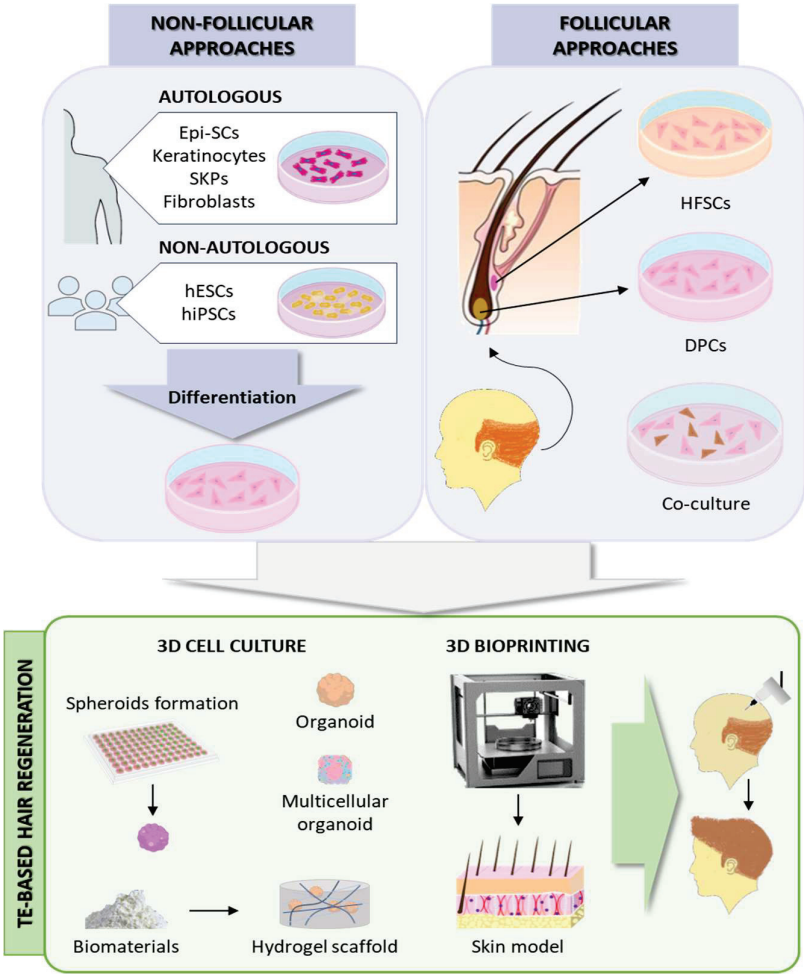


Figure 3. Hair follicle regeneration approaches. Epi-SCs: epidermal stem cells; hESCs: human embryonic stem cells; hiPSCs: human-induced pluripotent stem cells; SKPs: skin-derived precursors; HFSCs: hair follicle stem cells; DPCs: dermal papilla cells. Source of some vectors: https://scidraw.io/ (accessed on 17 June 2024).

3. Regenerative Cosmetics: A Transformative Alternative

OMIC technologies and tissue engineering have revolutionized regenerative cosmetics, marking a paradigm shift from symptomatic treatment to root-level correction. This revolution is driven by deep insights into the molecular mechanisms of skin aging, damage, and regeneration facilitated by OMIC approaches. This section explores three pivotal areas: the application of OMIC approaches in understanding and enhancing skin regeneration, the use of advanced skin modeling techniques to accelerate drug development, and innovative solutions for hair loss and promoting healthier hair growth. Together, these advancements offer promising avenues for the development of effective, safe, and personalized cosmetic treatments.

3.1. OMIC Approaches: A Key Tool for Regenerative Cosmetics

All kinds of OMIC approaches have been widely used to understand the intricate molecular landscape in a myriad of applications, including the development and characterization of novel and effective regenerative cosmetic approaches.

3.1.1. Proteomics

Proteomics, the comprehensive study of proteins, has emerged as a powerful tool to identify biomarkers in diseased skin [80], which could be potentially useful in developing targeted cosmetic therapies. Other applications in regenerative cosmetics include elucidating cellular processes involved in skin aging, regeneration, and damage [81] and characterizing biocompatible materials used in engineered skin substitutes [82]. In this regard, a recent publication highlighted the importance of the MSCs secretome in the improvement of anti-aging, whitening, and wound healing processes and its potential use as novel biomaterials for skin regeneration [83].

3.1.2. Metabolomics

Other OMIC technologies, such as metabolomics, have also been implemented in cosmetic applications and safety studies [84]. Specifically, the skin lipid structure and composition are known to be affected in many dermal diseases, impairing the barrier function [85]. Therefore, studying the lipidomic profile of known bioactive products provides insights into the molecular mechanisms behind their therapeutic effects [86]. It is well known that skin functionality is maintained by a complex system formed by the relationships between microorganisms and host cells [87]. In this regard, the use of metabolomics to elucidate these interactions is key for the development of innovative microbiome-based approaches to deal with skin conditions [88].

3.1.3. Multi-OMICs Integration

Integrating multiple OMIC approaches (multi-OMICs) plays a crucial role in understanding the mechanistic processes behind the use of cosmetics for skin regeneration. For example, a recent study integrated metagenomics and transcriptomics to study the role of eugenol in skin photoaging [89]. Additionally, advances in gene editing technologies, such as CRISPR-Cas9, enable targeting specific genetic factors underlying skin and hair conditions, paving the way for more precise and effective therapeutic interventions [90]. Moreover, the integration of regenerative medicine with Artificial Intelligence (AI) and machine learning algorithms holds promise for optimizing treatment protocols, predicting treatment outcomes, and enhancing patient satisfaction.

3.2. Skin Modeling Accelerates Drug Development

TE-based humanized animal models are crucial for the preclinical testing of novel therapeutic agents for skin conditions conditions [54]. By mimicking human skin physiology and pathology, including cellular composition, architecture, and immune responses, they offer a valuable platform for assessing the safety and efficacy of potential treatments before clinical trials in human patients [91], providing more clinically relevant data com-

pared to traditional animal models [92] and bridging the gap between preclinical and clinical research, enhancing dermatological care outcomes.

3D Skin-on-Chip Models and Microfluidics for Dermocosmetics

The integration of microfluidic devices in skin-on-chip (SoC) models enhances drug testing accuracy by closely mimicking the dynamic human skin microenvironment. These devices allow precise control of fluid flow, nutrient delivery, and waste removal, maintaining vascularized skin models for long-term studies. Microfluidic systems in skin chips replicate immune responses, inflammation, edema, and drug effects while also facilitating permeation and skin irritation tests. Additionally, they enable real-time monitoring of key parameters like oxygen levels, pH, and transepithelial/transendothelial electrical resistance, providing continuous feedback on cell viability and metabolic responses. Overall, incorporating microfluidic technology in skin models offers a more realistic and comprehensive platform for drug testing, advancing pharmaceutical research and skincare product development [93].

Current human skin models consist of epidermal keratinocytes and dermal fibroblasts integrated into a biocompatible scaffold, nourished by a culture medium. The 3D microfluidic systems, such as organ-on-a-chip (OoC) and skin-on-chip (SoC), revolutionize organ function simulation, offering realistic disease models and treatment testing [94,95]. They are faster and more cost-effective than traditional animal testing and can be used to investigate various skin phenomena, including wound healing, barrier function, and the effects of cosmetic products [96].

However, despite significant strides in creating an in vitro skin model, challenges persist, primarily because, until now, there are no SoC devices that fully incorporate all skin layers (the epidermal, dermal, and hypodermal layers) [97].

- **Materials:** The 3D SoC models require balancing cost and biomimicry through material selection. Synthetic polymers (PDMS, PCL, PLA) offer affordability and biocompatibility but lack the intricate structure of natural tissues. Hydrogels (alginate, collagen) mimic tissues but struggle with maintaining precise mechanical properties. Animal-derived materials (decellularized ECM, silk) provide the most biomimetic environment, closely resembling natural skin, but are expensive and raise ethical concerns [95,98–102]. Recent innovations like decellularized ECM and silk offer promising solutions, aiming to bridge the gap between affordability and biomimicry [15,103].
- **Challenges**: SoC models face challenges in controlling chemical gradients, technical sampling, and analysis. Integrating vasculature and microbiomes is crucial for physiological accuracy. Despite these, models like EpiDerm from MatTek Corporation or SkinEthic from L'Oréal show promise for dermocosmetics, offering safety and efficacy benefits over traditional methods [93,104,105].

3.3. Potential Solutions for Hair Loss and Promotion of Thicker, Healthier Hair Growth

Current treatments for alopecia typically involve oral or topical pharmacological treatments with minoxidil and finasteride, the only drugs approved by the Food and Drug Administration (FDA). However, these medications can cause side effects and do not always yield satisfactory results [10]. While surgical treatment by autologous hair transplant (by follicular unit extraction [FUE]) is considered the most effective method, it may be limited by the lack of enough follicles in the donor area in patients with advanced AGA [65,106].

Furthermore, to enhance the outcomes achieved with these therapies and promote better hair growth, some minimally invasive techniques have been used as complementary treatments (Figure 3), but a lack of standardization for these techniques is frequent:

- **Low-level laser therapy (LLLT)** stimulates hair growth with minimal side effects by exposing tissues to low-level light energy, showing a synergistic effect on promoting hair regrowth [107].

- **Mesotherapy**, involving the intradermal infusion of a mixture of factors, has demonstrated improvements in hair growth [93,108,109].
- **Carboxytherapy**, which entails the intradermic or subcutaneous insufflation of medical-grade sterile CO_2, enhances blood flow and nutrient delivery to the HF, potentially aiding in cases of alopecia [93,110,111].
- **Microneedling** promotes hair growth by inducing percutaneous wounds with sterile microneedles, stimulating the activation of HFSCs and the release of growth factors that promote wound healing and angiogenesis [112,113].
- **Autologous PRP** treatment, derived from the patient's blood, stimulates hair growth through the release of growth factors, cytokines, and chemokines, promoting cell proliferation, differentiation, and angiogenesis [114].
- **Nanoparticles** have been studied for drug delivery directly into the HF, minimizing the systemic adverse effects [107].

Different intricate intercellular signaling pathways regulate HF morphogenesis and cycle, including Bone Morphogenetic Protein (BMP), Ectodysplasin A Receptor (EDAR), Sonic hedgehog (Shh), Notch, or Transforming Growth Factor β (TGF-β). Among them, WNT/β-catenin is considered to be the main regulator of the HF cycle. Some HF regenerative medicine approaches aim to activate these signaling pathways [63].

Therapy with stem cells (derived from the HF, bone marrow, adipose tissue, or umbilical cord blood) has been demonstrated to improve hair growth, as they regulate hair cycles and regeneration through the secretion of proteins, extracellular vesicles, and nucleic acids, that play a role in paracrine factor signaling [115]. Similarly, exosome therapy could be a potential approach for hair loss, as they transport Wnt proteins [116]. Exosomes derived from DPC spheroids have been reported to stimulate proliferation, increase their trichogenic properties, and induce and prolong anagen in mice, promoting hair growth and regeneration [117]. Additionally, gene therapy emerges as a tool to introduce growth-promoting genes, replace defective ones, or reprogram surrounding cells for hair restoration [118].

4. Revolutionizing Beauty: The Convergence of Regenerative Medicine and Cosmetic Science

Regenerative medicine plays a pivotal role in the cosmetics industry, offering innovative solutions for skin and hair rejuvenation. Techniques such as stem cell therapy, TE, and growth factor application have shown promising results in promoting skin regeneration, reducing signs of aging, and treating hair loss conditions. For instance, stem cell-based therapies can stimulate collagen production, improve skin texture, and enhance elasticity, leading to a more youthful appearance [21,22]. Similarly, TE approaches enable the development of advanced skin substitutes and HF regeneration techniques, providing effective solutions for cosmetic enhancement. Moreover, the application of growth factors, such as PRP, accelerates tissue repair, promotes hair growth, and enhances the overall quality of skin and hair [58,114].

Looking ahead, the field of regenerative medicine presents exciting opportunities for further advancements in cosmetic applications. Future research efforts may focus on harnessing the regenerative potential of stem cells and TE to develop personalized cosmetic treatments tailored to individual needs and preferences.

Regenerative cosmetics hold promise for addressing skin and hair concerns through targeted biological interventions. They can stimulate collagen production, improve skin function, and mitigate oxidative stress for skin rejuvenation [26,27,31]. Additionally, engineered skin substitutes accelerate wound closure, minimize scar formation, and enhance scar tissue functionality [50]. Furthermore, advancements in TE offer potential solutions for hair loss and the promotion of thicker, healthier hair growth [63,115–118]. Lastly, developments in proteomics enable personalized cosmetic solutions tailored to individual needs and characteristics [81,82].

Overall, the intersection of regenerative medicine and cosmetics represents a dynamic and evolving field with vast potential to revolutionize the beauty industry and empower individuals to achieve healthier, more radiant skin and hair.

5. Challenges, Future Opportunities, and the Role of AI in this Field

As the field of regenerative cosmetics continues to evolve, there are several materials and areas that are likely to garner more attention from researchers in the coming years. However, the integration of tissue engineering (TE) in dermocosmetics presents a transformative approach to skin and hair care, yet it is not without its challenges.

The complexity of skin and hair physiology requires a multidisciplinary effort to replicate these structures accurately. One of the primary challenges lies in the scalability of TE products, ensuring they are accessible and affordable for widespread use. Additionally, the heterogeneity of human skin and hair types calls for personalized solutions that can adapt to individual variations. Standardization of tissue engineering-based substitutes could significantly reduce overall research and development costs by eliminating the need for animal testing.

Regulatory hurdles also pose a significant challenge, as the approval process for new biotechnological products can be lengthy and rigorous. Ensuring safety and efficacy through clinical trials is paramount, but it can delay the availability of innovative treatments to the market.

Looking to the future, the field holds immense potential. Advancements in 3D bioprinting could revolutionize the creation of skin and hair grafts, allowing for more precise and customized applications.

The exploration of novel biomaterials and growth factors promises to enhance the regenerative capabilities of cosmetic products. Furthermore, the integration of AI could streamline the designing and testing of new therapies, predicting outcomes, and personalizing treatments.

Artificial Intelligence in Regenerative Cosmetics

The integration of Artificial Intelligence (AI) can significantly enhance various aspects of regenerative cosmetics. Artificial intelligence (AI) is on the verge of becoming a standard tool in cosmetic dermatology. Presently, AI is being utilized to enable patients to participate more actively in their treatment choices through personalized skincare, augmented reality features, and home-based skin analysis instruments. Many dermatology practices have incorporated AI-powered skin analysis tools, which include the creation of three-dimensional facial reconstructions and predictive models for clinical results. This article underscores the existing and evolving uses of AI in cosmetic dermatology and offers a glimpse into prospective methods in this area. It is crucial for dermatologists to stay updated on these emerging technologies to effectively educate their patients and enhance their clinical practice [119]. AI can play a crucial role also in data analysis and drug discovery:

Data Analysis

AI algorithms can analyze large datasets from clinical trials and patient information to identify patterns and predict optimal treatment strategies for skin regeneration. This includes personalizing treatments based on an individual's unique genetic makeup and skin characteristics [16].

AI-Powered Drug Discovery

AI can accelerate the development of new drugs and therapies for skin regeneration. By analyzing vast chemical libraries, AI can identify potential drug candidates with targeted effects on specific cell types or signaling pathways involved in skin rejuvenation [120].

As research progresses, we may see a shift towards holistic regenerative solutions that not only address cosmetic concerns but also promote overall health and well-being. The ultimate goal is to harness the full potential of TE and AI to create dermocosmetic products that are not only effective but also sustainable and ethical.

6. Conclusions

The field of regenerative cosmetics, particularly skin TE, has made significant strides in anti-aging, repair, and hair restoration. Innovations like SoC models have revolutionized testing and understanding of skin responses. Oxidative stress and fibrosis, critical factors in skin aging, are now better managed through advanced application methods. OMIC technologies have provided deeper insights into skin biology. The application of stem cells, either directly or indirectly through their secreted factors, holds great potential for regenerative cosmetics. However, further research is needed to optimize stem cell delivery and differentiation, as well as to address safety concerns. As the field of regenerative cosmetics continues to advance, it is poised to revolutionize the way we care for our skin and hair, offering personalized and effective solutions for a wide range of skin and hair concerns.

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Article

Preliminary Experience with a Cleansing Mousse and a Non-Steroidal Emulsion for the Prevention and Treatment of Acute Radiation Dermatitis in Breast Cancer Patients Undergoing Adjuvant Radiotherapy

Anna Viola [1], Emanuele Martorana [2], Valentina Zagardo [1] and Gianluca Ferini [1,3,*]

[1] Radiation Oncology Unit, REM Radioterapia, Via Penninazzo 11, 95100 Viagrande, Italy; anna.viola@fondazioneiom.it (A.V.); valentina.zagardo@grupposamed.com (V.Z.)
[2] Istituto Oncologico del Mediterraneo, Via Penninazzo 7, 95029 Viagrande, Italy; emanuele.martorana@grupposamed.com
[3] Department of Medicine and Surgery, University Kore of Enna, 94100 Enna, Italy
* Correspondence: gianluca.ferini@unikore.it; Tel.: +39-095-789-9569

Abstract: Background: Radiation dermatitis (RD) is the most frequent side effect in patients undergoing adjuvant radiotherapy (RT) for breast cancer. Despite the skin-sparing benefits of new RT techniques, most patients develop RD. There is currently no standard treatment to prevent and soothe RD, which is generally managed with emollients, moisturizers, or corticosteroids. We conducted a prospective observational study to evaluate the rate and grade of RD with the application of a cleansing mousse and a non-steroidal emulsion during the adjuvant RT program in patients with breast cancer submitted to surgery. **Materials and Methods:** A cleansing mousse containing vegetable glycerin (12%), phytoextract of chamomile (0.5%), yarrow phytoextract (0.5%), sweet almond (0.1%), *Oenothera* oil (0.1%), and rice protein hydrolyzate (0.1%), and an emulsion containing micronized zinc oxide (3.7%), rapeseed phytosterols (1.7%), aloe (0.5%), 18-beta glycyrrhetinic acid (0.5%), alpha bisabolol (0.5%), and zanthalene (0.5%) were offered to breast cancer patients undergoing adjuvant RT to prevent the onset of RD and mitigate its severity. These specific ingredients were selected for their well-known anti-inflammatory, antioxidant, and moisturizing properties. Skin toxicities were recorded photographically and graded according to the RTOG scoring system. **Results:** From March 2023 to July 2023, a total of 24 patients with a median age of 59 years (range 42–75) were enrolled. Halfway through the RT treatment, 20 patients (83.3%) had G0 skin toxicity, three (12.5%) G1, one (4.2%) G2. None showed G3–G4 toxicity. At the end of RT, seven patients (29.2%) exhibited G0 skin toxicity, 14 (58.3%) G1, two (8.3%) G2, one (4.2%) G3. No patient developed G4 toxicity. Fifteen days after the end of RT, 13 patients (54.2%) had G0 skin toxicity, 10 (41.1%) G1, one (4.2%) G2, with none showing G3–G4 toxicity. **Conclusions:** Our data suggest that the tested topicals might be an effective option for preventing and alleviating RD. Further prospective randomized studies are needed to confirm our findings.

Keywords: breast cancer; radiotherapy; radiation dermatitis; side effects; cosmetics

1. Introduction

Adjuvant radiotherapy (RT) represents a cornerstone in the treatment path of women undergoing conservative surgery for early breast cancer (BC), having definitively supplanted demolitive mastectomy procedures in the management of small tumors [1]. RT could be omitted only under particular conditions carrying a very low risk of ipsilateral recurrence and cancer-specific death [2]. On the other hand, its administration is warranted even in some post-mastectomy cases depending on unfavorable tumor characteristics [3].

RT to the breast or chest wall is generally well-tolerated, with the risk for late cardiac events being the most feared and attempted to be reduced when treating left-sided BC

patients [4,5]. Indeed, radiation dermatitis (RD) is the most common side effect of breast RT. The underlying mechanisms likely responsible for the onset of acute damage are not entirely known. Several hypotheses have been proposed, with the most relevant suggesting that ionizing radiation stimulates cells such as fibroblasts, endothelial cells, and circulating immune cells. In response, these cells release cytokines and chemokines, including IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) [6]. This release occurs within a few hours of radiation exposure. In the following weeks, the hallmark of radiation-induced damage will be represented by the leukocyte infiltration in the irradiated skin [7].

Despite the skin-sparing effect related to the build-up depth of high-energy photon beams, approximately 50–60% of the prescribed dose is still deposited in the skin [8]. As such, approximately 90% of irradiated patients with breast cancer develop moderate to severe RD [9].

Clinically, the first reactive changes, mainly dryness, warmth, and mild erythema, may appear towards the middle of the treatment schedule and progress to moist desquamation and oedema in the following weeks. Additionally, patients may complain of burning, itching, and pain. These events are generally self-limiting and skin integrity is restored within a few weeks after the end of RT. If not, a few patients may develop serious toxicities, such as ulcers or skin necrosis. Most cases recover completely from the acute phase with no or negligible alterations. When this does not happen, a condition of chronic dermatitis can persist indefinitely, even for months or years, showing more fibrotic, drier skin, with telangiectasias and hypo- or hyperpigmentation [10].

Finally, exaggerated skin manifestations can occur quite unexpectedly when combining RT with certain drugs [11–13].

From this perspective, although non-life-threatening unlike radiation pneumonitis and ischemic cardiac events, RD emerges, at least, as an injury that can alter women's quality of life and treatment compliance [14]. Regarding the latter, in case of severe toxicity, it may even be necessary to discontinue the treatment to manage the toxicity and allow for recovery [15]. It is noteworthy to underline that any prolongation of overall treatment time due to unplanned interruptions following difficult-to-manage acute toxicity during RT may have a negative impact on prognosis [16].

Hence the need to effectively prevent and treat such a side effect. To this purpose, a wide variety of topical products exists, including emollients, moisturizers, and corticosteroids. Although several systematic reviews of the literature have attempted to clarify the benefits of one product over another [17,18], none of these preparations has proven to prevail over the others in terms of effectiveness and their choice is mainly based on patient preference and physician experience [19]. Nonetheless, RD is still an active field of research due to its frequency and impact on patients' lives and natural products are gaining more and more attention, being perceived as better tolerated than drugs [20].

Here we present a single-center prospective observational study on the use of a gentle cleansing soap and an emulsion for the prevention and alleviation of acute RD among BC patients undergoing adjuvant RT, with the aim of serving as a pilot experience eventually stimulating a comparison with other available topical agents.

2. Materials and Methods

From March 2023 to July 2023 women who underwent breast-conserving surgery or mastectomy for non-metastatic breast cancer and required adjuvant RT were offered enrollment in a prospective, observational, single-arm study testing the combination of two non-corticosteroid topicals for the prevention and alleviation of acute RD. Inclusion criteria were: age between 18 and 80 years old, Karnofsky performance status (KPS) $\geq$80, histopathological diagnosis of breast cancer, need for RT following conservative surgery or mastectomy. Exclusion criteria were: state of pregnancy, suffering from psychiatric disorders or cognitive deficits, or skin diseases (e.g., psoriasis, pemphigus, current sunburn, etc.), skin involvement by cancer (pT4), known hypersensitivity to one of the components of the medications tested, history of RT for previous thoracic malignancies (e.g., lymphoma,

etc.). Patients with relapsed BC were eligible for inclusion provided they did not receive any RT at first diagnosis.

2.1. Radiotherapy

A free-breathing computed tomography (CT) simulation with 3 mm thick slices was performed for each patient to plan RT delivery. An additional deep-inspiration breath-hold CT scan was acquired only for left-sided breast cancer patients to identify those benefitting most from respiratory gating for cardiac radiation dose reduction, as per our center's policy [21]. The target volume delineation on the CT simulation was conducted according to the ESTRO consensus guideline, i.e., cropping the clinical target volume for the residual breast tissue or chest wall (CTV_T) by 5 mm from the body surface. The CTV could also be extended to regional nodal basins (CTV_N) based on the cancer staging and risk of their involvement [22]. Forward treatment planning based on two opposite tangential fields (3D conformal radiation therapy, 3D-CRT) and field-in-field technique (where necessary to improve dose coverage) was used. Two dose prescriptions were allowed: normofractionation (NF) using 50 Gy in 25 fractions over 5 weeks (NF schedule) or hypofractionation (HF) delivering 40.05 Gy in 15 fractions over 3 weeks (HF schedule). A boost to the tumor bed could be planned in three (9 Gy in 3 days) or five fractions (10 Gy in 5 days).

Regarding the dosimetric goals, 95% of the prescribed dose had to cover at least 95% of the CTV. Dose inhomogeneity was kept within the 95–107% range of the prescribed dose according to the ICRU recommendations [23]. Where this was not possible, it was mandatory not to exceed the following dose-volume constraints: V105% < 30 cc (for both NF and HF), V107% < 3 cc (HF), V107% < 9 cc (NF), V107% < 28.6% and V110% < 5.13% (NF), as summarized in [24].

The photon beam energy was 6 MV for the CTV_T and, alternatively, 10 or 15 MV for the CTV_N and boost. No bolus was applied. To verify patient setup, the electronic portal imaging device (EPID) was used once a week for right CTVs and daily for left ones.

2.2. Skin Care

The recruited patients were supported by a gentle cleansing mousse and a non-corticosteroid emulsion for the prevention of acute RD.

The ingredients of the cleansing mousse were:

- Vegetable glycerin (12%): a natural compound that retains moisture and spread along the stratum corneum of the epidermis, thereby exhibiting moisturizing properties [25];
- Phytoextract of chamomile (0.5%): a medicinal plant with antioxidant and antibacterial activity [26];
- Yarrow phytoextract (0.5%): a flowering plant with powerful anti-inflammatory activity [27].
- Sweet almond (0.1%): an oil widely used for its moisturizing and anti-inflammatory properties [28];
- *Oenothera* oil (0.1%): it contains polyphenols, aliphatic alcohols, and fatty acids, which exhibit anti-inflammatory activity [29].
- Rice protein hydrolyzate (0.1%): known for its ability to hydrate and protect the skin. It works by increasing ceramide production, stimulating collagen production, and repairing cell damage [30].

The ingredients of the emulsion were:

- Micronized zinc oxide (3.7%): known as a topical protector against UV rays [31];
- Rapeseed phytosterols (1.7%): a plant with antioxidant and anti-inflammatory activity [32];
- Aloe (0.5%): known as a topical antibiotic with healing abilities [33].
- 18-beta glycyrrhetinic acid (0.5%): an anti-inflammatory, antibacterial, antioxidant herbal compound [34];

- Alpha bisabolol (0.5%): a herbal formulation with antimicrobial, antifungal, and analgesic activity [35];
- Zanthalene (0.5%): an anti-itching ingredient that works by inhibiting synaptic transmissions [36].

Neither cosmetic contained alcohol or perfume.

Patients were given instructions on how to apply the cleansing mousse and emulsion both orally and in writing. The emulsion was applied by the patients themselves on the irradiated skin area twice a day, 4 h before or immediately after RT and in the evening. Patients were required to use the cleansing mousse for daily personal cleansing of the irradiated skin area. The application of the emulsion and cleansing with mousse were started at the beginning of the RT schedule. Patients used the emulsion and cleansing mousse seven days a week during RT and continued the application for another 15 days after its completion or until the resolution of any skin manifestations. Patients were interviewed regularly during the observation period to ensure continued adherence to skin care instructions. They were also asked not to apply other topicals.

2.3. Aim of the Study and Toxicity Assessment

The study aimed to record the rate and grade of RD with the application of the two cosmetics described above among BC women treated with adjuvant RT. Skin toxicity was graded according to the radiation therapy oncology group (RTOG) scale [37] (Table 1) and photographically recorded at the first, mid-treatment, last day of RT program, and 15 and 30 days after its end. We investigated whether the grade of RD correlated with CTV size, type of surgery (conservative vs. mastectomy), boost delivery (yes vs. no), and dose fractionation (NF vs. HF). The flow-chart for the investigation methods is provided in Figure 1.

Table 1. RTOG score and corresponding skin changes.

RTOG Score	Skin Changes
0	No change over baseline
1	Follicular, faint or dull erythema/epilation/dry desquamation/decreased sweating
2	Tender or bright erythema, patchy moist desquamation/moderate edema
3	Confluent, moist desquamation other than skin folds, pitting edema
4	Ulceration, hemorrhage, necrosis

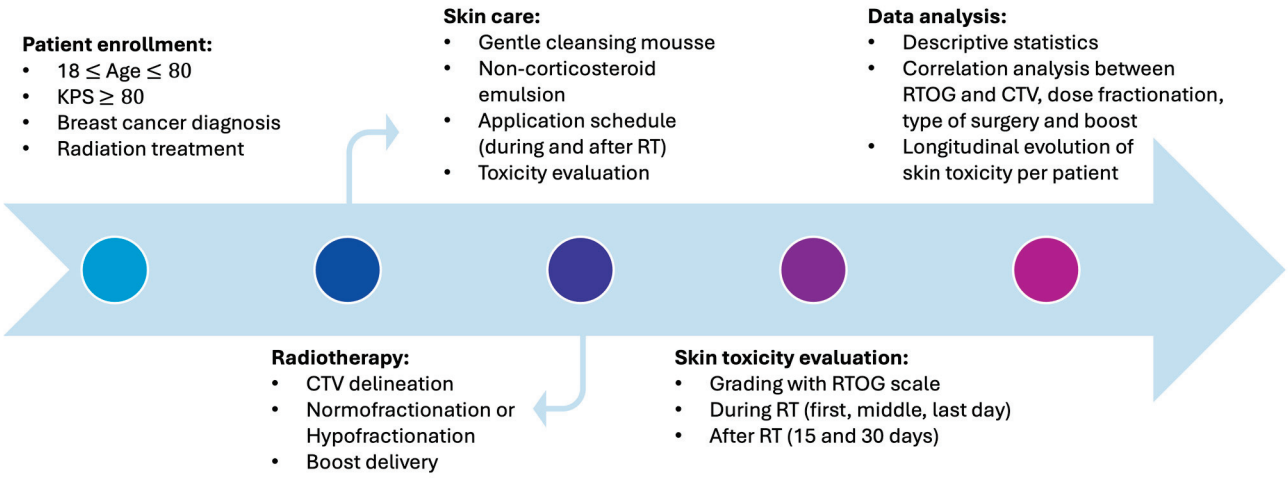


Figure 1. Study flow-chart.

2.4. Statistical Analysis

Correlation analysis between the five longitudinal toxicity assessments and CTV_T, RT target, dose fractionation, and boost delivery, as well as the longitudinal skin toxicity plot, were conducted using R [38] and RStudio [39]. Additional packages used for the analysis were heatmaply [40] and ggplot2 [41]. Evaluation of the relationship between continuous and categorical variables was performed using Kendall's rank correlation, whereas the categorical variables were evaluated using Fisher's Exact Test. The significance level to reject the null hypothesis was set at 5%.

3. Results

Twenty-four patients, aged between 42 and 75 years (median age 59 years), who underwent adjuvant radiotherapy (RT) at REM Radioterapia srl in Viagrande (Catania) participated in the study. Twenty-one and three patients had undergone breast-conserving surgery and mastectomy, respectively. Twelve patients were irradiated to the right side and 12 to the left side. The NF scheme was used for 18 patients, while six were treated with the HF scheme. Boost was delivered in 18 patients (10 Gy/5 fractions in 15 patients and 9 Gy/3 fractions in the remaining three). Six women were also irradiated to the CTV_N, including four on the left side. Median CTV_T size was 541 cc (IQR 406.1–763.5). Patient and treatment characteristics are summarized in Table 2. All treatment plans met the prespecified dosimetric requirements.

Table 2. Patient and treatment characteristics.

	Patients % (n)
Age, median (range) years	59 (42–75)
Sex	
Male	0
Female	100 (24)
Surgery treatment before radiotherapy	
Breast-conserving surgery	87.5 (21)
Mastectomy	12.5 (3)
Involved side	
Right breast	50 (12)
Left breast	50 (12)
CTV_T size, median (range) cc	541 (406.1–763.5)
Radiotherapy scheme	
Normofractionation	75 (18)
Hypofractionation	25 (6)
Boost delivery	75 (18)
10 Gy in 5 fractions	83.3 (15)
9 Gy in 3 frations	16.7 (3)

Halfway through the radiotherapy program, 20 patients (83.3%) had G0 skin toxicity, three (12.5%) G1, one (4.2%) G2. None of the patients had G3–G4 toxicity.

At the end of the RT course, seven patients (29.2%) showed G0 skin toxicity, 14 (58.3%) G1, two (8.3%) G2, one (4.2%) G3. No patient developed G4 toxicity.

Fifteen days after the end of RT, 13 patients (54.2%) had G0 skin toxicity, 10 (41.1%) G1, one (4.2%) G2, with none showing G3–G4 toxicity.

All toxicities resolved at the last assessment 30 days after the end of RT. The evolution of skin toxicities over time in all patients is represented in Figure 2. Figures 3 and 4 represent, respectively, a G1 RD and the only G3 toxicity reported by us.

No patient discontinued either the skin care program due to dissatisfaction with the tested products or RT due to skin toxicity.

The correlation analysis in Table 3 shows a small and statistically significant correlation between CTV_T and skin toxicity at timepoint T3 (after 15 days from the end of RT), T = 0.36, *p*-value = 0.03. All other toxicity assessments at different timepoints are not correlated with CTV_T. Furthermore, no relationships were found between the toxicity grade observed at each of the five timepoints and RT target (residual breast tissue versus chest wall), dose fractionation (NF versus HF), and boost delivery (yes or no), as shown in Table 3.

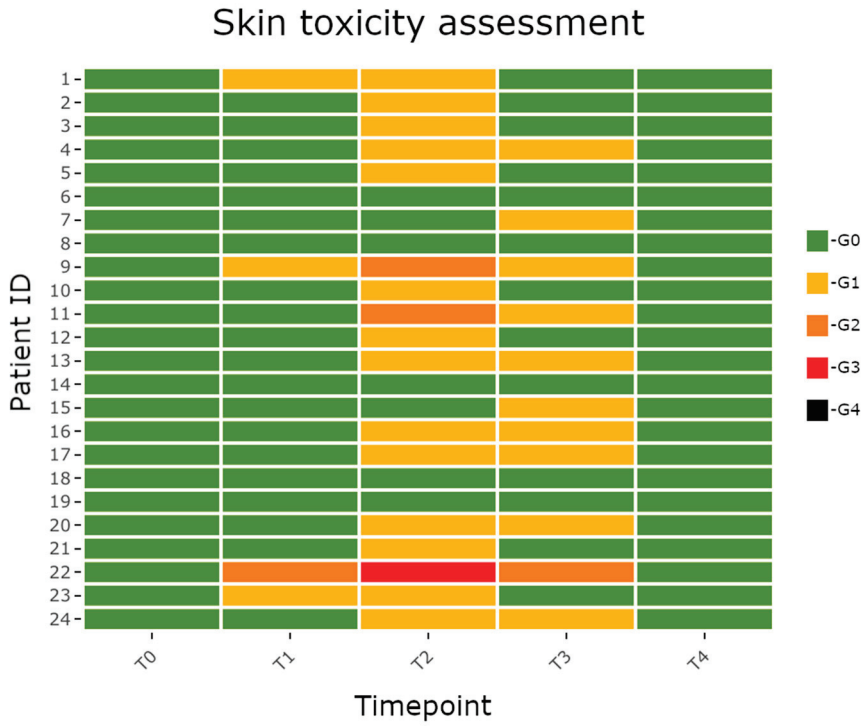


Figure 2. Evolution of skin toxicities over time in all patients. Here T0, T1, T2, T3, and T4 represent, respectively, the toxicity levels at RT start, half RT completed, end of RT, 15 and 30 days after RT completion.



Figure 3. Appearance of the skin breast before (**A**), on the last day of RT (**B**), and 15 days after its completion (**C**) in a patient who experienced G1 RD.



Figure 4. Appearance of the skin breast before (**A**), on the last day of RT (**B**), and 15 days after its completion (**C**) in the patient who experienced G3 RD.

Table 3. Relationship analysis between RD grade (T1, T2, T3) and CTV_T size, RT target, dose fractionation, and boost delivery.

Correlation Analysis		Toxicity T1	Toxicity T2	Toxicity T3
Kendall's correlation T (*p*-value)	CTV_T (cc)	0.26 (0.13)	0.29 (0.07)	0.36 (0.03)
Fisher's test *p*-value	RT target	0.15	0.17	0.06
	Dose fractionation	0.37	0.43	0.19
	Boost delivery	0.22	0.18	0.38

No unexpected events were recorded.

4. Discussion

The cosmetics tested in our study were found to be safe and satisfactory in the prevention and treatment of RD among women undergoing adjuvant RT for BC. Nearly 30% of patients did not develop signs of RD and only 12.5% had a G2–G3 exudative dermatitis with moderate oedema at the end of treatment, with no G4 toxicity recorded. Moreover, no toxicity during treatment was severe enough to threaten patient compliance with the RT program. Once RT was completed, skin integrity was rapidly restored over the following 15 days, still supported by the two cosmetics, scoring G0–G1 toxicity in 95.3% of patients. No patient showed unexpected skin reactions or complained about any aspect of the suggested topical treatment (e.g., frequency of administration, smell, consistency, stickiness, and greasiness of the two cosmetics, etc.). Overall, these findings promote our preliminary experience as worthy of further research.

Indeed, RD is still an extremely common side effect of adjuvant RT among patients with BC, although several therapeutic options are available for its prevention [42]. The natural compounds we tested have the advantage over topical corticosteroids of generating fewer concerns regarding possible late cutaneous effects, such as skin atrophy, telangiectasias, or striae [43]. Few studies are specifically dedicated to investigating this possible cause-and-effect relationship and are unable to produce conclusive evidence on the safety of corticosteroids due to small sample sizes [44].

Both the mousse and the emulsion contain a mix of molecules with a soothing, hydrating, anti-inflammatory, and anti-itch action, which replaced the need for real drugs. Our results join those obtained with other non-steroidal topical medications, among which hyaluronic acid stands out for its effectiveness also in other tissues damaged by radiation [45–48].

There is more and more attention towards the local side effects caused by breast RT due to the growing request from patients to preserve their physical appearance as much as possible [49].

In our study we noticed how good hydration and gentle cleansing of the irradiated area before, during, and after RT had a positive effect on the skin's tolerability to radiation.

In our clinical practice, we usually prescribe moisturizing creams to all patients with an indication for breast RT based on the experience that preventive therapy for skin side effects is more effective than treating them once they have occurred. This approach shows significant efficacy in reducing the onset of acute skin toxicity [50].

Acute RD can also affect both the quality of life and normal daily activities of patients [51], and, in some cases, can lead to unplanned treatment interruptions, thereby reducing the overall effectiveness of the therapy [16].

Apart from thorough cleansing of the skin and prevention of local trauma, there is no well-defined protocol to prevent radiation toxicity of the skin [52].

From the literature data, we know that there are some risk factors including the use of the bolus, smoking, and previous chemotherapy causing alteration of the small blood vessels [53].

The first pathophysiological stage of RD is determined by an overproduction of free radicals, responsible for damaging the stem cells in the basal layer of the epidermis, resulting in diminished turnover of epidermal cells. These cells, prompted into a state of senescence by ionizing radiation, subsequently release pro-inflammatory cytokines, responsible for initiating the inflammatory cascade. As a consequence of these effects, early RD may be observed clinically. The pathophysiology of moist desquamation involves the loss of keratinized layers and depletion of basal cell population as well. The erythema is the result of epidermal damage to basal cells and dilation of capillaries associated with increased vascular permeability [54].

These effects of radiation on the skin are influenced by several treatment factors, including, besides the total radiation dose, other dosimetric parameters (evaluable on dose–volume histograms), the dose fractionation (normofractionation vs. hypofractionation), the RT technique (3D-CRT vs. intensity-modulated RT, IMRT), and the use of an image-guided protocol for daily setup verification (IGRT). Keenan et al. provided some dose–volume constraints significantly associated with the onset of acute RD, suggesting in particular to minimize the skin volume exceeding 105% of the prescribed dose [24]. The rate of grade 2 or higher acute RD is also reduced by using hypofractionation rather than normofractionation [55]. Compared with 3D-CRT, IMRT decreases the risk of RD by improving dose homogeneity [56]. Furthermore, this reduction is greater the more complex the inverse treatment planning technique is [57]. Lastly, just as with other body targets [58], a daily setup verification by cone beam CT (CBCT) is more accurate than that by EPID and results in a significantly lower rate of acute RD, likely thanks to the more precise radiation delivery that avoids the generation of unplanned hotspots [59]. Large breast size is another known risk factor for skin toxicity [56]. However, our cohort showed no or mild skin toxicity in most cases, regardless of breast size, type of surgery (conservative vs. mastectomy), boost delivery (yes vs. no), and dose fractionation (normofractionation vs. hypofractionation). Although the level of toxicity achieved was mild for most patients, this was sufficient to draw a positive correlation (Table 3) between breast size and skin toxicity 15 days after the end of RT (T = 0.36, p-value = 0.03) highlighting a higher risk of toxicity resulting from increased breast size, according to what Freedman et al. observed [56]. Noteworthy for the same skin toxicity timepoint (T3) is the relationship with the RT target (p-value = 0.06) which is not statistically significant, probably due to the smaller size of the patients undergoing mastectomy, but which sheds light on a possible greater risk of skin toxicity for patients treated with mastectomy. No other relationship was found between the variables of interest in the study cohort; a larger and more diverse sample is mandatory to draw strong conclusions.

As regards our choice of using 3D-CRT, it should be noted that, compared to conventional tangential field irradiation, IMRT increases the integral dose to normal tissues and its potential advantage in reducing skin toxicity should be weighed against a possibly increased risk of more dreaded consequences, such as pneumonitis and secondary malig-

nancies due to a worse exposure of OARs to low radiation doses [60–62]. Accordingly, the American Society for Radiation Oncology (ASTRO) does not recommend routine use of IMRT to deliver breast RT [63].

Finally, CBCT-guided IGRT protocols, although effective in reducing skin toxicity, are time-consuming and may hinder the demands of managing high patient loads in RT departments [64].

Radiation dermatitis is still a dynamic area of research, where also the achievements from nanotechnology warrant attention. Indeed, nanocarrier-based dermopharmaceutical formulations penetrate deeper than traditional topicals, potentially resulting in improved efficacy. However, further studies are needed to establish safety and evaluate cost–benefit ratios [65].

This preliminary experience has several limitations. First, the heterogeneity (i.e., for dose fractionation, type of surgery, etc.) and small size of the cohort are underpowered to find any varying effectiveness across different subgroups. Second, the lack of a placebo control group, omitted for ethical reasons in a clinical context of exceptionally frequent skin side effects, prevents quantifying the extent of the benefit deriving from the use of the two cosmetics being tested. In this regard, our pilot study should be understood as a phase I/II trial that can transition to more mature research, hopefully involving the other topical medications available for comparison.

5. Conclusions

The combination of a cleansing mousse containing vegetable glycerin (12%), phytoextract of chamomile (0.5%), yarrow phytoextract (0.5%), sweet almond (0.1%), *Oenothera* oil (0.1%), rice protein hydrolyzate (0.1%), and an emulsion containing micronized zinc oxide (3.7%), rapeseed phytosterols (1.7%), aloe (0.5%), 18-beta glycyrrhetinic acid (0.5%), alpha bisabolol (0.5%), zanthalene (0.5%) may emerge as an effective option to prevent and treat acute RD among BC patients undergoing adjuvant RT. Larger prospective randomized studies are needed to substantiate this first impression.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available from the corresponding author upon request. The data are not publicly available due to privacy reasons.

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Review

Strategic Approaches in Formulation Development for Atopic Dermatitis

Alberto Keidi Kurebayashi [1], Khanh Phan [2], Ayyah Abdoh [2], Newton Andreo-Filho [1], Patricia Santos Lopes [1], Yousuf Mohammed [2,3,*] and Vania Rodrigues Leite-Silva [1,2,*]

[1] Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Departamento de Ciências Farmacêuticas, Universidade Federal de São Paulo, UNIFESP, Diadema 09913-030, Brazil; albertokeidi@uol.com.br (A.K.K.); newton.andreo@unifesp.br (N.A.-F.); patricia.lopes@unifesp.br (P.S.L.)
[2] Frazer Institute, Faculty of Medicine, University of Queensland, Brisbane, QLD 4102, Australia; k.phan@uq.edu.au (K.P.); a.abdoh@uq.edu.au (A.A.)
[3] School of Pharmacy, The University of Queensland, Brisbane, QLD 4102, Australia
* Correspondence: y.mohammed@uq.edu.au (Y.M.); vania.leite@unifesp.br (V.R.L.-S.)

Abstract: Atopic dermatitis (AD) is the most common chronic skin disease, significantly impacting patients' quality of life. One of the most effective management approaches for AD involves addressing the defective skin barrier by urging AD patients to regularly use suitable moisturizers. Therapeutic moisturizers designed for AD are precisely formulated with ingredients targeting critical and often early symptoms of AD (e.g., itch, inflammation, damaged skin barrier). Dermo-cosmetic products, which are rich in moisturizing and emollient agents contributing to recovery as well as strengthening the skin barrier, have proven to be excellent adjuncts in AD treatment. There are various galenic forms of dermo-cosmetics, such as lotions, gels, creams, foams, and sprays, requiring a rationale in choosing ingredients for the product formulation and development. In addition, the role of moisturizer and emollient therapy to address skin dryness linked to dermatological conditions is hugely dependent on varying chemistry and morphology in the deeper regions of the skin. There are also limits to the efficacy of treatments, corticosteroid side effects, and product sensory appeal, which may decrease patient acceptance and compliance. The objective of this review is thus to offer a comprehensive overview of the critical aspects involved in the development of cosmetic vehicles, as well as a detailed examination of the primary ingredients used in formulations for AD.

Keywords: atopic dermatitis; topical formulations; vehicle; moisturizer; dermo-cosmetic

1. Introduction

Studies have demonstrated that the skin acts as a vital interface between the body and external factors, providing major functions to support and protect the body against different physical and chemical risks [1–4]. The development of skin barrier abnormalities and immunological diseases is influenced by a complex combination of hereditary and environmental components [1]. Atopic dermatitis (AD) is considered a prime example of a chronic skin condition and has a complex pathophysiology [5]. The disease can provoke visible irritation (redness), itchiness, and discomfort on the skin [6]. It is also connected with other atopic conditions, including food allergies, allergic rhinitis, and asthma [7–10]. AD can affect individuals of all ages and is the most prevalent skin disease among children, affecting approximately 15–20% of infants and young children and 2–3% of the general population [5,11–13]. The disease typically begins by age 5, and early diagnosis and management are crucial to prevent complications and enhance quality of life [12,14,15]. Previously, individuals with AD were often described as having eczema, though "eczema" is a broader term encompassing various skin inflammatory conditions [8,16–18].

The objectives of AD treatment are to alleviate itching and burning, restore and maintain skin barrier function, reduce inflammation, interrupt the itch-scratch cycle, and address exter-

nal triggers and secondary infections that may exacerbate the condition [7,19,20]. One of the main treatments for AD includes applications of emollients and skin care products [20,21]. In randomized, double-blind studies involving healthy skin, specific emollients have been shown to enhance water gradients and improve hydration of the stratum corneum (SC). These emollients contain ingredients that can either replace or boost epidermal lipogenesis, thereby strengthening the SC barrier function [22,23]. Additionally, emollients enhance skin hydration and alleviate symptoms such as stinging and itching, as well as serve as a barrier against potential irritants [24,25].

Furthermore, dermo-cosmetic products have proven to be excellent adjuncts in the treatment of AD since they do not contain corticosteroids in their composition but offer a formulation, rich in moisturizing and emollient agents that can contribute to the recovery and strengthening of the skin barrier [26]. This work aims to review and evaluate different topical vehicles often used for drug delivery, focusing on their formulations, and intended impact on the skin, in order to enhance understanding of their qualities and facilitate informed decision-making for the treatment of AD.

2. Rationale for the Development of a Topical Vehicle for Drug Delivery

Considered as a delivery system to carry cosmetic or pharmaceutical active ingredients (APIs), a topical vehicle may also possess emollient properties that enhance skin hydration [27–30] and significantly affect the bioavailability of the active substances [29,31]. Topical therapeutic products are widely used to manage various dermatological conditions such as eczema, psoriasis, or xerosis [7,32]. In general, a final topical drug product is made by combining one or more APIs with different inactive ingredients that form the vehicle [31]. A vehicle can be a cream, lotion, gel, or ointment supporting the delivery of APIs across the stratum corneum (SC) into deeper regions, named the epidermis and dermis, of the skin [6]. The vehicle solubilizes insoluble drugs and provides a means for APIs to be applied evenly, remain protected, and reach unreachable strata of the skin. A vehicle is, thus, as important as the APIs because they both define the pivotal sensory characteristics favoring the adherence of treatment for skin conditions to the topical formulation [33]. There are different intrinsic and extrinsic factors that should be considered in structuring a vehicle, such as the solubility of the APIs in the formulation, the release form of the APIs at the time of application, the stability of the APIs, the vehicle itself (e.g., selection of excipients and formulation design), the hydration capacity of the SC, skin type, the application area, and importantly, the patient's preference for the vehicle [6,34]. Even though APIs are not present, applying a topical formulation to the skin may provide either eased (positive) or discomfortable (negative) feelings instantly, and the effects may persist for hours. Moreover, the commencement of clinical effects is usually gradual; hence, a well-tolerated formulation can contribute largely to patient compliance and the clinical success of topical drug products [35]. Additionally, assessing the current condition of the skin under environmental aspects such as ethnicity, gender, age, location, and disease status would be helpful in choosing a vehicle with good design quality [36].

In the context of AD, skin dryness and impaired barrier function (often with visible redness) are hallmarks of the skin diseases favoring invasion of microorganisms, allergens, and irritants [37]. The research of Danby et al. [32] indicated that so-called inert excipients within a topical formulation could induce molecular changes in the skin, promoting barrier repair, bolstering innate immune defenses, or offering therapeutic benefits for AD patients. The interaction between the vehicle and skin barrier is also crucial to addressing the condition's development and progression [6]. Therefore, a strategy is necessary in the formulation and development of vehicles that can calm and protect the skin while maintaining its pleasant sensory characteristics [5,38]. Many topical dosage forms contain ingredients with high emollient power that act as active ingredients on their own and have therapeutic potential for the product [32,39]. Proksch and Lachapelle [38] reported that the application of lipids, physiological lipids, and humectants could play a role in restoring

the lipid lamellae, improving skin biophysical properties such as skin hydration and skin elasticity, and thus reducing itching.

3. Key Vehicle Forms for Topical Drug Therapy

Key topical vehicle formulations, made from different excipients and ratios, can be distinguished based on their physical states such as liquids and semisolids [6,40]. Table 1 presents some main characteristics of the most common vehicles used in topical formulations, suggested areas of product application, and their possible effects.

3.1. Creams

As some of the most common topical semisolid dosage forms for dermatological applications, creams are characterized as emulsion system, consisting of a lipophilic phase with lipophilic (e.g., vegetable or mineral oils, alcohols, fatty acids, esters, and waxes) and an aqueous phase, combined through the action of emulsifiers [41,42]. According to the continuous phase (oily or aqueous), creams can be classified as water-in-oil (W/O) or oil-in-water (O/W) emulsions [43]. The O/W emulsions are particularly suitable for delivering water-soluble active substances, as their external phase exhibits hydrophilic properties. Conversely, W/O emulsions are optimal for lipid-soluble active substances, given that their external phase possesses lipophilic characteristics [44]. These kinds of emulsions exhibit no obstruction to heat dissipation owing to their miscibility with surface exudation. Cream emulsions impart a non-greasy sensation, a factor of paramount importance for certain patients [45,46].

3.2. Lotions

Lotions have a formulation structure similar to creams but may contain more than 50% water in their composition, resulting in greater fluidity [47]. Assessments of physical critical quality attributes (CQAs) such as rheological properties are often applied to discriminate cream and lotion products [31,48]. Due to their high-water content, lotions promote a pleasant cooling effect. Lotions are highly suitable for treating exudative dermatoses. Additionally, they can be applied easily in regions with hair like the scalp and demonstrate rapid spreadability [44].

Table 1. Characteristics of the most common topical vehicles.

Topical Formulations	Aspect	Application Area	Effect	Key Features
Creams [41,45]	semisolid emulsion	face and localized areas	moisturizer and a non-greasy sensation	no obstruction to heat dissipation
Lotions [44]	fluid emulsion	skin, body, and scalp	cooling effect	easy application
Ointments [43]	oily	specific area	occlusive effect	one phase vehicle
Gels [6,43]	thick gel	oily skin or areas with hair	film-like effect on the skin	easy removal
Foam [6,49]	aerated foam	sensitive skin	pleasant effect	reaches large areas
Spray [6,50]	solution aerosol	skin body, and scalp	cooling sensation	reaches large areas, may cause a burning and stinging sensation
Shampoo/Soap [41]	solid or liquid	skin, scalp	cleaning effect	reaches large areas
Paste [41]	semisolid, stiff consistency	skin, oral cavity, mucous membrane	occlusive and protective actions	skin protection and absorb secretions

3.3. Ointments

Ointments usually have a single phase, comprising lipophilic ingredients (e.g., soft paraffin), which could form an occlusive layer over the skin to prevent heat and water loss. Ointments may contain other ingredients dissolved in a co-solvent together with the lipophilic components [43]. Ointments are harder to spread or remove as compared to creams and lotions, but they are usually expected to play a barrier role as well as enhance the hydration of the skin [48]. They can demonstrate an occlusive action, effectively restricting the permeation of water and heat. The occlusive property also enhances the penetration and efficacy of the active drug, particularly in areas of thickened skin [6].

3.4. Gels

Gels are semisolid, transparent preparations. Gels are formed by a three-dimensional network created by macromolecules of gelling agents (e.g., hydroxyethylcellulose, carbomers, or carboxymethylcellulose), between which the liquid/solvent is distributed [43]. Based on the character of the solvent, gels can be classified as organogels (apolar phase) or hydrogels (polar phase). Organogels are characterized by their oily nature, whereas hydrogels possess a lighter sensory profile, making them easy to apply, non-greasy, and non-occlusive. Hydrogels are particularly suitable for application on hairy areas or in the treatment of skin conditions such as acne, AD, or seborrhea [43]. Gels may contain some alcohol content, providing refreshing feelings or a colling sensation when applying the product to the skin. The viscosity of gels is low, causing them to liquefy upon contact with the skin and possibly leave a film-like effect on the applied area. Gels are suitable for oily skin or areas with hair. However, due to their lighter characteristics, gels are easily removed with sweat or friction. Gels containing alcohol in their formulation may also cause a stinging sensation on damaged skin [6].

3.5. Pastes

Pastes are also semi-solid preparations. Pastes are characterized by a stiff consistency, achieved through a high concentration of dispersed solid ingredients. They are intended for applications on the skin, oral cavity, or mucous membranes. Due to their persistence on the skin, they have occlusive and protective actions. Pastes and ointments provide skin protection and can be used to absorb secretions [41].

3.6. Foam

Foam is considered a pharmaceutical formulation, typically comprising a substantial volume of gas dispersed within a liquid medium containing one or more APIs. This formulation is generated by the action of a propellant and may also include additional excipients in aqueous phases and/or oily phases [49]. Foams are particularly user-friendly, especially for sensitive skin, as their spreadability requires minimal force for product application [49].

3.7. Spray

Sprays are solution aerosols. They are composed of a solution combined with a propellant gas, which is often a mixture of nonpolar hydrocarbons. The solvents in these sprays may include ethanol, acetone, glycol ethers, and polyglycols. Sprays offer the advantage of effectively covering large areas of affected skin and can also provide a cooling sensation upon utilization. Sprays have a high safety profile because of their low product contamination [51]. However, sprays may cause burning or stinging sensations [6].

3.8. Shampoo/Soap

Soaps and shampoos are topical preparations available in solid or liquid forms. They are intended for application to the skin or scalp, followed by rinsing with water. These products can be classified according to emulsions or surfactant-based compounds that, when applied with water and friction, generate foams. Due to their characteristics, soaps

and shampoos have great ease in reaching a larger area of the body, which is helpful in treating skin conditions like AD [41].

4. Cosmetics as Adjuvants

In recent decades, the cosmetic industry has experienced substantial scientific and technical advancements, particularly in understanding skin physiology and the development of research techniques. These progressions have facilitated the safe utilization of novel APIs and carriers grounded in well-understood mechanisms of action [52,53]. Cosmetics have usually been formulated as products such as powders, lotions, and creams, designed to enhance personal appearance through direct skin application. However, recent scientific and technological progress has transformed comprehension of normal skin physiology and the ways in which cosmetics can alter its appearance through both physical modifications and biological activities [54].

Dermo-cosmetics has emerged as a subspecialty within dermatology, utilizing cosmetic products to treat various skin conditions. They are commonly employed to enhance photoprotection, treat dry or aging skin, and manage inflammatory skin conditions such as acne, rosacea, AD, psoriasis, and seborrheic dermatitis. Additionally, demo-cosmetics contribute to improving the quality of life of patients [55]. The products have proven to be excellent adjuncts in the treatment of AD, as they do not contain corticosteroids but offer formulations rich in moisturizing and emollient agents that aid in the recovery and strengthening of the skin barrier [56]. Furthermore, dermocosmetics has demonstrated the ability to decrease the side effects of pharmacological treatments, offer high levels of patient satisfaction, and increase adherence to treatment regimens. Collectively, reported evidence supports the incorporation of dermocosmetics into clinical practice [50].

5. Cosmetic Formulations

The primary objective in the development of cosmetic formulations across various galenic forms is to ensure the bioavailability of APIs while maintaining physicochemical and microbiological stability and safety and also providing a comfortable skin sensation under in-use conditions. The rationale behind developing an appropriate vehicle, aimed at achieving biocompatibility with the skin and the bioavailability of APIs, is crucial to obtaining an optimal balance between the ingredients of formulations and the skin [54].

The research of Külkamp-Guerreiro et al. [57] showed that consumers preferred free lipoic acid formulations, as this API brought the desired spreadability and residual oiliness. Additionally, the higher viscosity of the product conveyed a sensation of greater hydration for the applied skin area [57]. In psoriasis treatment, poor cosmetic properties have been identified as one of the significant reasons for non-adherence and incorrect application [45]. The sensory aspects of a product, thus, play a central role in users' acceptance and compliance. Accordingly, the sensory attributes as well as the safety and efficacy of the product should be taken into account in cosmetic formulation development.

6. Moisturizers

Healthy skin can improve quality of life and significantly contribute to social interactions and psychological well-being [6]. Skin irritation is the most frequent adverse effect, which can be perceived with or without signs and symptoms of skin inflammation [58].

Moisturizing power is considered one of the most important benefits of a skin product, and this hydration advantage could be divided into four actions: repairing the skin barrier, increasing the water content in the skin, reducing transepidermal water loss (TEWL), and maintaining the skin hydrated [59]. Moisturizers are essential components in any topical formulation for AD treatment, as they restore the ability of intercellular lipid bilayers to absorb, retain, and redistribute water [60]. Moisturizing agents can also penetrate the skin and contribute to the reorganization of skin layer structure. The work of Danby et al. [61] showed the positive hydration effect of emollient creams on the treatment of eczema or AD skin disease via both subjective and objective measurements.

However, even moisturizers, with their benefits, may be linked to cases of allergy, depending largely on the characteristics of the formulations as well as the immune response of each individual. Some possible adverse effects of moisturizers regarding the ingredients present in the formulation include skin irritation reactions (urea, acids, propylene glycol, solvents), contact dermatitis (lanolin, propylene glycol, methylchloroisothiazolinone (and) methylisothiazolinone, some preservatives, fragrances, sunscreens), occlusion folliculitis (lanolin, petrolatum, mineral oil), photosensitivity (fragrances, sunscreens, preservatives, acids), acne (occlusive ingredients), contact urticaria (sorbic acid, fragrances, balsam of Peru), poisoning (salicylic acid), among some examples [58,62,63].

Moisturizers, which are pivotal in topical formulations for AD treatment, can be categorized into primary and secondary types. Primary moisturizers are further divided into two groups: hydrophilic moisturizers (humectants) and lipophilic moisturizers (emollients and occlusives) (as shown in Figure 1) [6,64].

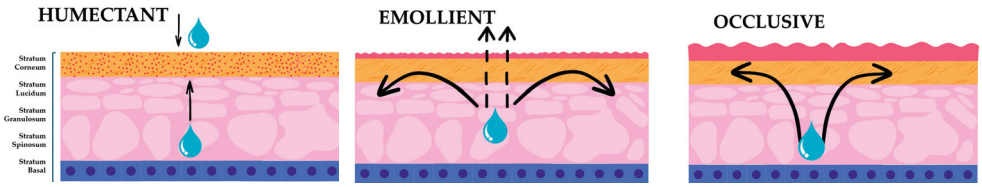


Figure 1. The mechanism of action of moisturizers on the skin [30–33].

Humectants are substances that mimic the natural moisturizing factor (NMF) of the skin [6], enhancing epidermal barrier function and hydration. They may also possess additional properties, similar to those of emollients. Common humectants used in topical formulations include glycerin, acid lactic, propylene glycol, urea, butylene glycol, panthenol, hyaluronic acid, sorbitol, glycolic acid, and sodium pyrrolidone carboxylic acid [6,64].

Emollients are agents that soothe, soften, and increase skin hydration levels. They are commonly incorporated into lotions, creams, ointments, or gels and function by filling the spaces between corneocytes, thereby protecting the skin from irritations, facilitating smooth application, or providing lubrication. Emollients enhance the overall appearance and texture of the skin and are frequently used in conjunction with emulsifiers [65]. Commonly used emollient ingredients in formulations include cholesterol, linoleic acid, squalene, oleic acid, stearic acid, fatty alcohols, and cocoa butter [6,66].

Occlusives emulate the sebum and natural lipids present in the skin. They form a hydrophobic barrier film on the skin's surface, thereby reducing TEWL and preventing water evaporation from the stratum corneum. Commonly used occlusive ingredients include liquid paraffin, lanolin, and white petrolatum, which provide hydration through their occlusive effects [6,64]. However, many occlusives are characterized by a greasy texture, odor, and potential allergenicity [59]. Kovács et al. [66] proposed developing a cream that offers smoothness and hydration benefits without using these traditional occlusive agents. Instead, they utilized sunflower oil and cocoa butter, resulting in a product with significant hydration potential without the occlusive effect of conventional ingredients [66].

7. Effects of Formulation Excipients

The development of topical vehicle formulations necessitates extensive knowledge and a tailored approach based on the physicochemical properties of the APIs, ensuring functionality in the target skin area [6]. A key consideration for formulators is to pay attention to the compatibility and stability of excipients and APIs, both for the product's commercial viability and for maintaining its safety and therapeutic efficacy [36]. "Excipients" are common non-active ingredients used in the formulation of topical vehicles, such as emulsifiers, thickeners, chelating agents, preservatives, pH adjusters, and fragrances. These excipients typically make up more than 90% of a topical product. Formulators

leverage the functionality of excipients to achieve specific performance attributes, for instance, improving solubility for API incorporation, controlling drug release and permeation, enhancing the product's aesthetic appeal to increase patient adherence, improving skin deposition, stabilizing active ingredients and vehicles, and preventing microbial growth and contamination [6,31,32].

7.1. Solvents

Solvents play multiple roles, including enhancing the solubility of APIs and facilitating their absorption through the skin. Solvents are also incorporated to dissolve other excipients, such as colorants and preservatives. Water is the most essential and commonly used solvent in cosmetic product formulations, requiring purity and freedom from contaminants and microbes [64]. Depending on the desired final formulation, other solvents may also be employed, such as propylene glycol, mineral oil, or hexylene glycol. Propylene glycol, in particular, is often used in formulations as a co-solvent to enhance drug permeation through the skin, acting as a skin penetration enhancer [32,34].

7.2. Consistency Agents

Hardening or consistency agents are excipients that form structure in topical semisolid formulations, used with the aim of imparting greater viscosity and stability to the formulations. Examples of these ingredients include beeswax, carnauba wax, white petrolatum, liquid paraffin, and lanolin. Topical formulations containing a high concentration of these consistency agents tend to form a protective occlusive barrier on the skin. This barrier shields the skin from harmful substances and aids in maintaining skin hydration by preventing TEWL [6,34].

7.3. Thickeners/Gelling Agents

Thickeners are critical excipients in topical vehicles, determining their final viscosity, which is directly related to skin retention and penetration of the APIs. Thickeners can be classified into four groups based on their origin:

1. Lipid thickeners (cetyl alcohol, cetearyl alcohol, stearic acid, and carnauba wax) are incorporated into the oily phase of the formulation due to their lipidic nature.
2. Natural thickeners (hydroxyethyl cellulose, carboxymethyl cellulose, guar gum, xanthan gum, gelatin, and sodium alginate) are polymers that absorb water thereby increasing the viscosity of the vehicle.
3. Mineral thickeners (silica, aluminum magnesium silicate, and bentonite) are of natural origin, absorbing water, and oils to increase the viscosity of the formulation.
4. Synthetic thickeners (carbomers, cetyl palmitate, and ammonium acryloyldimethyltaurate). Carbomers are one of the most commonly used thickeners due to their ability to form a transparent gel [6,34,61].

7.4. Silicones

Silicones are ingredients that can act as non-greasy occlusives, aiding in moisture retention, lubricating, and forming an occlusive film on the skin [61]. They function as emollients, providing a pleasant sensation on the skin and creating a desired smooth skin surface. Among various types of silicones, the most commonly used in topical formulations are dimethicone and cyclomethicone [6]. Hand creams, which are oil-in-water emulsions, typically contain between 15% and 40% oils, 5% and 15% humectants, and 45% and 80% water. By adding a specific silicone, due to its hydrophobic action, it is possible to achieve a water-resistant hand cream (through 4 to 6 washes). Most hand creams contain petrolatum, glycerin, waxes, or dimethicone [65]. Some silicones have occlusive characteristics, increasing the potential for allergenicity and imparting a greasy consistency [58].

7.5. Humectants

Humectants are crucial in formulations as they are substances that attract water, acting like sponges and keeping water in the skin. The ingredient content in the formulation must be sufficient to achieve the desired effect, because in lower dosages, it will be ineffective in hydration [65]. One of the most effective ingredients in this category is glycerol. As a humectant commonly used in topical moisturizers, glycerol has been shown to be able to accelerate skin barrier recovery. In the therapy of AD, glycerol is often used at doses varying from 20% to 40%. This is due to its beneficial benefits for the skin, such as hydrating the outermost layer of the skin and restoring the protective barrier. Youssef et al. [67] showed that concentrated glycerol, at a concentration of 85%, may serve as a viable option for patients in need of phototherapy since it effectively reduces staphylococcal presence on the skin and is also more cost-effective compared to the Narrow Band Ultraviolet B (NB-UVB) treatment [67]. In another research work, Danby et al. [61] demonstrated the superior efficacy of glycerol-based moisturizers compared to those lacking a humectant in restoring skin hydration and enhancing filaggrin expression by keratinocytes.

7.6. Emulsifiers/Solubilisers

Emulsifiers are directly related to the stability of topical vehicles, maintaining a uniform product throughout their period of use. Emulsifiers can be polar (hydrophilic) or nonpolar (lipophilic), resulting in greater or lesser affinity with water or oil. Their function is to reduce interfacial tension, thereby stabilizing emulsions and enhancing the wetting and solubility of hydrophobic ingredients [6]. Despite bringing structure benefits to producing good emulsions, emulsifiers can cause dermal irritation and corrosiveness and increase the potential for sensitization when applied in high concentrations [34].

7.7. Chelating Agents

Chelating agents such as ethylene diamine tetraacetic acid (EDTA) are used in formulations to improve the stability of a topical vehicle by binding to metal ions. In this way, they reduce undesirable effects in formulations arising from metal-catalyzed degradation. Chelating agents may also exhibit positive synergy with the effects of preservatives [6]. Their application is extensive, spanning the food and pharmaceutical industries, and they play an extremely important role, such as in countering heavy metal poisoning and reducing cholesterol levels in the blood. According to a comprehensive study conducted by experts from the Cosmetic Ingredient Review (CIR) to extensively examine the safety of chelating agents, various EDTA derivatives have been concluded to be safe for use in cosmetic formulations [68].

7.8. Acidifying, Alkalising, or Buffering Agent

The final pH of a product is crucial to optimizing its characteristics, especially in adapting to the skin's pH. Buffers are often employed to maintain the pH and other vital characteristics of a topical vehicle throughout their shelf life [6]. The pH regulators act to maintain a suitable pH for the delivery of APIs, with the most commonly used being citric acid, lactic acid, phosphoric acid, and sodium hydroxide. The pH of the vehicle is important for maintaining the stability and efficacy of APIs, thereby preventing their degradation. Values of pH can directly affect the effectiveness of the preservatives as well, compromising their preservative effect as well as impacting the viscosity of the product [34]. Nevertheless, products with high buffering capacity require careful consideration, as they have the potential to alter the skin surface, resulting in both positive and negative effects [32].

7.9. Antioxidants

Many topical formulations are susceptible to oxidative degradation. An antioxidant is, therefore, usually used as the important composition to protect the prepared formulation. In oily products, antioxidants prevent oils from becoming rancid. The most commonly used

antioxidants in topical formulations include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ascorbyl palmitate, and sodium ascorbate [6].

The concept of antioxidants is pertinent in the pursuit of benefits for topical applications. Reactive oxygen species (ROS) can be produced in the skin upon exposure to agents such as stress, UV radiation, environmental pollutants, and inflammation. These ROS cause damage to DNA, RNA, proteins, and lipid bilayers, resulting in skin aging and photocarcinogenesis. Thus, topical antioxidants have been utilized as a means of combating these damages. Chandan et al. [69] demonstrated the active role in the skin's defense mechanism of antioxidant enzymes (specifically superoxide dismutase (SOD) and catalase) in preventing oxidative damage caused by ROS. This could be a highly desired effect for products targeting skin conditions with inadequate oxidative repair, such as AD, psoriasis, and acne [69]. Zhang et al. [70] examined the antioxidant effects of natural extracts containing curly kale, apple, and green tea, which are rich in antioxidants. Their study illustrated that these formulations possessing high antioxidant capacity could exhibit anti-inflammatory effects in patients with mild to moderate AD. The formulations were also found to be able to reduce itching, decrease local SCORAD scores, and improve both barrier function and hydration of skin lesions [70].

7.10. Preservatives

Inclusion of preservatives in topical formulations, particularly those with high water content, is recommended to prevent contamination and microbial growth. In anhydrous formulations, such as ointments, the use of preservatives is less frequent. A preservative should exhibit efficacy against a broad spectrum of microorganisms. Several factors must be considered when selecting a preservative, including the formulation type, toxicity, potential for irritation, concentration within the formulation, and the presence of other preservatives in the ingredients. Some examples of widely used preservatives include alcohols (benzyl alcohol, ethanol, and phenoxyethanol), phenols (chlorocresol), hydroxybenzoates (all salts), and quaternary ammonium compounds (benzalkonium chloride and cetrimide) [6].

Due to their widespread use and significant risk of sensitization, preservatives remain a leading cause of contact allergies. Even the new generations of preservatives necessitate further studies before market introduction. Future preservatives aim to offer broad-spectrum antimicrobial activity while maintaining a high safety profile with respect to sensitization [71]. Preservatives containing chloromethylisothiazolinone (MCI) and methylisothiazolinone (MI) were widely used in cosmetic products due to their antimicrobial efficacy. However, since 1985, its sensitizing effect in cosmetics has been documented. Clinical symptoms primarily affect the hands and faces of patients using products such as gels, liquid soaps, and shampoos. This sensitization is intrinsically linked to multiple contact hypersensitivities, predominantly involving fragrances [72].

7.11. Fragrance

Fragrances are important for the attractiveness of cosmetic products. Their composition is complex, with a large number of ingredients, either from natural or synthetic origins. Fragrance allergens are the main cause of contact allergies, especially in children. A higher number of allergenic fragrances were found in facial products, with facial eczema being the most common scenario [73]. Besides, Travassos et al. [74] conducted a study with 11 different categories of cosmetic products containing non-perfumed allergens for the skin, hair, and body. Formaldehyde-based preservatives were found to be the major culprits for allergies, and the sunscreen ingredient octocrylene was also identified as an allergen [74].

Table 2 summarizes some of the usual inactive ingredients or excipients used in topical formulations.

Table 2. Excipients used in topical formulations.

Excipient	Role	Example	Other Function
Solvents [32,34,64]	vehicle, dissolve excipients	water, propylene glycol, mineral oil, and hexylene glycol	water, the most essential and commonly
Consistency agents [6,34]	hardening or consistency	beeswax, carnauba wax, white petrolatum, liquid paraffin, and lanolin	protective occlusive barrier
Thickeners/Gelling agents [6,32,34]	viscosity control	cetyl alcohol, cetearyl alcohol, carnauba wax, stearic acid, gelatin, hydroxyethyl cellulose, xanthan gum, carboxymethyl cellulose, guar gum, sodium alginate, bentonite, aluminum magnesium silicate, ammonium acryloyldimethyltaurate, silica, acetyl palmitate, carbomers	viscosity is directly related to skin retention and the penetration of actives
Silicones [6,32]	smooth skin surface	dimethicone, cyclomethicone	ingredients that can act as non-greasy occlusives
Humectants [65,67]	attract water	glycerin	accelerate skin barrier recovery
Emulsifiers/Solubilisers [6,34]	stability control	glycerol monostearate, cetostearyl alcohol, cetyl palmitate, sorbitan monostearate, polysorbate 20, polysorbate 60, polysorbate 80, propylene glycol monostearate, sorbitan monooleate, poloxamer, emulsifying wax, sodium lauryl sulfate, and diethylene glycol monoethyl ether	higher percentage can cause dermal irritation, corrosiveness, and an increased potential for sensitization
Chelating agents [6]	improve the stability of a topical vehicle	EDTA	may exhibit positive synergy with the effects of preservatives
Acidifying/Alkalising/ Buffering agent [6,32,34]	buffering, pH agent	citric acid, phosphoric acid, lactic acid, and sodium hydroxide	correct skin pH; help to delivery of actives; and maintaining the stability and efficacy of an active ingredient
Antioxidants [6]	composition protection	BHA, BHT, ascorbyl palmitate, and sodium ascorbate	prevent oil rancidity
Preservatives [6]	prevent contamination	benzyl alcohol, ethanol, phenoxyethanol, quaternary ammonium compounds (benzalkonium chloride, cetrimide), hydroxybenzoates (all salts), phenols (chlorocresol)	extend shelf life
Fragrance [6,73]	perfume the product	natural or synthetic origin	attractiveness of cosmetic products

8. Conclusions

A consensus exists that AD is recognized as a chronic condition, attributed to various factors, including a genetic component, requiring ongoing management throughout the patient's life. Therapeutic interventions may include oral medications, such as corticosteroids and immunosuppressants, alongside topical corticosteroids applied directly to

the skin. Additionally, the use of moisturizers as an adjunct therapy is recommended for deep hydration.

Several scientific studies have emphasized the importance of cosmetics as adjuncts in AD treatment, with their utilization and recognition of benefits gaining increasing strength. In addition to the hydration provided by cosmetic products, the final sensory experience delivered after application stands out as a crucial factor for patient adherence to AD treatment.

A cosmetic formulation for AD needs to be comprehensively balanced, and the significant contribution of a cosmetic lies in its ability to deeply hydrate compromised, dry, and fragile skin. This review places a greater emphasis on exploring hydration alternatives (emollient, humectant, and occlusive).

Essential concepts for the effective development of safe and modern cosmetics were also addressed in this review. This material aims to contribute to the dissemination of knowledge on aspects and concepts related to product development, providing strategic information for formulators to create products that enhance self-esteem, in addition to improving skin appearance and providing a pleasant sensation.

Despite extensive research highlighting the importance and benefits of cosmetic vehicles, testing standards for cosmetic products must evolve accordingly. It is crucial that assays evaluating the efficacy and safety of cosmetics and dermo-cosmetics adhere to rigorous, scientifically sound methodologies in line with the latest approved guidelines.

There is a lack of scientific studies addressing the quality and control of the water used in the development of topical products. Considering that water often constitutes the largest percentage in formulations, it is crucial for new studies to focus on this significant ingredient applied in formulations.

Several vehicles can alter the potency, sensory properties, safety, and efficacy of a formulation for AD. This scientific article addressed key points for the development of a cosmetic product for AD, thus contributing to assist the formulator in developing a safe and effective formulation.

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Review

Itch-Relieving Cosmetics

Ju Hee Han [1] and Hei Sung Kim [2,*]

[1] Department of Dermatology, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul 06591, Republic of Korea; alwaysmine8@gmail.com
[2] Department of Dermatology, Incheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul 06591, Republic of Korea
* Correspondence: hazelkimhoho@gmail.com; Tel.: +82-32-280-5105

Abstract: This review aims to explore the evolving role of cosmetics in alleviating itch, transcending their traditional aesthetic function. With a focus on formulations enriched with natural oils and other bioactive components, we examine the efficacy and safety of various cosmetic ingredients designed to control itch. Highlighted are ingredients such as colloidal oatmeal, postbiotics, menthol, peppermint, cryosim-1, capsaicin, asivatrep, polidocanol, pramoxine hydrocholoride, and palmitoylethanolamide, which are recognized to reduce itch. Special attention is also given to phytochemicals that can modulate the Janus kinase/signal transducer and activator of transcription signaling pathway and carry the potential as an itch-relieving cosmetic ingredient. This review encompasses clinical studies that verify the itch relieving effect of these cosmetic ingredients. By integrating current scientific evidence, we aim to shed light on the potential of anti-itch cosmetics as an adjunct to standard itch treatment, thereby broadening our understanding of their role in dermatological care.

Keywords: skin; anti-itch; cosmetics

1. Introduction

The Federal Food, Drug & Cosmetic Act (FD&C Act) characterizes cosmetics as "articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body for cleansing, beautifying, promoting attractiveness, or altering the appearance" (FD&C Act, sec. 201(i)). This category encompasses a variety of products, such as moisturizers, perfumes, lipstick, nail polish, eye and facial makeup preparations, shampoo, hair coloring, toothpaste, and deodorant, along with any material intended for use as a component of a cosmetic product [1]. The role of cosmetics in skin health has undergone significant changes over the years, expanding beyond aesthetic improvement and elementary skin safeguarding. As part of this shift, moisturizers are now being developed and marketed in multiple formulations that focus on different skin issues [1]. The market for products designed to address specific skin concerns, such as xerosis, acne, and atopic dermatitis (AD), is rapidly growing. These products aim to not only provide general skin care but also to alleviate the discomfort associated with these skin conditions. One notable example of this trend is the debut of itch-relieving cosmetics, which underscores the advanced and tailored approach to skincare. This review aims to take a closer look at the details of anti-itch cosmetics, focusing particularly on the common cosmetic products marketed for anti-itch effects and their active ingredients. In addition, we will identify the mechanisms by which the active ingredients exert their anti-itch effects and summarize the clinical studies supporting their efficacy. By doing so, we seek to provide a comprehensive understanding of how these products work and their potential benefits in skincare.

2. Methods

A search was conducted in two electronic databases, PubMed and Cochrane Library, consisting of search terms including "itch", "itching", "anti-itch", "pruritus', "pruritic",

"anti-pruritics", "moisturizers", "emollients", "cosmetics", "polyphenols", "phytochemicals", "eczema", "dermatitis", "dermatology". English-language articles that assessed the use of active ingredients in moisturizers for targeting itch were included. Non-English articles and drug ingredients not classified as cosmetic ingredients were excluded. We specifically included active ingredients that are currently widely marketed in cosmetics for itch relief. Preference was given to high-quality, peer-reviewed articles, and reference lists were reviewed to include relevant studies. Additionally, we considered clinical studies on the ingredients, whether they were RCTs, non-randomized controlled, or even uncontrolled.

3. Itch

Itch is an unpleasant sensation of the skin that provokes the desire to scratch [2]. With a lifetime prevalence reaching up to 25%, chronic itch (>6 weeks) is ubiquitous in the general population [3,4]. Elderly people are particularly prone to chronic itch, which is likely due to the anatomical, functional, and physiologic changes in aged skin [4,5]. Studies have shown that itch is associated with higher levels of stress and anxiety, and can disrupt one's sleep, concentration, and daily activities [6]. Not surprisingly, chronic itch exerts a substantial, negative impact on the quality of life (QoL) comparable to that of chronic pain [7].

Itch is often a symptom of a clinically evident skin condition such as AD, psoriasis, urticaria, scabies, bullous pemphigoid, and prurigo nodularis (PN) [8,9]. However, it may also be a prominent feature in many systemic diseases (i.e., acquired immunodeficiency syndrome, chronic kidney disease, liver disease, cholestatic disease, hematologic causes, thyroid disease, iron deficiency, malignancy), neuropathic disorders (i.e., nostalgia paresthetica, brachioradial itch, postherpetic itch), and psychologic conditions [3,10] (Table 1) Environmental pollutants and climate factors also trigger the itch sensation [5,11].

Table 1. Systemic etiologies for pruritus.

Autoimmune	Dermatomyositis Sjogren syndrome Dermatitis herpetiformis Linear immunoglobulin A disease
Hepatobiliary	Chronic Hepatitis Chronic pancreatitis Primary biliary cirrhosis Sclerosing cholangitis Drug-induced cholestasis Pancreatic carcinoma
Endocrine	Hyperthyroidism Hypothyroidism Hyperparathyroidism Diabetes mellitus
Renal	Chronic renal impairment
Hematologic	Iron deficiency Polycythemia vera Mastocytosis Multiple myeloma Lymphoma Leukemia
Neurologic	Postcerebral infarction Multiple sclerosis Cerebral tumor Notalgia paresthetica Brachioradial pruritus

Table 1. *Cont.*

Infectious	Acquired immunodeficiency syndrome Parasitic disease
Other	Drug Neuropsychiatric disorder Pregnancy Aging Dryness

The Itch Pathway

Itch involves complex crosstalk between keratinocytes, the immune system, and sensory nerves and is ultimately provoked by the interaction between the itch mediators (pruritogens) and the pruriceptors [12]. There are two major neuronal itch pathways: the histaminergic and the non-histaminergic, the latter of which involves a diverse array of receptors that respond to itch-inducing agents other than histamine [12–14]. Acute itch is primarily triggered by histamine, which is released by mast cells, basophils, and keratinocytes within the skin [14,15]. On the other hand, chronic itch, which is our focus, involves a wider variety of pruritogens, such as proteases, cytokines/chemokines, and amines [16]. There are primarily two types of itch receptors: the G protein-coupled receptor (GPCR), which is the main subtype, and the interleukin receptor (ILR) that is coupled to the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway. Both transmit itch signals downstream by activating the family of transient receptor potential (TRP) channels. The GRCRs include the histamine H1 and H4 receptors and the protease-activated receptors (PARs), mas-related G protein coupled receptors (MRGPRs), and neurokinin receptors. TRP ion channels, such as Transient Receptor Potential Vanilloid (TRPV) 1, Transient Receptor Potential Ankyrin (TRPA) 1, TRPV3, and TRPV4, play significant roles in both acute and chronic itch, as well as pain. The Nav1.7 sodium channel, essential for nerve signal transmission, is activated by TRPV1 and TRPA1 and conveys itch and pain signals [13,16–18].

Opioid receptors, particularly mu-opioid receptors (MOR) and kappa-opioid receptors (KOR), have also been implicated in the regulation of itch, with activation of MORs generally exacerbating itch, whereas activation of KORs tends to suppress itch [19]. Pro-opiomelanocortin (POMC), as a precursor molecule, is also related with the opioid-mediated modulation of itch. It is cleaved into several bioactive peptides, including β-endorphins that primarily activate MORs, which can provide pain relief but also potentially increase itch. Conversely, other derivatives of POMC can interact with KORs to help suppress itch signals. This demonstrates the complex balance of opioid receptor activation by POMC derivatives in the nervous system [20,21].

The sensation of itch is primarily transmitted through unmyelinated type C and thinly myelinated type Aδ nerve fibers in the skin [22]. The cell bodies of these pruriceptors are in the dorsal root ganglia and their axons continue into the spinal cord typically synapsing in the dorsal horn with activating or inhibitory interneurons [13,22]. Other than transmitting the itch signal, primary sensory neurons intensify itch by secreting itch mediators [23]. Activated sensory neurons release neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P (SP), which mediate mast cell degranulation and plasma extravasation. Cytokines and proteases released from keratinocytes and mast cells initiate a neurogenic inflammation further aggravating itch [24].

As for the immunomodulatory axis, type 2 cytokines interleukin (IL)-4, IL-13, and IL-31 are necessary in chronic itch settings [25–28]. Itch-induced scratching promotes TSLP and IL-33 release by epidermal keratinocytes, which perpetuates the itch-scratch cycle. Recently, the Th17/22 axis has also been implicated in the itch of psoriasis, AD [27,29–31], and PN [32,33], all of which are potential therapeutic targets.

4. Cosmetic Ingredients Tailored for Itch and Their Anti-Itch Mechanisms

Itch treatment aims at itch relief and beyond, which is the appropriate management of the underlying etiology. Due to its complex nature, most require multimodal treatment, utilizing both topicals and systemic agents [34]. The most commonly used topical medication is corticosteroids but the risk benefit ratio should be calculated if the intent is to treat large areas of the skin as topical corticosteroids can potentially suppress the hypothalamus-pituitary axis and cause skin thinning [35,36]. Alongside pharmaceuticals, consumers are increasingly drawn to cosmetic products that address itch while offering daily use convenience. Such a trend underscores the growing demand for safe and effective anti-itch formulations.

Dry skin increases intraepidermal nerve density and the expression of neuropeptides and cytokines, which enhances itch sensitivity through complex interactions with sensory neurons and keratinocytes [37]. Moisturizers, one of the most widely used preparations in cosmetics, restore the skin barrier and increase water content in the stratum corneum. Since dry skin and itch are intricately linked, the three main types of moisturizing ingredients (humectants, occlusives, and emollients) can help reduce itch. Additionally, moisturizers provide a barrier effect against environmental substances and leave the skin feeling fresh, further aiding in alleviating itch [38–40]. Beyond these functions, we will further focus on active ingredients with anti-itch properties (via targeting multiple components of the itch pathway) (Table 2) that may further supplement the itch-relieving effect of a regular moisturizer, with some already being commercialized (Table 3). By reviewing clinical studies that examine the itch relieving effect of these active ingredients, we offer insight into their potential therapeutic benefit (Table 4).

Table 2. Mechanism of active ingredients with anti-itch properties.

Ingredient	Mechanism
Colloidal oat [41,42]	Block the release of IL-8 Inhibit nuclear factor kappa B, arachidonic acid, and TNF- α Restore skin microbiome balance
Postbiotics (*Aquaphilus dolomiae* extract) [43,44]	Inhibit the activation of protease-activated receptor 2 Reduce the secretion of cytokines by T helper cells (Th1, Th2, and Th17)
Menthol and cryosim-1 [45–47]	Activate TRPM8 receptors Produce a cooling sensation Help alleviate itching by creating a counterirritant sensation
Capsaicin and asivatrep [48–51]	Analgesic, anti-inflammatory, and anti-pruritogenic activities Desensitization of the sensory neurons that express TRPV1 (Capsaicin) Reduce TRPV1 expression (Asivatrep) Inhibit the release of pruritogenic neuropeptides substance P and CGRP
Polidocanol [52]	Inhibit protease-activated receptor 2 Involved in the histamine-independent itch
Pramoxine hydrochloride [53]	Block voltage-gated sodium channels and prevent nerve signal transmission
Palmitoylethanolamide [54–58]	Inhibit TRPV1 ion channel Prevent the breakdown of lipids and boost their production in the stratum granulosum

TRPM8: Transient receptor potential cation channel subfamily member 8, TRPV1: Transient receptor potential vanilloid 1, CGRP: Calcitonin gene-related peptide.

Table 3. Commercialized cosmetic products with itch relief ingredients.

Company	Product	Active Ingredients
Aveeno (Skillman, NJ, USA)	Anti-itch concentrated lotion	Oatmeal
Aveeno (Boulogne, France)	Itch relief balm	Oatmeal
Dermal (Seoul, South Korea)	Anti-itch soothing lotion	Oatmeal, Menthol
Avène (Boulogne, France)	Eau thermale	*Aquaphilus dolomiae* extract
Eucerin (Hamburg, Germany)	Itch relief intensive calming lotio	Menthol
Head and shoulders (Cincinnati, OH, USA)	Itchy scalp shampoo	Menthol
Head and shoulders	Deep cleanse itch relief	Peppermint
Hair chemist (Orangeburg, NY, USA)	Peppermint scalp stimulator for dry scalp and anti-itch	Peppermint
Intrinsic (Seoul, South Korea)	IB spot serum	Cryosim-1
Greensations (Scotia, NY, USA)	Therma scalp anti-itch scalp spray	Capsaicin
Aestura (Seoul, South Korea)	Atobarrier Itching cream	Asivatrep
Ducray (Lavaur, France)	Physio-protective soothing body lotion	Polidocanol
Isdin (Barcelona, Spain)	UreadinCalm	Polidocanol
Eucerin (Hamburg, Germany)	Anti-itch spray	Polidocanol
CeraVe (New York, NY, USA)	Itch relief moisturizing lotion	Pramoxine hydrochloride
Gold bond (Chattanooga, TN, USA)	Anti-itch body lotion	Menthol, Pramoxine hydrochloride
Sarna (Johnson City, TN, USA)	Calm + Cool anti-itch lotion	Pramoxine hydrochloride, Menthol
Sarna	Sensitive anti-itch lotion	Pramoxine hydrochloride
GoodSense (Dublin, Ireland)	Clear anti-itch lotion	Pramoxine hydrochloride
Bioderma (Aix-en-Provence, France)	Atoderm intensive baume	Palmitoylethenolamide

4.1. OatColloidal Oatmeal, Produced by Finely Grinding the Oat and Boiling It to Extract the Colloidal Material, Has Been Recognized for Its Skin-Soothing and Protective Effects

Currently, colloidal oatmeal is extensively utilized for skin conditions and has been acknowledged by the U.S. Food and Drug Administration (FDA) as a natural and safe over-the-counter (OTC) skin protectant. It is included in the FDA's OTC monograph for skin protectant drug products, indicating its recognized safety and effectiveness. Recent evidence has indicated that colloidal oatmeal can also serve as a prebiotic, which could promote the growth of beneficial microorganisms, supporting the skin's microbial balance [59]. The formulations of colloidal oatmeal are offered in various forms such as bath treatments, cleansing bars, body washes, shampoos, lotions, creams, and shaving gels [59]. It is also a common ingredient in many commercial moisturizers that are designed to combat dry skin and associated itching, and it is especially beneficial for conditions such as AD [60].

Removing potentially sensitizing proteins from oats might be crucial, especially for individuals with AD [61]. The development of Rhealba Oat Plant Extract is one example of this approach. Specifically designed for atopic skin by being free of oat proteins, Rhealba oat extract can minimize the risk of allergic reactions [61,62]. Oats primarily consist of starch, along with proteins, including enzymes, lipids, and fibers and beta-glucans. Within oats, avenanthramides are a group of phenolic compounds, about 20 different types, which are low in molecular weight and soluble. They are found in oats at concentrations of up to 300 parts per million, or 0.03% [41]. Many studies demonstrated that avenanthramides, bioactive compounds found in oats, exhibit anti-inflammatory, anti-proliferative, vasodilative, cytoprotective, and antioxidant properties as well as anti-itch activity [60,63].

Further research is needed to elucidate the mechanisms through which anti-pruritic actions are manifested; however, a recent in vitro study has revealed that components in oatmeal extract, notably avenanthramides, block the release of IL-8, thereby reducing inflammation and itch [41]. Additionally, it inhibits nuclear factor kappa B (NF-kB), arachidonic acid, and TNF-α, which are key in inflammatory skin diseases [41]. Interestingly, avenanthramides' structural similarity to the antihistamine drug tranilast may suggest their itch-relief might stem from hindering histamine signaling. Applied topically at low concentrations, avenanthramides are reported to be effective in managing inflammation and itch, indicating their value as adjuvant as dermatological treatments [41].

Studies have also shown that oatmeal in skin products can help by acting as a natural buffer, keeping skin moist and forms a protective layer to lock in moisture [64]. In line with these findings, there is ongoing research into cosmetic products with 1% colloidal oat designed to alleviate itching by restoring the natural balance of the skin's microbiome [65]. A previous randomized, open-label trial study has shown effectiveness of oat extract-based emollient in managing chronic pruritus in elderly patients with xerosis by comparing treatment to non-treatment in 30 participants [42]. Additionally, previous studies have demonstrated the topical corticosteroids sparing effect of emollient containing oat extracts, as well as its effectiveness in reducing the severity of AD and minimizing flare-ups in patients with the condition [66,67]. Matheson et al. evaluated the efficacy of liquid paraffin with 5% colloidal oatmeal in comparison with other contained liquid paraffin in the management of patients with burn injuries, and reported that product contains liquid paraffin with 5% colloidal oatmeal significantly decreased itching and patients requested significantly less antihistamine [68].

Additionally, the impact of Rhealba oat plantlet extract on chronic pruritus was assessed in a randomized crossover study involving elderly patients aged 60 and above. The emollient containing Rhealba oat plantlet extract provided significant relief from pruritus, and there were notable improvements in sleep quality and xerosis [42].

4.2. Postbiotics (Aquaphilus dolomiae, Vitreoscilla filiformis, Streptococcus thermophilus, and Lactobacillus johnsonii Extract)

Research has shown that creams containing *Aquaphilus dolomiae* extract (ADE-G1), derived from a type of cyanobacteria in Avène Thermal Spring Water, have been successful in alleviating itchiness and dry skin across various skin and systemic conditions [69]. In vitro research indicates that ADE-G1 has multiple beneficial effects for the management of pruritus and xerosis, such as immunomodulatory and anti-inflammatory actions that adjust the levels of various inflammatory markers [43]. Moreover, ADE-G1 has been shown to possess anti-itch properties, inhibiting the activation of PAR2 and reducing the secretion of cytokines by T helper cells (Th1, Th2, and Th17) [43]. A controlled clinical trial using the cowhage-itch model further validated the anti-itch properties of an emollient containing ADE-G1, also demonstrating its targeted inhibition of the non-histaminergic itch pathway [44]. Research has highlighted the effectiveness of topical lotions or creams with bacterial extracts from *Vitreoscilla filiformis*, *Streptococcus thermophilus*, and *Lactobacillus johnsonii* in alleviating symptoms of AD [70–72]. These benefits are possibly related to reducing the harmful impacts of *S. aureus* and *Malassezia*, leading to improved clinical outcomes such as reduced erythema, scaling, and itching.

Numerous research efforts focusing on microbiome cosmetics to enhance skin health and address associated itching suggest that the area of microbiome dermocosmetics could emerge as a promising field in the future.

4.3. Menthol and Cryosim-1

Transient receptor potential cation channel subfamily M member 8 (TRPM8) channels, located in various organs and particularly in sensory neurons, are responsible for sensing non-noxious cold temperatures and are activated by cooling compounds like menthol [73]. Cooling has been proven effective in reducing the discomfort of itches, positioning TRPM8

channels as promising targets for developing itch-relief therapies [74]. In previous double-blind, randomized study, a cooling lotion with TRPM8 agonists was effective for relieving chronic itching in people with dry skin, with up to 84% of users reporting better results than a standard lotion [47].

Menthol, an organic compound derived from peppermint (*Mentha piperita*) and other mint plants, is widely recognized for its cooling sensation when applied to the skin. This cooling effect can help alleviate itching by creating a counterirritant sensation that distracts from the itch [45]. Menthol works by activating TRPM8 receptors in the skin. These receptors are part of the body's temperature-sensing system. When activated, they produce a cooling sensation, which can help to soothe and distract from the sensation of itching [45,46].

Previous research evaluated a moisturizing cream with 3% menthol and ceramides for its safety and anti-itch efficacy. Among 60 volunteers, including those with AD, the cream significantly reduced itch intensity with no adverse effects reported except for one individual who experienced a stinging sensation. The improvement in itch scores was noted as early as one week and sustained through a month of use, indicating the cream's effectiveness and safety for dermatitis-related itch relief [75].

In addition, a study has shown that a cream with menthoxypropanediol, an activator of the TRPM8 channel, rapidly alleviates itching in individuals with AD. Most patients reported significant itch relief shortly after application, with over 90% satisfaction after 15 min. Despite some side effects, the study supports the use of TRPM8 agonists in managing itch and suggests potential mechanisms, including direct effects on nerve endings and involvement of Merkel cells, which are known to express TRPM8 [76]. Another study found that topical application of peppermint oil significantly improved chronic pruritus due to underlying hepatic, renal, and diabetic cause compared to petrolatum, suggesting it to be effective in managing chronic pruritus [77].

Cryosim-1, a synthesized compound that stimulates TRPM8 receptors, is being utilized as a component in anti-itch cosmetic products. In previous study, cryosim-1 gel led to a reduction in itching for individuals with conditions like AD and urticaria, enhancing their QoL [78]. In a clinical study involving 30 subjects with PN, by the eighth week, those treated with Cryosim-1 gel reported a notable decrease in itching and sleep disturbances compared to those given a placebo, along with greater satisfaction with the treatment [79]. A randomized controlled trial (RCT) demonstrated that cryosim-1 significantly reduced itch in patients with eczema and urticaria, providing rapid relief within minutes and maintaining anti-itch effects for up to two hours, indicating its potential as an effective treatment for pruritus [80]. In another RCT, Cryosim-1 gel for scalp pruritus significantly lowered Numerical Rating Scale scores within two hours of application compared to a control treatment, offering quick itch relief. This improvement also led to enhanced QoL for the participants [78].

The use of topical menthol is recommended fort management of chronic pruritus in current European S2k guideline [34].

4.4. Capsaicin and Asivatrep

Pruriceptive nociceptors that respond to histamine or non-histamine pruritogens often express TRPV1, a channel that reacts to capsaicin and heat. Capsaicin, a compound found in capsicum plants like chili peppers, jalapeños, and cayenne pepper, is renowned for its sharp taste and irritation of nerve endings [81]. It boasts various effects, including analgesic, anti-inflammatory and anti-pruritogenic activities [48]. Capsaicin has been demonstrated to trigger the release of substance P, a neuropeptide known for causing a severe burning and pricking that can be felt as either pain or itchiness. Paradoxically, prolonged use of capsaicin results in pain relief and itch reduction, as it causes desensitization in the sensory neurons that express TRPV1 [48,49].

Various OTC capsaicin creams with differing concentrations have been suggested to be effective in managing skin-related neuropathic conditions like brachioradial pruritus, uremic pruritus, hemodialysis-related pruritus, aquagenic pruritus, PN, and AD [82–88].

A study with 33 patients explored how applying capsaicin cream, in concentrations from 0.025% to 0.3%, could treat PN. Applied up to six times daily for as long as ten months, the treatment completely relieved itchiness within 12 days suggesting effectiveness of capsaicin for this condition [86]. Several studies have reported the impact of topical capsaicin cream, in 0.025% or 0.03% concentrations, applied four times a day for the treatment of chronic kidney disease-associated uremic itching, compared against a placebo cream. Findings suggest that capsaicin cream application likely offers significant relief from symptoms of uremic itching compared to placebo [82,83,89]. In addition, the Cochrane Review on interventions for itch in people with advanced chronic kidney disease concluded that topical capsaicin likely reduces uremic pruritus, even though it noted that additional high-quality evidence is needed before a definitive conclusion [90]. Treatment of localized chronic pruritus with capsaicin may be considered according to current European S2k guidelines, especially in case of brachioradial pruritus and notalgia paresthetica treatment [34].

Asivatrep is a highly potent and selective antagonist of TRPV1. In AD-like murine models, topical application of asivatrep cream effectively reduced inflammation by lowering levels of type 2 cytokines IL-4 and IL-13 [91]. This in turn, might inhibit the release of pruritogenic neuropeptides substance P and CGRP, thereby reducing itch [50]. It also improves skin barrier function by promoting the production of key epidermal differentiation markers such as loricrin, filaggrin, involucrin, and other differentiation-related keratins [91]. The anti-itch effect of asivatrep can be attributed to its ability to reduce TRPV1 expression, which helps to alleviate dermatitis and pruritus while strengthening the skin barrier [51]. In a phase 2b clinical trial, asivatrep decreased pruritus and inflammation in AD patients [92]. In addition, a phase 3 study demonstrated that asivatrep cream significantly improved clinical signs and symptoms of AD, including pruritus, compared to a vehicle [51].

Although there are limited laboratory and clinical studies on asivatrep, it is included as an active ingredient in moisturizers for its itch control effects and is expected to be applied to a wider range of conditions associated with pruritus in the future.

4.5. Polidocanol

Polidocanol, a polyethylene glycol ether of lauryl alcohol, have been investigated by cosmetic industries for its antipruritic and local anesthetic properties [93,94]. Polidocanol formulations are currently available in various commercial products, including a topical aqueous leave-on tonic, lotions, and creams. Previous studies have shown that polidocanol is beneficial in managing urticarial pigmentosa [93]. In a previous study on anti-itch effect of polidocanol involving 45 subjects, applying 3% topical polidocanol for 1 h significantly reduced cowhage-induced itch not affecting histamine-induced itch [95]. Although the exact mechanism behind the anti-itch effects of polidocanol is not yet fully understood, its association with PAR-2 receptors is suggested. These receptors, found on keratinocytes and small unmyelinated C-nerve fibers, are involved in the histamine-independent itch pathway, also known as cowhage-sensitive C fibers [52]. Enhanced PAR-2/PAR-4 signaling, which is associated with cowhage-induced itch, has been linked to AD [96]. Neurons activated by cowhage have higher conduction velocity and discharge rates compared to histamine-responsive neurons, making them more susceptible to inhibition by lower concentrations of local anesthetics [97,98]. Further research on the precise mechanism of anti-itch action of polidocanol and its association with PAR-2 receptors is needed.

4.6. Pramoxine Hydrochloride

Pramoxine hydrochloride is a topical local anesthetic agent that shows relatively fast onset of action, providing itch relief within 3 to 5 min [53]. It works by blocking voltage-gated sodium channels, reducing sodium ion permeability, and preventing nerve signal transmission, which provides both anesthetic and antipruritic effects. It is used off-label in dermatology to manage various conditions with pruritus. In a study involving 200 patients with various itchy skin conditions, pramoxine effectively controlled pruritus in 57% of cases, with the cream formulation being more effective and preferred over the

gel [99]. Study on hemodialysis patients with uremic pruritus showed a significant reduction in itch intensity by using pramoxine hydrochloride lotion compared to the control group [100]. In addition, combination of lactic acid and pramoxine hydrochloride significantly improved skin hydration and reduced dryness and itch among 24 women [101]. Ceramide-containing pramoxine hydrochloride formulations provided rapid and long-lasting relief in AD, with itch severity reduced by 24.6% two minutes post-application and 58.0% eight hours post-application, comparable to ceramide cream with hydrocortisone [102]. Another combination of hydrocortisone acetate and pramoxine cream showed significant itch reduction after a single day of use, with a mean percentage reduction in itch intensity of 31.74% [103].

4.7. Palmitoylethanolamide (PEA)

PEA is a bioactive lipid similar to endocannabinoids naturally produced in mammalian tissues [104]. It is commonly utilized in the management of acute or chronic status, recognized for its effectiveness in regulating various systems involved in inflammation, itching, and pain [56,57,104]. Research has indicated that PEA can significantly reduce itch by inhibiting the TRPV1 ion channel, primarily present in nociceptive neurons of the peripheral nervous system [54,55]. Preclinical studies have shown that the effectiveness of PEA relies on the peroxisome proliferator-activated receptor-alpha receptor, which is known to play a significant role in regulating inflammatory processes [58,104,105]. Moreover, the application of phospholipids like PEA may help prevent the breakdown of lipids and boost their production in the stratum granulosum, potentially aiding in the fight against dry skin and reducing associated itching [56].

One study showed that cream containing PEA decreased itch symptoms drastically in patients with specific skin conditions with pruritus including prurigo and lichen simplex [106]. Additionally, PEA-based cream has shown promising results in alleviating symptoms of AD, offering an affordable alternative to traditional treatments [107]. Another study on topical Levagen+, a novel PEA formulation, showed significantly reduced eczema symptom severity, including redness, dryness, and total Patient-Oriented Eczema Measure score, compared to a standard moisturizer over a 4-week period [104]. In patients with AD including pediatrics, PEA-containing lamellar matrix cream also led to a significant reduction in the severity of symptoms including erythema, pruritus, lichenification, and dryness over a period of 4–6 weeks [108]. PEA is included as an active ingredient in various moisturizers on the market for its anti-itch effects.

Table 4. Application of itch relief cosmetics in skin conditions (summary of clinical studies).

Author	Formulation	Study Detail	Key Findings
		Oat	
Theunis et al. (2017) [42]	Topical emollient containing Rhealba® oat extract	30 patients aged $\geq$ 60 years with xerosis associated with chronic pruritus Applied for 2 weeks RCT	Improvement of pruritus and xerosis
Mengeaud et al. (2015) [67]	Topical emollient cream containing oat plantlets	108 children (ages 6 months-6 years) with moderate AD Applied twice daily for 3 months Absence of control group, open-label	Improvement of AD clinical symptoms (SCORAD and PO-SCORAD indexes) Fewer flare-ups Less topical corticosteroid use
Grimalt et al. (2006) [66]	Topical emollient containing Rhealba® oat extract	173 infants under 12 months old with AD Applied twice daily for 6 weeks RCT	Reduced the high-potency topical corticosteroid consumption in infants with AD

Table 4. *Cont.*

Author	Formulation	Study Detail	Key Findings
		Postbiotics	
Deleuran et al. (2020) [69]	Topical emollient containing an *Aquaphilus dolomiae* extract	5910 patients of all age with a range of dermatologic and systemic diseases with pruritus and xerosis Applied for 7 days Absence of control group, real world study	Improvement of pruritus, xerosis and quality of life
Blanchet-Rethore et al. (2017) [72]	Topical lotion containing the heat-treated *Lactobacillus johnsonii* NCC 533	31 patients with mild to moderate AD Applied for 3 weeks Absence of control group	Reduction in S. aureus load Decrease in local objective SCORAD
Gueniche et al. (2008) [109]	Topical cream contining *Vitreoscilla filiformis* lysate	75 patients with mild AD Applied for 4 weeks RCT	Improvement of SCORAD and pruritus
Gueniche et al. (2008) [110]	Topical cream containing *Vitreoscilla filiformis* extract	10 patients with mild to moderate AD Applied for 4 weeks Left-right comparison study	Improvement of AD lesions and pruritus
Di Marzio et al. (2003) [70]	Topical cream containing sonicated *Streptococcus thermophilus*	11 patients with AD Applied for 2 weeks Compared with healthy volunteers	Improvement of AD (erythema, scaling, pruritus)
		Menthol and cryosim-1 (TRPM8)	
Choi et al. (2024) [79]	Topical serum containing cryosim-1	30 patients with prurigo nodularis RCT	Improvement of pruritus Reduced sleep disorder
Kang et al. (2022) [78]	Topical gel containing cryosim-1	In part A, 31 patients with recalcitrant scalp itch In part B, 25 subjects with scalp itch RCT	Improvement of scalp pruritus and quality of life
Jung et al. (2021) [80]	Topical gel containing cryosim-1	39 patients with recalcitrant itch (eczema and urticaria) RCT	Improvement of pruritus and quality of life
Misery et al. (2019) [76]	Topical cream containing menthoxypropanediol	22 patients with AD Applied for 7 days Absence of control group	Improvement of pruritus
Ständer et al. (2017) [47]	Topical lotion containing the TRPM8 agonist	70 dry skin patients with pruritus Applied twice daily for 4 weeks RCT	Improvement of severe pruritus
Elsaie et al. (2016) [77]	Topical peppermint oil	50 subjects with chronic pruritus due to hepatic, renal, or diabetic cause Applied twice daily for 2 weeks	Improvement of chronic pruritus
		Capsaicin and asivatrep (TRPV1)	
Park et al. (2022) [51]	Topical asivatrep cream 1.0%	240 AD patients between age of 12–70 Applied for 8 weeks RCT	Improvement of AD severity and pruritus
Lee et al. (2019) [91]	Topical asivatrep cream 0.1%, 0.3% and 1.0%	194 mild to moderate AD patients Applied for 8 weeks RCT	Improvement of AD severity and pruritus
Makhlough et al. (2010) [89]	Topical capsaicin 0.03%	34 patients on hemodialysis with uremic pruritus Applied for 4 weeks RCT	Improvement of pruritus
Ständer et al. (2001) [86]	Topical cream with capsaicin 0.025% to 0.3%	33 patients with prurigo nodularis Applied 4 to 6 times daily for 2 weeks up to 10 months Absence of control group	Improvement of skin lesions and pruritus

Table 4. *Cont.*

Author	Formulation	Study Detail	Key Findings
Capsaicin and asivatrep (TRPV1)			
Lotti et al. (1994) [84]	Topical cream with capsaicin 0.025%, 0.025%, 0.5% or 1.0%	5 patients with aquagenic pruritus Applied three times daily for 4 weeks Absence of control group	After treatment, contact with water did not evoke pruritus
Tarng et al. (1996) [83]	Topical cream with capsaicin 0.025%	19 hemodialysis patients with idiopathic, moderate to severe pruritus Applied four times daily Double-blind, placebo-controlled, crossover study	Improvement of pruritus
Breneman et al. (1992) [82]	Topical cream with capsaicin 0.025%	29 patients undergoing long-term hemodialysis with pruritus Applied four times daily for 6 weeks Open-label, uncontrolled (21 patients) and double-blind, vehicle-controlled study (7 patients)	Improvement of pruritus
Pramoxine hydrochloride			
Zirwas et al. (2017) [102]	Topical ceramide-containing cream with pramoxine hydrochloride 1%	66 patients with history of AD RCT	Improvement of pruritus
Young et al. (2009) [100]	Topical lotion with pramoxine hydrochloride 1%	28 moderate to severe uremic pruritus patients receiving hemodialysis Applied for 4 weeks RCT	Improvement of pruritus
Grove et al. (2004) [101]	Topical cream with lactic acid 12% neutralized with ammonium hydroxide and pramoxine hydrochloride 1%	24 women with a history of dry itchy skin Applied for 7 days Open-label, observer-blinded, controlled trial	Improvement of pruritus and dryness
Noojin et al. (1954) [99]	Topical cream and gel with pramoxine hydrochloride 1%	200 dermatosis patients with localized or generalized pruritus	Improvement of pruritus
Palmitoylethanolamide			
Rao et al. (2023) [104]	Topical palmitoylethanolamide (Levagen+)	72 patients with AD Applied for 4 weeks RCT	Improvement of AD severity
Yuan et al. (2014) [56]	Topical cream with N-palmitoylethanolamine and N-acetylethanolamine	60 patients with AD Applied for 4 weeks RCT	Improvement of pruritus and skin barrier function
Eberlein et al. (2008) [108]	Topical cream with a unique lamellar matrix containing N-palmitoylethanolamine	2456 patients with mild to moderate AD Observational, non-controlled, prospective cohort study	Improvement of pruritus, loss of sleep and quality of life
Ständer et al. (2006) [106]	Topical cream containing N-palmitoyl ethanolamine	22 patients with prurigo, lichen simplex and pruritus Open label observational study	Improvement of pruritus

5. JAK-STAT Signaling Pathway and Itch

The JAK–STAT pathway plays a central role in modulating multiple immune axes involved in the immunopathogenesis of inflammatory skin diseases, including AD. Furthermore, it is involved in maintaining the epidermal barrier and modulating peripheral nerves related to the sensation of itch. Hyperactivation of JAK1 has been reported to be associated with overexpression of cutaneous serine protease, resulting in skin barrier dysfunction [111]. STAT3, a key transcription factor, regulates keratinocyte differentiation and maintains skin

integrity. In a study using a murine dry skin model, the JAK inhibitor delgocitinib, which blocks JAK1, JAK2, and JAK3, was shown to inhibit STAT3 activation and improve skin barrier function by increasing levels of epidermal proteins such as filaggrin, loricrin, and natural moisturizing factors [112].

Regarding itch, the activation of STAT3 mediates astrogliosis in the spinal dorsal horn, which amplifies itchiness and leads to chronic itch [113]. In this signaling pathway, lipocalin-2 is reported as distinctly upregulated factor that enhances itch signals and triggers the vicious itch-scratch cycle [114]. Notably, IL-31 stimulates nerve elongation and directly communicates with primary cutaneous afferent neurons, which pruritogens require to transduce itch signals [25]. IL-31 is known to trigger the production of pro-inflammatory cytokines and chemokines through the activation of the JAK1/JAK2 and STAT3 pathways [115]. In an AD-like murine model, topical delgocitinib has shown to suppress IL-31-promoted nerve elongation in vivo and the morphological changes of peripheral nerves from the dorsal root ganglion in vitro [116]. Additionally, it significantly reduced scratching behavior in AD mouse models.

Recently, various JAK-STAT inhibitors have been applied in the treatment of AD and inflammatory diseases. Moreover, clinical applications and research on natural polyphenol phytochemicals that could potentially modulate the JAK/STAT signaling pathway are actively being conducted (Table 5). These polyphenol phytochemicals hold potential for use as cosmetic ingredients to manage itch.

Table 5. Effects of itch relief cosmetic ingredients associated with JAK-STAT pathway).

Ingredient	Effect
Apigenin [117–119]	Regulate balance between Th1 and Th2 cells through the downregulation of the NF-κB pathway Decrease histamine level, IFN-γ, IL-4, 5,13, 31, IgE, and STAT6
Quercetin [120–122]	Suppress the expression of Th2-related cytokines, such as TSLP and thymus and activation-regulated chemokine Suppress the dysregulated JAK-STAT signal pathway and excessive production of IL-4, 5, 13.
Curcumin [123–126]	Regulate SOCS protein expression via JAK-STAT pathways Reduce the levels of cytokines associated with the TH2 immune response and inhibit the STAT6 activation. Suppress pro-inflammatory mediators including lipoxygenase, cyclooxygenase-2, and inducible nitric oxide synthase, and cytokines such as TNF, IL-1, IL-6, IL-8 Inhibit NF-κB
Resveratrol [127–130]	Upregulate SOCS1 production Block STAT3 signaling Reduce chemokine and cytokines such as IL-6, 31, and IgE

NF-κB: Nuclear Factor Kappa B, TSLP: Thymic Stromal Lymphopoietin, ROS: Reactive Oxygen Species, SOCS: Suppressor of Cytokine Signaling.

5.1. Polyphenol Phytochemicals on the JAK-STAT Signaling Pathway

5.1.1. Apigenin

Apigenin is a plant-derived flavonoid that is abundant in parsley, chamomile, celery, vine spinach, artichokes, and oregano [131]. Apigenin has been reported to possess numerous biological applications including anti-inflammatory, antioxidant, anti-cancer, anti-allergic, neuroprotective, and cardioprotective effects, and its application in cosmetics has been increasing recently [132]. Specifically, apigenin has also been reported in various studies to have itch-relieving effects [132]. Research has shown that apigenin reduced IL-31 expression in human mast cells and mouse skin with AD itch model by suppressing mitogen-activated protein kinase (MAPK) and NF-κB pathways [117]. In a study using BALB/c mice with ovalbumin-induced allergic rhinitis, apigenin regulated a balance between Th1 and Th2 cells through the downregulation of the NF-κB pathway, while also decreasing histamine levels, IL-4, 5, 13, IgE, and STAT6 [118]. Moreover, orally applied apigenin was found to decrease levels of IgE and IFN-γ in the serum of NC/Nga mice and mitigate skin damage caused by picrylchloride [119]. It also inhibited the phosphorylation of STAT6 in IL-4 stimulated mouse spleen cells, indicating suppression of inflammatory

pathways [119]. Collectively, apigenin may ease the symptoms of AD and itching by inhibiting the MAPK, NF-κB, and JAK-STAT pathways, which leads to a reduction in pro-inflammatory cytokines, including IL-31 and IL-33, suggesting its potential applicability in itch relief cosmetic formulations.

5.1.2. Quercetin

Quercetin, a flavonoid found in apples, onions, red wine, red grapes, tea (Camellia sinensis), capers, broccoli, lovage, and various berries, serves as one of the most important natural antioxidants utilized in cosmetics [133]. Along with its antioxidant activity, quercetin is also known for its effects on the skin, including anti-inflammatory, anti-aging, anti-bacterial, skin whitening, anti-allergic, and anti-itching. Previous studies demonstrated anti-AD effects of quercetin by activating the nuclear factor erythroid 2-related factor 2 and heme oxygenase pathways [120]. Additionally, studies with mouse models showed that quercetin could suppress the expression of Th2-related cytokines, such as thymus and activation-regulated chemokine and TSLP and 12-O-tetradecanoylphorbol-13-acetate-induced skin inflammation [121,134]. Furthermore, it is suggested to play a significant role in regulating the dysregulation of the JAK-STAT pathway and excessive production of IL-4, 5, and 13 in skin conditions with inflammation [122].

5.1.3. Curcumin

Turmeric, specifically its bioactive compound curcumin, is extensively researched for its therapeutic potential in skin conditions due to its anti-inflammatory, antioxidant, antiviral, and antifungal properties [135]. Curcumin's effectiveness stems from its ability to modulate various inflammatory pathways and cytokines, suggesting a promising organic option for dermatological treatments amid concerns over side effects from conventional medications [136]. Curcumin is known for its potent anti-inflammatory properties. Previous studies have indicated that curcumin regulates suppressor of cytokine signaling (SOCS) protein expression via JAK/STAT pathways in various experimental models [123,135]. It has shown anti-inflammatory properties by suppressing the expression of SOCS1 and SOCS3 in lipopolysaccharide-stimulated murine macrophages, suggesting its potential therapeutic use in chronic inflammatory conditions [137].

In a recent study involving ovalbumin-induced AD mice, curcumin treatment significantly reduced the levels of cytokines associated with the TH2 immune response and inhibit the STAT6 activation, showing its potential benefit in treating AD and associated infections [124]. In addition, it could suppress pro-inflammatory mediators including lipoxygenase, cyclooxygenase-2, and inducible nitric oxide synthase, and cytokines such as TNF, IL-1, IL-6, IL-8 [125,138,139]. Additionally, it inhibits NF-KB, a key factor in activating pro-inflammatory genes. Overall, curcumin exhibits broad-spectrum anti-inflammatory capabilities [126].

Curcumin has also been researched for its anti-pruritic effects. Two RCTs assessed curcumin tablets for pruritus treatment. In one study, patients with pruritus from sulfur mustard saw significant improvements after taking 500 mg of curcuminoids with 5 mg piperine for 4 weeks, including a 30% reduction in pruritus score [140]. Another RCT with end stage renal disease patients found a 56.9% reduction in pruritus scores after 8 weeks of 1500 mg turmeric tablets, significantly better than the placebo [141].

5.1.4. Resveratrol

Resveratrol, a natural bioactive polyphenol and antioxidant, is present in various plants, such as teas, berries, grapes, grains, and nuts [135]. Pharmacologically, resveratrol is known to have antitumor, anti-inflammatory, antioxidant, and cardioprotective effects [142–144]. Additionally, resveratrol can aid in treating various dermatological issues such as acne, exfoliative eczema, psoriasis, and UV-induced skin damage [127].

In a prior study, resveratrol was shown to enhance production of SOCS1 in LPS-stimulated RAW 264.7 macrophages by suppressing micro RNAs, which are known to

play a crucial role as negative regulators of cytokine signaling by inhibiting the JAK-STAT pathway [128]. Additionally, resveratrol was found to block STAT3 signaling through the induction of SOCS1 in head and neck squamous cell carcinoma cells [129]. In models of AD, resveratrol delayed the onset of skin lesions and alleviated inflammation induced by 2,4-dinitrochlorobenzene (DNCB) in mice by decreasing levels of pro-inflammatory cytokines and chemokine [127,130]. A different study demonstrated that treatment with rice enriched with resveratrol decreased scratching and reduced the secretion of cytokines, including IL-6, 31, and IgE in mice with DNCB-induced AD-like lesions [130]. These findings suggest that resveratrol could be applied as an adjuvant treatment for managing chronic itch as well as skin inflammations associated with AD.

6. Limitations

There are several limitations to this review. First, we assessed only English-language papers, excluding studies published in other languages. Second, ingredients included in the review were primarily those with anti-itch efficacy as their main function in cosmetics, and other ingredients were not included. Finally, the specific search queries used in this review were not documented, and thus are not reported.

7. Conclusions

This review highlights the expanding role of cosmetics in managing itch, emphasizing the beneficial potential of active ingredients. While these ingredients show promise in alleviating itch with high patient acceptability and minimal adverse effects, further research is needed to identify new active ingredients and optimize existing formulations. Unexplained issues such as the long-term effects of these ingredients and their interactions with other treatments warrant deeper investigation. As the demand for non-pharmacological treatments grows, itch-relieving cosmetics could become a pivotal part of dermatological care, offering a holistic approach to skin health and comfort.

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Review

Cosmetic Packaging: European Regulatory Aspects and Sustainability

Silvia Morel [1,*], Giulia Mura [2], Marina Gallarate [3] and Simona Sapino [3,*]

[1] Dipartimento di Scienze del Farmaco, Università del Piemonte Orientale A. Avogadro, Largo Donegani 2/3, 28100 Novara, Italy
[2] Scuola di Scienze del Farmaco e dei Prodotti della Salute, Università di Camerino, Via Madonna delle Carceri, 9, 62032 Camerino, Italy; giuliamura97@libero.it
[3] Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, Via P. Giuria 9, 10125 Torino, Italy; marina.gallarate@unito.it
* Correspondence: silvia.morel@uniupo.it (S.M.); simona.sapino@unito.it (S.S.)

Abstract: This review aims to provide a comprehensive overview of various aspects related to cosmetic product packaging, highlighting both advancements and challenges in the field. Initially, it offers a general description of the main materials used in cosmetic containers, including plastic, glass, paper, and aluminum. This is followed by an analysis of the existing EU legislative frameworks that govern cosmetic packaging, encompassing chemical, food, and waste regulations. The paper also discusses recent EU regulatory proposals and guidelines from trade associations aimed at enhancing the sustainability of cosmetic packaging materials. Additionally, the role of recycled and bio-based packaging materials in promoting environmental sustainability is analyzed. Overall, this review aims to provide insights for experts in the field on how to balance safety, functionality, and environmental responsibility in cosmetic packaging.

Keywords: cosmetic packaging; EU legislation; guidelines; container materials; sustainability

1. Introduction

Packaging plays a vital role in protecting and transporting goods, as well as facilitating the communication and use of industrial products such as foods, cosmetics, and pharmaceuticals. Packaging materials are typically classified into primary, secondary, and tertiary categories, each fulfilling specific yet interconnected roles within the logistics and distribution chain.

Understanding the composition and properties of packaging materials is essential to guaranteeing the efficient delivery and storage of goods. Typically, the decision-making process concerning packaging is complex, requiring input from various stakeholders, fulfillment of various functions, meeting diverse requirements, and careful consideration of various conditions [1]. For instance, when developing packaging for a cosmetic product, adherence to technical specifications such as product protection, compatibility with the product, and performance in the filling process is essential. However, the ultimate selection of packaging largely relies on the manufacturer's discretion and the desired brand image.

The most important types of materials employed to produce packaging are glass, paper, metal and overall plastics [2]. In the last few years, however, the surge in global consumption patterns has intensified the environmental footprint of packaging waste, with significant repercussions on ecosystems and human health, underscoring the urgent need for sustainable innovations in packaging materials. Alternatives such as paper, Paper bamboo or innovative materials such as bioplastics have started to be proposed as an innovative approach to packaging, aligning with the growing demand for green and sustainable products. However, all these innovative sustainable materials must still ensure safety standards, and current regulations often lag behind these advancements.

Consequently, as a result, cosmetic and packaging manufacturers often have to refer to non-specific but related regulations, such as those in the food industry. Indeed, despite their specificity, food and cosmetic packaging share numerous similarities and analogous risks: both food and cosmetic products come into direct contact with the packaging materials, raising concerns about potential chemical migration and the safety of the end consumer.

Focusing on the European industry of packaging, despite the pressing need for uniform regulations, there is currently a significant variance in the regulatory strategy adopted by the Member States of the EU. This divergence presents substantial challenges, hindering the smooth operation of the internal market for packaging. For this purpose, Directive (EC) n.62/94 was enacted with the aim of standardizing national regulations concerning packaging materials and waste management, promoting reuse, recycling, and other methods of packaging waste recovery, thus fostering the transition towards a circular economy [3]. In alignment with the principles set by this EU Directive, a strategy was launched in January 2018, the EU Circular Economy Action Plan (CEAP), including a focus on plastics [4]. In particular, this strategy is designed to enhance the sustainability of plastics by promoting recycling, reducing the use of single-use plastics, and fostering innovation in plastic production and design. The importance of this focus lies in the fact that plastic is widely used throughout various industrial sectors, including cosmetics packaging, due to its versatility, cost-effectiveness, and durability. Nevertheless, despite its widespread utility, plastic poses significant environmental and health risks [5].

Plastic packaging can introduce contaminants or impurities into the cosmetic product, compromising its safety and efficacy. Meanwhile, consumers expect cosmetics to be safe for use, both in terms of efficacy and potential health risks.

In view of the premises, it is clear that regulatory bodies and industry stakeholders play a key role, not devoid of consistent challenges, in the transition to packaging sustainability. As a result of these challenges, the EU Commission has recently proposed a new regulation aimed at reducing the environmental impact of specific plastic products [6], and it is still working on providing a more detailed legislative framework. This review aims to provide an overview of the various aspects related to cosmetic product packaging, offering insights into potential advancements and challenges.

Initially, a general description of the main cosmetic container materials, such as plastic, glass, paper, and aluminum, will be given. Afterwards, a description of existing EU legislation frameworks governing the packaging of cosmetic products will be done, including chemical, food and waste regulations. The paper also discusses recent EU regulatory proposals as well as guidelines from trade associations for improving the sustainability of cosmetic packaging materials. Additionally, it will examine the role of recycled and bio-based packaging materials in promoting environmental sustainability, with a particular emphasis on the migration of substances from the container to the formulation.

2. Cosmetic Packaging

Cosmetic packaging is all about keeping the product safe against light and microbiological contamination, while also serving as a key element in marketing strategies. The type of container impacts both user convenience and consumer safety by affecting the accuracy of the delivered dose [7].

Primary packaging constitutes the immediate enclosure of the product, coming into direct contact with its contents. It serves as the first line of defense against external factors such as moisture, light, and air, which may compromise the product's stability and efficacy. Common materials used in primary packaging include glass, plastics, metals (mainly aluminum), and composite materials designed to meet specific product requirements. These materials must exhibit suitable barrier properties, inertness, and compatibility with the product to prevent contamination and maintain its quality throughout its shelf life. Primary cosmetic packaging comes in a variety of shapes and sizes, including attractive cylindrical tubes for lip gloss, compact square jars for creams, elegant pump bottles for lotions, and convenient stick sizes for deodorants and solid perfumes.

Secondary packaging encompasses the outer layer surrounding primary packaging, providing additional protection during handling, transportation, and storage. Its primary function is to enhance the structural integrity of the primary containers, safeguard against mechanical damage, and facilitate efficient handling and identification. Cardboard, corrugated board, paperboard, and various types of plastics are commonly employed in secondary packaging due to their durability, flexibility, and printability. Additionally, secondary packaging may incorporate features such as cushioning materials, seals, and labeling to further ensure product safety and compliance with regulatory standards. Secondary cosmetic packaging typically includes a range of sizes and shapes, such as rectangular boxes designed to snugly fit perfume bottles, square cartons for creams and serums, and cylindrical tubes for mascara, all crafted to enhance protection, display, and branding appeal.

Tertiary packaging involves the consolidation and containment of multiple units of primary or secondary packaging for bulk transportation and distribution. It serves as a protective barrier against external hazards such as moisture, temperature fluctuations, and physical impacts during transit. Tertiary packaging materials are typically robust and cost-effective, prioritizing efficiency and sustainability in supply chain logistics. Common materials include pallets, stretch wrap, shrink wrap, corrugated fiberboard containers, and reusable shipping containers, selected based on factors such as weight, volume, and mode of transportation [8]. Figure 1 illustrates the three primary types of cosmetic packaging.

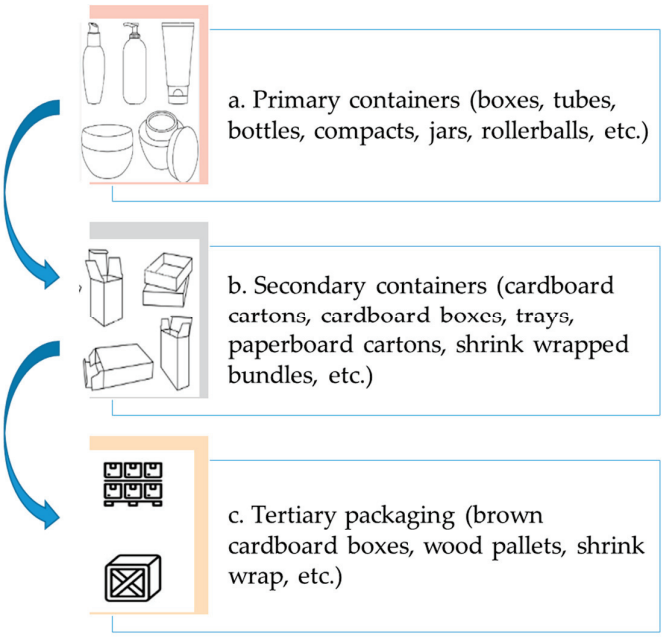


Figure 1. Types of cosmetic packaging: (**a**) primary packaging, which directly contains the product such as bottles, tubes, and jars; (**b**) secondary packaging, which includes the outer wrapping or box that encases the primary package for additional protection and branding; (**c**) tertiary packaging, used for bulk handling, storage, and distribution, typically including larger boxes and pallets.

Special attention must be given to the interactions between the content and packaging, commonly referred to as container–content interactions (CCI). CCI studies, prevalent in the food and pharmaceutical sectors, examine the potential migration of molecules from packaging to product, which can affect product quality, efficacy, and consumer safety. Hence, food product legislation plays a pivotal role in the cosmetic industry by aiding in container selection [9].

2.1. Glass

Glass, being an inert material, is the preferred choice for cosmetic packaging due to its zero migration properties, ensuring the integrity of the cosmetic formulation. Its transparency, color variety, decorative potential, impermeability, chemical integrity, design flexibility, heat stability, microwave compatibility, etc. further justify its extensive use in the food and beverage industry [10]. Moreover, its unique attributes position glass as an excellent material for pharmaceutical packaging [11]. In the European Pharmacopoeia, there is a chapter on glass containers for pharmaceutical use [12].

From a recycling standpoint, it is also an excellent material, as glass can be recycled indefinitely without losing its properties. Recycling glass saves energy and environmental resources. However, despite its advantages, glass packaging has several drawbacks, the first of which is its weight, which makes it heavier than plastic and cardboard, resulting in heavier transport, which causes more emissions of CO_2. Another inconvenience arises when transporting fragile glass bottles or jars, as they might be subjected to rough handling and could potentially shatter, leading to messy spills and the risk of injuries. Furthermore, glass packaging may not be ideal for products like face creams or lotions that are frequently used in the bathroom, where moisture and humidity can accumulate, leading to slippery surfaces and increasing the likelihood of accidents. In addition, glass containers are not suitable for items for babies or pets that need to be lightweight and safe from breakage, just as in the food industry.

2.2. Paper

Cosmetic companies are increasingly turning to paper as a material for cosmetic product packaging due to growing consumer demand for eco-friendly alternatives. Paper packaging is perceived as a green and low-impact solution because it is easily recyclable and biodegradable. However, while paper offers certain advantages, it also presents challenges and considerations that must be addressed.

Paper packaging aligns with consumers' desire for sustainable and environmentally friendly products. Brands that adopt paper packaging can enhance their image as environmentally responsible companies, potentially attracting environmentally conscious consumers. However, it is essential for companies to ensure that their paper packaging truly embodies sustainability principles, addressing issues such as recyclability, sourcing, and environmental impact throughout the entire lifecycle of the product. Indeed, while paper containers may seem eco-friendly, they often require a plastic film lining to ensure product integrity and prevent contamination. This plastic layer adds complexity to the recycling process and raises questions about the overall environmental impact of paper-based packaging solutions. Cosmetic companies must balance the need for product protection with their sustainability goals when choosing packaging materials.

Notably, recycling paper packaging involves significant water consumption and energy usage, particularly in the pulping process [13]. Moreover, the presence of plastic components complicates the recycling process, as separating the paper from the plastic can be technically challenging and economically unfeasible. Cosmetic companies need to invest in innovative recycling technologies and work collaboratively with waste management facilities to improve the recyclability of paper-based packaging. Moreover, to address concerns about deforestation and habitat destruction, cosmetic companies must ensure that the paper used for packaging comes from responsibly managed forests certified by organizations like the Programme for the Endorsement of Forest Certification (PEFC) or the Forest Stewardship Council (FSC). Sourcing paper from certified forests helps mitigate environmental impacts and supports sustainable forestry practices [14,15].

Overall paper packaging has inherent limitations compared to other materials like plastic and glass. It is not waterproof, which can pose challenges for products that are sensitive to moisture or require airtight sealing. Additionally, paper packaging is generally not reusable, unlike some plastic or glass containers that can be repurposed or refilled, leading to potential waste generation. However, ongoing research and innovation in paper

packaging technology have recently expanded the possibilities for cosmetic companies. New advancements in barrier coatings and material engineering are improving the performance of paper-based packaging, making it more suitable for a wider range of cosmetic products while maintaining its sustainability credentials [16].

2.3. Aluminum

Aluminum is an excellent packing material, offering a formidable shield against air, light, temperature fluctuations, moisture, microorganisms, and odors. It remains neutral in taste, capable of housing volatile components and preserving aromas. Its lightweight nature facilitates ease of handling; indeed, approximately 11% of global aluminum production finds application in packaging, of which 75% is dedicated to food, 8% to cosmetics, 7% to pharmaceutical products, and 10% is allocated to various other industries [17]. In cosmetic packaging, aluminum tubes are ideal for creams and lotions, while aluminum bottles are commonly used for shampoos and conditioners.

Aluminum jars preserve thicker products like waxes, while aluminum cans offer lightweight protection for aerosol-based items. Aluminum compacts provide sleek packaging for powders and eyeshadows. Bottles with spray pumps maintain the quality of perfumes, while aluminum sachets offer convenience for single-use or sample-sized products. Aluminum foil packaging ensures stability by creating barriers against moisture, light, and air. These diverse options demonstrate aluminum versatility in meeting the packaging needs of the cosmetics industry. Notably, aluminum is highly recyclable, making it an environmentally friendly choice. Additionally, aluminum packaging helps extend the shelf life of cosmetic products by protecting them from external factors that can degrade their quality.

While aluminum packaging offers numerous benefits, it also carries drawbacks, particularly concerning its environmental and social impact. Mining for aluminum can result in deforestation, pollution, and human rights violations in countries like Malaysia and Guinea. The energy-intensive process of bauxite extraction damages local habitats and can lead to habitat loss, soil erosion, and water contamination. Additionally, waste generated during aluminum production poses bioaccumulation risks for aquatic life, affecting the broader ecosystem and potentially human health through the food chain. Moreover, the production processes and energy consumption associated with aluminum packaging contribute significantly to its "global-warming cost" compared to plastic alternatives [18]. Another drawback relies on the fact that aluminum, as a metal-based packaging material, is not entirely inert to food and cosmetic products; therefore, it requires the application of protective lacquers and coatings to prevent interaction between the container and the content, as well as the migration of metal components [19]. These coatings face challenges in maintaining integrity over long storage periods, meeting stringent regulatory requirements, and ensuring product visual appeal. Contamination can occur through extraction, interaction with substances, or delamination from the metallic substrate. Compounds such as alcohols and low-molecular-weight substances pose adhesion problems and may compromise product quality. Compliance with FDA regulations and European Parliament standards is essential, demanding coatings with chemical resistance, ductility, and adhesion properties. While liquid organic coatings currently dominate the market, the shift towards powder coatings is driven by environmental concerns and regulatory pressures. Powder coatings offer solvent-free application, shorter processing times, and potential cost savings, presenting a promising alternative for the future of cosmetic packaging [20].

Overall, innovations in barrier coatings and material engineering are elevating the capabilities of aluminum cosmetic packaging, ensuring its suitability for diverse product ranges while upholding its sustainability attributes.

2.4. Plastic

Plastics are ideal for packaging due to their lightweight, convenience, and affordability, as well as their many desirable properties, such as transparency, softness, heat seal ability,

and a good strength-to-weight ratio. Even for cosmetic products, plastic is widely used for rigid and flexible packaging. Globally, over 30% of plastic production is used for disposable packaging [21].

In the packaging industry, petrochemical-based plastics such as polyethylene terephthalate (PET), polyvinyl chloride (PVC), polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyamide (PA) have long dominated due to their abundance, affordability, reliable mechanical performance, and heat resistance. However, there is a growing interest in renewable alternatives like PLA, bio-PE, and bio-PET, reflecting a broader environmental consciousness. Yet, the cosmetics sector presents unique challenges demanding more than just eco-friendly or biodegradable materials. As in the food industry, packaging in the cosmetic industry must contend with product instability, requiring bioplastics with tailored functionalities [22,23].

More than 30 types of plastics are employed as packaging materials, often enhanced with additives to confer specific characteristics [7]. For instance, UV light protection additives are incorporated into transparent packaging to prevent product degradation through photoactivation processes. Dyes and pigments are included to enhance packaging aesthetics, while processing aids or antioxidants facilitate manufacturing processes and improve durability. Plasticizers can also be added to enhance flexibility. Recent research has explored the integration of nanoparticles, particularly nanocomposites of phyllosilicates, into plastic materials to bolster their barrier properties [24].

Additives, due to their lack of chemical bonding with the polymer, can potentially migrate from the container to its contents, posing a potential leachable. As a result, the potential release of plastic additives into cosmetic products during storage becomes significant especially if they can be dangerous for human health [25]. Additionally, elements like oxygen and water vapor can permeate packaging materials from the surroundings to the product.

Careful selection of packaging materials is crucial, taking into account their barrier properties against various compounds that may migrate either from the product to the packaging or vice versa. Such migrations can lead to alterations in product formulation, potentially impacting its efficacy. Furthermore, migration from product to container can affect the container's properties, compromising its strength and durability [9]. Moreover, the transfer of a compound from the container to its contents under normal usage conditions or extreme circumstances also includes Non-Intentionally Added Substances (NIAS) that have not been deliberately included for technical purposes and that can be potentially harmful. These substances may include impurities, degradation byproducts or environmental contaminants.

Given these premises and considering that cosmetic products are utilized daily by individuals of all age groups, careful monitoring of plastic additives in hygiene and beauty items must be carried out to ensure consumer welfare. According to Regulation (EC) n.1223/2009, toxicologists are responsible for evaluating packaging risks and potential contaminants in cosmetic products, which poses a significant challenge for the industry. However, it does not provide detailed guidance on the specific tests required for such evaluations. Therefore, appropriate strategies must be introduced to address these risks. This can be achieved by developing specific analytical tests and standard procedures that facilitate comprehensive toxicological assessments, thereby ensuring consumer safety.

It is evident how the legislation for materials in contact with food is of great assistance to the cosmetic sector, as it serves as a guide to protect consumer health even for cosmetic products that share similar chemical and physical characteristics with food. Similarly to food packaging, cosmetic packaging can also leach diverse contaminants based on content type, temperature, and duration of contact. Hence, tests for assessing material release properties and potential migration in cosmetic packaging are predominantly analytical and usually derive from standardized food packaging assessments. These tests aim to detect if plastic production additives, potentially harmful to human health, migrate into the product. A notable example is bisphenol A (BPA), a chemical compound primarily

used in the manufacturing of various plastics that, being similar to 17β-estradiol, interacts with human estrogen receptors and, for this reason, has been banned in European cosmetic products and restricted in food items. Several methods for determining BPA in foodstuffs have been developed in the last 10–15 years [26] and can also be applied for monitoring the phenomenon of migration from cosmetic matrices. To assess the safety of contact materials by simulating real-world conditions and to predict the transfer of chemicals from the packaging into the product, both the food and cosmetic industries employ substitutes useful to mimic interactions with different types of products. Guided by regulations such as those set by the European Union, common simulants include water for aqueous environments, 3% acetic acid for acidic foods, 10% ethanol for alcoholic foods, and refined olive oil for fatty foods [27]. This approach is extended to cosmetic research, aiming to find substitutes that represent worst-case scenarios accurately while ensuring compliance with safety standards.

An illustrative example of a CCI study was conducted recently by P. Murat and colleagues. In this study, eleven potential cosmetic packaging materials (made up of PET, PE, PP and SAN) were evaluated using different simulating liquids. These types of packaging were put in contact with five simulants chosen to mimic cosmetics behavior to reinforce the safety evaluation of the cosmetic containers. Leachable was analyzed using a GC-MS method developed to screen for different phthalates. Some phthalates and BPA were detected in several samples, but only one contaminant, diisobutyl phthalate, was found to be above the set concentration threshold [9].

Other similar tests are reported in the literature aimed at checking levels of polycyclic aromatic hydrocarbons or heavy metals or carcinogenic, mutagenic, or toxic for reproduction (CMR) substances [25,28–30].

The plastics industry has historically prioritized production over recycling or reusing, leading to significant waste accumulation, particularly in packaging. Poor waste management contributes to millions of tons of plastic ending up in terrestrial and oceanic environments, with land-based sources being the primary contributors. Microplastic particles, formed through degradation, pose risks to marine life and can enter the food chain, potentially carrying harmful contaminants. Plastic pollution also has substantial economic costs, affecting industries like tourism, fishing, and shipping [31]. Even in recent years, there has been an increase in single-use plastics. However, the massive production of plastic packaging has led to environmental concerns, resulting in a shift to a circular economy of plastics that emphasizes reuse and recycling, a topic that will be discussed in more detail in Section 4 below.

3. Regulatory Aspects of Cosmetic Packaging in Europe

Regulation (EC) n.1223/2009 governs all aspects related to the composition, presentation, and requirements necessary for the production, sale, and import of cosmetic products. It consists of 40 articles and 8 annexes, including Annex I, which concerns the safety of cosmetic products and packaging [31]. Among all the articles, article 3 is one of the most important, stating that "*a cosmetic product made available on the market shall be safe for human health when used under normal or reasonably foreseeable conditions of use*". Article 10 indeed mandates that the Responsible Person ensure cosmetic products undergo a safety assessment in accordance with Annex I before being placed on the market. According to article 11, the Responsible Person must compile the Product Information File (PIF). The essential element of the PIF is the Cosmetic Product Safety Report (CPSR), which serves to demonstrate that a cosmetic product complies with Article 3 of the Regulation. Information on packaging, reported in the CPSR, is present in Annex I, part A, point 4 [31].

Packaging must not negatively impact the safety of the cosmetic formulation. It must also comply with the Packaging and Waste Directive and the requirements relating to EU legislation on chemical products [3,32–34].

Although there are no specific rules for the packaging of cosmetics, point 4 of Annex I, part A, requires the Safety Assessor (SA) to consider the potential impact of packaging

on product safety. The SA must evaluate the potential migration of substances from the packaging to the formulation. However, since migration cannot always be avoided, Article 17 of Regulation (EC) n.1223/2009 allows for the unintentional presence of traces of a prohibited substance, provided Article 3 of the regulation and good manufacturing practices are observed [31]. The undefined concept of unintentional traces has posed challenges for manufacturers in drafting the CPSR, prompting the European Commission to publish guidelines on Annex I in 2013 [35].

In analyzing the regulatory aspects of packaging, it is necessary to also refer to EU regulations concerning the packaging of the substances and chemical mixtures used to create the finished product.

Regulation (EC) n.1272/2008 on the classification, labeling and packaging of substances and mixtures (CLP) applies to the raw materials that constitute cosmetic products, as these are considered chemicals. This regulation harmonizes the classification of substances and mixtures, as well as the rules related to labeling and packaging, to ensure a high level of protection for human health and the environment and the free circulation of the substances. The CLP Regulation requires cosmetics manufacturers to classify the substances in their products based on the hazard criteria established by the regulation itself, thereby guaranteeing consumer safety [32].

The fundamental elements of packaging containing chemical substances or mixtures, as specified in article 35 of the CLP, are resistance and robustness. In certain cases, the container must be equipped with a child-proof closure, and in others, it must bear a tactile danger warning [32]. These attributes ensure that the packaging provides a solid seal and does not loosen under the stresses encountered during handling. Additionally, the packaging must not attract children's attention or cause confusion among adults, as also reported in the Regulation 2023/988/UE on General Product Safety. Packaging, in fact, must not compromise the health and safety of the consumer; therefore, cosmetics must not be confused with food products due to their packaging [33].

Regulation (EC) n.1907/2006, concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), regulates the production, import and use of chemical substances, including cosmetic raw materials. Safety is of paramount importance as cosmetic products must be safe for use. REACH aims to enhance the protection of human health and the environment from the risks posed by chemical substances while also promoting the competitiveness of companies within the EU. Only substances registered in accordance with REACH are authorized for use in cosmetic products that can be marketed in the European Union [34].

Cosmetic products must also comply with Directive 75/324/EEC, which pertains to the harmonization of laws among Member States regarding aerosol dispensers. The person responsible for marketing cosmetic aerosol dispensers must affix the symbol 3″ (an inverted epsilon) to the dispensers, indicating that they meet the requirements of this Directive and its Annex [36].

3.1. Food Legislation as a Guide for Cosmetic Packaging

European legislation has established several regulations to define interaction and migration studies on packaging, depending on the type of product to be marketed. These include the European Pharmacopoeia [12] for medicinal products, Regulation (EC) n.745/2017 for medical devices [37], Regulation (EC) n.10/2011 [27] and Regulation (EC) n.213/2018 [38] for food and food contact packaging materials [8]. In contrast, the cosmetic sector lacks specific migration limits in its regulations. Consequently, as suggested in the guidelines on Annex I [35], cosmetic companies often rely on the food regulations regarding primary packaging namely on regulation EC n.1935/2004 and on regulation EU n.10/2011. Regulation (EC) n.1935/2004 concerns materials and articles intended to come into contact with food during production, processing, storage, preparation and serving to ensure their safety. For instance, primary packaging must not release or transfer its components into the food

in a manner that could endanger the consumer's health or negatively impact the quality of the food itself [39].

Regulation (EU) n.10/2011 on food contact materials (FCMs) and materials and objects in contact with food addresses the specific safety of plastic materials and objects intended for food contact [27]. It includes a positive list of substances authorized for use in plastic materials and articles and imposes restrictions on migration limits (article 12), specifying the maximum allowed amount of migration. Many substances used in the production of FCMs have already undergone toxicological evaluation, primarily concerning ingestion. The scientific community agrees that ingestion poses a greater risk than skin contact. The regulation also includes the use of food simulants, substances that mimic the behavior and characteristics of certain classes of food. For cosmetic products, it is crucial to report the food simulants that have been used [27].

When compliance with food legislation cannot be guaranteed due to a non-permitted component or because it exceeds pre-established limits, this does not necessarily mean that the packaging is unsuitable for the cosmetic product in question. In such cases, the suitability assessment falls to the SA, who may use other standards, such as pharmaceutical ones, to verify whether the chosen packaging material is compatible with the cosmetic formulation. Indeed, cosmetic products often involve complex matrices that can differ significantly from food. Moreover, if manufacturers wish to use new and/or original containers, a more thorough evaluation of the materials and the container/formulation interaction is required. One critical aspect to investigate is microbiological safety, as plastics derived from waste are highly likely to be microbiologically contaminated. Recycled plastic must meet the same safety standards and composition requirements as virgin plastic and must comply with the migration limits and restrictions established by Regulation (EU) n.10/2011. Therefore, plastic waste must be decontaminated before reuse, using appropriate recycling technology to ensure that these materials meet safety standards [27].

Regulation (EU) n.1616/2022, concerning the marketing of materials and objects intended to come into contact with food products, includes specific provisions regarding the use of recycled plastic and new recycling technologies. Notably, plastic materials suitable for food contact can also be considered for cosmetic use. However, the use of recycled plastic materials is recommended only if strict safety requirements are met. This regulation specifies the recycling technologies appropriate for producing recycled plastic intended for food contact [39]. It also introduces a voluntary labeling system for food products packaged with recycled plastic, enabling consumers to easily identify such products and make informed choices [40].

Regulation (EU) n.1616/2022 is crucial given the priority of addressing environmental pollution from plastic, with the packaging sector being a major producer. Packaging is considered the main source of waste globally, often having a very short useful life, such as in the case of disposable plastic [41,42].

3.2. Guidelines from Industrial Associations

It must be noted that primary packaging is generally not produced by the cosmetic company itself but by external suppliers who are expert packaging manufacturers. Therefore, continuous interactions between the packaging industry and cosmetic companies is essential to ensure consumer safety.

To address regulatory gaps in cosmetic packaging, cosmetic industry associations have provided guidelines to manufacturers. In particular, in 2019, Cosmetic Europe published the Advisory Document on "Information Exchange on Cosmetic Packaging Materials Along the Value Chain in the Context of the EU Cosmetics Regulation (EC) n.1223/2009". This document aims to clarify and define the requirements of the regulation, enabling the SA to effectively carry out the safety report [25].

This advisory document proposes a methodology for the exchange of information between the cosmetics and packaging industries. There is no legal obligation for cosmetic manufacturers to use this information. According to this advisory document, all items

and/or materials used to create the packaging must be accompanied by their general chemical composition. This allows the SA to evaluate the impact of the packaging on the safety of the cosmetic product.

It is essential to assess the characteristics of the packaging material, the interactions between the product and the packaging material, the barrier properties of the packaging material, and the migration of substances to and from the packaging material. These factors indicate potential dangers and allow the SA to estimate the associated risks. The SA must consider the composition of the material, including additives, technically unavoidable impurities, and possible migration from the packaging. Migration can also depend on storage conditions.

Cosmetics Europe has stated that it may be effective to refer to Regulation (EC) n.1935/2004. They note, "The materials developed for food packaging have often already been tested, and relevant information on stability and migration may be available. Consequently, further experiments may not be necessary. However, further evaluation may be required for new packaging" [25].

Indeed, it is mandatory for cosmetic packaging to comply with EU legislation on chemicals and packaging waste [3,32,34]. According to REACH, packaging companies must declare the presence of substances of very high concern (SVHC) by providing their identity if they are present in quantities equal to or greater than 0.1% w/w. They must also confirm that there are no heavy metals exceeding the limit of 100 ppm, as stipulated by the Packaging Waste Directive for the sum of the concentration levels of lead, cadmium, mercury, and hexavalent chromium. Additionally, packaging companies must provide appropriate documentation to the cosmetics company regarding banned substances or those subject to restrictions under Annex II and III of Regulation n.1223/2009, as well as substances classified as skin sensitizers.

To facilitate the preparation of the Product Information File (PIF), a packaging supplier is required to provide information on the packaging material, including the component name, type of material, and general chemical description [25]. Even in the case of migration, the cosmetics company can refer to tests already existing in the food sector, with specific limits set by EU or national legislation based on toxicity data for specific substances.

The rules for plastic materials used to create primary packaging in direct contact with food are established by Regulation (EU) n.1245/2020 of the Commission, which amends the 2011 regulation [43].

For materials such as paper, rubbers and printing ink, the rules for migration tests are established by national regulations or industry guidelines.

Migration tests can be performed on foods, food simulants, migration models, or estimated based on conservative assumptions or models. The SA must determine which method to use for migration testing and whether the results can be applied to the cosmetic formulation.

In the cosmetic field, certain chemical formulas, such as alkaline preparations used in hair care, cannot be represented by food simulants. When no appropriate simulants exist or if the migration evaluation with food-suitable simulants results in values exceeding the Overall Migration Limit and/or Specific Migration Limit, the "safe for food, safe for cosmetics" argument cannot be applied. In such cases, the packaging cannot be assessed as compliant with food contact regulations. Therefore, it is clear that a cosmetic container may not comply with food contact standards if it contains a substance not authorized for materials in contact with food or if the substance is used outside the restrictions established for such use. When cosmetics are not assigned to a food simulant, the cosmetics company relies on the opinion of an expert to assign an appropriate food simulator. The cosmetic industry can also refer to pharmaceutical standards as well as food and feed additives [43].

In general, because European legislation is still developing, member states have a certain degree of autonomy that allows for guidelines from industry associations. For example, in Italy in 2016, the Italian Packaging Institute presented the "Guidelines for the Definition of Packaging Characteristics as Part of the Safety Assessment of the Cosmetic

Product: Operational Suggestions for the Obligations Required by Regulation 1223/2009 on Packaging". These guidelines aim to provide SAs with the most suitable tools necessary for risk assessment and the compilation of the CPSR. The migration tests conducted with the alternative simulants proposed by the Cosmetic Packaging Commission take into account the actual conditions of use and packaging of the relevant cosmetic products [44].

More recently, Aliplast, a company specializing in the collection and recycling of plastic waste, in collaboration with Cosmetica Italia (the Italian Association of Cosmetic Companies), developed a white paper titled "Plastic Packaging in the Cosmetics Sector—Guide for More Circular Production". These guidelines provide recommendations for designing each type of plastic container (such as PET, PP, PE, etc.) to ensure maximum recyclability at the end of its life, in accordance with the standards set by RecyClass and SPICE. (Sustainable Packaging Initiative for Cosmetics), European initiatives by plastic recyclers and the cosmetics industry. This concise guide, intended for producers in the cosmetics industry, contains practical indications for designing easily recyclable cosmetic product packaging. The objective of the white paper and the partnership between Aliplast and Cosmetica Italia is to promote the development of aesthetically refined packaging that enhances the functionality of the product while being useful for waste elimination. While in the past, cosmetic product packaging struggled to balance aesthetic, commercial, and environmental considerations, today, the principles of a circular economy guide much of the innovation in the beauty sector [45].

The European Commission has adopted new EU Ecolabel criteria for cosmetics, providing consumers across the EU with reliable evidence of genuinely eco-friendly brands and supporting the transition to a clean, circular economy [46]. To assist consumers in properly disposing of cosmetic packaging, European legislation mandates that manufacturers include alphanumeric coding on the packaging. As chemical products, cosmetic packaging must also comply with the European recyclability requirements outlined in Decision (EC) No. 129/97 [47].

Given the complex legislative landscape, Table 1 has been created to provide a concise summary of all the different EU legislation frameworks governing cosmetic packaging.

Table 1. EU legislation frameworks regulating packaging of cosmetic products.

EU Legislative Framework	Regulation	Subject	[Ref]
COSMETICS	Regulation (EC) n.1223/2009	Regulation on Cosmetic Products - Annex I (Cosmetic Product Safety Report) - Annex II (Substances Prohibited) - Annex III (Restricted Substances)	[31]
	European Decision 25/11/2013	Decision on Guidelines on Annex I to Regulation (EC) n.1223/2009	[35]
	Industrial Guidelines	Cosmetic Europe (2019): Document on information exchange on cosmetic packaging materials along the value chain in the context of the EU cosmetics regulation EC n.1223/2009	[25]
		Italian Packaging Institute (2017): open work table of safety of cosmetic packaging	[44]
		Aliplast (2024): Cosmoprof 2024	[45]
	Regulation (EU) n.988/2023	General product safety	[33]
CHEMICALS	Regulation (EC) n.1907/2006	Regulation concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)	[34]
	Regulation (EC) n.1272/2008	Regulation on classification, labeling and packaging of substances and mixtures (CLP)	[32]
	Directive (EEC) n.324/1975	Aerosol dispensers	[36]

Table 1. *Cont.*

EU Legislative Framework	Regulation	Subject	[Ref]
FOOD	Regulation (EC) n.1935/2004	Regulation on materials and articles intended to come into contact with food	[39]
	Regulation (EU) n.10/2011	Regulation plastic materials and articles intended to come into contact with food (FCMs)	[27]
	Regulation (EU) n.1245/2020	Regulation on plastic materials and articles intended to come into contact with food	[43]
	Regulation (EU) n.1616/2022	Regulation on recycled plastic materials and articles intended to come into contact with foods	[40]
WASTE	Directive (EC) n.62/1994	Directive on packaging and packaging waste	[3]
	Proposal for a Regulation 2022	Proposal on packaging and packaging waste	[6]

4. Sustainable Cosmetic Packaging

In recent decades, the expansion of the cosmetics industry has led to the generation of a greater amount of waste at all stages of production and disposal. Consequently, the impact of the cosmetics industry on a sustainable environment is significant. Most cosmetic packaging is made of plastics and non-recyclable composites, which exacerbate the environmental impact. As these plastic materials degrade, they break down into microparticles known as microplastics. This issue is further compounded by the fact that in some types of products, microplastics can also originate from the ingredients themselves. The pervasive presence of microplastics in ecosystems poses potential harm to wildlife and human health, underscoring the urgent need for appropriate management of cosmetic waste [48].

For these reasons, there is a growing interest in sustainable cosmetic packaging solutions, such as biodegradable materials, recycled plastics, and minimalistic designs that reduce waste. For instance, some companies have pioneered the use of "naked" packaging, offering products like shampoo bars and solid lotions that require no packaging at all. Additionally, other brands utilize glass containers and recycled plastics for their products. Other practical examples are offered by circular shopping platforms that collaborate with important cosmetic brands to provide reusable packaging that consumers can return for cleaning and refilling. These initiatives not only help in reducing landfill waste but also conserve resources and promote a circular economy, reflecting a commitment to environmental responsibility and sustainability within the cosmetics industry. However, adopting circular economy principles requires several actions throughout the supply chain of cosmetic products, including reducing consumption, designing reusable and recyclable products, making production energy efficient, and improving recycling practices. The predominance of plastic in cosmetic packaging suggests that both bioplastics and plastic recycling can substantially contribute to sustainable packaging.

Currently, recycling faces challenges related to sorting and contamination, which impact the safety and cost of recycled materials. Advances in nanomaterial science offer promising solutions; rapid developments in recent decades suggest that nanoparticles could revolutionize packaging materials [49]. However, overcoming the economic barriers to recycled materials requires continued investment and legislative support. High costs are also attributed to the need for comprehensive management of environmental impacts, which involves considering the entire life cycle of products from sourcing to production. Educating consumers and businesses on life cycle thinking is also essential for promoting sustainable practices. Digitalization, a key component of the fourth industrial revolution, supports the transition towards a circular economy by digitizing the product life cycle through intelligent, interconnected web-based platforms. This approach enhances the productivity and efficiency of packaging from production to consumption, thus improving sustainability [50]. Moreover, the reuse and refill of packaging present viable alternatives to landfilling [22]. In the post-consumer phase, recycling, reusing, or refilling used packaging

is preferable to landfill disposal. To create a circular economy, it is also necessary to reduce overall production, though this approach remains underutilized due to safety concerns [51]. Therefore, the adoption of circular economy principles involves a comprehensive strategy across the entire plastics supply chain, encompassing consumption reduction, product redesign, energy-efficient production, and improved recycling practices [52]. However, this approach is still underused due to safety uncertainties.

The EU Commission intends to ensure that by 2030 all packaging is reusable or recyclable in an economically sustainable way while substantially reducing waste production. In November 2022, the European Commission presented a proposal for a regulation amending Directive (EC) n.62/94 on Packaging and Packaging Waste, provided for by the "New Action Plan for the Circular Economy for a cleaner and more competitive Europe", presented in March 2020 [6]. This new regulation updates the regulatory framework respecting the rules relating to environmental protection and waste, reduces the use of virgin plastic, and develops a strategic framework for bio-based, biodegradable, and compostable plastic. This EU proposal aims to reduce packaging waste and encourage the reuse of packaging, through standardization of packaging formats and clear labeling of reusable packaging. It also aims to eliminate packaging, defined as "unnecessary", create mandatory return systems for plastic bottles and aluminum cans and mandate rates of recycled content that manufacturers will have to include in new plastic packaging.

An overview of the main aspects associated with the transition towards sustainable cosmetic packaging is shown in Figure 2.

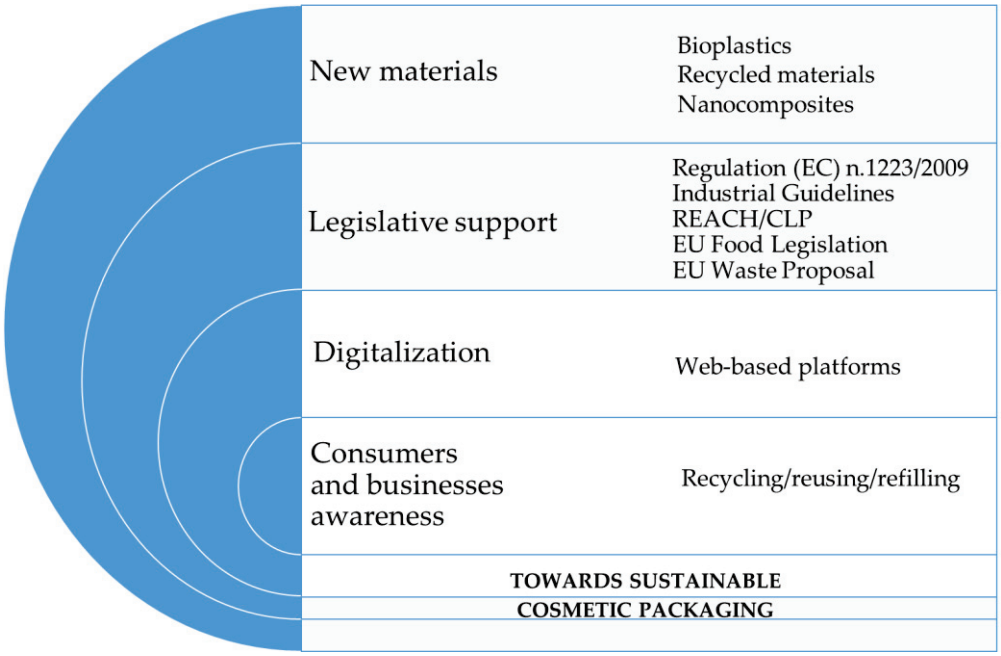


Figure 2. Key elements involved in the transition towards sustainable cosmetic packaging.

In the following two paragraphs, we explore sustainable packaging solutions, focusing particularly on the merits and challenges of bioplastics and recycled materials.

4.1. Bioplastics and Biopolymers

Bioplastics play a pivotal role in revolutionizing food and cosmetic packaging by offering sustainable alternatives to traditional plastics, thereby reducing environmental impact, and promoting the use of renewable materials. The term bioplastics covers a wide range of definitions and production techniques, including deriving monomers from renewable biomass, extracting polymers from biomass, biodegradability of the polymer, production via biological processes, or a combination of these factors. It is important to avoid labeling fossil-derived, degradable plastics as bioplastics.

Employing more precise terminology can improve comprehension. For example, bio-based durable PE is sourced from biomass but exhibits low biodegradability, whereas PBS is derived from fossil fuels yet is biodegradable. Furthermore, PHAs are both biodegradable and bio-based when cultivated from biomass-grown microorganisms. Additionally, PHAs are both biodegradable and bio-based when produced from biomass-grown microorganisms [53].

It is important to distinguish between biodegradation and composting, as compostability depends on specific microbial and chemical conditions. Additionally, it is crucial to understand that bio-based plastics are not inherently more environmentally friendly than fossil-based plastics. Sustainability depends on various factors throughout the product life cycle, such as how the raw materials are sourced, processed, and disposed of, which requires comprehensive life cycle assessments. Bioplastics are plastics made from natural materials, like plants, instead of fossil fuels. Bioplastics, derived from natural materials like plants, are perceived as environmentally superior but are often costlier due to a scarcity of resources and higher production energy. Farming for bioplastic plants can harm the environment, and disposal poses challenges due to inadequate recycling and composting infrastructure. In addition, competition with food sources is possible since some bioplastics use food crops, raising concerns about shortages.

In an ideal circular economy, plastics are sourced from renewable or recycled materials and produced in biorefineries able to convert biomass into valuable chemicals, categorized into first-generation (e.g., fermentable sugars from corn) and second-generation (non-edible biowastes) feedstocks [22]. However, a full transition from fossil-based to biomass plastics is presently improbable due to resource constraints, underscoring the necessity of decreasing consumption and enhancing recycling efforts.

Completely substituting global packaging plastics with bioplastics would demand significant agricultural resources, emphasizing the necessity for sustainable solutions that strike a balance between environmental impact and resource utilization. Moving towards a circular plastic economy entails utilizing renewable energy for both production and recycling processes. Advanced recycling methods transform plastic waste into valuable materials, utilizing biomass and pyrolysis oils as feedstocks. Future biorefineries strive to repurpose agricultural and food waste into resources, incorporating seaweed and plant-based monomers. Overcoming challenges involves enhancing conversion efficiency and refining processing techniques. Research focuses on improving pretreatment methods and developing robust microorganisms to streamline the transition towards renewable resources in biorefineries. Several methods have been explored for obtaining vinyl monomers, carboxylic acids, alcohols, amides, and rubbers from biomass [54]. Additionally, aliphatic polyesters, such as PLA, PBS, and PHAs, constitute a prominent category of bioplastics renowned for their degradability, rendering them suitable for various applications [55].

Despite their environmental benefits, challenges persist in scaling up bioplastic production and optimizing their properties for commercial viability. Nevertheless, ongoing research and advancements in biorefinery technologies hold promise for widespread adoption of bioplastics across various industries [16].

4.2. Recycled Packages: Strategies, Challenges and Future Directions

The ability to recycle a material depends on its inherent characteristics. Recycling can be categorized into four types based on the quality of the material produced (Figure 3). Primary recycling involves mechanically processing material into a product with identical properties to the original material. For instance, PET from used bottles can be used to manufacture new bottles. Secondary recycling yields a product with lower quality properties compared to the original material, such as producing synthetic fibers from PET bottles. Tertiary recycling, also referred to as chemical recycling, entails using the material to generate chemicals; for example, degrading PET into monomeric units for the production of virgin PET. Quaternary recycling involves utilizing waste material for energy production through incineration [56].

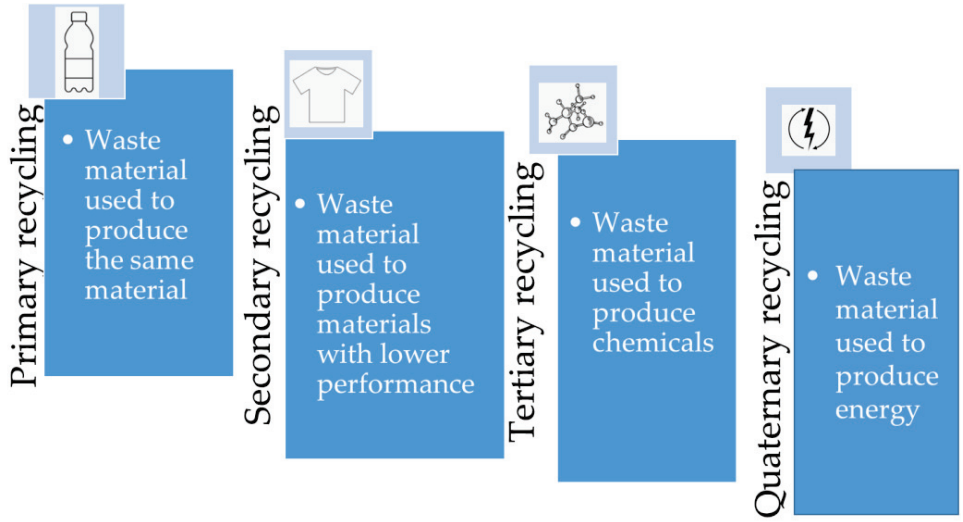


Figure 3. Recycling categories.

Metal and glass containers are permanently recyclable since their properties remain unchanged throughout the recycling process [22]. Materials like thermoplastics, paper, and paperboard undergo chemical alterations during recycling, potentially constraining their recyclability. For instance, recycled PET progressively experiences a reduction in some mechanical properties with each recycling cycle [13]. Nevertheless, recycling remains a crucial strategy for alleviating environmental degradation by lessening the need for new materials. The effectiveness of recycled plastic depends heavily on the quality and purity of the recycled compounds, which often change, affecting their usability. To meet functional requirements, recycled materials are frequently combined with new ones. However, recycling encounters significant challenges, including inefficient waste management systems, low recycling rates, and the presence of multiple components in recycled materials, all of which compromise their safety and usability. Another layer of complexity is added by the migration of chemicals from packaging to contents. This process involves diffusion through packaging materials and subsequent transfer to the product, influenced by factors such as initial concentration, size, solubility, and environmental conditions. In the realm of food safety, stringent regulations are in place, with specific limits established to safeguard consumers. These regulations aid scientists in studying the properties of cosmetic packaging in correlation with the behavior of the product it contains, which must possess similar characteristics to food. Current regulations governing recycled plastics in food contact materials are under review to ensure equal safety standards for both recycled and new materials. Compliance with these limits is verified using food or regulated food simulants. These simulated foods mimic real ones and possess different properties, such as being hydrophilic (water-attracting) or lipophilic (fat-attracting). Researchers test a variety of foods, including those with low fat content and high fat content. They have discovered that foods with higher fat content tend to allow certain substances, such as small particles and oily matter, to migrate into them more readily. Additionally, lighter molecules migrate faster, particularly under conditions of heat and prolonged storage. Paper and cardboard packaging generally facilitate greater substance migration into food compared to plastics like PE and PP, possibly due to their higher porosity. Among plastics, PET and PA exhibit lower rates of substance migration because they offer better barriers. However, even within the same type of plastic, molecules may migrate differently depending on the shape of the packaging. For instance, PET trays allow more migration of smaller particles compared to PET bottles, likely due to differences in shape. Thinner packaging also tends to facilitate faster migration. In October 2020, the European Commission unveiled a chemicals strategy aimed at enhancing the safety of recycled materials and products. This involves eliminating harmful substances, including endocrine disruptors, from everyday items like food packaging [57]. Two primary methods are commonly utilized to analyze the

contents of food packaging: targeted and non-targeted approaches. In targeted analysis, scientists specifically search for predetermined substances, while in non-targeted analysis, they seek substances that were not previously anticipated, such as non-intentionally added substances (NIAS). NIAS occurrences are more common when recycled materials are utilized, as various components may become mixed during recycling processes or undergo degradation during recycling.

Recently, Van Velzen and colleagues investigated the contents of PET bottles made from different types of PET, including some newly produced and some recycled [58]. They combined recycled PET pellets with new ones in various proportions to manufacture the bottles. The analysis involved examining the composition of both pellets and bottle pieces for metals and chemicals. Antimony, cobalt, and chlorine were detected in the pellets, while chemicals like acetaldehyde and benzene were found in the bottles, primarily originating from PET heating during bottle production. Furthermore, certain chemicals inherent to PET manufacturing were identified. Interestingly, bottles made from new PET exhibited similar chemical levels, suggesting minimal impact from recycled PET. Another study by Horodytska and colleagues explored the composition of recycled pellets from plastic bags and cleaning product containers [59]. The analysis unveiled a diverse array of chemicals, derived from plastic additives and other contaminants. Moreover, contact between certain foods and packaging materials resulted in chemical breakdown and alteration over time [60]. As with recycled food packaging, since packaging plays a crucial role in the preservation of cosmetics, evaluating the quality of recycled cosmetic packaging is of paramount importance. This assessment entails identifying and measuring the materials as well as any unintended substances that may originate from production or recycling processes.

5. Conclusions

With the rise in environmental consciousness among consumers, the cosmetic industry is increasingly seeking more sustainable packaging options, such as bioplastics or recycled materials. This shift towards eco-friendly packaging highlights that sustainable solutions not only reduce waste but also enhance brand trust and loyalty. Nonetheless, this transition poses challenges in terms of material availability, safety and cost-effectiveness. Additionally, standardized labeling and certification for sustainable packaging are essential to ensure transparency and consumer safety.

Packaging safety for cosmetics is concisely outlined in Regulation (EC) n.1223/2009. Currently, to choose the appropriate packaging, regulations on chemical substances, food regulations, and industrial guidelines assist cosmetic and packaging companies with regulatory aspects. The EU Commission is also working on proposed regulations for the recycling and reuse of packaging to promote sustainability while maintaining competitiveness and innovation.

Therefore, the transition of the cosmetics industry to sustainable packaging practices and safety standards requires close collaboration among regulatory bodies, trade associations, and companies. To achieve this goal, it is crucial to establish rigorous standards and certification processes.

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Abbreviation

BPA	Bisphenol A
CCI	Container-Content Interactions
CEAP	Circular Economy Action Plan
CLP	Classification, Labeling and Packaging
CMRCPSR	Carcinogenic, Mutagenic, or toxic for ReproductionCosmetic Product Safety Report
FCMs	Food Contact Materials
FDA	Food and Drug Administration
FSC	Forest Stewardship Council
NIAS	Non-Intentionally Added Substances
PA	Polyamide
PBS	Polybutylene succinate
PE	Polyethylene
PEFC	Programme for the Endorsement of Forest Certification
PET	Polyethylene terephthalate
PHAs	Polyhydroxyalkanoates
PIF	Product Information File
PLA	Polylactic acid
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinyl chloride
REACH	Registration, Evaluation, Authorisation and restriction of Chemicals
SA	Safety Assessor
SAN	Styrene acrylonitrile copolymer
SVHC	Substances of Very High Concern

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The Inhibition of Oxidative Stress-Mediated Cell Apoptosis by the Caspase Inhibitor (S)-3-((S)-2-(6-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)-1-oxoisoindolin-2-yl)butanamido)-4-oxo-5-(2,3,5,6-tetrafluorophenoxy)pentanoic Acid in Human Dermal Papilla Cells

Bomi Park [1], Daeun Kim [1], Yurim Lee [2], Seihyun Choi [2], HeeDong Park [2], Sanghwa Lee [2] and Jaesung Hwang [1,*]

[1] Department of Genetics & Biotechnology, Graduate School of Biotechnology, College of Life Sciences, Kyung Hee University, Youngin 17104, Republic of Korea; pbm1208@khu.ac.kr (B.P.); kpkdy0107@khu.ac.kr (D.K.)
[2] Innovo Therapeutics Inc., 507 38, Mapo-daero, Mapo-gu, Seoul 04174, Republic of Korea; yrlee@innovothera.com (Y.L.); seihyun@innovothera.com (S.C.); hdpark@innovothera.com (H.P.); shlee@innovothera.com (S.L.)
* Correspondence: jshwang@khu.ac.kr

Abstract: Alopecia is traditionally viewed as androgen-dependent, but emerging evidence has implicated oxidative stress in the pathogenesis of hair loss. Current treatments for alopecia have limited efficacy, leading to the need for new therapies. Human dermal papilla cells (hDPCs) play a pivotal role in hair follicle (HF) development and hair growth regulation. In this study, we investigated the potential of (S)-3-((S)-2-(6-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1-oxoisoindolin-2-yl)butanamido)-4-oxo-5-(2,3,5,6 tetrafluorophenoxy) pentanoic acid (THPA), a pan-caspase inhibitor, to reduce ROS-induced cellular damage and apoptosis in hDPCs. Our study revealed that THPA effectively suppressed hydrogen peroxide-induced apoptosis while also attenuating activated caspase signaling. Additionally, THPA restored the down-regulated expression of β-catenin, a key mediator of the Wnt/β-catenin pathway, in hDPCs exposed to hydrogen peroxide. Furthermore, significant alterations in Akt/mTOR/p70S6K signaling were observed following THPA treatment. Notably, THPA treatment led to a reduction in the expression of Dickkopf-1 (DKK-1), an inhibitor of the Wnt/β-catenin pathway implicated in hair follicle regression. Moreover, THPA treatment decreased the expression of the cell senescence markers p21 and p16, suggesting a potential role in preserving hDPC function and delaying hair follicle regression. Collectively, our findings highlight the therapeutic potential of THPA in preventing hair loss by protecting hDPCs against oxidative stress damage.

Keywords: alopecia; apoptosis; caspase; reactive oxygen species; human dermal papilla cells; β-catenin; Akt; senescence

1. Introduction

Alopecia is a common form of hair loss affecting both men and women. Up to 80% of men and 50% of women are affected by alopecia, which becomes more common and severe as people age [1]. While alopecia may not directly damage the body, it can cause patients to experience severe psychological stress that can lead to negative feelings such as inferiority, anxiety, and despair and negatively impact their quality of life and personal social activities [2]. Consequently, it is critical to manage alopecia appropriately. Topical minoxidil and oral finasteride are two Food and Drug Administration-approved therapies. Minoxidil is known as a direct-acting arteriolar vasodilator that expands blood flow to induce growth in hair follicles, and finasteride is a 5-alpha reductase inhibitor that prevents testosterone from converting into dihydrotestosterone (DHT), thus affecting hair loss [3]. However, these medications can partially stop hair loss but do not fully

stimulate hair growth. Moreover, negative side effects and the challenge of adhering to daily treatment regimens may further limit their use [4,5]. Therefore, recent research has explored therapeutic approaches such as pharmaceuticals and nutritional supplements like vitamins and plant extracts, highlighting the essential need for new treatments to increase hair growth [6].

Hair follicles consist of keratinocytes and a specialized mesenchymal cell population known as dermal papilla cells (DPCs) [7,8]. Human dermal papilla cells (hDPCs) are pivotal in follicle development, regeneration, and hair growth cycling. hDPCs stimulate follicular keratinocytes to maintain and regenerate the hair follicle cycle [9]. By secreting growth-promoting signals, hDPCs play essential roles in regulating the onset of anagen and the transition from the anagen to catagen phases [10]. Recently, DPCs have been used in in vitro screening models to assess the efficacy of agents that target hair growth.

Alopecia is characterized as an age-related or androgen-dependent mechanism in hDPCs [11]. However, clinical reports have demonstrated that alopecia does not occur solely through androgen-dependent pathways; it also involves androgen-independent mechanisms, such as those induced by chemotherapy and stress [12–14]. Recent studies have shown that apoptotic and senescent levels are markedly elevated in balding DPCs compared to non-balding DPCs [15,16]. Interestingly, reactive oxygen species (ROS) have emerged as crucial inducers to both androgen-dependent and androgen-independent alopecia [12], with higher ROS levels detected in the dermal papilla of balding scalps than in non-balding scalps [17,18]. Elevated ROS levels correlate with reduced DPC motility [15] and heightened cell senescence in balding-scalp DPCs [15]. Recent studies have linked cisplatin-induced alopecia to ROS production and ROS-mediated apoptosis in DPCs [19]. These findings suggest that targeting ROS is a promising and necessary approach in the development of alopecia prevention and treatment strategies.

The Wnt/β-catenin signaling pathway plays a crucial role in the development of hair follicles [6,20,21]. Following activation in adult tissues, β-catenin, the primary mediator of the Wnt/β-catenin signaling pathway, aggregates within the cytoplasm before translocating to the nucleus. Within the nucleus, it forms complexes with transcription factors from the lymphoid enhancer factor/T-cell factor (Lef/Tcf) family [22]. This complex orchestrates the transcription of target genes that are crucial for regulating fundamental cellular functions such as proliferation and migration [23]. Also, exposure to H_2O_2 decreased the length of human hair follicles through the modulation of the GSK-3β/β-catenin signaling pathway [24].

Apoptosis, a highly regulated programmed cell death process, plays a pivotal role in various physiological and pathological conditions. It is essential for maintaining tissue homeostasis by eliminating damaged or unwanted cells. Considering the widespread use of H_2O_2 to induce oxidative stress-related apoptosis, we adopted an in vitro model to simulate oxidative stress using H_2O_2 [25,26]. Research has indicated that H_2O_2, along with superoxide and hydroxyl radicals, plays a role in damaging hDPCs, ultimately contributing to hair loss [24,27].

In this study, we developed a novel pan-caspase inhibitor, (S)-3-((S)-2-(6-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1-oxoisoindolin-2-yl)butanamido)-4-oxo-5-(2,3,5,6-tetrafluorophenoxy)pentanoic acid (THPA). We aimed to demonstrate that THPA could ameliorate hair loss by modulating cell apoptosis and regulating the associated signaling pathways. This was confirmed by inducing oxidative stress with H_2O_2 in hDPCs.

2. Materials and Methods

2.1. Synthetic Method of (S)-3-((S)-2-(6-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1-oxoisoindolin-2-yl)butanamido)-4-oxo-5-(2,3, 5,6-tetrafluorophenoxy)pentanoic Acid

(1) Synthetic scheme of Compound **6**

To the solution of 4-Benzyl N-(tert-Butoxycarbonyl)-L-aspartate (25 g, 77.3 mmol) and N-methylmorpholine (9.35 mL, 85.0 mmol) in tetrahydrofuran (250 mL) under nitrogen

gas at −15 °C, we added isobutyl chloroformate (10.53 mL, 81.2 mmol) dropwise. After stirring at −15 °C for 30 min, the mixture was warmed to 0 °C.

Freshly prepared excess diazomethane (5.0 eq) in ether was added to the solution at 0 °C under nitrogen (until the yellowish color did not disappear). The reaction mixture was stirred at 0 °C for 30 min.

The reaction mixture was treated with 5 N HBr/glacial acetic acid (1:1) (30.9 mL, 2 eq, 154.6 mmol) slowly. After stirring at 0 °C for 30 min, the mixture was partitioned between ethyl acetate and water. The organic phase was washed successively with water, saturated sodium bicarbonate, and saturated sodium chloride. The solution was dried over anhydrous sodium sulfate, filtered and evaporated to dryness. Hexane was added and the solid was filtered and washed with hexane to provide Compound **4** (25 g, 80.8% over three steps).

To a solution of Compound **4** (25 g, 62.5 mmol) in DMF (250 mL), we added potassium fluoride (9.068 g, 156.3 mmol) and 2,3,5,6-tetrafluorophenol (11.408 g, 68.8 mmol). The reaction mixture was stirred at room temperature for 2 h and then partitioned between ethyl acetate and water. The organic layer was washed with sat. ammonium chloride and brine. The organic layer was dried over sodium sulfate, filtered, and concentrated. The crude product directly went to the next step.

Compound **5** was treated with 4 N HCl in dioxane (155 mL), and the reaction mixture was stirred at r. t. for 1 h. The solution was concentrated under reduced pressure. The remaining one was stirred in anhydrous diethyl ether for 1 h. The solid was filtered, washed with EtOAc, and dried to produce the desired Compound **6** (23.6 g, 90% over two steps) (Figure 1).

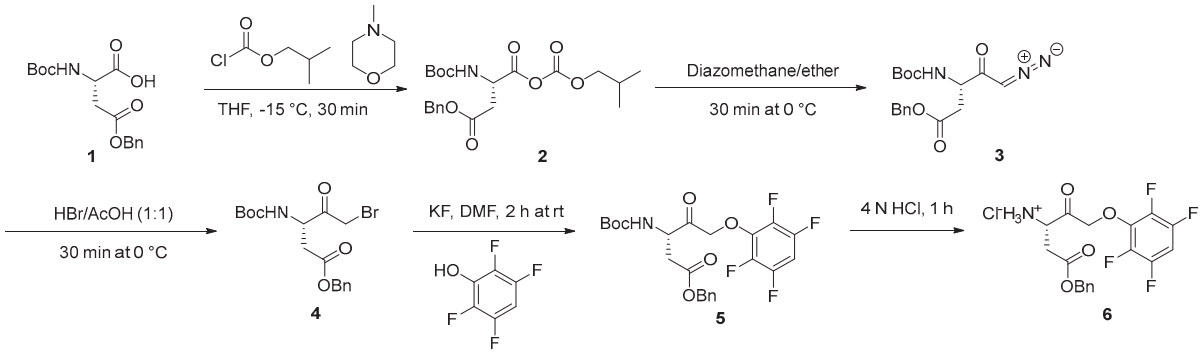


Figure 1. Synthetic scheme of Compound **6**.

(2) Method used to make diazomethane

N-methylurea (86 g, 1.162 mol) was dissolved in water (715 mL). Sodium nitrite (88.2 g, 1.278 mol) was added, and the mixture was cooled to 0 °C in an ice bath. Then, 33% aq. hydrochloric acid (170 mL, 1.51 mol) was added dropwise over a period of 1 h. The mixture was left to stir for 30 min at the same temperature. The precipitate was filtered off, washed with water, and dried in vacuo. N-methyl-N-nitrosourea (74.8 g, 62%) was obtained as a light-beige powder (it was stored at −28 °C).

N-methyl-N-nitrosourea was introduced into an Erlenmeyer flask containing diethyl ether and 40% aq. KOH previously cooled to 0 °C in an ice bath. The mixture was left to stand for 30 min at 0 °C, carefully shaking it several times. The organic phase containing the generated diazomethane was decanted and dried (KOH pellets) at 0 °C for 3 h.

(3) Synthetic scheme of (S)-3-((S)-2-(6-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1-oxoisoindolin-2-yl)butanamido)-4-oxo-5-(2,3,5,6-tetrafluorophenoxy)pentanoic acid

To the solution of Compound **7** (Methyl 5-Bromo-2-methylbenzoate) (25 g, 109.14 mmol) and N-bromosuccimide (19.424 g, 109.14 mmol) in CHCl3 (100 mL), we added dibenzoyl peroxide (1.322 g, 5.46 mmol). The reaction mixture was stirred at 75 °C for 1 h and then

diluted with dichloromethane and water. The organic layer was washed with water (three times) and brine, then dried with sodium sulfate and concentrated under reduced pressure to produce Compound 8.

To the solution of Compound **8** (from the previous reaction) and tert-butyl (2S)-2-aminobutanoate hydrochloride (21.287 g, 108.78 mmol) in acetonitrile (300 mL), we added diisopropylethyl amine (DIPEA) (56.84 mL, 326.33 mmol). The reaction mixture was stirred under reflux for 5 h. The solution was cooled to room temperature and then diluted with ethyl acetate. The solution was washed with water, saturated ammonium chloride, and brine; dried over sodium sulfate; and concentrated under reduced pressure. The crude product was purified by crystallization with ether and hexane to produce Compound **9** (25.1 g, 65%).

To the solution of Compound **9** (10.0 g, 28.32 mmol) in 1,4-dioxane (100 mL), we added Tetrakis(triphenylphosphine)palladium (3.26 g, 2.83 mmol), 2 M potassium carbonate solution (28.2 mL, 56.5 mmol) and 2,3-dihydro-1,4-benzodioxin-6-ylboronic acid (5.58 g, 29.73 mmol) The reaction mixture was degassed for 10 min. The reaction solution was refluxed at 100 °C for 10 h and cooled to room temperature. The solution was diluted with ethyl acetate and water and filtered through a Celite pad. The organic layer was washed with saturated ammonium chloride, brined, dried over sodium sulfate, and concentrated using a rotary evaporator. The crude product was used for the next step without any purification.

Compound **10** was treated with 4 N HCl in dioxane (20 eq, 142 mL), and the reaction mixture was stirred at room temperature for 8 h. The solution was concentrated under reduced pressure. The crude product was washed with ether (200 mL) and ethyl acetate (200 mL) to produce Compound **11** (9.7 g, 96% over two steps)

Compound **11** (5.0 g, 14.15 mmol) and Compound **6** (5.96 g, 1 eq, 14.15 mmol) were dissolved in DMF (50 m) under N2 gas. HATU (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-Oxide Hexafluorophosphate) (6.99 g,18.40 mmol) and DIPEA (9.86 mL, 56.59 mmol) were added. The reaction mixture was stirred at room temperature for 5 h. The solution was diluted with ethyl acetate and washed with water (x 2), saturated ammonium chloride, saturated sodium bicarbonate, and brine; dried over magnesium sulfate; and concentrated under a rotary evaporator. The crude produce was purified by a silica gel column (40% EtOAC in Hex) to produce Compound **12** (7.4 g, 72%).

To a solution of Compound **12** (7.0 g, 1.38 mmol) in THF:AcOH (70 mL:3.5mL) was added 10% palladium on carbon (0.05 eq). The reaction solution was degassed and charged with hydrogen gas several times. The reaction mixture was stirred at room temperature for overnight. The solution was filtered through Celite pad and concentrated. The crude product was purified by Prep-HPLC (70% ACN in H_2O with 0.035% TFA) to produce "THPA" (3.7 g, 60%) (Figure 2).

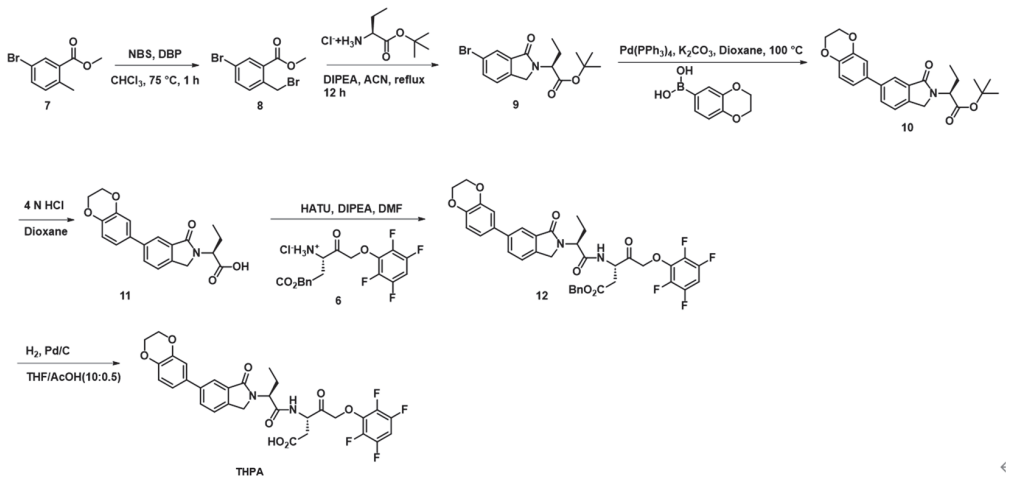


Figure 2. Synthetic scheme of THPA.

1H NMR (400 MHz, DMSO-d6) δ12.51 (s, 1 H), 8.85 (dd, J = 11.2, 7.5 Hz, 1H), 7.91–7.82 (m, 2 H), 7.68 (d, J = 7.8 Hz, 1 H), 7.66–7.52 (m, 1 H), 7.23 (d, J = 8.0 Hz, 2 H), 7.00 (d, J = 8.2 Hz, 1 H), 5.27 (d, J = 4.1 Hz, 2 H), 4.81 (dd, J = 9.7, 5.7 Hz, 1 H), 4.74–4.62 (m, 2 H), 4.53 (d, J = 17.7 Hz, 1 H), 4.33 (s, 4 H), 4.07 (q, J = 7.1 Hz, 1 H), 2.80 (dd, J = 16.9, 6.0 Hz, 1 H), 2.62 (dd, J = 16.9, 7.0 Hz, 1 H), 2.03 (s, 2 H), 1.84 (dq, J = 9.5, 7.2 Hz, 1 H), 1.21 (t, J = 7.1 Hz, 1 H), 0.88 (t, J = 7.3 Hz, 3 H). MS (ESI, LR) Calculated for $C_{31}H_{27}H_4N_2O_8$ (MH+): 631.17, found: 631.2.

2.2. Cell Culture

Human dermal papilla cells (hDPCs) were purchased from Promocell (Promocell, Heidelberg, Germany). Cells were cultured using the growth medium kit in a humidified incubator at 37 °C under 5% CO_2 according to the instructions provided by Promocell. All experiments used cells between passage three and five. Before treatment with THPA and H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA), the medium was replaced with fresh DMEM (WelGENE, Daegu, Republic of Korea) containing 1% FBS (WelGENE, Daegu, Republic of Korea), and cells were incubated for 24 h to reduce the influence of serum and growth factors. Subsequently, THPA and H_2O_2 were treated according to the experimental conditions.

2.3. Cell Viability

Relative cell viability was examined using the WST assay (Daeil Lab Service, Seoul, Republic of Korea) following the manufacturer's protocol. Briefly, hDPCs (5×10^3/well) were seeded into 96-well plates and cultured for 24 h. The medium was replaced with DMEM supplemented with 1% FBS, and the cells were pre-treated with THPA for 4 h. Following H_2O_2 treatment for 24 h, the culture medium was replaced with fresh DMEM media containing 10% WST regent. Absorbance was detected using a microplate reader (Tecan, Mannedorf, Switzerland) at 450 nm.

2.4. Lactate Dehydrogenase Assay

Cytotoxicity was assessed using a Cytotoxicity LDH Assay kit (Dojindo Molecular Technologies, Kumamoto, Japan) following the manufacture's protocol. hDPCs (5×10^3/well) were plated in 96-well plates and incubated for 24 h. hDPCs were pre-treated with THPA for 4 h and exposed to H_2O_2 for 24 h. Following the treatment, the medium was replaced with the working solution. After 30 min, a stop solution was added. The absorbance of the solution in each well was determined using a microplate reader at 490 nm.

2.5. Cellular ROS Detection Assay

The detection of ROS production was performed using the 2′,7′-dichlorofluorescein diacetate (DCFDA)-Cellular ROS Detection Assay kit (Abcam, Cambridge, UK). According to the manufacturer's instructions, hDPCs (5×10^3/well) were seeded into a 96-well microplate. After overnight incubation, the media were removed and cells were washed with buffer. Subsequently, cells were incubated with 25 μM DCFDA at 37 °C for 45 min. After removing DCFDA, supplemented buffer containing THPA and H_2O_2 was added. Fluorescence intensity was measured using a microplate reader at Ex/Em = 485/535 nm.

2.6. TUNEL Staining

Apoptosis was determined using the Click-iT^{TM} plus terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kits for in situ apoptosis detection (Invitrogen) according to the manufacturer's instructions. In brief, hDPCs (3×10^4/well) were seeded in a 24-well plate. Following treatment with THPA and H_2O_2, cells were fixed with 4% paraformaldehyde, permeabilized with a Triton X-100, and incubated with a TdT reaction mixture at 37 °C for 1 h. Counterstaining was performed using DAPI, followed by analysis with fluorescence microscopy (Nikon, Tokyo, Japan).

2.7. Wnt Reporter Assay

WRHEK293A cells were purchased form Amsbio (Amsbio, Abingdon, UK). Cells were seeded in white 96-well plates and incubated overnight. After treatment with H_2O_2 and THPA, cells were lysed using 50 μL of lysis buffer (Thermo Scientific, Waltham, MA, USA). GFP fluorescence was measured using a microplate reader at Ex/Em = 488/510 nm. Subsequently, 50 μL of luciferase substrate solution (Promega, Madison, WI, USA) was added to each well, and luciferase activity was measured. The measured values were normalized using GFP fluorescence.

2.8. Real Time Quantitative Polymerase Chain Reaction

Total RNA was extracted using RNAiso Plus (Takara, Shiaga, Japan). Subsequently, cDNA synthesis was achieved using RevertAid Reverse Transcriptase (Thermo Scientific). Quantitative polymerase chain reaction (qPCR) analysis was conducted utilizing the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and FastStart Essential DNA Probes Master (Roche, Basel, Switzerland), following standard protocols.

2.9. Western Blot

After washing the cells in DPBS, protein was extracted using RIPA lysis solution supplemented with 1 mM PMSF and a protease inhibitor cocktail (Sigma-Aldrich, Missouri, USA). Extracted proteins were equally separated on NuPAGE^{TM} 12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA) and transferred onto a PVDF membrane. Following overnight incubation with the appropriate antibodies (Cell Signaling Technology, Danvers, MA, USA) in TBS-T, the membranes were blocked with 5% BSA and subsequently probed with a horseradish peroxidase-conjugated anti-mouse secondary antibody (Bio-Rad).

2.10. Statistical Analysis

Significant F-value items in an ANOVA were evaluated for significance at the $p < 0.05$ level using the Tukey test, which was conducted using SPSS software as the statistical analysis tool.

3. Results

3.1. H_2O_2 Induced Cell Death in Cultured hDPCs

H_2O_2 induces hair loss through its cytotoxic effects and the ability to induce oxidative stress [28]. In this study, H_2O_2 was used to induce oxidative stress in hDPCs. To evaluate the cytotoxicity of H_2O_2 on hDPCs, cells were treated with 125 μM–1.5 mM of H_2O_2. The viability of hDPCs was measured using the WST assay after 24 h of treatment. The viability of the hDPCs decreased by approximately 50% at a 1 mM concentration (Figure 3A). In addition, the LDH assay showed a significant increase starting at a 1 mM H_2O_2 concentration (Figure 3B). Based on these results, 1 mM of H_2O_2 was used in subsequent experiments to induce cell death.

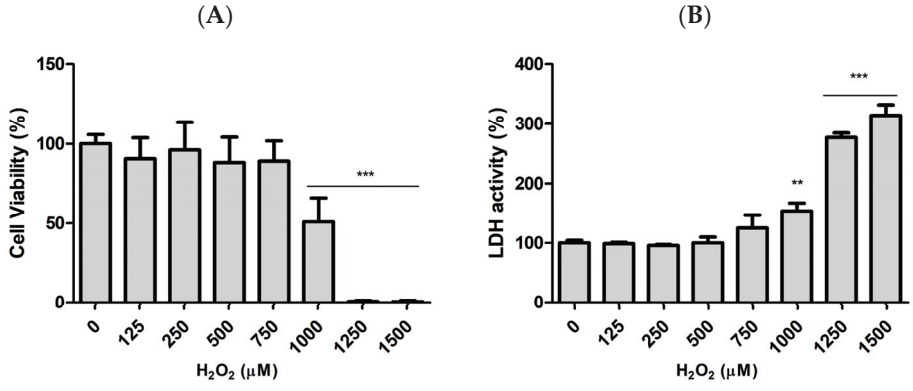


Figure 3. H_2O_2 induces cell apoptosis. Viability (**A**) and LDH (**B**) assays of hDPCs after exposure to different concentrations of H_2O_2 for 24 h, $n = 4$. ** $p < 0.01$, *** $p < 0.001$.

3.2. THPA Protected hDPCs from Cell Apoptosis and Oxidative Damage Induced by H_2O_2

We developed a pan-caspase inhibitor that inhibited all types of caspases, thereby preventing cell apoptosis and oxidative damage induced by H_2O_2. THPA showed no cytotoxicity to the cells at concentrations ranging from 1 nM to 10 uM (Figure S1). THPA was pre-treated with THPA for 4 h prior to H_2O_2 treatment. THPA pre-treatment prevented cell death in hDPCs induced by H_2O_2 in a concentration-dependent manner (Figure 4A). We examined the cellular ROS levels after THPA and H_2O_2 using 2′,7′-dichlorofluorescin diacetate (DCFDA) staining. While 1 mM H_2O_2 significantly increased the cellular ROS level, 5 μM THPA reduced ROS levels (Figure 4B). Furthermore, we performed a TUNEL assay to investigate the inhibitory effect of THPA on the apoptosis of hDPCs. There was a significant increase in the number of TUNEL-positive cells, whereas THPA-treated hDPCs exhibited a reduction in the number of TUNEL-positive cells (Figure 4C).

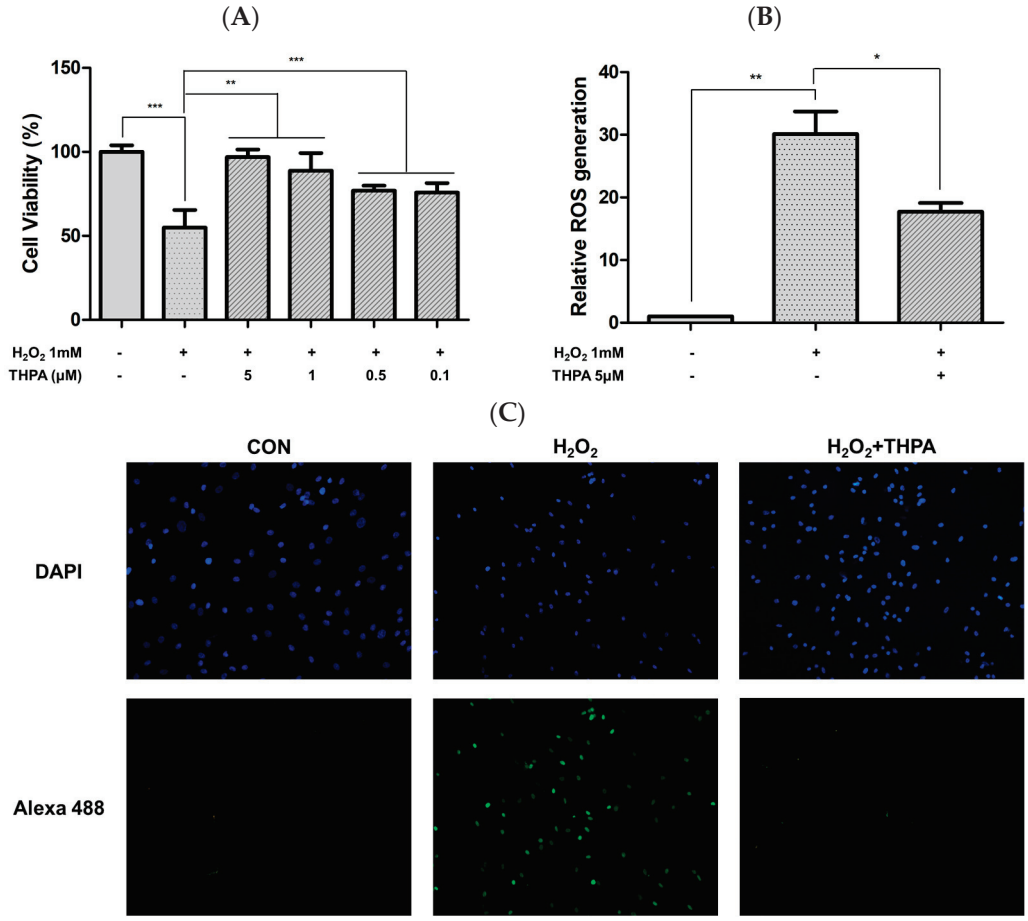


Figure 4. THPA reduced H_2O_2-induced cell apoptosis and oxidative damage. THPA alleviated H_2O_2 effects on hDPCs viability (**A**), ROS level (**B**), apoptosis (**C**), $n = 4$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3. THPA Inhibited Caspase Activation Induced by H_2O_2

Caspase-3 and caspase-7, which lead to cell apoptosis, are regarded as catagen markers. During catagen, these proteins are highly expressed in the hair matrix and participate in cell fragmentation. Additionally, they mediate the effector mechanisms of apoptosis in human hair follicles [29,30]. PARP is known to respond to DNA damage by activating DNA repair mechanisms and aiding in cell survival. However, when cellular damage is severe or repair is not feasible, PARP promotes cell apoptosis [31]. We investigated the inhibition of apoptosis by measuring the protein expression levels of caspase-3, caspase-7, and PARP following THPA treatment (Figure 5A). Similar to the Western blotting results, THPA significantly blocked the H_2O_2-induced increase in caspase-3/7 activity (Figure 5B).

These results indicated that THPA could prevent catagen entry and cellular damage by preventing apoptosis in hDPCs.

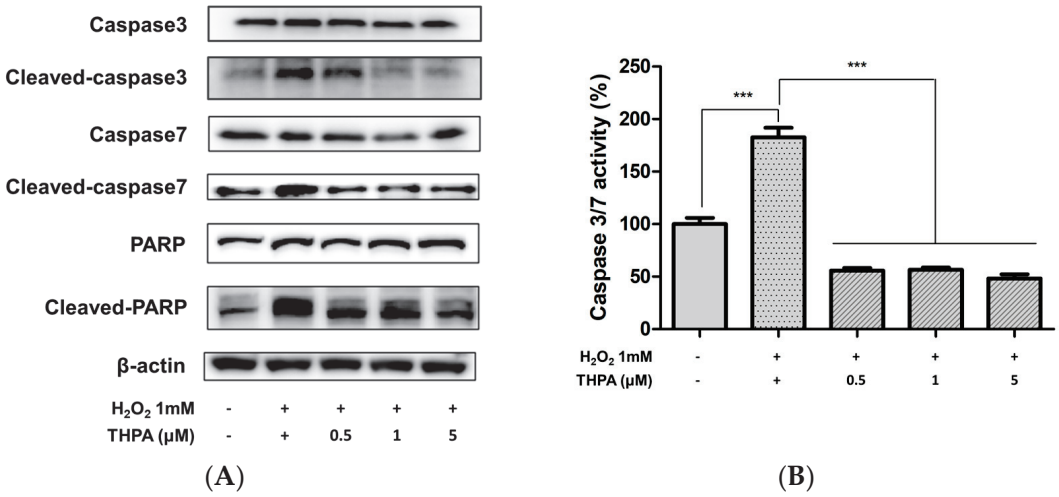


Figure 5. THPA inhibited H_2O_2-induced caspase activity. THPA inhibited H_2O_2-induced caspase activity in hDPCs (**A**,**B**), $n = 3$. *** $p < 0.001$.

3.4. THPA Restored the Reduced β-Catenin Level

The Wnt/β-catenin pathway plays a crucial role in the hair regeneration process [32]. Studies have shown that the inhibition of β-catenin hinders the hair growth cycle, whereas its activation in hDPCs promotes hair growth and prolongs the anagen phase [33–35]. To investigate the effects of H_2O_2 and THPA treatment on Wnt/β-catenin signaling, we examined the protein expression of β-catenin. As shown in Figure 6A, H_2O_2 reduced the protein expressions of β-catenin. This indicated that H_2O_2 treatment affected hair growth and cycling. However, this decrease induced by H_2O_2 was prevented by THPA pre-treatment. Also, a stable Wnt reporter cell line was used to investigate the preventive effects of THPA on the Wnt/β-catenin pathway. Treatment with H_2O_2 decreased Tcf/Lef-mediated reporter activity. However, THPA increased luciferase activity (Figure 6B). This result indicated that THPA stimulates hair regeneration and growth by preventing the decrease in β-catenin levels induced by H_2O_2.

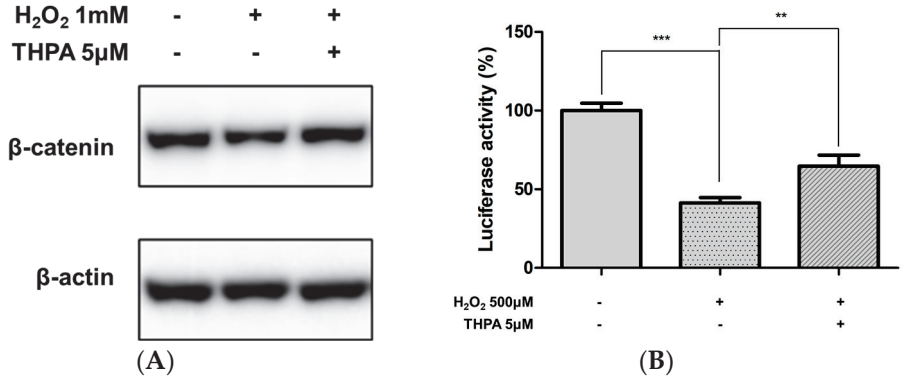


Figure 6. THPA restored the reduced Wnt/β-catenin level. THPA modulates the β-catenin protein expression in hDPCs (**A**) and the activity of Tcf/Lef transcription factor in WRHEK293A cell line (**B**), $n = 3$. ** $p < 0.01$, ***$p < 0.001$.

3.5. Akt/mTOR/p70S6K Signaling Pathway Is Required for Mediating the Effects of THPA on the Inhibition of H_2O_2-Induced Apoptosis

To elucidate the molecular mechanism of action of THPA, we investigated the MAP kinase molecules, which are crucial for cellular signaling cascades. MAP kinases phospho-

rylation was analyzed using a dot blot assay. The phosphorylation of Akt, mTOR, and p70S6K showed the most significant changes (Figure S2). There are studies showing that excessive oxidative stress activates Akt signaling in various cell lines [36–38]. Oxidative stress triggers cell death by inducing Akt activation [39]. Therefore, we further confirmed the changes in the phosphorylation of Akt, mTOR, and p70S6K in response to H_2O_2 and THPA treatment using Western blot. We found that H_2O_2 significantly increased the phosphorylation of Akt, mTOR, and p70S6K, whereas THPA restored their phosphorylation levels (Figure 7A,B).

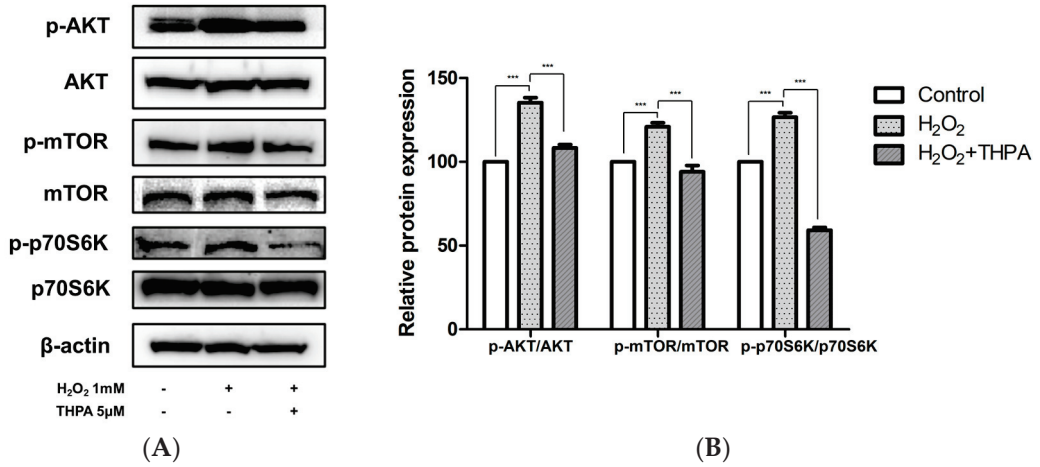


Figure 7. Akt/mTOR/p70S6K signaling is required for mediating the effects of THPA on the inhibition of H_2O_2-induced apoptosis. Protein expression levels of p-AKT, p-mTOR and p-p70S6K were detected by Western blot analysis in hDPCs (**A**,**B**), $n = 3$. *** $p < 0.001$.

3.6. THPA Reduced Catagen and Senescence Markers in H_2O_2-Treated hDPCs

To determine whether THPA prevents catagen and cell senescence induced by H_2O_2, the mRNA expression of the catagen marker DKK-1 and the protein levels of cell senescence markers p16 and p21 were measured. Dickkopf-1 (DKK-1), recognized as a Wnt antagonist and an inducer of catagen, facilitates the regression of hair follicles by inhibiting Wnt/β-catenin signaling [40]. THPA significantly decreased the elevated mRNA level of DKK-1 induced by hydrogen peroxide (Figure 8A). Protein expressions of p21 and p16 were significantly reduced in 5μM of THPA treatment, indicating that cell senescence could be prevented by THPA (Figure 8B).

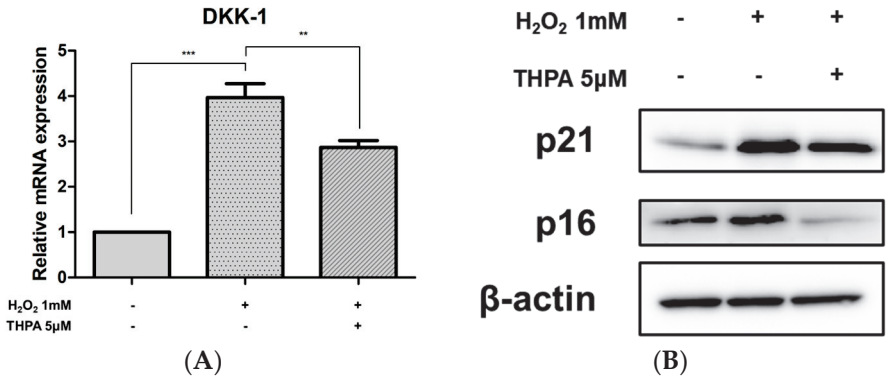


Figure 8. THPA reduced catagen and senescence markers in H_2O_2-treated hDPCs. The mRNA expression of DKK-1 (**A**) and the protein expression of p21 and p16 (**B**) were reduced in H_2O_2-treated hDPCs by THPA. ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

In the present study, we provided in vitro evidence that a pan-caspase inhibitor compound (THPA) can support hair regeneration by preventing of ROS-induced cell damage in hDPCs.

Caspases are a family of genes that are important for maintaining homeostasis by regulating cell death and inflammation, and their inhibition contributes to the prevention of cell death [41]. We found that apoptosis was induced by H_2O_2 and confirmed that THPA prevented it in hDPCs by regulating caspase signaling, which was activated by oxidative stress. Furthermore, the effects of THPA are related to its anti-oxidative effect. The up-regulation of intracellular ROS levels mediated by H_2O_2 was dramatically inhibited following THPA pre-treatment. Recent reports have demonstrated H_2O_2 was reported to induce premature senescence in rat dermal papilla cells, resulting in the inhibition of cell proliferation in follicular keratinocytes [42], and induce cell senescence by cell cycle arrest in hDPCs [43]. These findings suggest that THPA is a novel and potent therapeutic agent against oxidative stress-induced cell dysfunction in hDPCs.

The activation of the Wnt/β-catenin pathway is crucial for the regeneration of hair follicles and the growth of hair shafts. Studies have shown that hair follicles fail to develop when β-catenin, a pivotal signaling molecule within this pathway, undergoes mutation in the hair substrate of mice. Introducing this mutation into normal mice prevented hair follicles from transitioning into the subsequent hair growth cycle. [33] Furthermore, diminished β-catenin activity directly obstructs the differentiation of hair follicle stem cells (HFSCs) from hair follicle cells. [44,45]. As expected, the Wnt/β-catenin pathway was negatively affected by high levels of H_2O_2 in cultured hDPCs. We have found that the decreased expression of β-catenin in cultured hDPCs was restored by THPA.

We conducted a MAPK array to further demonstrate the role of THPA in molecular mechanisms and confirmed significant changes in Akt signaling. It has been reported that Akt activation and downstream signaling involve many pathways implicated in several biological functions, including hair proliferation, regeneration, and survival [46,47]. However, as previously mentioned, there are research findings indicating that Akt activation increases in response to oxidative damage induced by H_2O_2 [39,48]. According to research findings, the PI3K/Akt pathway is involved in various apoptotic signaling pathways. It is believed that the activation of the PI3K/Akt pathway during the early stages of cell apoptosis is understandable. As cell apoptosis progresses, cells exhibit preconditioning properties, where they are protected against apoptosis by a transient insult. Additionally, following withdrawal from a transient apoptotic insult, cells demonstrate self-sustained apoptosis, a remodeling process of apoptosis, that contributes to cell survival. During this apoptotic remodeling process, PI3K/Akt signaling can be inhibited through a negative feedback mechanism, thus providing further protection for cell survival [49,50]

In this context, THPA can prevent the apoptosis of hDPCs by ROS-induced Akt/mTOR/p70S6K activation, indicating that the Akt/mTOR/p70S6K signaling cascade may be the potential mechanism for hair loss.

In both murine and human models, DKK-1, a well-known inhibitor of Wnt/β-catenin signaling, induces apoptosis in hair follicle cells and restricts hair follicle enlargement during regeneration, thereby promoting the transition of anagen hair follicles to the catagen phase [50]. Moreover, the expression of DKK-1 in balding-scalp tissues was elevated compared to non-balding-scalp tissues [51]. Therefore, our data suggest that THPA promotes hair growth by potentially inhibiting the entry into the catagen phase through down-regulating the level of DKK-1 protein.

THPA treatment also significantly reduced the protein expression of the cell senescence markers p21 and p16. Cell senescence involves a state of cell cycle arrest, with key the involvement of regulatory proteins like p21CIP/WAF1 and p16 INK4a. These cyclin-dependent kinase inhibitors play critical roles in controlling the G1/S cell cycle checkpoint [52,53]. In our study, we observed that exposure to H_2O_2 resulted in a signifi-

cant increase in the protein expression levels of p21 and p16. Conversely, THPA treatment reduced the H_2O_2-induced expression of both p21 and p16.

5. Conclusions

Our research offers insights into the cellular and molecular mechanisms by which the caspase inhibitor THPA may protect hair survival in cultured hDPCs. To date, a hair loss treatment utilizing a caspase inhibitor has not been commercialized. Taken together, our data demonstrate that THPA could be a new treatment by preventing cell senescence and premature catagen entry by protecting hDPCs against oxidative stress. Therefore, we propose that THPA should be further investigated in animal and clinical trials to substantiate its potential for preventing hair loss by elucidating its underlying mechanisms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cosmetics11040105/s1, Figure S1: THPA is non-toxic to cells from 1 nM–10 μM; Figure S2: Analysis of phosphorylation MAPK array.

Author Contributions: Conceptualization, J.H. and S.L.; methodology, J.H. and B.P.; validation, J.H., S.L. and B.P.; formal analysis, B.P. and S.C.; investigation, B.P.; resources, J.H. and H.P.; data curation, J.H. and Y.L.; writing—original draft preparation, B.P.; writing—review and editing, S.L., J.H. and B.P.; visualization, B.P. and D.K.; supervision, J.H. and S.L.; project administration, J.H. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

hDPCs	human dermal papilla cells
HF	hair follicle
DHT	dihydrotestosterone
THPA	(S)-3-((S)-2-(6-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1-oxoisoindolin-2-yl)butanamido)-4-oxo-5-(2,3,5,6 tetrafluorophenoxy) pentanoic acid
DKK-1	dickkopf-1
ROS	reactive oxidative species
Lef/Tcf	lymphoid enhancer factor/T-cell factor
DCFDA	2′,7′-dichlorofluorescein diacetate
Ex/Em	Excitation/Emission
qPCR	quantitative polymerase chain reaction
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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Chemical Profile and Antioxidant and Tyrosinase Inhibitory Activity of *Chamaemelum nobile* L. Green Extracts

Luciana Maria Polcaro [1,2], Antonietta Cerulli [1], Francesco Montella [3], Elena Ciaglia [3], Milena Masullo [1,*] and Sonia Piacente [1,4]

[1] Dipartimento di Farmacia, Università degli Studi di Salerno, Via Giovanni Paolo II, 84084 Fisciano, SA, Italy; lpolcaro@unisa.it (L.M.P.); acerulli@unisa.it (A.C.); piacente@unisa.it (S.P.)
[2] Ph.D. Program in Drug Discovery and Development, Università degli Studi di Salerno, Via Giovanni Paolo II n. 132, 84084 Fisciano, SA, Italy
[3] Dipartimento di Medicina, Chirurgia e Odontoiatria "Scuola Medica Salernitana", Università degli Studi di Salerno, Via S. Allende, 84081 Baronissi, SA, Italy; fmontella@unisa.it (F.M.); eciaglia@unisa.it (E.C.)
[4] National Biodiversity Future Center (NBFC), 90133 Palermo, PA, Italy
* Correspondence: mmasullo@unisa.it; Tel.: +39-089968149

Abstract: The request for skin-whitening agents and bioactive principles able to control hyperpigmentation disorders is continuously growing. Chamomile (*Matricaria chamomilla*) is used as a remedy for skin diseases, but little is known about the ability of Roman chamomile (*Chamaemelum nobile*) to act as a skin-whitening agent. With the aim to investigate antioxidant and lightening potential, fresh aerial parts of *C. nobile* were extracted by maceration, ultrasound-assisted extraction, and solid–liquid dynamic (SLDE-Naviglio) extraction using EtOH/H_2O mixtures. Moreover, 32 metabolites (flavonoids, sesquiterpenoids, amides, and polar fatty acids) were identified by liquid chromatography/mass spectrometry. Principal component analysis revealed how the extract EtOH/H_2O 50% (Naviglio and long maceration), along with the extract EtOH/H_2O 60% (maceration) were richest in flavonoids. All extracts were tested by TEAC and DPPH assays, and to determine their in vitro antioxidant activity, the DHR 123 probe–intracellular ROS assay in HaCaT cells, for some extracts, was performed. Moreover, their ability to exert a whitening effect was tested by analyzing their tyrosinase inhibitory activity. The quantitative determination of apigenin, known as a natural tyrosinase inhibitor, was performed by LC-ESI/QTrap/MS/MS using the multiple reaction monitoring (MRM) method. These results are promising for selecting an extraction method to obtain a sustainable product rich in bioactives.

Keywords: green extracts; *C. nobile*; tyrosinase assay; LC-MS analysis

1. Introduction

Chamomile boasts a rich history of traditional medicinal uses dating back to ancient civilizations such as those of Egypt, Greece, and Rome. The Greeks, including Hippocrates and Dioscorides, as well as the Romans, like Pliny the Elder, recognized its therapeutic properties and documented its use for various ailments. Galen, a prominent physician in ancient Rome, also praised chamomile for its medicinal benefits. Throughout history, chamomile has been utilized in diverse preparations to address a wide range of health issues. Some of the ailments include abrasions, abscesses, acne, anorexia, anxiety, arthritis, asthma, catarrh, contact dermatitis, convulsions, cough, dry skin, hepatic disorders, hysteria, insomnia, menstrual cramps, psoriasis, and more others. The reverence for chamomile extended beyond its medicinal applications. Ancient Egyptians, in particular, held it in high esteem, viewing it as a sacred gift from the Sun God. Chamomile flowers were appreciated for their ability to alleviate high fevers and sunstroke, demonstrating their significance in ancient cultures not only as a medicinal herb but also as a symbol of divine healing [1].

Chamomile is represented by two main species: Roman chamomile (*Chamaemelum nobile*) and German chamomile (*Matricaria chamomilla*). Chamomile flowers are commonly used in traditional medicine and modern medicine for their content in flavonoids, coumarins, volatile oils, terpenes, sterols, organic acids, and polysaccharides, responsible for pharmacological activities such as anti-infective, anti-inflammatory, and antioxidant activities; along with their use as medicine, their applications in cosmetics are relevant [2,3]. In this field, chamomile is reported to have a soothing and softening effect on skin (it repairs sensitive skin, eliminates acne, and improves skin dehydration) and also has use in hair preparations (shampoos and rinses) like lightening and conditioning [4]. Therefore, it has been used as an ingredient in skincare products [5].

Roman chamomile is the common name of *Chamaemelum nobile* (L.) All. (syn. *Anthemis nobilis* L. and *Chamomilla nobilis* Godr.), a perennial herb belonging to the Asteraceae family that presents flowers with double petals and soft stems, and it has a green apple fragrance. In fact, it is also called "the apple of the ground" [6].

Considering the traditional use of chamomile in the treatment of skin imperfections and the new trend of "Green Cosmetics", which include formulations containing active ingredients derived from plants produced in eco-sustainable ways [7], and that in the literature, only one paper on a green hydroalcoholic extract of *C. nobile* with a conventional technique is reported [8], an LC-HRMS-based metabolomics study of different eco-sustainable extracts of *C. nobile* was carried out. In detail, *C. nobile* was submitted to conventional extraction methods like macerations (MACs) and unconventional extraction methods like ultrasound-assisted extraction (UAE) and solid–liquid dynamic extraction (SLDE-Naviglio) using ethanol (EtOH 100%) and ethanol–water (EtOH/H_2O) at different percentages (50%, 60%, 75%) as "green" solvents. Since to date in the literature there are no reports about non-conventional extraction procedures of *C. nobile*, with the aim to select the best technique and percentage of EtOH/H_2O for the extraction of bioactives, the extracts were analyzed by UHPLC-HRMS analysis using ultra-high-performance liquid chromatography coupled with a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (UHPLC-Q-Orbitrap) equipped with a heated electrospray ionization probe (HESI), in positive ion mode, allowing the identification of specialized metabolites such us flavonoids and sesquiterpenoids; thereafter, the UHPLC-HRMS of the "green" extracts of the *C. nobile* were compared by multivariate statistical analysis.

The "green" extracts of *C. nobile* were submitted to different spectrophotometric assays; in particular, considering the capacity to inhibit various ROS formation factors mainly ascribable to flavonoid derivatives [9], thus preventing skin ageing, total phenolic content, DPPH, and TEAC assays of the extracts were performed.

Moreover, *M. chamomilla* is known for its ability to treat sensitive skin, eliminate acne, and improve skin, ascribable to its capacity to inhibit tyrosinase, a key enzyme in the synthesis of melanin [10,11]. To the best of our knowledge, no information is reported in the literature about the capacity of *C. nobile* to inhibit tyrosinase enzyme; therefore, herein, for the first time, the evaluation of the anti-tyrosinase activity of *C. nobile* "green" extracts was performed. The literature data show how apigenin is a marker of chamomile responsible for antioxidant and lightening properties [3]; thus, its content was determined in the different extracts by LC-ESI/QTrap/MS/MS using the multiple reaction monitoring (MRM) method.

2. Materials and Methods

2.1. Reagent and Solvents

For the reagents and solvents used, see the Supplementary Materials.

2.2. Sample Preparation

Fresh plants of *Chamaemelum nobile* L. (aerial part) were provided by the Fitomedical company (Binasco, MI, Italy), which purchased them from Azienda Agricola Bio Il Ramerino, Pitigliano (GR), a certified company in the cultivation of officinal plants. The

plant was cut to reduce the solvent–drug contact surface. In addition, the water content already present in the fresh plant was evaluated by calculating the dry yield. A known quantity of fresh *C. nobile* (100 g) was dried in the oven at 120° for 90 min. The weight of the fresh plant minus the weight of the dried plant (37 g) corresponded to the water content in the plant. The water content was taken into account for the preparation of EtOH/H_2O mixtures.

So, the fresh plant of *C. nobile* was submitted to different extraction techniques like maceration, long maceration (21 days long), UAE, and SLDE-Naviglio using EtOH 100% and EtOH/H_2O mixtures at different percentages (50%, 60%, 75%). The extracts were subsequently filtered with filter paper and a funnel. Each extracted sample consisted of three technical homogeneous replicates.

2.3. Extraction Methods, Conventional and Non-Conventional Extraction Techniques

The fresh plant of *C. nobile* L. was submitted to conventional extraction procedures like macerations and long macerations. The macerations were performed 3 times following the EP pharmacopeia guidelines using 50 g of the fresh plant and 210 mL of four different mixtures of solvent, made up of EtOH 100% and EtOH/H_2O (50%, 60%, 75%), for 3 days. The long macerations were performed using 50 g of the fresh plant and 210 mL of four different mixtures of solvent, made up of EtOH 100% and EtOH/H_2O (50%, 60%, 75%), for 21 days. After the filtration and evaporation of the solvent to dryness in vacuo, dried extracts were obtained.

The fresh plant of *C. nobile* was also extracted using non-conventional extraction procedures, UAE and the SLDE-Naviglio technique. EtOH 100% and EtOH/H_2O (50%, 60%, 75%) were used for each extraction procedure. For UAE, 20 g of the fresh plant of *C. nobile* was extracted with 84 mL of solvent for 15 min in an ultrasonic bath. The extractions were repeated three times and the solutions were filtered with filter paper and dried by a rotavapor. SLDE-Naviglio was performed using a Timatic micro series Naviglio extractor, with 200 g of the fresh plant, 840 mL of the solvent, and an extractive protocol of 20 extractive cycles of 12 min (9 min in the static phase and 3 min in the dynamic phase) [12].

2.4. UPLC-HRMSMS Analysis

All obtained extracts were analyzed using liquid chromatography coupled with electrospray ionization and a high-resolution mass spectrometer. For the experimental details, see the Supplementary Materials.

2.5. Multivariate Data Analysis of LC-MS Data

To highlight differences between extracts and understand how the extraction methods and also the solvent can affect the chemical profile, LC-ESI/QExactive/MS/MS chromatograms of all obtained extracts were subjected to the chemometric study of a multivariate statistical approach such as principal component analysis (PCA). For details, see the Supplementary Materials [13].

2.6. Total Phenolic Content, TEAC and DPPH Assays

The total phenolic content of the extracts was determined by the Folin–Ciocalteu assay, as previously reported by Cerulli et al. [14]. The radical scavenging activity of the green extracts of *C. nobile* and apigenin was determined by two different spectrophotometric assays, DPPH and TEAC, as reported in the Supplementary Materials [15].

2.7. Cell Culture

HaCaT cells were cultured in DMEM (high glucose) with 10% heat inactivated FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, and 100 U/mL/100 µg/mL penicillin/streptomycin in an atmosphere of 5% CO_2 at 37 °C. Cells were subcultured by trypsinization at intervals of 3–4 days.

2.8. DHR 123 Probe—Intracellular ROS Assay

HaCaT cells were cultured in a 96-well plate at 30.000 cells/well and 200 µL of medium for 24 h. The next day, the cells were treated with apigenin at 1.35 µg/mL and with different extracts at 10 µg/mL. After 16 h of treatment, 2 mM of H_2O_2 for 2 h was added. At the end of the treatment, the cells were washed and incubated with 100 µL of DHR probe at 5 µM for 30 min in the dark at 37 °C and 5% CO_2 [16]. After washing, the fluorescence (λexc: 500 nm/λem: 536 nm) was measured with TECAN Infinite M200Pro (v.1.10).

2.9. Statistical Analysis of Data

Data are reported as means ± mean standard error (SEM) of at least three independent experiments. Each experiment was conducted in triplicate. The results were analyzed using GraphPad Prism 8.0 software with one-way ANOVA followed by Dunnett's multiple comparison test. A *p*-value less than 0.05 was considered significant.

2.10. Tyrosinase Inhibition Assay

The tyrosinase inhibitory activity was evaluated using a method described by Oh et al. [17] with slight modifications, as reported in the Supplementary Materials. Kojic acid, a known tyrosinase inhibitor, was used as a positive control.

2.11. LC-ESI/QTrap/MS/MS Analysis

Quantitative analysis was performed on an LC-ESI/QTrap/MS/MS system using the MRM method, as reported in the Supplementary Materials.

3. Results and Discussion

3.1. Metabolite Fingerprint of C. nobile "Green Extracts" by UPLC-HRMSMS Analysis

In traditional use, the most common species of chamomile (*M. recutita*) is reported for the treatment of acne, light-scattering properties, and other skin imperfections [18]. Therefore, to evaluate the possibility of using Roman chamomile (*C. nobile*) in cosmetic formulations, simple and fast extraction methods based on the use of cheap and relatively non-toxic solvents were carried out. The extraction procedures can significantly influence the kind of metabolites present in the extracts; thus, the fresh plant material of *C. nobile* was submitted to different extraction protocols like conventional extraction procedures (maceration and long maceration) and non-conventional extraction procedures (UAE and SLDE-Naviglio). EtOH was selected as the "green" solvent, considering its good solvent properties and safety for human consumption. Herein, EtOH 100% and different mixtures EtOH/H_2O (50%, 60%, 75%) were used. Successively, to identify specialized metabolites, which are likely responsible for antioxidant and light-scattering activities, and to highlight the metabolite differences occurring among eco-sustainable extracts, UPLC-HRMSMS analysis was carried out (Figures S1–S4).

By the careful analysis of accurate masses, characteristic fragmentation patterns, and the literature data, the occurrence of 32 metabolites belonging to flavonoids (compounds **1**–**6**, **10**–**12**, **17**, and **18**), sesquiterpenoids (**7**–**9**, **13**–**15**, **19**–**22**, **27,** and **28**), amides (**16**, **29**, and **32**), polar fatty acids (**23**–**26**, **30**, and **31**) were tentatively identified (Figure 1, Table 1).

In the UPLC-HRMS profiles of *C. nobile*, two main classes of specialized metabolites were detected. The first main class was represented by flavonoids; in particular, flavone derivatives apigenin (**10**), hispidulin (**11**), luteolin-*O*-rutinoside (**1**), apigenin-*O*-hexoside (**2**), and chamameloside (**5**) and flavonol derivatives isorhamnetin (**6**), 5,7-dimethyl-quercetin (**12**), kumatakenin (**18**), bracteoside (**3**), 5,7-dimethyl-quercetin-*O*-hexoside (**4**), and 3-flavanol (**17**) were putatively identified.

Among the flavonoids identified by UPLC-HRMS, compounds **1**, **2**, **4**, **5**, **6**, **10**, and **11** were previously reported in *C. nobile* [19,20]. By contrast, compounds **3**, **12**, **17**, and **18** were previously reported only in *Matricaria recutita* L.; therefore, to the best of our knowledge, herein, compounds **3**, **12**, **17**, and **18** are reported for the first time in *C. nobile* [2,21].

The second class of specialized metabolites was represented by sesquiterpenoids. In detail, guaianonobilin (**8**), 8-tigloylhydroxyisonobilin (**9**), hydroxyisonobilin (**13**), nobilinon A/B/C (**14**), and nobilin (**15**), belonging to germacranolide type, were identified as distinctive compounds of *C. nobile*, as previously reported by De Mieri et al. [22]. Among the identified sesquiterpenes, achillin (**19**), linderazulene (**20**), chamazulene carboxylic acids (**21** and **22**) and dehydrocostus lactones (**27** and **28**) were previously reported in *M. recutita* L. [23–25] but never in *C. nobile*; thus, herein, they are reported for the first time in this species.

The accurate analysis of the ULPC-HRMS profiles of "green" extracts also highlighted the occurrence of primary metabolites belonging to amide (**16**, **29**, and **32**) and polar fatty acid (**23**–**26**, **30**, and **31**) derivatives; among the amide derivatives, myristic diethanolamide (**16**), oleamide (**29**), and erucamide (**32**) were putatively identified. The above-mentioned primary metabolites were reported before in other genera of the Asteraceae family; therefore, in this paper, they are reported for the first time in *C. nobile.*

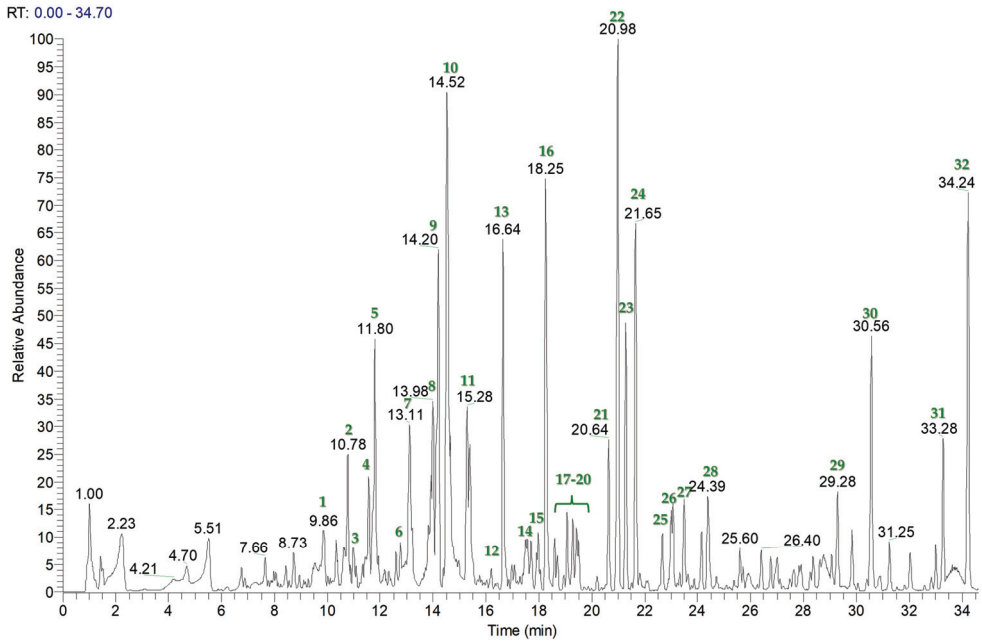


Figure 1. UPLC-HRMS base peak profile (in positive ion mode) of *C. nobile* EtOH/H_2O 50% extract obtained by long maceration.

Table 1. Metabolites identified in *C. nobile* extracts by LC-ESI/QExactive/MS/MS analysis [a].

	R_t	$[M + H]^+$	$[M + Na]^+$	Mol Formula	Δppm	MS/MS	Name
1	9.86	595.1652		$C_{27}H_{30}O_{15}$	−0.98	287.0550 ($C_{15}H_{11}O_6$), 449.1061 ($C_{21}H_{21}O_{11}$)	Luteolin-*O*-rutinoside
2	10.78	433.1128		$C_{21}H_{20}O_{10}$	−0.37	271.0599($C_{15}H_{11}O_5$)	Apigenin-*O*-hexoside
3	11.45	477.1026		$C_{22}H_{20}O_{12}$	−0.38	301.0704 ($C_{16}H_{13}O_6$), 286.0470 ($C_{15}H_{10}O_6$)	Bracteoside
4	11.56	493.1334		$C_{23}H_{24}O_{12}$	−1.28	331.080 ($C_{17}H_{15}O_7$), 316.0577 ($C_{16}H_{12}O_7$)	5,7-dimethyl quercetin -*O*-hexoside
5	11.80	577.1536		$C_{27}H_{28}O_{14}$	−2.81	271.0597 ($C_{15}H_{11}O_5$)	Chamaemeloside
6	13.11	317.0650		$C_{16}H_{12}O_7$	−1.92	302.0420($C_{15}H_{10}O_7$)	Isorhamnetin
7	13.83	263.1276		$C_{15}H_{18}O_4$	−0.85	245.1171 ($C_{15}H_{17}O_3$), 227.1067 ($C_{15}H_{15}O_2$)	Helenalin
8	13.92	345.1693		$C_{20}H_{24}O_5$	−1.07	245.1171 ($C_{15}H_{17}O_3$), 227.1067 ($C_{15}H_{15}O_2$)	Guaianonobilin
9	14.20		385.1616	$C_{20}H_{26}O_6$	−0.57	303.1209 ($C_{15}H_{20}O_5Na$), 285.1098 ($C_{15}H_{18}O_4Na$)	8-tigloylhydroxyisonobilin
10	14.52	271.0595		$C_{15}H_{10}O_5$	−2.28	119.0492 (C_8H_7O)	Apigenin
11	15.29	301.0702		$C_{16}H_{12}O_6$	−1.47	286.0472 ($C_{15}H_{10}O_6$)	Hispidulin
12	15.38	331.0809		$C_{17}H_{14}O_7$	−1.35	316.0575 ($C_{16}H_{12}O_7$), 303.0512 ($C_{15}H_{11}O7$)	5,7-dimethyl quercetin
13	16.64		385.1616	$C_{20}H_{26}O_6$	−0.57	303.1209 ($C_{15}H_{20}O_5Na$), 285.1098 ($C_{15}H_{18}O_4Na$)	Hydroxyisonobilin
14	17.49		383.1459	$C_{20}H_{24}O_6$		283.0945 ($C_{15}H_{16}O_4Na$), 239.1050 ($C_{14}H_{16}O_2Na$)	Nobilinon A/B/C
15	17.90		369.1671	$C_{20}H_{26}O_5$	−0.28	269.1140 ($C_{15}H_{18}O_3Na$), 251.1039 ($C_{15}H_{16}O_2Na$)	Nobilin
16	18.25	316.2838		$C_{18}H_{37}O_3N$	−2.65	280.2634 ($C_{18}H_{34}ON$), 262.2528 ($C_{18}H_{32}N$)	Myristic diethanolamide
17	18.70	227.1064		$C_{15}H_{14}O_2$	−0.99	199.1119 ($C_{14}H_{15}O$), 181.1010 ($C_{14}H_{13}$)	3-Flavanol
18	18.95	315.0862		$C_{17}H_{14}O_6$	−0.33	300.0627 ($C_{16}H_{12}O_6$)	Kumatakenin
19	19.28	247.1326		$C_{15}H_{18}O_3$	−1.05	229.1221 ($C_{15}H_{17}O_2$), 201.1274 ($C_{14}H_{17}O$)	Achillin
20	19.42	211.1118		$C_{15}H_{14}O$	−0.71	196.0884 ($C_{14}H_{12}O$), 193.1013 ($C_{15}H_{13}$), 183.1168 ($C_{14}H_{15}$)	Linderazulene
21	20.64	229.1219		$C_{15}H_{16}O_2$	−1.82	227.1064($C_{15}H_{15}O_2$), 199.1117($C_{14}H_{15}O$)	Chamazulene carboxylic acid isomer
22	20.98	229.1218		$C_{15}H_{16}O_2$	−1.81	211.1116 ($C_{15}H_{15}O$), 183.1167 ($C_{14}H_{15}$), 157.1010 ($C_{12}H_{13}$), 143.0854 ($C_{11}H_{11}$)	Chamazulene carboxylic acid
23	21.27	353.2677		$C_{21}H_{36}O_4$	−2.56	261.2212 ($C_{18}H_{29}O$), 92.0911 ($C_3H_8O_3$)	Linolenoylglycerol
24	21.65	353.2676		$C_{21}H_{36}O_4$	−2.82	261.2209 ($C_{18}H_{29}O$)	Linolenoylglycerol isomer

Table 1. *Cont.*

	R_t	[M + H]$^+$	[M + Na]$^+$	Mol Formula	Δppm	MS/MS	Name
25	22.10	293.2109		$C_{18}H_{28}O_3$	−0.92	219.1381 ($C_{14}H_{19}O_2$)	Oxo-octadecatrienoic acid
26	23.00		359.2186	$C_{20}H_{32}O_4$	−1.89	273.1453 ($C_{15}H_{22}O_3Na$), 259.1668 ($C_{15}H_{24}O_2Na$)	Dihydroxy-eicosatetraenoic acid
27	24.16	231.1377		$C_{15}H_{18}O_2$	−1.84	213.1272 ($C_{15}H_{17}O$), 195.1170 ($C_{15}H_{15}$), 159.1167 ($C_{12}H_{15}$)	Dehydrocostus lactone
28	24.39	231.1375		$C_{15}H_{18}O_2$	−2.10	213.1273 ($C_{15}H_{17}O$), 185.1325 ($C_{14}H_{17}$), 159.1167 ($C_{12}H_{15}$)	Dehydrocostus lactone isomer
29	29.28	282.2785		$C_{18}H_{35}ON$	−2.27	265.2520 ($C_{18}H_{33}O$), 247.2420 ($C_{18}H_{31}$)	Oleamide
30	30.56	593.2745		$C_{27}H_{45}O_{12}P$	3.94	413.2088($C_{21}H_{34}O_6P$), 277.2161 ($C_{18}H_{29}O_2$)	Polar fatty acid
31	33.28	637.3007		$C_{29}H_{49}O_{12}P$	3.57	441.2402 ($C_{23}H_{38}O_6P$), 305.2475 ($C_{20}H_{33}O_2$)	Polar fatty acid
32	34.24	338.3416		$C_{22}H_{43}ON$	−0.56	321.3149 ($C_{22}H_{41}O$), 303.3046 ($C_{22}H_{39}$)	Erucamide

[a] R_t and Δppm refer to the profile of *C. nobile* EtOH/H_2O 50% extract obtained by long maceration.

3.2. Multivariate Statistical Analysis of LC-ESI/QExactive/MS/MS Profiles

Targeted principal component analysis (PCA) has been carried out to elucidate the impact of the preparation method on the metabolite profiles of the extracts of *C. nobile*, analyzed in triplicate. The raw data were first filtered using MZ mine 2.38 software and then analyzed using SIMCA-P$^+$ software 12.0. The result of the validation test further emphasized the significance and predictability of the model; in particular, PC1 contributed to 51.0% of the variance, and PC2 contributed to 13.5%. As observed from the PCA score plot (Figure 2), the distribution of the extracts was mainly related to the extraction technique; in fact, in the upper part of the plot, the extracts obtained using conventional extraction techniques, like maceration and long maceration, were grouped, while in the lower part of the plot, the extracts obtained by non-conventional extraction techniques, UAE and SLDE-Naviglio, were mainly located.

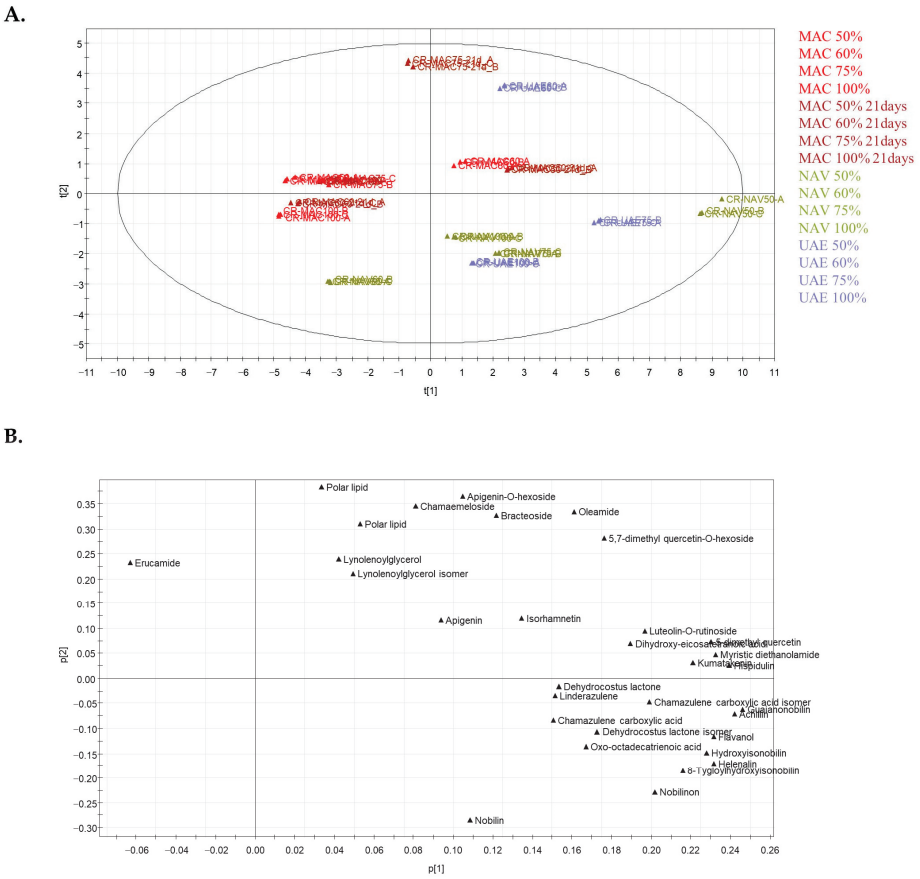


Figure 2. Principal component analysis of specialized metabolites in *C. nobile* extracts obtained by UPLC-HRMS targeted analysis: (**A**) PCA score scatter plot; (**B**) PCA loading plot.

To visualize the metabolites responsible for the distribution of the "green" extracts in different areas of the score plot, a PCA loading plot (Figure 2B) was carried out.

The extract EtOH/H_2O 50% obtained by SLDE-Naviglio and long maceration, along with the extract EtOH/H_2O 60% obtained by maceration, showed flavonoids, natural compounds promising in the prevention and treatment of skin diseases [26], as characteristic compounds. In detail, these extracts were richest in flavonoids such as luteolin-O-rutinoside (**1**), isorhamnetin (**6**), hispidulin (**11**), 5,7-dimethyl quercetin (**12**), and kumatakenin (**18**). The EtOH/H_2O 60% and EtOH/H_2O 75% extracts obtained by SLDE-Naviglio, along with the EtOH/H_2O 75% extract obtained by UAE showed a higher content of lactonic sesquiterpenoids like camazulene carboxylic acid and its isomer (**21**, **22**), 8-tigloylhydroxyisonobilin (**9**), hydroxyisonobilin (**13**), and nobilinon (**14**).

Apigenin (**10**) was placed in a fairly central position in the plot, with the highest occurrence in the extract obtained by long maceration with EtOH/H_2O 50% and maceration with EtOH/H_2O 60%.

3.3. Evaluation of Total Phenolics Content of C. nobile

The skin is constantly exposed to reactive oxygen species (ROS) from various sources, including endogenous metabolic processes within cells and external factors such as ultraviolet (UV) radiation and air pollutants. Endogenous ROS are natural byproducts of cellular metabolism, primarily generated during mitochondrial respiration and other metabolic pathways. These molecules play essential roles in cellular signaling and homeostasis. However, excessive ROS production or insufficient antioxidant defenses can lead to oxidative stress, causing damage to cellular components, including proteins, lipids, and DNA. Extrinsic sources of ROS, such as UV radiation from sunlight and pollutants like cigarette smoke, automobile exhaust, and industrial emissions, further contribute to oxidative stress in the skin [27,28]. Polyphenols work by donating electrons to stabilize free radicals, thus preventing them from causing harm to skin cells. By neutralizing free radicals, antioxidants help to protect the skin from oxidative stress and the associated damage. So, a phenolic content assay was performed using the Folin–Ciocalteu method [14]. Regarding the mixture of solvents used, the results of the total phenolic content determination indicated a better phenolic content for the EtOH/H_2O 50% and 60% extracts. By contrast, considering the extraction techniques, SLDE-Naviglio (NAV) was the best one in terms of phenolic content. Consequently, SLDE-Naviglio (NAV) extracts obtained using EtOH/H_2O 50% and 60% showed GAE (milligrams of gallic acid equivalents for gram of extract) values corresponding to 223.07 and 235.07, respectively (Figure 3).

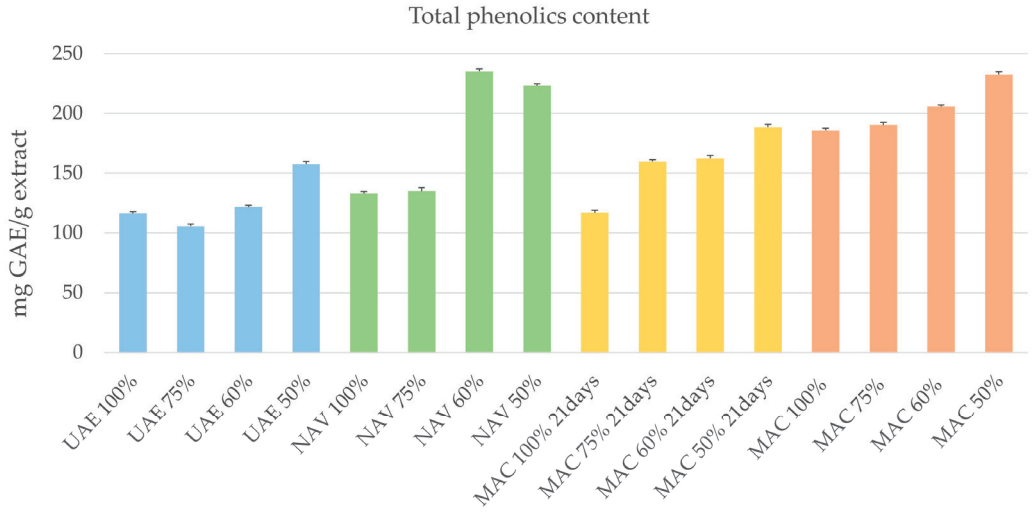


Figure 3. Total phenolics content assay result of green extracts of *C. nobile*.

3.4. Evaluation of the Antioxidant Activity of C. nobile

Based on the UPLC-HRMS profile of Roman chamomile, which attested to the presence of antioxidant compounds like flavanols, flavones (mainly apigenin and derivatives), and sesquiterpenoids, the radical scavenger activity of *C. nobile* " green" extracts was tested by TEAC and DPPH assays [29,30]. Also, the antioxidant activity of apigenin was tested. The TEAC assay highlighted the highest free radical scavenging activity for hydroalcoholic extracts obtained using EtOH/H_2O 50% and 60% (Figure 4A). In detail, the EtOH/H_2O 50% extracts displayed a radical scavenging activity in the range of 1.63 to 1.73 mM compared to quercetin (3.70 mM), which was used as a reference compound. According to the total phenolic content and TEAC results, the DPPH assay highlighted how EtOH/H_2O 50% and 60% extracts exerted moderate antioxidant activity (Figure 4B). In particular, the EtOH/H_2O 60% extract obtained by SLDE-Naviglio showed the strongest activity (IC_{50} = 295.54 µg/mL),

followed by the EtOH/H_2O 50% extract obtained by SLDE-Naviglio (IC_{50} = 310.12 µg/mL), the EtOH/H_2O 50:% extract obtained by UAE (IC_{50} = 311.22 µg/mL), the EtOH/H_2O 50% extract obtained by long maceration (IC_{50} = 338.75 µg/mL), and the extract EtOH/H_2O 60% obtained by long maceration (IC_{50} = 346.56 µg/mL). The weakest antioxidant activities were shown by the extracts obtained using EtOH/H_2O 75% and EtOH 100%, confirming the results obtained by the TEAC assay (Figure 4A) (Table S1). The TEAC and DPPH values of the chamomile extracts compared to the corresponding total phenolic content (TPC) values were correlated using the Pearson method (Table S2). A positive correlation was found between TEAC and TPC. Regarding DPPH, the increase in TPC could be related to the increase in antioxidant activities, as indicated by a lower IC_{50} of DPPH; in fact, TPC was negatively correlated with DPPH IC_{50}.

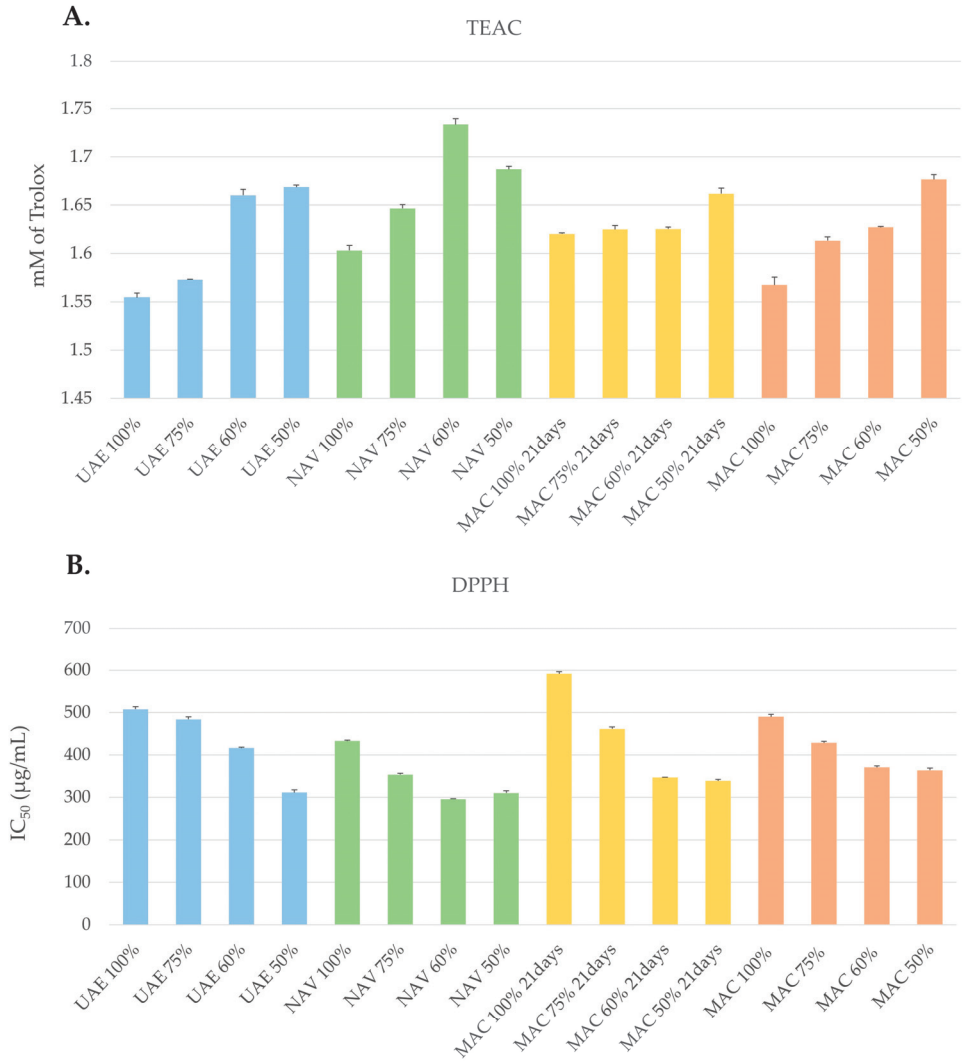


Figure 4. TEAC (**A**) and DPPH (**B**) assays of "green" extracts of *C. nobile*.

3.5. Effect of Extracts and Apigenin on ROS Production

Based on the TEAC and DPPH assays, the extracts MAC 60% at 21 days, MAC 50% at 21 days, MAC 50%, MAC 60%, NAV 50%, and NAV 60% were selected to further evaluate their in vitro antioxidant activity. HaCaT cells, a spontaneously immortalized human keratinocyte line widely used for studies of skin biology [31], were used. To determine the antioxidant activity of the selected extracts and apigenin, the cell permeant reagent dihydrorhodamine 123 (DHR 123) was used for the detection of reactive oxygen species such as peroxide and peroxynitrite. Indeed, after cell uptake, DHR 123 is oxidized by

ROS into a fluorescent compound measured by a fluorimeter. In our setting, the 16 h pretreatment of HaCat cells with apigenin and the extracts resulted in a lower production of DHR oxidation, following 2 h of H_2O_2 exposition. In detail, as shown in Figure 5, the extracts obtained by long maceration with EtOH/H_2O 60% and EtOH 100% and the extract obtained by maceration with EtOH/H_2O 50% displayed statistically significant activity. These data are in agreement with the TEAC and DPPH activities shown by these extracts.

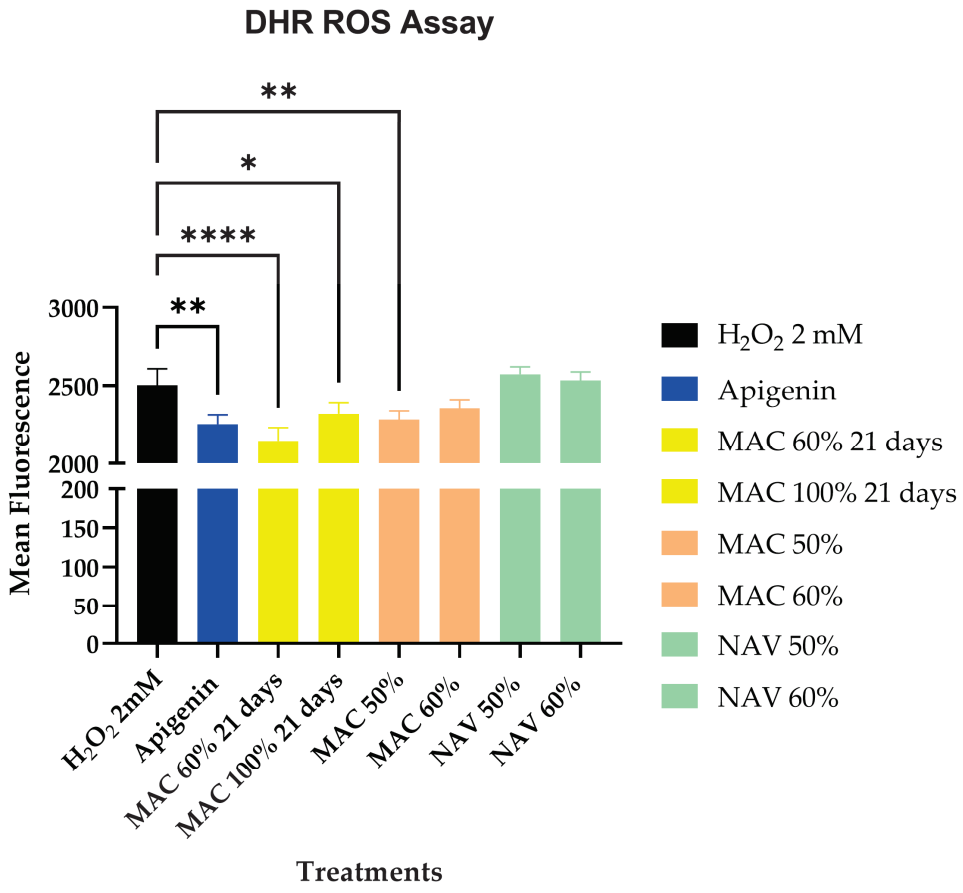


Figure 5. Effect of extracts of *C. nobile* (10 µg/mL) and apigenin (1.35 µg/mL) on ROS formation in HaCaT cells stimulated with H_2O_2. The data were analyzed using one-way ANOVA followed by Dunnett's test as the post test. (*): $p < 0.05$; (**): $p < 0.01$; (****): $p < 0.0001$.

3.6. Evaluation of the Lightening Activity of C. nobile

Continuing our research on the cosmetic potential of *C. nobile*, the attention was focused on its lightening activity by evaluating the inhibition of the tyrosinase enzyme. Tyrosinase is the key enzyme in melanin biosynthesis, which is the process responsible for the production of pigment in the skin, hair, and eyes. The enzyme catalyzes two key reactions in this process: ortho-hydroxylation (tyrosinase converts the amino acid tyrosine into 3,4-dihydroxyphenylalanine (DOPA)) and the oxidation of DOPA (tyrosinase further oxidizes DOPA to ortho-quinone). These reactions are essential steps in the production of melanin, which provides colour to the skin, hair, and eyes. However, excessive melanin production can lead to skin pigmentation disorders such as hyperpigmentation, an uneven skin tone, and age spots [32,33]. Because of its central role in melanin synthesis, tyrosinase is a target for various skincare and cosmetic products [33]. As reported before, *Matricaria chamomilla* was used as a remedy for skin diseases, but little is known about the ability of *C. nobile* to act as a skin-whitening agent.

To explore the capacity of *C. nobile* extracts to inhibit the tyrosinase enzyme and offer potential alternatives to synthetic products commonly used in skincare, which have some safety concerns associated with long-term use [34], the tyrosinase inhibitory activity of

C. nobile "green" extracts was tested by a spectrophotometric assay. Kojic acid, a known tyrosinase inhibitor, was used as a positive control (IC_{50} = 39.44 µg/mL ± 3.81). The obtained results (Figure 6) showed how all *C. nobile* extracts inhibited the tyrosinase enzyme in a range from 56.15 to 70.36 µg/mL in terms of IC_{50} values (Table S3). Also, the apigenin, considered a marker of chamomile, was tested. On the basis of tyrosinase inhibitory activity, all the extracts presented the potential ability to regulate melanin synthesis, providing benefits for skin health and appearance. Considering the mixture of solvents, there were no significant differences related to the percentage of ethanol used as the extraction solvent, on the contrary to what was observed for antioxidant activity. Also, the extracts EtOH/H_2O 75% and EtOH 100% presented good inhibition, possibly due to the polar fatty acid content [32]. Considering the extraction technique used, however, SLDE-Naviglio turned out to be the most efficient. In particular, the extract EtOH/H_2O 50% obtained by SLDE-Naviglio showed the highest inhibition of the tyrosinase enzyme (IC_{50} = 56.15 µg/mL ± 4.74).

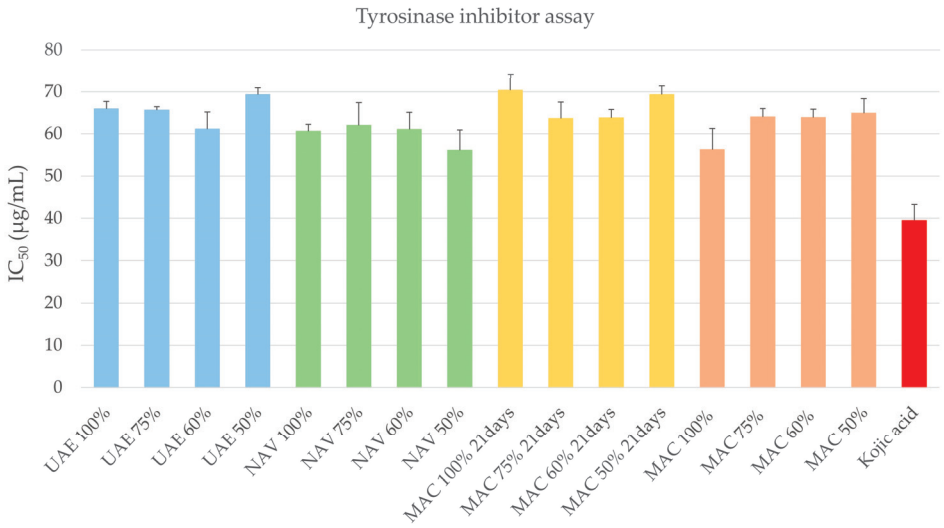


Figure 6. Tyrosinase inhibition assay of green extracts of *C. nobile*.

*3.7. Quantitative Analysis of Apigenin (**10**)*

Apigenin is considered a marker of chamomile. Moreover, it is known for its own antioxidant and lightening potential [3]; therefore, it was quantitatively analyzed in all "green" extracts by LC-ESI/QTrap/MS/MS using multiple reaction monitoring (MRM) method. LC-ESI/QTrap/MS/MS is characterized by a hybrid triple–quadrupole linear ion trap that is particularly suited for quantitative analysis [35]. The analysis was led in positive ion mode, highlighting the transition 271 → 119 due to the opening of the C ring via the retro Diels–Alder mechanism [36]; consequently, the above-mentioned transition was selected as the key transition for MRM experiments. In this way, the amount (mg/g of extract) of apigenin in the "green" extracts was determined (Table 2). Apigenin occurred in the concentration range of 0.20–4.86 mg/g extract, displaying the highest concentration in the EtOH/H_2O 50% extract obtained by SLDE-Naviglio and long maceration (21 days), according to the PCA loading plot results.

Table 2. Quantitative results of apigenin (**10**) (mg/g extract ± SD) in green extracts of *C. nobile*.

Extracts	UAE	NAV	MAC 21days	MAC
EtOH/H_2O 50%	1.50 ± 0.02	4.86 ± 0.29	4.78 ± 0.31	3.12 ± 0.18
EtOH/H_2O 60%	1.22 ± 0.03	2.98 ± 0.12	2.22 ± 0.23	3.62 ± 0.24
EtOH/H_2O 75%	1.01 ± 0.91	2.66 ± 0.18	2.10 ± 0.12	0.23 ± 0.03
EtOH 100%	0.20 ± 0.14	2.31 ± 0.23	0.23 ± 0.11	0.33 ± 0.02

4. Conclusions

Nowadays, there is increasing attention being placed on issues such as ecology and sustainability, and consumers prefer to choose products that are environmentally friendly, hoping that they are not harmful to health and reduce pollution. This phenomenon is also occurring in the cosmetic field, defining a new challenge of "Green Cosmetics", which include formulations (creams, makeup, and beauty products) containing active ingredients derived from plant products produced in an eco-sustainable way. This manuscript is placed in this context, offering a study about Roman chamomile extracted through different "green" procedures. With the aim to select an extraction technique and a mixture of solvents which lead to obtaining an effective and bio-sustainable product, *C. nobile* was subjected to different extraction techniques, conventional (maceration and long maceration) and, for the first time, non-conventional (SLDE-Naviglio and UAE) techniques, using different mixtures of bio-solvents made up of EtOH 100% and EtOH/H_2O (50%, 60%, 75%). The chemical investigation of the extracts, performed by LC-HRMS analysis, highlighted the presence of 32 metabolites belonging to flavonoids, sesquiterpenoids, amides, and polar fatty acids. The PCA revealed how the extracts EtOH/H_2O 50% obtained by SLDE-Naviglio and long maceration, along with the extract EtOH/H_2O 60% obtained by maceration, were richest in flavonoids. The extracts EtOH/H_2O 60% and EtOH/H_2O 75% obtained by SLDE-Naviglio, along with the extract EtOH/H_2O 75% by UAE, showed a higher content of lactonic sesquiterpenoids. Moreover, considering the importance of antioxidant activity in skin disease, the total phenolic content, TEAC, and DPPH of the "green" extracts were tested by spectrophotometric assays, highlighting how EtOH/H_2O 50% and 60% extracts exerted a higher antioxidant activity than the extracts obtained using EtOH/H_2O 75% and EtOH 100%. Based on the TEAC and DPPH assays, the antioxidant activity of some extracts was performed by the DHR 123 probe–intracellular ROS assay in HaCaT cells. The results showed that the extracts obtained by maceration and long maceration displayed statistically significant activity. Lastly, considering that no information is reported in the literature about the capacity of *C. nobile* to inhibit tyrosinase enzyme, to have information about the skin-lightening potential of *C. nobile,* all "green" extracts were subjected to a tyrosinase inhibition assay. All the extracts showed a potential good regulation of melanin synthesis, inhibiting the tyrosinase enzyme in a range from 56.15 to 70.36 μg/mL, with the possibility to exert beneficial effects for skin spots and hyperpigmentation problems. A quantitative analysis of apigenin, a marker of chamomile and known as a natural tyrosinase inhibitor, was performed, displaying the highest concentration in the EtOH/H_2O 50% extracts obtained by SLDE-Naviglio, maceration, and long maceration. Also, a good content of apigenin was detected in the extract EtOH/H_2O 60% obtained by maceration. Altogether, these results show how it is preferable to use a bio-solvent mixture made up of ethanol and water (in a ratio of 50% and 60%). SLDE-Naviglio and also maceration represent methods that allow one to obtain a final extract with interesting antioxidant and anti-tyrosinase activities.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cosmetics11030094/s1. Experimental data of UPLC-HRMSMS analysis, multivariate data analysis of LC-MS data, total phenolic content, TEAC and DPPH assays, tyrosinase inhibition assay, and LC-ESI/QTrap/MS/MS analysis; Figures S1–S5: UPLC-HRMS base peak profiles of *C. nobile* extracts; Table S1: Phenolic content and antioxidant activity of "green extracts" of *C. nobile*; Table S2: Correlation between TPC evaluated by Folin–Ciocalteu and antioxidant activity evaluated by the ABTS and DPPH methods; Table S3: Tyrosinase inhibition activity of "green extracts" of *C. nobile*.

Author Contributions: Conceptualization, M.M. and S.P.; methodology, A.C., L.M.P. and E.C.; software, A.C. and L.M.P.; validation, L.M.P., M.M., A.C. and F.M.; formal analysis, L.M.P. and F.M.; investigation, L.M.P., A.C. and F.M.; resources, L.M.P. and M.M.; data curation, M.M.; writing—original draft preparation, L.M.P., A.C. and E.C.; writing—review and editing, M.M. and S.P.; supervision, M.M. and S.P.; funding acquisition, S.P. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

Sample Availability: Samples of the extracts are available from the authors.

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Towards the Development of a Cream with Antiviral Properties Targeting Both the Influenza A Virus and SARS-CoV-2

Philippe Mangeot [1,2,3,4], Kristell Lazou [5], Aurelia Blin [5], Valerie Gorzelanczyk [5], Olivier Jeanneton [5], Chantal Kurfurst [5], Karl Pays [5], Bruno Bavouzet [5], Carine Nizard [5], Theophile Ohlmann [1,2,3,4,*] and Anne-Laure Bulteau [5,*]

1 Centre International de Recherche en Infectiologie (CIRI), 69007 Lyon, France
2 Institut National de la Santé et de la Recherche Médicale (INSERM) Unité U1111, 69007 Lyon, France
3 Ecole Normale Supérieure de Lyon, Université de Lyon (UDL), 69622 Lyon, France
4 Centre National de la Recherche Scientifique (CNRS), Unité Mixte de Recherche 5308 (UMR5308), 69007 Lyon, France
5 LVMH Recherche, 185 Avenue de Verdun, 45800 Saint Jean de Braye, France; ablin@research.lvmh-pc.com (A.B.)
* Correspondence: theophile.ohlmann@ens-lyon.fr (T.O.); abulteau@research.lvmh-pc.com (A.-L.B.)

Abstract: Objective: Many severe acute respiratory infections are caused by viral pathogens, and viruses are responsible for a large number of deaths worldwide. Among the most common respiratory viruses are the influenza A virus (IAV) and, more recently, the SARS-CoV-2 that emerged in 2019 and caused the most significant human pandemic of the beginning of the 21st century. Both IAV and SARS-CoV-2 share clinical features and a common transmission route through the emission of viral particles via aerosols and droplets. These penetrate the host after entry from the nose and mouth or an indirect mode of transmission via contact contamination of different media. These facts prompted us to investigate the possibility of designing a soft cream with a virucidal activity targeted against IAV and SARS-CoV-2. Methods: We first investigated the action of chemical compounds known to have antiviral properties such as cyclodextrin, or algae extracts containing sulfated polysaccharides, on cultured cells infected with lentiviral viral particles pseudotyped (VP) with either proteins HA (hemagglutinin) and NA (neuraminidase) from IAV or the G protein from the vesicular stomatitis virus or spike-bearing particles in order to select molecules with antiviral activities in human embryonic kidney (HEK293T) cells. Results: Our results show that some cyclodextrin-containing creams can significantly reduce the stability of HANA- and spike-bearing particles when they are applied prior to challenge with a viral inoculum on skin. Conclusions: We observed some specificities of these creams towards either IAV or SARS-CoV-2, indicating that the neutralization of viral activity is correlated with the mechanism of receptor interaction and entry of these two pathogens.

Keywords: SARS-CoV-2; virucides; cyclodextrins; cell culture; formulation; microbiology

1. Introduction

Respiratory viruses are responsible for the most common causes of acute respiratory infections in humans. Throughout human history, outbreaks of virally induced respiratory diseases have been reported and have caused severe death tolls. Among the many respiratory viruses, influenza, and more recently coronaviruses, are major concerns for human health and have caused millions of deaths over the last century.

The influenza type A virus pandemic (H1N1 subtype), also referred to as the 'Spanish Flu', was one of the most devastating viral pandemics, with an approximate death toll of about 50 million people worldwide in less than 2 years, between 1918 and 1920 [1]. More recently, the new pulmonary disease (COVID-19) caused by the severe acute respiratory

syndrome coronavirus 2 (SARS-CoV-2) has spread worldwide since December 2019 and was declared a pandemic by the World Health Organization (WHO) in March 2020 [1].

Influenza viruses belong to the orthomyxovirus family of RNA viruses, which is composed of three groups (A, B and C), with type A being the most virulent [2]. Human-to-human transmission of influenza mainly occurs through the air via respiratory droplets or aerosols, but it can also be contracted via contact with contaminated surfaces. IAV infection causes a spectrum of clinical symptoms, from a benign upper respiratory tract infection to fulminant pneumonia, which mostly strikes the elderly and immunocompromised patients [3]. SARS-CoV-2—in addition to MERS-CoV and SARS-CoV-1—belongs to the beta coronavirus family and is considered to have emerged from bats and spread to humans very recently. The mode of transmission from human to human occurs through multiple routes that mainly include the emission and inhalation of droplets and aerosols, but also indirect contamination via hand contact with surfaces and objects [4]. In addition to the fact that both SARS-CoV-2 and influenza viruses are enveloped and contain a single-stranded RNA genome, they also share several common features such as the transmission route, clinical symptoms, viral shedding and the serial interval of disease. For both pathogens, vaccines are available, and although they have considerably restricted the progression of the COVID-19 pandemic and attenuated the severity of the clinical symptoms, they cannot completely eliminate the spread of and infection with viruses [2].

In order to invade and infect their hosts, both SARS-CoV-2 and influenza need to establish initial contact with the upper respiratory tract through aerosols and droplets that are inhaled through the nose and mouth. Thus, a first line of defense against these pathogens consists of wearing facial masks, and this strategy has been adopted worldwide as a simple and efficient measure for slowing down the progression of the pandemic. However, indirect contamination can also occur through contact transmission of the pathogens from various media on which they can settle. As such, virally contaminated droplets can be deposited on the surface of various objects, such as tissues, coins and bank notes, that can be touched by hand. This, in turn, can contact the mucous membranes of the mouth, nose and eyes that then mediate viral entry to and infection of the patient. As for the influenza H1N1 strain, it has been shown [5] that viruses could persist and remain infectious on stainless steel surfaces for 7 days. Recent experiments with SARS-CoV-2 [6] have also demonstrated the presence of viable virus for up to 72 h, depending on the environmental surface conditions tested. This was confirmed in several general studies [7,8] that showed that the vast majority of respiratory tract viruses, such as coronaviruses, influenza and rhinovirus, could persist on inanimate surfaces for a few days, thus concluding that fomite transmission for influenza and SARS-CoV-2 is likely to occur and form part of the infectious process.

Given that contact transmission is a substantial risk factor in the spreading of both influenza and SARS-CoV-2, this emphasizes the need to employ proper hand hygiene to prevent the transmission of these pathogens, and this was officially recommended by the WHO as one of the first preventative measures to slow down the propagation of SARS-CoV-2 [7,9,10]. Although frequent hand washing can be easily performed, this is not the case for the face, which remains exposed in the absence, or inappropriate wearing, of a facial mask. Furthermore, the frequent use of antiviral disinfectants is a major cause of skin irritations, especially in healthcare workers. As such, the prolonged use of these measures induces various skin reactions, with a prevalence ranging from 43% [11] to 97% [12] among healthcare workers exposed to infected patients.

These facts formed the rationale for investigating the design and manufacture of a cream that could be applied to both the face and hands and that would exhibit antiviral activities. Surprisingly, this approach has not been thoroughly investigated since the beginning of the SARS-CoV-2 outbreak, as to our knowledge, there has been only one study published testing the virucidal activity of a cream/lotion on human skin [13]. This is even more surprising if one considers that better face protection would certainly have a significant impact on person-to-person transmission. Therefore, this established the rationale for this work, which was to conceive and produce a daily applicable skin cream

with virucidal activity. As highly versatile vehicles, lentivectors are widely used for gene delivery purposes, since they allow for stable integration of transgenes into cell lines and differentiated cells. Interestingly, they can be engineered to incorporate envelopes from different viral origins that modulate their tropism for recipient cells [14] and authorize their use for serological investigations [15] or the identification of antiviral reagents [16]. In our attempt to compose a cream with viral protective activity, we first tested the ability of different chemical and natural compounds to inactivate lentiviral-based pseudo-particles pseudotyped with the proteins HA (hemagglutinin) and NA (neuraminidase) from influenza or the G protein from the vesicular stomatitis virus in human embryonic kidney (HEK293T) cells. Among these compounds, cyclodextrins have been shown to possess virucidal effects against many viruses cultivated in vitro, including the herpes simplex virus (HSV), respiratory syncytial virus (RSV), dengue virus and Zika virus [17–20]. In addition, sulfated polysaccharides that can be extracted from natural plants or fungi can interfere with several steps in the virus' life cycle without presenting adverse biological effects [21]. Dipotassium glycyrrhizinate (DG), a natural triterpene that can be isolated from the roots of licorice, has been shown to inhibit the replication of hepatitis B virus and human immunodeficiency virus [22,23]. We chose two sulfated polysaccharides from algae extracts: One was furcellaran, a sulfated polysaccharide (carrageenan), which was extracted from the cell wall matrix of red seaweed belonging to the genus *Furcellaria*. The second was an extracellular sulfated polysaccharide (EPS) from the genus *Porphyridium* (*Porphyridium cruentum* extract). The typical antiviral mechanism of most sulfated polysaccharides against enveloped viruses can be explained as follows: These viruses attach to host cells through the interaction between their glycoproteic envelope and the heparan sulfate receptor on the cell surface. The formation of the virus–cell complex primarily relies on ionic interactions between the negatively charged (mostly sulfate) groups in this polysaccharide and the basic amino acids within the glycoprotein [18]. This suggests that the antiviral effect occurs by effectively neutralizing the positively charged sites on the viral envelope glycoproteins, preventing the viral adsorption process. We also tested beta-cyclodextrin (KLEPTOSE®). Cyclodextrins (CDs) are occurring glucose derivatives with a rigid cyclic structure, consisting of (1–4)–linked glucopyranoside units. Beta-cyclodextrin has previously been reported as displaying antiviral activity against a number of enveloped viruses [19]. We also tested calcium D pantetheine (CAD) and DG. We initially tested these compounds in a model of cultured cells challenged with the pseudotyped VLPs containing HA-NA from IAV or the G protein from the vesicular stomatitis virus and expressing a reporter gene in order to assess the virucidal effects of the compounds. In the second part of this study, we used human skin explants derived from donors on which diverse versions of the cream were applied. In this experiment, in addition to HA-VPs, we added lentivectors pseudotyped with the envelope of SARS-CoV-2 into the viral inoculum, and in particular a spike variant, to assay the virucidal activity of our formula on IAV and SARS-CoV-2.

Our results show that some compounds are effective at inactivating VLPs bearing HANA and spike and significantly reduce their infectious potential. Moreover, when incorporated into a cream formulation, these compounds remain as active as in the cell culture. This suggests that they can be developed to be incorporated into a face or a hand cream formulation.

2. Materials and Methods

2.1. Algae Materials and Chemical Molecules Tested

The algae extract (AE) was sourced from the red seaweed *Furcellaria lumbricalis*, located in the Baltic Sea. The Baltic Sea has a salinity of 10 $g \cdot L^{-1}$, very close to physiological serum, which could well explain its affinity for the skin. This extract was obtained from Codif (Saint-Malo, France). Furcellaran is a sulfated polysaccharide that is extracted from the cell wall matrix of red seaweed belonging to the genus *Furcellaria*. The furcellaran was then depolymerized using a patented process. The structure of furcellaran is similar to kappa carrageenan and has been described as a hybrid of kappa–beta carrageenan complex. The

essential difference is that kappa carrageenan has one sulfate ester residue per two sugars, whereas furcellaran has one sulfate ester residue per three or four sugar residues. The AE master solution (10 mg/mL) was prepared in water heated at 70 °C for complete dissolution. The working solutions were prepared rapidly through dilution of the master solution in a warm culture medium. The extracellular sulfated polysaccharide (EPS) extract was developed by Givaudan Active Beauty Marine (Toulouse, France). Red unicellular microalgae from the genus *Porphyridium* (*Porphyridium cruentum* extract) produce an extracellular sulfated polysaccharide (EPS) with acidic characteristics and with potential applications in cosmetics, as an inhibitor of hyaluronidase and having anti-allergic and antiviral properties. Betacyclodextrin (KLEPTOSE®) was developed by Roquette (La madeleine, France). The master solutions for KLEPTOSE® (10 mg/mL), dipotassium glycyrrhizinate (50 mg/mL) and calcium D pantetheine-S-sulfonate (3 mg/mL) were prepared in culture medium. All solutions were filtered through a 0.2 µm-pore-sized filter, except for AE. Hydrapatch® is a unique combination of three polysaccharides: pullulan (repeating maltotriose units), alginate (polymer of D-mannuronic and L-glucuronic units) and hyaluronic acid. Hydrapatch® can regulate the rate and kinetics of absorption of active ingredients and was developed by BASF (Levallois Perret, France). Liposkin® was developed by Lucas Meyer (Massy, France) and is a complex of C12–16 alcohols, hydrogenated lecithin, palmitic acid, phytosphingosine and cholesterol.

2.2. Composition of Formulations for Testing on Skin Explants

Formulations for testing on skin explants were composed as follows:

Formulation a: CD pantetheine 0.7%, KLEPTOSE® 0.1%;

Formulation b: CD pantetheine 0.7%, KLEPTOSE® 0.1%, EPS 5%, AE 2%;

Formulation c: CAD pantetheine 0.7%, KLEPTOSE® 1%, EPS 10%, AE 10%;

Formulation d: CAD pantetheine 0.7%, KLEPTOSE® 1%, EPS 10%, AE 10%, Hydrapatch® 3%;

Formulation b': CAD pantetheine 0.7%, KLEPTOSE® 0.1%, EPS 5%, AE 2%, Liposkin® 2%;

Formulation c': CAD pantetheine 0.7%, KLEPTOSE® 1%, EPS 10%, AE 10%, Liposkin® 2%.

For each formulation, 100 mg of cream was prepared.

2.3. Viability and Confluency Assays

Cell viability was measured using the Premix WST-1 Cell Proliferation Assay System (Takara, St. Germain en Laye, France). In brief, 5000 human embryonic kidney (HEK293T) cells (ATCC, Manasas, VA, USA) were plated in a 96-well plate and treated with the different compounds for 48 h. For each condition, the viability was measured in 8 independent dishes ($n = 8$). The confluency of the treated cells was measured using Inucyte-S3 (Sartorius, Dourdan, France).

2.4. Preparation of Lentiviral Vectors

We first tested the ability of different chemical and natural compounds to inactivate lentiviral-based pseudo-particles pseudotyped with the proteins NAHA (hemagglutinin) and NA neuraminidase) from influenza or the G protein from the vesicular stomatitis virus in HEK293T cells. The lentiviral particles were produced from HEK293T cells plated at 4×10^6 cells in a 10 cm diameter dish. The day after plating, cells were transfected using Jet-Optimus (Polyplus, Illkirch-Graffenstaden, France) with 4 µg of PAX-2 (packaging plasmid; Addgene; #12260), 0.3 µg of pCI-M2 (Addgene; #44170), 1 µg of pNAHA (Addgene; #44169) and 3.7 µg of SWINGY (reporter construct bearing the YFP gene driven by a CMV early promoter derived from GAE) [24]. For VSV-G pseudotyped particles, pNAHA and PCI-M2 were replaced with a phCMV-G plasmid (1 µg). Spike-VPs were produced using HDM-SARS2-spike-del21-D614G (Addgene #158762) as an envelope plasmid (1 µg) and an RFP lentivector (3.7 µg).

Particle-containing supernatants were harvested 48 h after transfection and clarified with 5 min of centrifugation at 500× *g* before filtration through a 0.45 µm-pore-sized filter. Next, the particles were ultra-centrifugated at 20,000 rpm in an SW41 rotor (Beckman, Villepinte, France) at 7 °C for 80 min. The sedimented lentivectors were resuspended into cold PBS to obtain a 100×-fold concentration and frozen at −80 °C.

2.5. Titration

To calculate the titer of the G-VP and HA-VP preparations, 2×10^5 HEK293T were transduced using serial dilutions of a lentiviral preparation. The FACS analysis of transduced cells 72 h after transduction allowed for the measurement of the percentage of transduced cells and the calculation of titers as a number of transducing units per mL (TU/mL).

To measure the transduction efficiencies of VPs harvested from the skin, target ACE2-expressing cells were prepared by transfecting 1×10^5 HEK293T cells with 0.75 µg of ACE2 (Addgene; #145033) and 0.25 µg TMPRSS2 (Addgene; #53887) in a 12-well plate. The day after transfection, ACE2-expressing cells were transduced using VPs retrieved from the skin and analyzed 48 h later using FACS. The percentage of cells expressing YFP revealed an efficient transduction with HA-VPs, and the percentage of RFP-expressing cells revealed efficient transduction with spike-VPs.

2.6. Treatment of Particles and Living Skin Explants with Reagents

2.6.1. Treatment of Viral Particles with Compounds

To evaluate the antiviral activity of compounds in a transduction assay, reagents were added at the given concentration into the medium of cultivated HEK293T cells plated in a 96-well plate (2.5×10^4 per dish). After 3 h of culture, diluted viral particles (40 µL) were added to obtain a final volume of 140 µL. The viral dilutions were performed in a reagent containing PBS to avoid modification of reagent concentrations in the final transduction medium. Then, 72 h later, the transduced cells were trypsinized, and the means of fluorescence (MFI) were analyzed using FACS.

2.6.2. Treatment of Skin with Compound-Containing Creams

The skin experiments were performed using NativeSkin® kits (Genoskin, Toulouse, France). This system consists of round skin biopsies prepared from surgical residues from healthy adult volunteers. Written informed consent was obtained from the donors, in full respect of the Declaration of Helsinki and Article L.1243-4 of the French Public Health Code. This method does not require any prior authorization from an ethics committee for sampling and using surgical waste.

The skin explants were cultivated in a 12-well plate format and embedded in a nourishing medium that kept them alive for several days. In this system, the skin surface was exposed and equipped with a silicon ring, allowing for the deposit of a liquid droplet that was maintained on the surface of the skin until evaporation. To evaluate the virucidal effect of creams, NativeSkin® systems were cultivated at 37 °C for 24 h before administration of reagent-containing mixtures. The formulations were added using a polypropylene micro-pestle (Sigma Aldrich, St. Quentin Fallavier, France) previously immersed in the cream preparation and used to spread the mixture on the skin surfaces. An excess of cream was avoided, and complete absorption of cream by the skin explants was ensured for all tests. We estimated that this treatment administered close to 2 mg of cream per cm^2.

After the formulation treatment, the skin explants were left for 3 h at room temperature before being deposited at the center of a silicon ring of a 50 µL inoculum containing HA-VPs and spike-VPs. After 30 min at room temperature, VLP-containing droplets were harvested from the skin surfaces. Next, the titration of retrieved particles was performed on ACE2-HEK293T cells.

2.7. Statistical Analysis

The results were expressed as mean ± SEM and analyzed using GraphPad Prism 8.4.3 software. Student's *t*-tests were used to compare data sets.

3. Results

3.1. Exploring Compounds That Have the Potential to Prevent Viral Infections

In our attempt to compose a cream with viral protective activity, we tested several compound candidates that were previously identified as natural broad-spectrum antivirals [17–19,21]. Hence, our study first focused on the test of one beta-cyclodextrin, KLEPTOSE® (K), and two sulfated polysaccharides, one being furcellaran, which is similar to kappa carrageenan and has been described as a hybrid of a kappa–beta carrageenan complex from an algae (AE), and one being an exopolysaccharide (EPS) from *Porphyridium cruentum* extract combined with dipotassium glycyrrhizinate (DG) and calcium D pantetheine (CAD). In essence, our study explored different associations of these reagents, whose protective effect against viral infection was measured in HEK293T cells used to titer the residual viral activity. Thus, we first investigated the potential toxicity of each candidate compound when tested on growing HEK293T cells. For this purpose, HEK293T cells were cultivated with increasing concentrations of calcium D pantetheine (CAD), dipotassium glycyrrhizinate (DG), KLEPTOSE® (K), algae extract (AE) and one exopolysaccharide (EPS). After 48 h of culture in serum-containing medium, the cell viability and confluency were measured for each compound.

We next sought to identify whether the five compound candidates had an effect on viral infectivity, and we developed a method for measuring this. For this purpose, we chose a transduction assay where compounds were added into a transduction medium, and efficiencies of infection were monitored after 3 d (Figure 1A). To optimize this assay, we chose to exploit lentiviral vectors that can be engineered to transfer a reporter transgene such as the yellow fluorescent protein (YFP), which facilitates the measurement of infection efficiency. Furthermore, these defective lentiviral particles can be pseudotyped using diverse fusogens, including envelopes from the vesicular stomatitis virus (VSV-G) or the influenza virus (HANA) (Figure 1B). This experimental design allowed us to investigate the antiviral properties of compounds using two different modes for cell entry. Two viral batches of VSV-G-pseudotyped particles (G-VPs) and HANA-pseudotyped particles (HA-VPs) were generated, purified and titrated using human permissive HEK293T cells. A range of low viral non-saturating doses of pseudoparticles corresponding to a multiplicity of infection (MOI) of 3.8 and 0.33, respectively, were applied (Figure 1C). Human HEK293T target cells permissive for both pseudotypes were next transduced with G-VPs and HA-VPs diluted in either phosphate-buffered saline (PBS) for the control or a selection of the compounds of interest at the concentration previously defined. In this assay, we applied pseudoparticles at low doses and verified that that the corresponding multiplicities of infection (number of theoretical particles per target cell: 3.8 for VSVG and 0.33 for HANA) were non-saturating, as illustrated in Figure 1C, in order to detect and measure the effects of tested compounds. The results are expressed as the means of fluorescence measured in recipient cells 3 d after transduction and are plotted in Figure 1D. We noted that most compounds have an impact on the YFP-transfer, with the highest effect obtained with CAD, which significantly inhibited both G-VP and HA-VP transductions (60% and 66% inhibition, respectively). Interestingly, the addition of AE, KLEPTOSE® and, to a lesser extent, EPS in the cell medium also decreased transduction for both pseudotypes, with a marked inhibition for HA-VPs. This test also illustrates some differences between the pseudotypes with a specific inhibition of HA-VP-mediated transduction using dipotassium glycyrrhizinate (DG), which did not affect G-VP transduction in this assay.

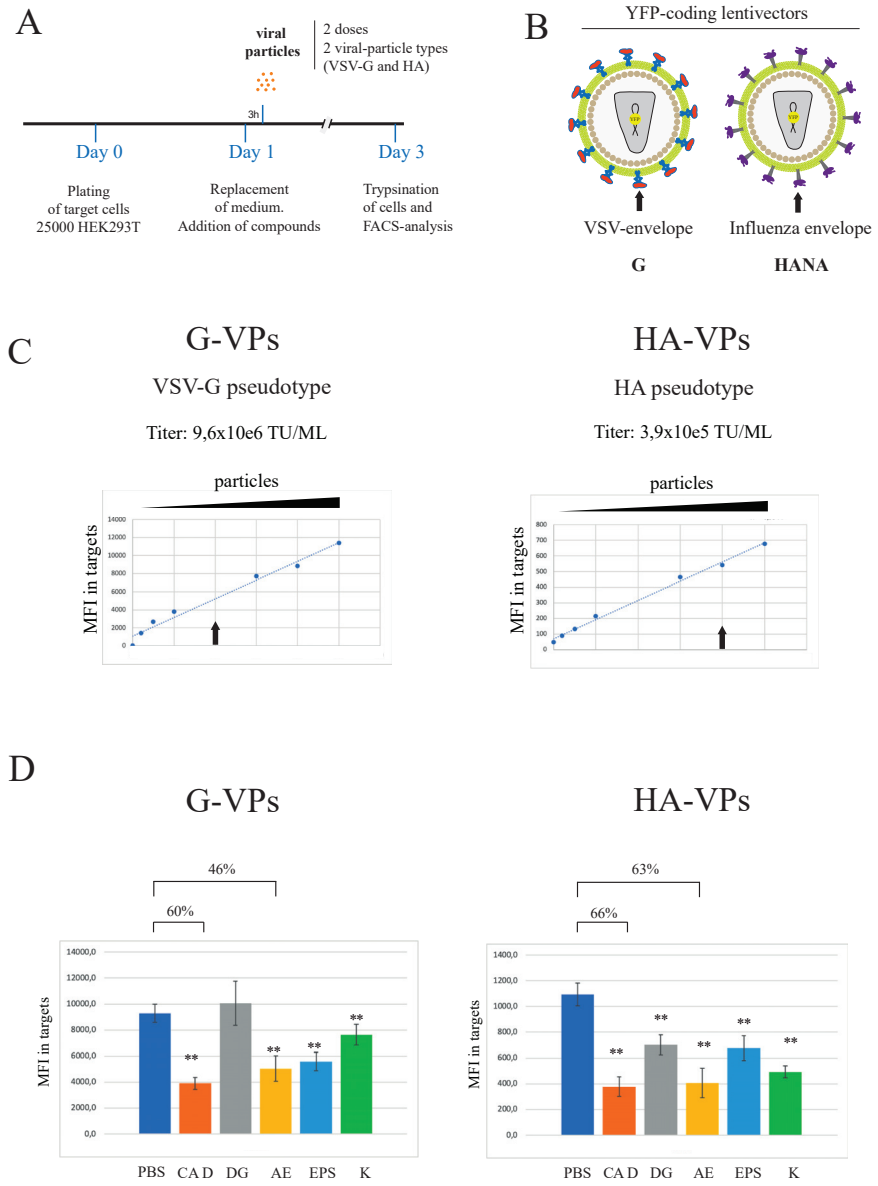


Figure 1. Identification of compounds inhibiting infection in a cell culture transduction assay: (**A**) principle of the transduction assay. HEK293T-target cells plated at d 0. At d 1, the culture media were replaced and supplemented with compounds before infection with two doses of viral particles (VP). At d 3, the means of fluorescence in transduced cells (MFI) were measured using FACS. (**B**) Scheme of VPs used: G-VPs and HA-VPs consist of YFP-encoding lentiviral vectors pseudotyped with VSV-G and HANA, respectively. (**C**) Characterization of particle batches. Graphs represent the dynamic of transduction efficiencies measured for both VP types. Increasing amounts of particles led to a linear increase in means of fluorescence (MFI) in target HEK293T cells. Titers of VP preparations are indicated. Arrows show the doses used in the assay. (**D**) Transduction assays using VPs-G and VPs-HA in HEK293T target cells treated with different reagents: Phosphate-buffered saline (PBS), CAD pantetheine sulfonate (CAD), dipotassium glycyrrhizate (DG), one algae extract containing a hybrid of kappa–beta carrageenan complex (AE), one extracellular sulfated polysaccharide (EPS) extract from the genus *Porphyridium* (*Porphyridium cruentum* extract) and KLEPTOSE® (K). MFI of target cells 48 h after transduction are given for each treatment. Bars indicate percentages of transduction inhibition measured for cells treated with CAD pantetheine sulfonate (CAD) and AE. Statistical analysis is expressed vs. phosphate-buffered saline (PBS) (mean ± SEM; $n = 3$; ** $p < 0.01$, with Student's *t*-test).

3.2. Identification of Cream Formulations Conferring a Protective Effect against Viral Infection

From these data, we next decided to extend our remit and formulate creams in which the active compounds were mixed at different concentrations. To test these formulations, we used the skin explant model, NativeSkin®, a patented and standardized technology that maintains human skin biopsies alive for up to 7 d to enable efficacy and safety testing. In our test, the stratum corneum of the skin explants were first treated through the homogenous application of the cream formulations on the surface, and then its exposure to a viral inoculum containing lentivectors that express fluorescent proteins was measured. After a short period of incubation (30 min) to mimic the natural exposure of human skin to a viral droplet, the inoculum was retrieved from the skin surface, and its infectivity was measured using titration on human cells.

In this experiment, in addition to HA-VPs, we added lentivectors pseudotyped with the envelope of SARS-CoV-2 into the viral inoculum, and in particular, a spike variant identified as an optimal envelope for neutralization assay [25]. The HA-VPs encode YFP, and the spike-VPs were engineered to package a lentiviral construct expressing the red fluorescent protein (RFP). This allows the same FACS assay to be used to measure how the creams affect both HA-VPs and spike-VPs through monitoring the fluorescence of YFP and RFP in the cell population used for titration of the retrieved inoculum. This experimental framework is summarized and depicted in Figure 2A. Briefly, viral inoculum retrieved from treated skins was next used to infect new permissive cells. Should a particular cream affect viral integrity or interfere with a given viral envelope, the transduction abilities of a viral inoculum exposed to the treated skin should be decreased. Thus, our final readout is the level of YFP and RFP into these target cells, with any decrease in fluorescence revealing a potential antiviral effect.

As a positive control, we verified that such an assay could detect the antiviral effect of a hydroalcoholic solution (HS) that destroyed more than 90% of VPs. We also checked for the absence of conflict between HA-VP transduction and spike-VP transduction through measurement of a non-biased proportion of double-positive (YFP + RFP) transduced cells, indicating that the same cell can be transduced with both pseudotypes.

In total, four creams were created containing a combination of cyclodextrins (calcium D pantetheine sulfonate and KLEPTOSE®), as in our previous tests, we identified them as potential antiviral compounds. The addition of AE and EPS to the formulation was also evaluated, since both of these reagents could exhibit a potential antiviral activity. The results are summarized in Figure 2B, and they show the transduction efficiencies of HA-VPs and spike-VPs retrieved from untreated skin (NT), skin treated with a placebo cream (Pl) or with the four different formulations that are labeled from (a) to (d). We observed that treatment of skin explants with a cream containing calcium D pantetheine sulfonate combined with KLEPTOSE® (formulation a) inhibited the viral transduction of HA-VPs by 27% but had no significant effect on spike-VP-mediated transduction. The further addition of AE and EPS at a low concentration did not affect the magnitude of inhibition (formulation b). However, we obtained a significant effect when the formulations with a higher concentration of KLEPTOSE® (1%) and hygroscopic compounds (AE 10% and EPS 10%) were tested, as they decreased the amount of active HA-VPs measured in the retrieved inoculum by about 2-fold (Figure 2B; formulation c and d). This effect was even further increased to 59% inhibition of HA-VPs following the addition of Hydrapatch®. Hydrapatch® is an active ingredient that is a unique combination of three polysaccharides that regulate the rate and kinetics of absorption of active ingredients. The idea was to use it to help maintain the antiviral ingredients in the stratum corneum of the skin explants and prevent their penetration, to improve their reaction with the viral particles.

Likewise, formulations c and d, which are inhibitory to HA-VPs, did not affect the spike-containing viral particles. This suggests that these mixtures have a specific effect on the stability or the entry mechanism of particles using the HA envelope.

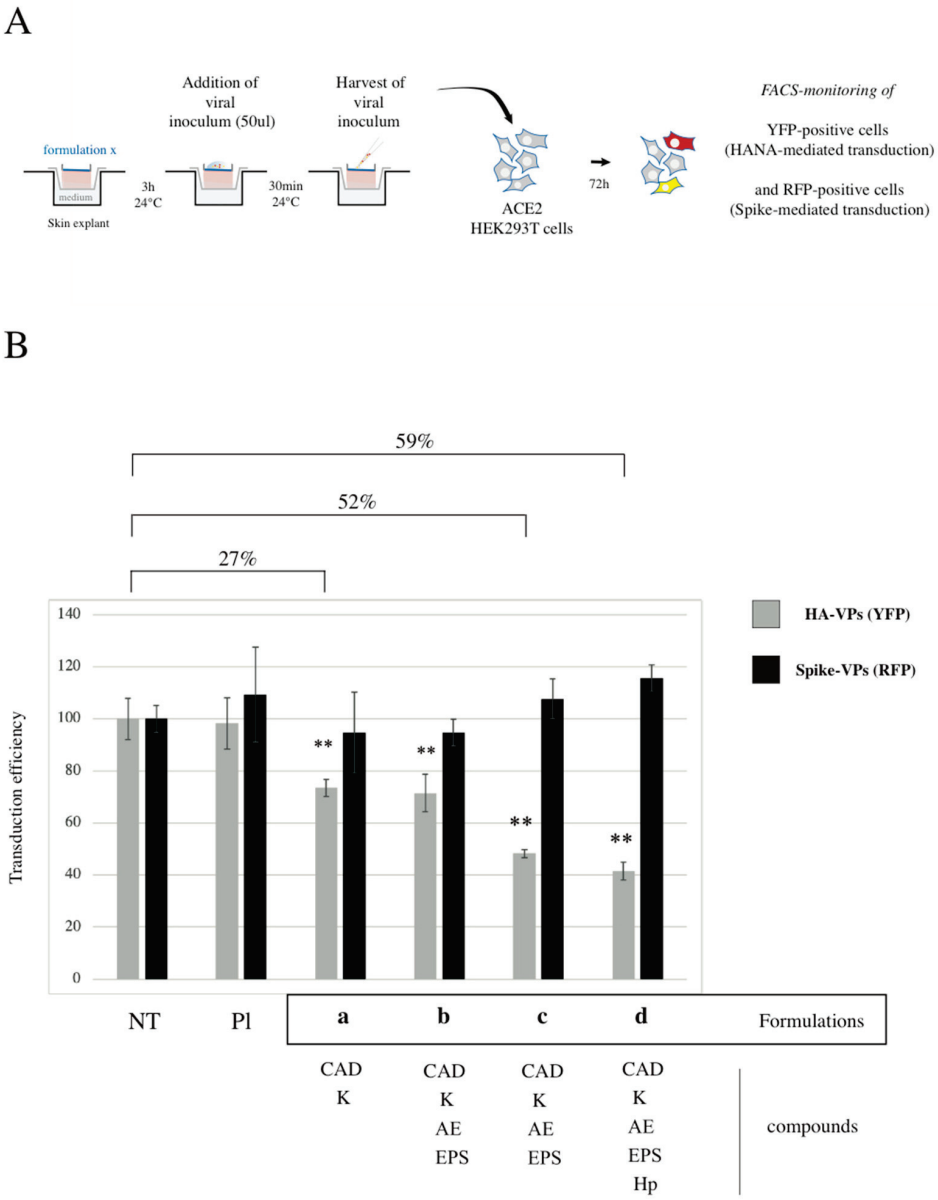


Figure 2. Identification of formulations conferring an antiviral protection to living skin: (**A**) Framework of the living skin assay. Skin explants were cultivated at 37 °C and treated with formulated creams before the addition of a viral inoculum on the skin surface. The viral solutions contained both HA-VPs and spike-VPs. After 30 min of exposure, VP solutions were retrieved from skin surfaces and titered on HEK293T cells expressing ACE2. (**B**) Effect of cream mixture on viral stability. Transduction efficiency measured for non-treated skin (NT) was fixed at 100%. Placebo (Pl) and compound-containing mixtures are indicated. CAD pantetheine sulfonate (CAD), dipotassium glycyrrhizate (DG), AE, EPS, KLEPTOSE® (K) and Hydrapatch® (Hp) concentrations are provided in the Materials and Methods Section. (+) indicates higher concentrations for K, AE and EPS. Results are shown as the infection efficiencies measured for VPs-HA (in grey) or spike-VPs (in black). Analyses were performed 48 h after transduction with harvested inoculum (n = 3). Statistical analysis is expressed vs. NT (mean ± SEM; n = 3; ** $p < 0.01$, with Student's t-test).

3.3. Supplementation of Creams with Liposkin® Decreased the Infectious Capacities of Spike-VPs Retrieved from Treated Skin

Natural emulsifier may considerably improve the efficacy of active ingredients as essential oils or cosmetic products [26,27]. In our effort to develop a protective cream, we tested whether the addition of an emulsifying compound could enhance the antiviral

properties of our candidates. Liposkin® (LIPS) contains hydrogenated lecithin, a natural emulsifier that has also skin-hydrating properties. Though the cream formulations containing LIPS did not show relevant antiviral activity on HA-VPs, we surprisingly observed that the spike-VPs were sensitive to skin treated with LIPS. These results are depicted in Figure 3 and illustrate the transduction efficiencies of spike-VPs after they have been collected from skin surfaces. In all formulations in which LIPS was added (formulations b' and c'), as well as the LIPS–placebo (Pl'), we noted that the infectious capacity of spike-VPs was decreased by almost 40%. This value was not increased significantly for creams supplemented with the active compounds CAD, K, AE or EPS, suggesting that the action of LIPS does not synergize with other antiviral compounds.

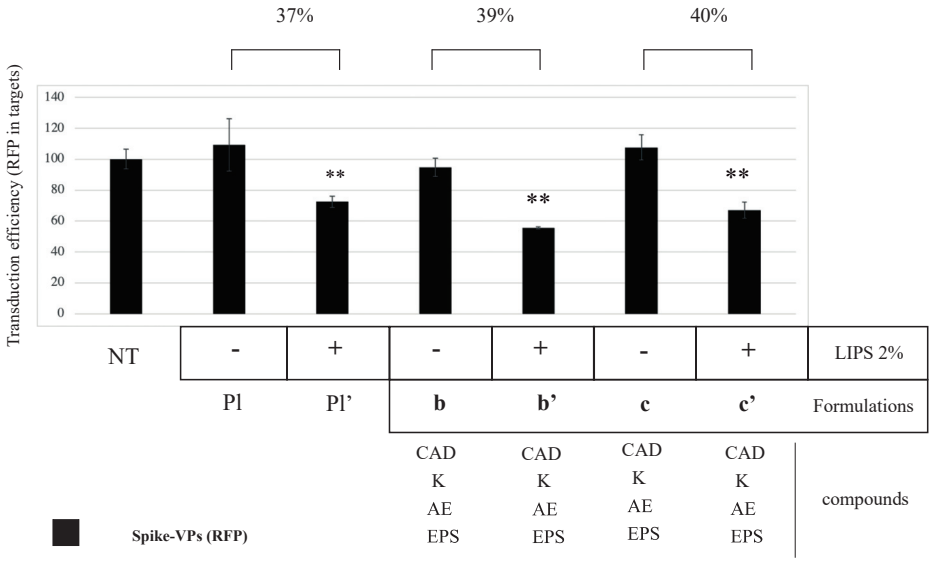


Figure 3. Treatment of skin with Liposkin® decreases the infectivity of exposed SARS-CoV-2 VPs. Cream formulations supplemented with Liposkin® (LIPS) were tested for their capacity to affect the infectivity of spike-VPs. Placebo (Pl) and formulations b and c were supplemented with Liposkin® (LIPS): these new mixtures are noted as Pl', b' and c', respectively. The results show transduction efficiencies achieved with spike-VPs in reporting ACE2-HEK293T (RFP signal) after retrieving of inoculum from treated skin. Compounds in formulation are noted. (+) indicates a 10-fold higher concentration of KLEPTOSE® (K) AE and EPS. Bars indicate the level of transduction inhibition observed for conditions supplemented with Liposkin® (LIPS) as compared with the non-supplemented condition. (n = 3). Statistical analysis is expressed vs. NT (mean ± SEM; n = 3; ** $p < 0.01$, with Student's t-test).

4. Discussion

Both IAV and SARS-CoV-2 have been shown to be remarkably resistant on these surfaces and can remain transmissible and infectious for more than 3 days. As such, this is a major concern for the health professionals who are highly exposed in their working environment. In this context, the development of virucidal creams that can be applied to the hands and face without the adverse effects seen with an HS solution could constitute an efficient first line of defense against viral transmission.

This established a framework for our work in order to develop and identify cream formulations that confer skin protection against the transmission of respiratory viruses. For this purpose, we first focused on the use of previously described antiviral compounds, including cyclodextrin-derived reagents that interfere with the entry of a variety of viruses and limit their transmission 18 Ferreira, 2023 [18,20]. To measure the antiviral effect of these reagents and how they can affect entry of IAV and SARS-CoV-2, we chose to use reporter-coding lentivectors pseudotyped with a HANA envelope or with the spike envelope from SARS-CoV-2. Those defective particles exploit the same entry route and cellular receptors as the parental virus but are not pathogenic to humans and can be manipulated with

minimal safety requirements (BSL2 facilities). We first showed that transduction with these lentivectors encoding fluorescent proteins can be accurately monitored and quantified with fluorescence-activated cell sorting (FACS) analysis (Figure 1). Furthermore, these lentivectors can be pseudotyped with a broad range of viral envelopes, and they provide a safe and rapid method for the evaluation of viral stability. Therefore, this technology could also be effectively adapted to the study of numerous enveloped viruses, including novel emerging strains of SARS-CoV-2.

Initial toxicity assays were performed, which indicated the optimal concentrations at which the compounds should be used to guarantee the appropriate balance between activity and cell viability. Then, our data first indicated that the addition of calcium D pantetheine sulfonate and KLEPTOSE® had the effect of reducing the viral infectivity of both HANA and VSVg pseudoparticles (Figure 1D). This effect was confirmed when the two compounds were added to the cream formulation; those molecules remained active and limited the entry of HA-VPs, especially when the KLEPTOSE® concentration was raised to 1% (Figure 2B; formulations c and d). However, it remains to be determined how these compounds interact with particles on the skin surface. One possibility would be that the exposure of VPs to cyclodextrins could sequester cholesterol from the viral particles, which may, in turn, disrupt lipid rafts and alter the fusogenic properties of the viral envelope. This most probably disorganizes the integrity of HA-VPs, as previously described [28,29]. At the same time, the same compounds only slightly affect the infectivity of the spike-VPs, suggesting that the SARS-CoV-2 envelope is resistant to these compounds. On the other hand, we noted that LIPS, an emulsifying agent used in cosmetic creams to create an oil–water interface on the skin and that can optimize the activity of reactive biomolecules, specifically altered the capacity of spike-VPs to infect new recipient cells after exposure to LIPS-treated skin (Figure 3). This surprising result showed that HANA and spike particles, though both enveloped, do not exhibit the same biological properties nor the same resistance to external reagents, even if those reagents are not specifically designed to target their respective viral envelope.

In addition to the identification of cream formulations that can serve as a base for future developments, our results show the first proofs of concept of a strong experimental design that can be used to test lotions or creams on human skin and assess their interactions with human pathogens.

In this work, we have assayed the potential anti-viral activities of several creams on human skin explants derived from donors. Our results showed different anti-viral specificities of these creams towards either IAV or SARS-CoV-2 envelopes, indicating that viral activity is correlated with receptor interaction and entry of these two pathogens.

Author Contributions: Conceptualization, A.-L.B. and T.O.; methodology, P.M. and A.B.; validation, A.-L.B. and T.O.; formal analysis, A.B. and A.-L.B.; investigation, P.M. and K.L.; resources, V.G.; data curation, O.J.; writing—original draft preparation, P.M., A.-L.B. and T.O.; writing—review and editing, T.O. and A.-L.B.; visualization, P.M.; supervision, C.K.; project administration, C.N.; funding acquisition, B.B. and K.P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was performed on biopsies, obtained from surgical residues after written informed consent from the donor, in full respect of the Declaration of Helsinki and article L.1243-4 of the French Public Health Code. The latter does not require any prior authorization by an ethics committee for sampling and using surgical residues.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: P.M. and T.O. declare a conflict of interest. This work was part of collaborative work between CIRI/INSERM/CNRS/ENS/UDL and LVMH Recherche. The remaining authors are employed by LVMH Recherche.

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A Randomized Controlled Prospective Cohort Study on the Efficacy of a Witch Hazel Extract Cream for the Eyelids and Eye Contour Area and a Cleansing Face Cream in Dermatitis of the Eyelids

Federica Veronese [1], Elia Esposto [1], Chiara Airoldi [2], Nunzia Di Cristo [3], Pamela Paganini [3], Paola Savoia [3,*] and Elisa Zavattaro [3]

[1] SCDU Dermatologia, AOU Maggiore della Carità di Novara, 28100 Novara, Italy
[2] Department of Translational Medicine, University of Eastern Piedmont, 28100 Novara, Italy
[3] Department of Health Sciences, University of Eastern Piedmont, 28100 Novara, Italy; elisa.zavattaro@med.uniupo.it (E.Z.)
* Correspondence: paola.savoia@med.uniupo.it; Tel.: +39-0321-3733387

Abstract: Background: Eyelid dermatitis is a common, multifactorial, chronic, and/or relapsing condition with a considerable impact on life quality that represents a diagnostic and treatment challenge. Methods: We carried out a single-blind, controlled, randomized, parallel-group study to evaluate the efficacy of two dermo-cosmetics (cream for the eyelids and eye contour area and cleansing face cream) in which the key ingredient was witch hazel extract, compared to generic cream, in the treatment of patients affected by eyelid dermatitis. Enrolled subjects were treated and followed-up for 4 weeks; dermatological evaluation was objectively performed using the DLQI, OSDI, NRS–itch, and EDSI indicators. Results: The products tested proved to be more effective than the placebo in ameliorating the dermatitis in the treated patients, based on all considered dermatological scores. In the treatment arm, we observed a reduction in both DLQI score and in the indicators related to the extension of dermatitis, greater than that observed in the control arm. Conclusions: This comparative study demonstrates the role of dermo-cosmetic products tested not only as a support treatment, but also as a first-choice clinical approach.

Keywords: eyelid dermatitis; irritative contact dermatitis; allergic contact dermatitis; inflammation; dermo-cosmesis

1. Introduction

Eyelid dermatitis is a common dermatologic discomfort condition with acute, chronic, or relapsing skin manifestations that can be due to different causes and have a considerable impact on patients' quality of life. Moreover, eyelid dermatitis is often a challenge to diagnose and treat [1,2].

Indeed, the eyelids constitute one of the areas of thinnest skin (0.55 mm compared with about 2 mm in other facial areas) [3] and greatest abundance of sebaceous glands, and are continually exposed to external contaminants [1,4]. For this reason, eyelids are the elective site for both irritative (irritative contact dermatitis—DIC) and allergic phenomena. In particular, cosmetics and personal hygiene products, along with numerous airborne allergens, drugs for topical use, and eye drops frequently cause allergic contact dermatitis (ACD) in this specific location [5–8].

Furthermore, the eyelids represent the elective site of numerous inflammatory chronic relapsing dermatoses, such as psoriasis, seborrheic dermatitis, and, especially, atopic dermatitis [4,9]. In the latter condition, typical lesions appear on the eyelid(s), compatible with the clinical aspects of chronic eczema, especially in adolescent and adult patients, with

an incidence that increases with the age of the affected subjects [9,10]. Also, ocular adverse events are reported in patients undergoing biologic systemic treatments, despite the good response of dermatitis in other anatomical sites [11]. The visual impact of skin changes (eyelid edema, erythema, weeping, and scale) and the itching and burning sensation that frequently accompany these pathological conditions have significant impact on the daily lives of affected patients [5,12]. Furthermore, the multifactorial nature of eyelid dermatitis and the need to reduce the use of corticosteroids in this site complicates the treatment [13].

In this context, the identification of topical products (emollients and cleanser) that allow a rapid regression of clinical manifestations and that can be well tolerated even by patients with polysensitization and/or inflammatory chronic skin diseases is essential.

In our dermatologic clinic in a Northern Italy hospital, we carried out a study to evaluate the efficacy of Rilastil Difesa® cream for the eyelids and eye contour area and Rilastil Difesa® cleansing face cream, compared to generic cream, in the treatment of adults with eyelid dermatitis of an irritant, allergic, or inflammatory nature in terms of reduction in dermatological score (clinical and related to quality of life).

2. Materials and Methods

2.1. Study Design

This is a single-blind, controlled, randomized, parallel group, single-center study. Subjects were enrolled (T0) and followed-up over time after two (T1) and four (T2) weeks.

The study was conducted between November 2022 and May 2023 in a dermatologic clinic of a Northern Italy hospital (AOU Maggiore della Carità, Novara, Italy); demographic and clinical characteristics of the patients involved were recorded. Participants were randomly assigned to receive treatment vs. control in a 1:1 ratio. To ensure blindness, an independent statistician generated a random allocation sequence with random block sizes of four and six patients for each disease. Then, during each follow-up visit, dermatological scores were calculated.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Ethics Committee of Comitato Etico Interaziendale A.O.U. "Maggiore della Carità"-ASL BI, ASL NO, ASL VCO (N° CE148/2022, 29 July 2022). All the patients enrolled in this study signed an informed consent form.

Inclusion and Exclusion Criteria

The inclusion and exclusion criteria for this study are listed below:

Inclusion criteria:

- Aged over 18 years.
- Signature of informed consent.
- Patients able to understand and respond to the questionnaires required by the study.
- Patients able to understand the application method of the cream (independently at their home) and who can guarantee the regularity of the checks.

Exclusion criteria:

- Absence of consent to participate.
- Absence of clinical signs compatible with eyelid dermatitis.
- Inability to understand the questionnaires and impossibility of autonomous application of the product.
- Failure to guarantee regularity in follow-up visits.
- Concomitant treatment with topical or systemic corticosteroids or other immunosuppressive drugs.

2.2. Treatment

The product tested in our study, i.e., Rilastil Difesa® cream for the eyelids and eye contour area, is a soothing and emollient cream indicated for subjects with sensitive, intolerant, and allergy-prone skin on eyelid and periocular areas.

Its key ingredient is witch hazel extract. This compound is extracted from the bark of witch hazel, is known to be rich in soluble tannins, and is a traditional remedy for some different inflammatory skin diseases. Witch hazel extract promotes the stabilization of keratinocyte cell membranes; hence, in in vitro models, it has been shown to reduce the production of proinflammatory cytokines [14]. Moreover, trehalose is a further active ingredient of the tested product. It is a sugar composed of two glucose units and its peculiarity is the capability to be more effective, when compared to other sugars, in preserving the functionality of biological molecules under stress conditions, thus avoiding their destabilization and denaturation. Trehalose has demonstrated anti-inflammatory and photoprotective activities in keratinocyte cultures submitted to UVB radiation through the induction of autophagy [15,16]. The cream was tested together with Rilastil Difesa® cleansing face cream, a cleanser containing the same active ingredient, suitable for delicately cleansing and removing make-up from sensitive, intolerant, and allergy-prone skin. The complete composition of both treatment and placebo products are listed below.

Treatment Group		**Placebo Group**
Cream	**Cleanser**	**Cream**
Aqua (Water) • Caprylic/capric Triglyceride • Glycerin • Pentylene glycol • Trehalose • Hydroxyethyl Cellulose • Plukenetia Volubilis Seed Oil • Methylpropanediol • Arachidyl alcohol • Hamamelis Virginiana (Witch Hazel) Bark/Twig Extract • Behenyl Alcohol • Arachidyl Glucoside.	Aqua (Water) • Glycerin • Pentaerythrityl Tetraethylhexanoate • Pentylene Glycol • Methyl Glucose Sesquistearate • Hydroxyethylcellulose • Xanthan Gum • Tetrasodium Glutamate Diacetate • Methylpropanediol • Lactic Acid • Hamamelis Virginiana (Witch Hazel) Bark/Twig Extract • Sodium Hydroxide.	Aqua (Water), Caprylic/Capric Triglyceride, Glycerin, Pentylene Glycol, Hydroxyethylcellulose, Arachidyl Alcohol, Behenyl Alcohol, Arachidyl Glucoside

Patients were randomized 1:1 to receive the study products (treatment group) or a placebo (control group). The placebo administered to patients belonging to the control group was a product for topical use containing the same excipients as the Rilastil Difesa® cream for the eyelids, but without the active ingredients. The products have been packaged in identical tubes, so as not to make it possible for the patients to identify the product contained in them, and to ensure the blinding of the study. However, the patients belonging to the control group did not receive a specific detergent and were invited to use the usual one.

The cream and cleanser and the placebo were supplied free of charge by the cosmetic manufacturer.

Patients enrolled in both study arms were educated to cleanse once a day and apply the cream twice a day for four weeks.

A schematic flow-chart of the study protocol is reported below (Figure 1). The total number of enrolled patients in both arms is reported.

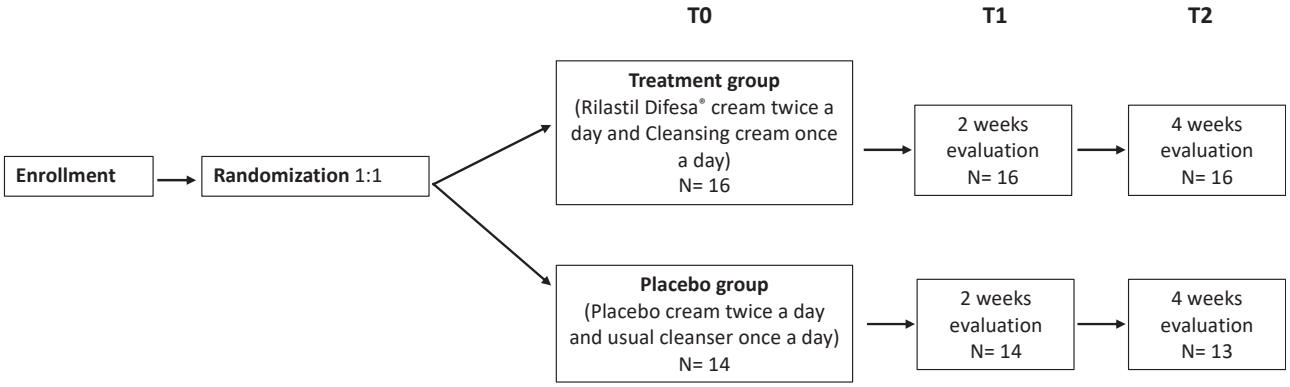


Figure 1. Schematic flow-chart of the study protocol.

2.3. Dermatological Variables

Dermatological evaluations were performed by a group of experienced dermatologists, using the following indicators:

- OSDI (Ocular surface disease index): evaluates the dry eye disease severity and effect on vision-related function, its scores vary from 0 to 100 (the higher scores representing greater disability) [17].
- NRS–itch (Numerical rating scale for pruritus): measures the intensity of itch using a scale from 0 (no itch) to 10 (worst imaginable itch) [18].
- EDSI (Eyelid dermatitis severity index): evaluates eyelid dermatitis in terms of erythema, papules, and scaling. It ranges from 0 to 9 (modified by [19]).

Furthermore, since we decided to evaluate the impact of the dermatitis in the daily quality of life in both the groups, we used the following score:

- DLQI (Dermatology life quality index): estimates patients' perception of the impact of skin diseases in different aspects of their health-related quality of life in the last week. Score assumed values between 0 (no effect at all on patient's life) and 30 (extremely large effect on patient's life) [20].

2.4. Primary Outcome

The primary outcome of this study is represented by the evaluation of the effectiveness of the tested product in reducing the clinical signs and symptoms related to eyelid dermatitis and the possible impact on the quality of life of affected patients.

2.5. Sample Size

Sample size was estimated using a repeated measures model to evaluate the difference of dermatological score in time (one pre-randomization observation and 2 post-randomization observations); assuming an intercorrelation among subjects of 0.9 and an effect size of 0.4, with a I type error of 0.05 (two-tailed) and a power of 0.80, at least 15 subjects for groups are needed. So, we enrolled a total of 30 subjects.

2.6. Statistical Analysis

Study data were collected and managed using REDCap electronic data capture tools hosted at Università del Piemonte Orientale. REDCap (Research Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies.

A descriptive analysis was conducted considering subjects overall and separately for the treatment group. Absolute and relative percentages were reported for categorical variables while mean and standard deviation (SD) or median and interquartile range [IQR] were reported for numerical ones, as appropriate.

First, to assess if the randomization worked well, characteristics at baseline among treatment and the non-treatment group were compared using the chi-square or Fisher test and *t*-test or non-parametric alternative (Wilcoxon), without reporting the *p*-values.

Second, differences in time (T1 and T0 and T2 and T0) were calculated for dermatological scores, separately for the two groups. For categorical data, we counted the subjects who had an improvement (reduction in eczema, vesicles, extension, changes in the dermatitis extension from bilateral to unilateral, and/or changes in the position from under-over-palpebral to under/over-palpebral), while for continuous ones (DLQI, OSDI, NRS, and EDSI), the difference of score in time was calculated and graphical representations were performed. Appropriate tests (as described above) were used to compare the treatment and control groups in terms of clinical improvement or reduction in the dermatological score. Finally, repeated measures models were used to consider all time measurements together; each clinical score was used as outcome in a separate model and time and treatment were used as covariates. Beta and 95% confidence intervals [95% CI] were reported for all covariates.

A *p*-value of 0.05 (two tails) was considered statistically significant and all the analysis were conducted using the intention-to-treat approach. The software used was SAS 9.4 and R.4.3.2.

3. Results

3.1. Patients

Thirty subjects were enrolled in this randomized controlled trial: 16 in the treatment group and 14 as controls; only one patient (belonging to the control group) was lost to the closure follow-up visit (T2). Most patients were female (n = 20, 66.7%), and their age ranged from 19 to 84 years, with a median age of 44.2 [IQR 34.7; 59.1] years.

Most patients enrolled had a previous diagnosis of atopic dermatitis (AD) (21 patients, 72.41%) or irritant or allergic contact dermatitis (8 patients, 27.59%). Among the subjects affected by concomitant comorbidities, the majority suffered from allergies (14 patients 46.67%) and allergic rhinitis (10 patients, 33.33%); among allergies, 10 were caused by food, 10 were environmental-related, and 3 were by contact origin.

Twenty-two patients (78.57%) reported previous skin manifestations like those presented at the time of enrollment and 23 (76.67%) had been suffering from dermatitis for more than 6 months. Among the local treatments used before the enrollment, we noticed topical emollients (22), steroids (16), antibiotics plus steroids (5), delicate cleaners (5), and 1 other unspecified topic product. Most patients (25 patients, 83.33%) had previously undergone dermatologic examination for the same reason.

3.2. Clinical Characteristics at Baseline

Tables 1 and 2 report the descriptive statistics of subjects enrolled in this study, at baseline.

The eyelid dermatitis was bilateral in 76.67% of cases (23 patients) and in 83.33% of cases (25 patients) affected both the under- and the over-palpebral area. The lesions extended to more than 75% of the eyelid area in 11 subjects (36.67%).

Regarding clinical signs of eyelid dermatitis, we evaluated edema and vesiculation separately since these signs are not included in the EDSI score. At baseline, 40% of patients presented edema, and the EDSI score showed a mean of 2.17 (range 0–3) for erythema and a mean total score of 4.73 (range 0–9). These values were slightly higher in the treatment group compared to the control group. Other signs (i.e., vesiculation, papules, and scaling) were less represented.

No statistical difference (p-value > 0.10) among demographic and clinical characteristics between treatment and control groups was observed, indicating that randomization worked well (p-values are not reported in the tables).

Table 1. Descriptive statistic of subjects, separately for treatment and control group.

Variable	Level	Overall (*n* = 30)	Control (*n* = 14)	Treatment (*n* = 16)
Gender	Male	10 (33.33)	5 (35.71)	5 (31.25)
	Female	20 (66.67)	9 (64.29)	11 (68.75)
Age, years				
Median [Q1–Q3]		45.87 [31.77; 60.98]	47.97 [23.10; 60.98]	44.23 [34.71; 59.14]
Exposure at work	Yes	5 (16.67)	2 (14.29)	3 (18.75)
Diagnosis	Atopic dermatitis	21 (72.41)	12 (85.71)	9 (60)
	DIC/DAC	8 (27.59)	2 (14.29)	6 (40)
Comorbidities	Allergic asthma	7 (23.33)	4 (28.57)	3 (18.75)
	Allergic rhinitis	10 (33.33)	8 (57.14)	2 (12.5)
	Allergic conjunctivitis	4 (13.33)	2 (14.29)	2 (12.5)
	Known allergies	14 (46.67)	8 (57.14)	6 (37.5)
Dermatitis onset	<6 months	7 (23.33)	2 (14.29)	5 (31.25)
	6+ months	23 (76.67)	12 (85.71)	11 (68.75)
Previous lesions	Yes	22 (78.57)	12 (92.31)	10 (66.67)
Topics use	Yes	26 (89.66)	12 (92.31)	14 (87.5)
Face wash per day	0–1	6 (20)	2 (14.29)	4 (25)
	2+	24 (80)	12 (85.71)	12 (75)
Previous dermatologic visit	Yes	25 (83.33)	12 (85.71)	13 (81.25)

Table 2. Signs, symptoms, and scores.

Variable	Level	Overall (*n* = 30)	Control (*n* = 14)	Treatment (*n* = 16)
Edema	Yes	12 (40)	5 (35.71)	7 (43.75)
Vesiculation	Yes	5 (16.67)	1 (7.14)	4 (25)
Dermatitis type	Unilateral	7 (23.33)	2 (14.29)	5 (31.25)
	Bilateral	23 (76.67)	12 (85.71)	11 (68.75)
Position	Over palpebral	5 (16.67)	1 (7.14)	4 (25)
	Under and over palpebral	25 (83.33)	13 (92.86)	12 (75)
Extension	1–25%	4 (13.33)	1 (7.14)	3 (18.75)
	26–50%	6 (20)	2 (14.29)	4 (25)
	51–75%	9 (30)	6 (42.86)	3 (18.75)
	76–100%	11 (36.67)	5 (35.71)	6 (37.5)
DLQI				
	Median [Q1–Q3]	4.00 [1.00; 10.00]	5.50 [1.00; 10.00]	3.00 [1.00; 8.50]
OSDI				
	Median [Q1–Q3]	22.92 [6.25; 35.42]	19.79 [6.25; 29.17]	22.92 [9.38; 37.50]
NRS–itch				
	Median [Q1–Q3]	7.00 [5.00; 8.00]	6.00 [3.00; 8.00]	7.00 [5.00; 8.00]

Table 2. *Cont.*

Variable	Level	Overall (*n* = 30)	Control (*n* = 14)	Treatment (*n* = 16)
EDSI erythema				
	Median [Q1–Q3]	2.00 [1.50; 3.00]	2.00 [1.50; 3.00]	2.25 [1.25; 3.00]
EDSI papules				
	Median [Q1–Q3]	0.50 [0.50; 1.50]	0.50 [0.00; 1.00]	0.50 [0.50; 2.25]
EDSI scaling				
	Median [Q1–Q3]	1.50 [0.50; 2.00]	1.50 [0.50; 2.00]	1.50 [0.50; 2.25]
EDSI total				
	Median [Q1–Q3]	4.00 [3.50; 7.50]	3.75 [3.50; 5.50]	4.00 [3.00; 7.50]

3.3. Response to Treatment

As shown in Table 3, a reduction in the extension of dermatitis was observed in 13 patients (43.3%) at T1 vs. T0 and in 15 patients (53.6%) at T2 vs. T0, with overlapping results between the two groups (treatment and control).

Table 3. Time variation. Absolute and relative percentage of improvement from T1 vs. T0 and T2 vs. T0. Results are reported overall and separately for the control and treatment groups, both with *p*-value obtained by Chi-square or Fisher tests.

Variable	Overall (*n* = 30)	Control (*n* = 14)	Treatment (*n* = 16)	*p*-Value
Improvement T1 vs. T0				
Edema	2 (6.67)	1 (7.14)	1 (6.25)	>0.999
Vesiculation	3 (10.34)	0 (0.00)	3 (18.75)	0.23153
Dermatitis type	0	0	0	
Position	2 (6.9)	1 (7.69)	1 (6.25)	>0.999
Extension	13 (43.33)	6 (42.86)	7 (43.75)	>0.999
Improvement T2 vs. T0				
Edema	4 (13.79)	2 (15.38)	2 (12.5)	>0.999
Vesiculation	3 (10.34)	0	3 (18.75)	>0.999
Dermatitis type	0	0	0	
Position	5 (17.24)	2 (15.38)	3 (18.75)	>0.999
Extension	15 (53.57)	7 (58.33)	8 (50)	0.71768

Only a few patients had an improvement in terms of reduction in edema (2, 6.7% at T1 and 4, 13.8% at T2 vs. T0) and vesiculation (3, 10.3% at T1 and 3, 10.3% at T2 vs. T0); no differences were observed between the two groups for the parameter "edema", whereas all the patients in which we observed a reduction in the parameter "vesiculation" belonged to the treatment group.

No patient ameliorated from bilateral to unilateral dermatitis; in 2 patients (6.9%) at T1 and 5 (15.2%) at T2 vs. T0, we observed a reduction in the extension of the areas involved (from both upper and lower eyelid to upper eyelid alone), with better results in the treatment group compared to the controls (18.75% vs. 15.38%), even if no statistically significant differences were observed among the two groups.

Finally, a reduction in the extension of dermatitis was observed in 13 (43.3%) and 15 (53.6%) patients at T1 and T2 vs. T0, respectively, with overlapping results between the two groups (treatment and control)

In Table 4 we show that overall, patients in both groups had an improvement in terms of dermatological score, as the differences among T1 and T0 and T2 and T0 were negatives (high scores indicate a worse situation). A slightly greater reduction in DLQI (−1.06 at T1 vs. T0, −2.75 at T2 vs. T0), disease severity (EDSI) (−1.03 at T1 vs. T0, 1.81 at T2 vs. T0), and itch (1.27 at T1 vs. T0 and −2.38 at T2 vs. T0) was observed in the treatment group than in control one (DLQI: −0.14 and −1.62, EDSI: −0.75 and −1.15, itch: −0.64 and −1.54, for T1 vs. T0 and T2 vs. T0, respectively). Particularly, the score in which we found the most significant reduction after treatment was OSDI (−6.20 and −4.51 in treatment group, and −1.24 and −2.94 in control group for T1 vs. T0 and T2 vs. T0, respectively). Despite this trend of improvement in treatment groups rather than control one, we were not able to reach the statistical significance as all the *p*-value were higher than 0.05 (Tables S1 and S2).

Table 4. Time variation. Mean and standard deviation of score difference in time (T1 vs. T0 and T2 vs. T0). Results are reported overall and separately for the control and treatment groups, both with *p*-value obtained by Student *t*-test.

Variable	Overall	Control	Treatment	*p*-Value
	Difference T1 vs. T0			
DLQI	−0.63 (3.77)	−0.14 (3.63)	−1.06 (3.96)	0.5150
OSDI	−3.89 (11.08)	−1.24 (10.2)	−6.20 (11.61)	0.2275
NRS	−0.97 (2.47)	−0.64 (1.91)	−1.27 (2.94)	0.5069
EDSI total	−0.9 (2.39)	−0.75 (2.73)	−1.03 (2.14)	0.7543
	Difference T2 vs. T0			
DLQI	−2.24 (4.52)	−1.62 (3.2)	−2.75 (5.42)	0.5118
OSDI	−3.81 (8.99)	−2.94 (6.83)	−4.51 (10.6)	0.6489
NRS	−1.96 (2.93)	−1.54 (3.1)	−2.38 (2.81)	0.4732
EDSI total	−1.52 (2.66)	−1.15 (2.98)	−1.81 (2.43)	0.5173

Generally, the reduction was clearer after 4 weeks than after 2 weeks (compared to baseline) (Figure 2). The results argue in favor of a major activity of the treatment compared to the control but in the absence of statistically significant results (Figure 3).

Finally, repeated measurement models were performed considering as outcome the dermatological scores (DLQI, OSDI, NRS–itch, and EDSI) and as covariate the time and the treatment. The results are reported in Table 5. First, we observe that time had a significant impact in terms of score reduction and the difference is more marked after 4 weeks than 2 weeks. The impact of treatment, adjusted for time, did not have a statistically significant effect.

Table 5. Repeated measures models.

	Beta [95% CI]	***p*-Value**
DLQI		
T1 vs. T0	0.63 [−1.96; 0.69]	0.3498
T2 vs. T0	−2.01 [−3.66; 0.37]	0.0165
Treatment vs. control	**−1.42 [−4.55; 1.70]**	0.3725
OSDI		
T1 vs. T0	−3.99 [−7.79; 0.01]	0.0505
T2 vs. T0	−3.25 [−6.56; 0.07]	0.0551
Treatment vs. control	**1.34 [−11.22; 13.90]**	0.8341
NRS		
T1 vs. T0	−0.96 [−1.83; −0.09]	0.0301
T2 vs. T0	−1.63 [2.77; −0.49]	0.0049
Treatment vs. control	**−0.19 [−1.90; 1.52]**	0.8245
EDSI		
T1 vs. T0	−0.90 [1.74; −0.06]	0.0361
T2 vs. T0	−1.44 [2.39; −0.49]	0.0030
Treatment vs. control	**0.01 [−1.21; 1.23]**	0.9859



Figure 2. Eyelid dermatitis at baseline (**a**,**d**) and after 2 weeks (**b**,**e**) and 4 weeks (**c**,**d**) of treatment. Left side (**a**–**c**) treatment arm; right side (**d**–**f**) control arm.

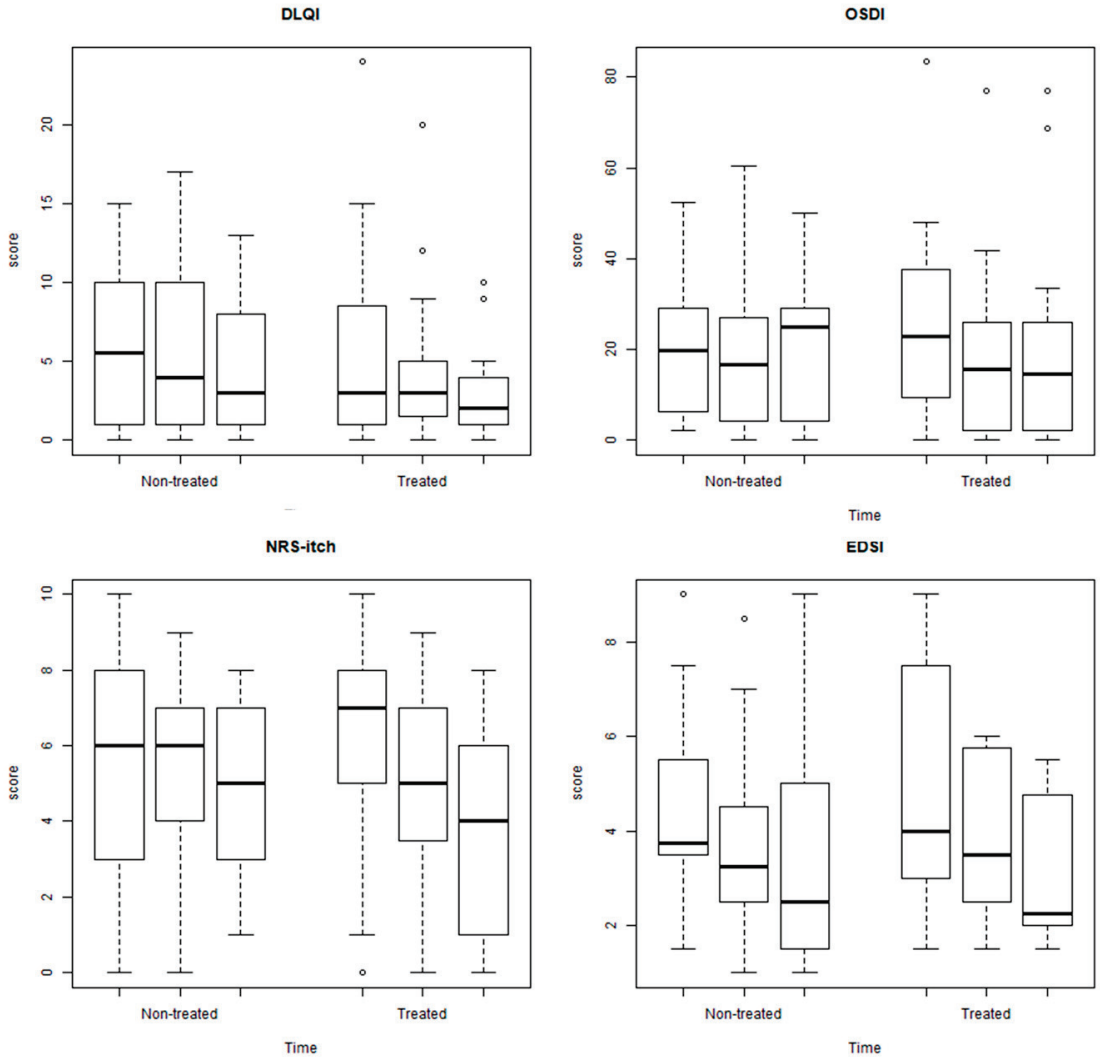


Figure 3. Box plots of DLQI, OSDI, NRS–itch, and EDS separately for non-treated and treated in different time points. The ° indicates the outliers.

4. Discussion

This study aims to provide evidence of the effectiveness of two dermo-cosmetic products (Rilastil Difesa® cream for the eyelids and eye contour area and Rilastil Difesa® cleansing face cream) in subjects suffering from various types of eyelid dermatitis. The patients enrolled in the treatment arm were compared with a control group, which received a placebo consisting of only the excipients and continued cleansing with products already in use.

Eyelid dermatitis has always been a diagnostic and therapeutic challenge for the dermatologist, as the presence of skin lesions in a visible location has a significant impact on the patient's quality of life and because exposure can lead to continuous interaction with irritating and/or allergenic agents, that contribute to the maintenance of dermatitis [21–24]. Furthermore, the distinguishing structure of this anatomical region [3], and the thinness of the skin, increases the risk of side effects caused by topical treatments, including poor tolerability and steroid-induced atrophy [25,26]. So, it is of utmost importance to identify topical products capable of inducing rapid regression of clinical manifestations and symptoms, and that can be well tolerated by patients, with the slightest adverse effects.

Few studies are published in the literature comparing, in patients affected by eyelid dermatitis, emollient medical devices with topical steroids or topical calcineurin inhibitors [27], such as the product's characteristics and the efficacy of different dermo-cosmetics or "dermatologist-recommended" moisturizers [28–30]. To the best of our knowledge, this is the first study which highlights the results obtained in the treatment of eyelid eczema with specific dermo-cosmetic products, in comparison with a placebo.

The products tested (Rilastil Difesa® cream for the eyelids and eye contour area and Rilastil Difesa® cleansing face cream) have proven to be more effective than the placebo in determining an improvement in the treated patients, based on all considered dermatological scores. In detail, regarding the general impact of dermatitis on the patient's quality of life, we observed a greater reduction in scores obtained by the DLQI questionnaire in the treatment than in the control group, both for the single time intervals considered (treatment: −1.06 at T1 and −2.75 at T2; control: −0.14 at T1 and −1.62 at T2) and through the repeated measurement model (treatment vs. control: −1.42). These results are relevant, considering the high burden on life-quality caused by chronic eyelid dermatitis [12].

Also, we observed in the treatment arm a greater improvement in the scores related to the extension of dermatitis (treatment: EDSI: −1.03 at T1 and −1.81 at T2; OSDI: −6.2 at T1 and −4.51 at T2; control: EDSI −0.75 at T1 and −1.15 at T2; OSDI: −1.24 at T1 and −2.94 at T2), and for the intensity of itching (NRS–itch: treatment 1.27 at T1, 2.38 at T2; control: −0.64 at T1 and −1.54 at T2). Similarly, all patients (3.18, 75%) in whom a reduction in the vesiculation parameter was observed at T2 belonged to the treatment arm. These parameters, such as the "edema" parameter, were evaluated separately, as they are not included as an integral part in the ESDI and OSDI scores. Regarding edema, no changes were evident between T0, T1, and T2, neither in the treatment arm nor in the control arm; however, this parameter has proven to be more difficult to modify with the use of dermo-cosmetics, even when used in association with corticosteroids [29]. It should also be noted that for all the considered parameters, the improvement in the treatment arm was achieved more quickly than in the control arm, with clinical and symptomatic improvement evident only 2 weeks after starting. This is a critical point, as it facilitates the patient's adherence to the treatment, guaranteeing its continuation and therefore the possibility of obtaining even better results. The comparison of the scores obtained at T1 and T2 versus the baseline values, considered separately for the treatment group and the control group, allowed us, in fact, to achieve statistically significant results both with regards to the means and the medians for the OSDI, NRS, and ESDI parameters.

In the treatment group, we also observed a greater reduction in the extent of the affected areas (18.75% in the treatment vs. 15.38% in the control group), although these differences did not reach statistical significance. These patients presented sub- and supra-

palpebral dermatitis at T0 and a single supra-palpebral localization at T2, while the dermatitis did not change from bilateral to unilateral in any patients.

To reinforce the relevance of our results, it should be noted that most patients included in this study were suffering from chronic (76.67%) and/or recurrent dermatitis (78.57%), predominantly associated with allergic comorbidities (46.67%). Furthermore, 83.33% had already received at least one previous dermatological evaluation and had undergone at least one line of treatment before study inclusion, to which they were unresponsive or refractory. Among these, 21 patients had used a topical steroid (alone or in combination with antibiotics) before enrollment in our study; despite the planned two week wash-out period, a possible "rebound" effect deriving from the suspension of these treatments, with a consequent worsening of dermatitis during the observation period, must be taken into consideration, as well as the "trigger" effect on dermatitis carried out by the possible contact, even during the study, by airborne and/or ingested allergens [6,31–33]. These confounders may have contributed to reducing the significance of the observed differences.

One of the main strengths of this study is represented by the standardized and validated dermatological scores used for the evaluation of the clinical progress of dermatitis, its symptoms, and its relative impact on the patient's quality of life [17–20]; these scores allow the response to treatment to be objectively assessed. Another strong point is represented by the independent randomization of the patients enrolled in the study into the two treatment/control arms. The descriptive statistics of the population at baseline demonstrate the absence of statistically significant differences both in terms of demographic aspects and clinical aspects between the two groups, proving the correct randomization process. To the best of our knowledge, this is also the first comparison study on the effectiveness of a dermo-cosmetic on eyelid dermatitis.

A possible limitation is represented by the relatively low sample size; the number of patients eligible for inclusion in the study was lower than expected, perhaps because of the unusual climatic conditions of recent seasons, which have reduced the incidence of "seasonal" dermatitis (seborrheic dermatitis, irritant contact dermatitis, etc.). Even though we applied corrections in the statistical processing of the results, as explained in the "Sample size" section, this could have influenced the statistical significance of the results. Another limitation is represented by the risk that some patients have not correctly applied the provided topical products. Indeed, it should also be remembered that the first draft of the study envisaged that the patients enrolled in the control group would also receive the cleansing cream, in addition to the topical placebo product. This draft was subsequently modified, for the fear that even the detergent alone could have a specific effect in the control group; thus, we evaluated the synergistic effect of both treatment and the cleansing cream in the treatment group. However, the indication for patients enrolled in the control group to use the habitual cleanser could have effectively led to an "opening" of the study, making it easier for patients to recognize that they had received a placebo rather than the product under study, and increasing the risk of interference with self-medication (always a risk for studies conducted on products for topical use, as well as that of inappropriate or insufficient application of the topical itself).

5. Conclusions

Our experience confirms the deep impact of chronic eyelid dermatitis on the quality of life of affected patients and the need to identify targeted therapeutic approaches. This comparative study demonstrates the role of dermo-cosmetic products tested, not only as a support treatment, but also as a first-choice clinical approach.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/cosmetics11030083/s1: Table S1. *p*-value obtained comparing mean values for T1 vs. T0 and T2 vs. T0, separately for treatment and controls using paired *t*-test. Table S2. *p*-value obtained comparing median values for T1 vs. T0 and T2 vs. T0, separately for treatment and controls using Wilcoxon signed rank.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent was obtained from the patient(s) to publish this paper.

Data Availability Statement: The dataset presented in this study is available on request from the corresponding author.

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Review

Deciphering the Effects of Different Types of Sunlight Radiation on Skin Function: A Review

Sophia Letsiou *, Elpida Koldiri, Apostolos Beloukas, Efstathios Rallis and Vasiliki Kefala

Department of Biomedical Sciences, School of Health and Care Sciences, University of West Attica, GR-12243 Athens, Greece; hope_c2@hotmail.com (E.K.); abeloukas@uniwa.gr (A.B.); efrall@otenet.gr (E.R.); valiakef@uniwa.gr (V.K.)

* Correspondence: sletsiou@uniwa.gr or sletsiou@gmail.com

Abstract: Sunlight radiation is a fundamental component of our daily lives. Specifically, blue light as well as UV light appear to play a role in the development of oxidative stress, DNA damage, photoaging, and pigmentation through the chromophores in skin tissues. However, several skin problems like psoriasis, eczema, and atopic dermatitis can be avoided with short-duration exposures to low-energy blue light radiation or UV radiation. In addition, exploring the effects of blue light as well as UV radiation on skin is quite essential for the development of minimally invasive antiaging strategies and for the design of innovative cosmetic formulations in modern aesthetics and cosmetology. Thus, in this review, we present the advantages as well as the disadvantages of light radiation, with a special focus on blue light and UV radiation activity on the human skin. We also discuss the molecular action of blue light and UV radiation on human skin. Other types of light radiation are included to holistically approach the effect of light on human skin.

Keywords: blue light; UV light; skin protection; photoprotection; skin photoaging

1. Introduction

Daily exposure to several environmental stresses and insults occurs on the skin [1]. Indeed, air pollutants, both indoors and outdoors, affect the environment and pose an increasing global health risk to people. Since these pollutants enter the skin through transdermal and systemic pathways, the skin is one of their primary targets as they may penetrate both its superficial and deeper layers [2].

The sun is the most important source of ultraviolet, visible, and infrared radiation [3]. Solar radiation, and especially ultraviolet radiation (UV), is thought to be the primary factor causing photoaging [4,5]. In addition, blue light is a form of radiation that causes a series of damages to the skin as we are exposed to it daily due to the increasing use of electronic devices.

Thus, this review's objective is to provide an overview of the most recent findings on the impact of sunlight irradiation on skin function, with a special focus on blue and UV light, as these two types of sunlight are currently being scrutinized by many researchers. For this, recent studies based on different sources of blue light or UV light exposure are presented, focusing on their effects on the human skin. The publications were selected according to a search on PubMed using the terms "light radiation", "blue light", and "UV light" and filtering only for human skin during the last five years. The search yielded 119 publications that were manually filtered to select only the most relevant publications, which are presented in this review. Of note, we did not intend to perform systematic research here. We therefore intentionally limited our literature search to PubMed and the term "human skin cells". In addition, the molecular mechanisms underlying the observed biological skin changes, such as the signs of skin aging, are also described. Specifically, in this review, the subcategories of solar radiation and the common sources of visible light and blue light are presented. This is followed by a section highlighting the advantages of UV

light but also the unpleasant effects of exposure to it due to the molecular changes that are induced in cells. The role of matrix metalloproteases, the concept of photoaging, and the process of pigmentation are analyzed to comprehend the phenotypic changes in the skin. In the following sections, the known biological effects of the individual colors of visible radiation are discussed, focusing especially on blue light, its mechanism of action, its skin penetration depth, and its similarities and differences with UV radiation. In addition, the therapeutic effects of blue light and UV light are presented, whether they concern skin conditions or other diseases such as cancer. Finally, the damaging effects of blue light and UV light on human skin are highlighted.

2. Sunlight

The general composition of sunlight is as follows: 44% visible light (400–780 nm), 53% infrared radiation (700–1440 nm), and 3–7% ultraviolet (UV) energy [3]. Ultraviolet radiation consists of three zones of different energies known as UVA, UVB, and UVC, with different impacts on human health (Figure 1). Of note, ozone has an excellent function in mitigating hazardous UVC rays that pass through the stratosphere [3]. Numerous positive impacts of solar radiation are present in our lives, including its crucial role in the photosynthesis of microbial algae and plants, the control of circadian rhythms, and the sensation of sight [3].

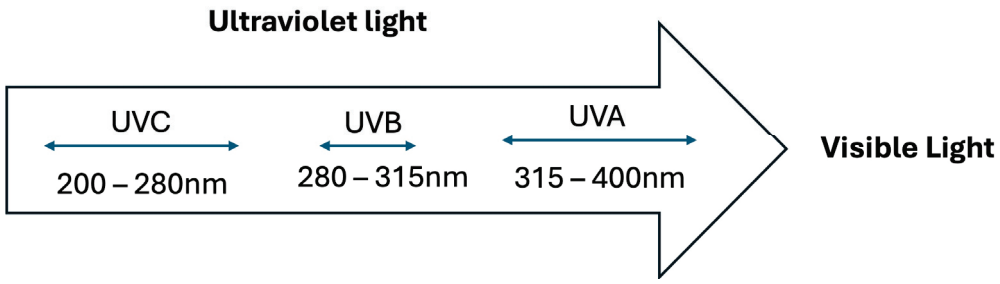


Figure 1. Three zones of ultraviolet light.

2.1. Visible Light

About half of the solar energy that reaches the Earth's surface at sea level is in the form of visible light, which has a wavelength between 400 and 700 nm [6–8]. A reference color is assigned to each range of wavelengths [9,10] (Table 1).

Table 1. Light wavelengths with the reference color.

Wavelength (nm)	Color
<400	Ultraviolet (UV)
380–449	Violet
449–466	Violet/Blue
466–478	Blue/Violet
478–483	Blue
483–490	Blue/Green
490–510	Green/Blue
490–560	Green
560–630	Yellow/Orange
630–700	Red
700–3000	Infrared NIR (near infrared)

2.1.1. Visible Light Sources

Sunlight is the primary natural source of visible light [11], but artificial sources include fluorescent lamps, lasers, LED (light-emitting diode) lights, cell phones, television or computer monitors, and contemporary smart watches, all of which emit a full visible light spectrum [12].

2.1.2. Blue Light Sources

The sun, which also gives a blue hue to the sky, and all digital screens found in electronic devices are the two primary sources of blue light radiation [13].

In order to enhance the brightness and clarity of the electronic display, a large number of widely used digital devices produce blue light (400–480 nm) from their back-illuminated light-emitting diode (LED) display [14]. It is noteworthy that blue light is emitted by specialized medical equipment, such as incubators, to cure jaundice [15]. Currently, many researchers are very interested in studying the effects of blue light on the skin since its wavelength spectrum is quite close to UVA radiation.

Moreover, LED lights are present in a plethora of products that come into contact with the skin, including lamps for indoor and outdoor illumination, medical indications, and lamps used in extremity care facilities [11,16–18]. In addition, the maximum emission range for televisions and computer monitors is between 400 and 490 nm [19], whereas the range for tablets and smartphones is between 452 and 456 nm [20].

2.2. Recent Data on Light Exposure

Although artificial blue light emission from electronics is significantly lower than that of natural blue light—at least two to three times lower—the biological effects of this light on skin are still not well known [21].

The ratio of the solar spectrum's irradiance to that of digital displays is predicted to be less than 1/200, although the average irradiance of the blue portion of the solar spectrum is 8 mW/cm^2 [22]. As a result, blue light (420–490 nm) from screens on computers, TVs, and cell phones has a relative intensity that is 99–1000 times lower than sunlight. In addition, Rascalou et al. [22] found that the irradiance of a number of digital gadgets was 36 $\mu W/cm^2$. The most powerful television displays have a blue light irradiance of 30 $\mu W/cm^2$, according to a report by Passeron's group [23]. In their work, Jo et al. [20] calculated the daily dose of blue light exposure using measurements of sunshine taken in March 2019 and August 2019, and the result was 38,790 J/cm^2. The computed dose of blue light exposure indoors, excluding sunlight, was 6993 J/cm^2.

More specifically, the dose of fluorescent lamps was calculated at 6.529 J/cm^2/day, from computer screens at 0.383 J/cm^2, from televisions at 0.022 J/cm^2, from tablets at 0.031 J/cm^2, and from mobile cell phones at 0.029 J/cm^2. Based on the above data, Jo et al. concluded that individuals are frequently subjected to blue light, mainly from sunlight during outdoor activities but also from digital devices during indoor activities [20].

Nowadays, incandescent lighting is being phased out in favor of so-called low-energy technologies like light-emitting diodes (LEDs) and compact fluorescent lamps (CFLs) as a result of the current requirement to reduce energy usage [24]. However, it seems that 90% of human life involves activities in an indoor environment [13]. The recent increase in the use of modern technology has led to various types of electrical devices emitting electromagnetic fields in both the home environment and the workplace [3]. It has been reported that an office worker's typical exposure is 1597 J/cm^2 over approximately 27 days, 958 J/cm^2 over approximately 16 days, and 447 J/cm^2 over approximately 8 days [20]. Recent reports have shown a rapid increase in the use of smartphones and electronic devices in general during the COVID-19 pandemic [25]. In addition, according to recent research, 60% of adults use digital devices for up to six hours every day [14]. Indeed, nowadays, people are busy browsing the Internet, using social media, watching TV programs, playing online games, chatting with friends or even family, etc. Therefore, the rise in the use of electronic devices has led people to be more and more exposed to blue light, to which the eyes and

facial skin are particularly exposed [19]. Depending on the source, this blue light usually has a peak emission between 440 and 460 nm, covering a spectrum from 420 to 490 nm [26].

Another survey based on blue light exposure to workers aged between 20 and 49 years has shown that the average time of smartphone use per day is 118.97 min, of tablet use is 63.61 min, of television use is 182.58 min, and of fluorescent light exposure is 1046.34 min, while on weekends, the average computer time is 579.6 min and sunlight exposure is 165.39 min [20]. According to this, the amount of time spent using electronic devices is constantly rising. The idea of healthy aging is being shaken by this modern phenomenon known as "digital pollution", as numerous studies have shown ailments linked to it [27,28].

3. Effects of UV Irradiation on the Skin

3.1. UVA Radiation (320–380 nm)

Chronic UVA exposure causes epidermal hyperplasia by changing the thickness of the stratum corneum [29,30]. Specifically, UVA causes DNA damage indirectly through the formation of melanin and its byproducts. UVA2 wavelengths between 320 and 340 nm cause the distribution of melanin granules in the epidermis's superficial layers, whilst UVA1 wavelengths between 340 and 400 nm increase melanin density in the basal layer [31–33]. UVA may act indirectly through photon absorption by endogenous photosensitizers, which subsequently trigger photo-oxidation processes, resulting in ROS [34,35]. Furthermore, it has been claimed that UVA radiation penetrates the skin 3 to 4 mm and directly damages cellular components, potentially contributing to skin aging and carcinogenesis [35].

3.2. UVB Radiation (280–320 nm)

UVB induces direct damage to cellular DNA by generating thymine dimers [33,36], which increases melanin synthesis [37]. It also interacts with skin cells, as opposed to UVA, which penetrates the skin's deeper layers and impacts immune cells [38,39]. UVB exposure stimulates proinflammatory markers, including TNF-α, IL-6, iNOS, and COX-2 [40]. It has been observed that these proinflammatory and inflammatory molecules generate a variety of pathophysiological changes that worsen skin damage by increasing ROS generation [40,41].

3.3. Positive Effects of UV Radiation

Human skin benefits greatly from UV light in several ways, including antibacterial action, wound healing promotion, prevention of jaundice, and the creation of active vitamin D [40,42–44]. Moreover, a low UVR dose of 1.5 kJ/m^2 on 4 consecutive days may find therapeutic use in individuals in need of local immunosuppressive treatment, such as contact hypersensitivity [45]. In addition, psoriasis and vitiligo may benefit from the UVB rays at 311–312 nm [46,47], and a moderate dose of UVA1 (340–400 nm, 50 J/cm^2) can be helpful in treating atopic dermatitis and scleroderma [48,49]. Although the detailed mechanisms underlying the successful treatment of these refractory skin diseases remain unclear, the effectiveness of these phototherapies is thought to depend on their respective action spectra. These findings suggest that wavelengths greater than 296 nm are effective for the phototherapy of skin conditions [10].

3.4. Negative Effects of UV Radiation

Long-term exposure to UV radiation poses the danger of skin damage, accelerated aging of the skin, wrinkles, sagging, sunburn, inflammation, and even mutations that lead to the development of various types of skin cancer [3]. It must be emphasized that large dosages of UV radiation cause changes in the cellular redox balance and, as a result, oxidative alterations of lipids, nucleic acids, and proteins, which interfere with cellular metabolism by triggering photoreaction processes [50]. Regular and often unavoidable exposure to solar ultraviolet (UV) radiation makes it one of the most critical factors that cause DNA damage in human cells [51]. Recent studies have also demonstrated that skin exposure to UV radiation causes collagen damage and elastin degradation due to induced

oxidative stress [35,52,53]. Indeed, oxidative stress plays an essential role in skin aging, and induced ROS also have a major role in stimulating skin pigmentation. Finally, in vitro reports have shown that UVA radiation demonstrates a more substantial effect than UVB radiation on collagen destruction at 330 nm [54].

3.5. Physiological Function of UV Radiation

The main negative effects of UV radiation on skin have been documented to include photoaging, inflammation, sunburn, and skin cancer [55,56]. According to Reichrath and Rass (2014), prolonged exposure to UVA rays causes epidermal hyperplasia and modifies the stratum corneum's thickness [57]. Reactive oxygen species (ROS) and DNA damage are two negative consequences of UVB exposure [29,30]. Numerous investigations have shown that UVR typically stimulates activator protein-1 (AP-1)-mediated transcription in the skin and activates a number of kinases, including p38 MAP kinase and c-Jun N-terminal kinase (JNK) [58]. As a result, shielding the skin from UV radiation can prevent skin aging. UVR, however, is not limited to its detrimental effects on skin. According to Piotrowska et al., UVR interacts with 7-dehydrocholesterol, a cholesterol precursor in the skin that is eventually transformed into vitamin D [43,44]. In order to form bones, vitamin D helps with calcium absorption. Since food only provides 50% of the necessary amount of vitamin D, getting enough UVR exposure is crucial for good health. Furthermore, for patients requiring local immunosuppression therapy, such as in contact hypersensitivity, a low dose of UVR (1.5 kJ/m^2) may have therapeutic uses [45]. Psoriasis and vitiligo can be improved by narrowband UVB (311–312 nm) [46].

According to Rodenbeck et al., atopic dermatitis and scleroderma, which cause the skin to tighten and harden, may benefit from a medium dosage of UVA1 (340–400 nm, 50 J/cm^2) [48]. According to these findings, skin-related illnesses may benefit from therapeutic UVR exposure.

4. Skin Molecular Biology Mode of Action under UV Radiation

UV photons damage the skin through two mechanisms: direct absorption and photosensitization. In the direct absorption method, cellular chromophores absorb UV radiation and transform the absorbed energy into a biochemical signal, activating subsequent biological responses [59]. Cellular chromophores that absorb UVB radiation are nucleic acids, amino acids (such as tryptophan and tyrosine), kinins, flavins, porphyrins, and urocanic acid (UCA), while UCA absorbs UVA radiation [59]. The primary UV-induced damages are cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine pyrimidine (6-4PP). These photoproducts impede RNA transcription, activating the p53 gene and causing apoptosis in irradiated keratinocytes [60].

However, the second pathway, photosensitization, is indirect. Endogenous or exogenous sensitizers absorb UV radiation and trigger reactions that result in the creation of ROS and reactive nitrogen species (RNS), which can reach the nucleus and cause oxidative DNA alterations and strand breaks [3,10,18,56]. The primary ROS produced by UV light are the hydroxyl radical, superoxide anion, peroxyl radical, and their active precursors, which include singlet oxygen, hydrogen peroxide, and ozone. The primary RNS generated by UV radiation are nitric oxide and nitrogen dioxide. Excess free radicals cause DNA alteration and aberrant gene expression, resulting in the loss of cellular integrity [61].

In response to ROS accumulation, keratinocytes release melanocyte-activating factors, resulting in pigmentation [3]. After UVA and UVB exposure, the intracellular generation of ROS and inflammatory mediators is significantly increased, resulting in oxidative stress and an imbalance in the antioxidant defense system. These modifications induce structural and functional changes such as breakdown of extracellular matrix (ECM) proteins and overexpression of MMPs, which promote skin photoaging, sunburn development, inflammatory cell activation, and melanogenesis [62,63]. UV exposure also damages other essential macromolecules in the skin, such as proteins and lipids [64,65]. It is also responsible for causing phototoxic reactions associated with certain pharmaceutical preparations [64,65].

4.1. Matrix Metalloproteinases (MMPs)

MMPs are a category of zinc- and calcium-dependent endopeptidases that degrade extracellular matrix (ECM) components such as collagen, elastin, and fibrillin-1 [66]. Increased MMP and decreased collagen production cause a loss of collagen content in the skin, which eventually causes wrinkle formation. MMPs are induced by severe oxidative stress and inflammatory reactions [66,67] and break down different types of collagen (Figure 2).

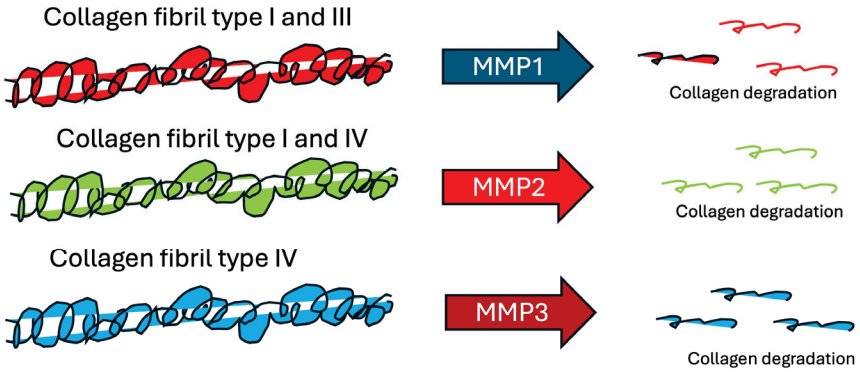


Figure 2. Collagen tbreakdown by different *matrix metalloproteinases*.

4.2. Photoaging

Skin photoaging has phenotypic traits such as wrinkle formation and roughness. Furthermore, a recent study revealed other epidermal characteristics related to skin photoaging, such as epidermal hyperplasia and damage or disruption of collagen fibers [3]. Skin photoaging is also linked to an increase in metalloproteinases (MMPs) in dermal fibroblasts in response to UV exposure [67,68].

4.3. Pigmentation Process

ROS are produced by a wide range of stimuli and metabolic processes, and they consume electrons from other molecules (oxidation) to form additional ROS in a chain reaction. The excessive synthesis of oxidizing chemicals is known as oxidative stress, and it destroys lipids, proteins, and nucleic acids. ROS and oxidative stress play a role in sunburn, carcinogenesis, inflammatory disorders, aging, and pigmentation [69,70]. Both visible and UVA1 radiation cause pigment alterations in three phases: *Immediate Pigment Darkening (IPD)*, which lasts up to 2 h after irradiation; *Persistent Pigment Darkening (PPD)*, which lasts for 24 h; and *Delayed tanning (DT)*, which begins about 5–7 days after irradiation and can extend for weeks to months. Specifically, UVR and visible radiation cause IPD, PPD, and DT, especially in the darkest FST phototypes (III–VI). Visible light-induced melanogenesis is most evident in phototypes IV to VI, with reports of both transitory and long-lasting (up to 8 weeks) pigmentation in human skin, depending on the total dose. It has been proposed that the creation of rapid pigmentation after exposure to visible light is photochemical in origin, while delayed pigmentation arises from neomelanogenesis [6]. The major stimulant of melanogenesis, the alpha-melanocyte-stimulating hormone (a-MSH), lowers UV-induced hydrogen peroxide levels in irradiated melanocytes, indicating the relationship between pigmentation and oxidative stress. Furthermore, short-wavelength visible light stimulates melanogenesis via the G protein-coupled membrane receptor opsin-3 [29]. Furthermore, a recent study found that oxidation of pre-existing melanin appears to contribute to enhanced pigmentation after UVA exposure; blue light may have a similar impact [70].

5. Effects of Visible and Near-Infrared Irradiation on Skin Cells

5.1. Violet Light (400–450 nm)

According to a recent study, UV irradiation (410 nm, 10–50 J/cm^2) dramatically decreases the expression of cell differentiation factors [71]. Specifically, at 410 nm and at 30 J/cm^2, it lowers the mRNA expression levels of antimicrobial peptides (AMPs),

which are important for epithelial defense. Furthermore, UV irradiation stimulates ROS production, inflammatory cytokine release, and cellular DNA damage, and it also prevents early keloid formation [72].

5.2. Blue Light (450–490 nm)

Blue light (450–490 nm) has both beneficial and detrimental effects on the skin. Mignon et al. found that human dermal fibroblasts exhibit inhibitory effects on metabolic activity when exposed to 450 nm blue light at low intensities (<30 J/cm^2), while cytotoxicity occurs at higher intensities (>30 J/cm^2) [73]. Furthermore, blue light irradiation promotes oxidative stress in the mitochondria of cultured human keratinocytes, while from 430 nm to 510 nm, it slows epidermal barrier restoration following injury [74].

5.3. Green Light (490–560 nm)

Simoes et al. found that green light (520 $\pm$ 30 nm, 240 J/cm^2) promotes angiogenesis and myofibroblast development, crucial for the repair of third-degree burns [75]. However, further research is needed into the biological impacts of green light.

5.4. Yellow/Orange Light (560–630 nm)

Yellow/orange light radiation at 590 nm lowers UVA-induced free radicals and MMP-1 expression in human fibroblasts, while at 595 $\pm$ 2 nm, it increases collagen type I and MMP-1 expression in human dermal fibroblasts [76,77]. Furthermore, yellow light at 590 nm dramatically decreases triglyceride levels via autophagy-related lysosomal degradation and may thus be therapeutically effective for lowering excess body fat [78].

5.5. Red Light (630–700 nm)

Red light radiation at 660 nm has been demonstrated to decrease MMP-1 expression, enhance collagen I expression in skin, expedite wound healing, protect against DNA damage induced by UVB radiation, and contribute to the protection of human dermal fibroblasts from UVB radiation [79–82]. These findings indicate that red light may be helpful to the skin and potentially useful in photomedical applications such as expediting the regeneration of wounded skin and antiaging treatments.

5.6. Near-Infrared Light (700–3000 nm)

According to a study by Akhalaya et al., near-infrared spectroscopy (NIRs) promotes the generation of oxygen free radicals (ROS) and destroys skin collagen in a manner similar to that seen with UVR [83]. However, a clinical investigation found that the majority of patients (around 51–75%) who received NIRs (830 nm) for approximately six months had better skin tone and reduced roughness [84]. Another study found that NIR (830 nm) significantly increases the amounts of collagen and elastin fibers in all experimental groups, and low-intensity NIR radiation (810 nm) enhances collagen accumulation and promotes cell proliferation and complete re-epithelialization, concluding that NIR can be used clinically to improve wound healing through photomodulation therapy [79].

6. Biological Effects of Visible Radiation

UVA and visible light radiation penetrate deeper into the dermis, affecting cellular and extracellular components. Until recently, visible light was assumed to be relatively innocuous because of its low energy [85] in comparison to ultraviolet and infrared radiation. Blue light is less hazardous than UV light due to changes in photon energies, yet it can trigger cell alterations [13].

Recent research and experimental approaches have revealed the ability of visible light to cause erythema in fair skin and pigmentary alterations in individuals with darker skin phototypes [12]. As a result, both the medical profession and social media users have been alarmed regarding its possible detrimental skin consequences [12].

Blue light radiation has the potential to generate biological effects similar to those of UVA radiation because of its proximity to the UVA spectrum. However, according to new studies, blue light has been linked to the effects of UVA radiation on photoaging and carcinogenic adverse effects in the long run [12,14]. The visible light spectrum accounts for more than half of solar radiation, and these wavelengths reach the lower dermis layer. Electron spin resonance spectroscopy has demonstrated that visible light and infrared radiation generate 50% of the skin's total oxidant burden, contributing to accelerated skin aging via oxidative pathways [21]. Furthermore, another study indicated that skin fibroblasts are more harmed by exposure to this range of radiation than keratinocytes or melanocytes [86].

Interestingly, visible light radiation causes approximately 50% of the overall oxidative stress caused by sunshine; however, the oxidation efficacy of blue light is just a quarter of that of UVA radiation, implying a greater share of blue light emission in visible light [87–89].

6.1. Molecular Mechanism of Action of Visible Radiation in Skin

Photoreceptor chromophores, such as melanin, heme, and opsins, absorb photons from visible light, activating and transmitting energy to the chromophores and altering the function of the skin [90].

Enzymes, aromatic amino acids, urocanic acid, tryptophan, tyrosine, NADPH, NADH cofactors, riboflavins, porphyrins, melanin precursors, melanin, protoporphyrin IX, bilirubin, hemoglobin, carotene, or water molecules are examples of molecules that absorb light and are referred to as chromophores [91]. Thus, a variety of chromophores (photoreceptors) determine how blue light behaves. The most significant types of photoreceptors include nitrosated proteins (such as S-nitro-albumin), flavones, opsins, and porphyrins [92].

The main endogenous chromophore that is activated by blue light is flavins. In addition, the epidermis contains G protein-coupled receptors called opsins, which are light-sensitive [26,93]. Photoreceptors react to different wavelengths of sunlight; opsins (OPNs) are photoreceptors linked to the blue spectrum [26]. Opsins are classified into various types based on where they are expressed [93].

Human skin, melanocytes, and keratinocytes express short-wavelength OPN1, OPN2 (rhodopsin), and non-optical OPN3 (panopsin or encephalopsin) [94]. Human melanocytes contain OPN2, which is activated by UVA and leads to the synthesis of melanin. It has been discovered that both the anagen hair follicle and the skin express OPN2 (rhodopsin) and OPN3 (panopsin, encephalopsin). Buscone et al. have demonstrated that blue light radiation (3.2 J/cm, 2453 nm) can prolong the regenerative phase of hair follicles. The OPN3 sensor detects visible light with shorter wavelengths, such as blue light [95]. It works as a calcium-dependent melanocyte photoreceptor, activating the melanogenesis-related enzymes that cause hyperpigmentation [26]. It has been demonstrated that OPN4 is expressed in human fibroblasts, melanocytes, and keratinocytes. Blue light stimulates calcium entry and the phosphorylation of extracellular kinases in a dose-dependent manner [96].

Blue light has been shown to directly alter melanocytes and to influence melanogenesis via OPN3. Interestingly, the stimulation of other blue light photoreceptors causes the creation of ROS [92,97].

6.2. Penetration Depth of Visible Light Radiation

The depth of visible light penetration is determined by chromophores in the skin, the Fitzpatrick skin type (FST), reflection, scattering, and absorption, all of which are controlled by the skin's natural barrier [6].

The principal components of visible light scattering and absorption in the skin include hemoglobin, melanin, keratin, bilirubin, carotene, lipids, and other structures such as filamentous proteins and cell nuclei. Melanin and keratins are the major pigments in the epidermis, scattering and absorbing light. In the epidermis, hemoglobin is the predominant absorber, and collagen is the primary scatterer of visible light, accounting for 18% to 30% of the layer volume [6,92].

Hemoglobin's highest absorption occurs in blue radiation at 418 nm and in yellow/orange radiation at the 542 nm/577 nm wavelength bands; nevertheless, the two are highly connected with the concentration of erythrocytes [98].

Furthermore, various cell types in the epidermis and dermis, including melanocytes, keratinocytes, fibroblasts, and hair follicle stem cells, have been found to contain opsin receptors. Different spectrum sensitivities in the visible and UV light ranges are caused by differences in the amino acid sequences of opsins in the chromophore binding pocket, as well as the kinetics of binding to the chromophore [99]. Carotenoids, including α-carotene, γ-carotene, β-carotene, lycopene, lutein, and zeaxanthin, and their isomers absorb and scatter light in the skin. Carotenoids absorb light between 440 and 490 nm and reflect it between 458 and 472 nm. The distribution of carotenoids in human skin depends on the areas of skin studied, varies greatly between people, and is related to the skin's antioxidant ability [100].

6.3. Positive Effects of Blue Light

Phototherapy is frequently used to treat a variety of common skin disorders. The effect of blue light radiation relies on its wavelength, frequency, and method of action, as well as the radiation period and dose [91]. In vitro data on cell survival, cell morphology, and mitochondrial function demonstrate that LED phototherapy is safe, with no negative consequences during or after treatment [12].

Photo-Bio-Modulation therapy (PBM) is a new and minimally invasive approach to the management of hypertrophic scars and keloids in combination with existing treatments [101]. It is a more recent term for low-level laser treatment (LLLT), which comprises applications utilizing visible, near-infrared, and infrared energy [8].

6.4. Blue Light for Clinical Applications

Blue light is characterized by its anti-inflammatory [102] and anti-proliferative effects [103]. Thus, it could be effective in different inflammation-related skin states, including psoriasis or eczema [104,105]. Other dermatological disorders treated with blue light include cutaneous T-cell lymphoma, rosacea, acne, and precancerous and cancerous skin lesions. Blue light radiation has also been found to alleviate itching and stimulate hair growth, and it can be utilized in acne therapy [11,30]. In addition, blue/red light combinations have been used to treat acne, scurvy, and seborrheic dermatitis [12].

Furthermore, blue light is utilized to treat neonatal jaundice, nonhyperkeratotic actinic keratosis, seasonal affective disorders, depression, and neurodermatitis [13,106]. Additionally, photodynamic therapy is utilized to treat cancer [107]. However, more research is needed to design protocols and monitor long-term safety [107].

6.5. Phototherapy

Phototherapy is the use of light for medical purposes. For safety reasons, light with a specific wavelength identified by the corresponding chromophore should be used. As such, it is critical to identify the photoreceptors in the skin and comprehend the fundamental mechanisms that underpin them [10].

6.6. Photorejuvenation

When used correctly, photodynamic blue light treatment for photorejuvenation is thought to be both effective and safe. By maximizing absorption selectivity and limiting the duration and penetration depth of blue light, one can reduce injury to adjacent healthy tissues [108]. The most common adverse effects are erythema, edema, pruritus, epithelium desquamation, hyperpigmentation, and discomfort [109].

6.7. Psoriasis

A short clinical research study on psoriasis treatment revealed that blue light radiation at 420 nm and 453 nm was helpful and had low and infrequent adverse effects [110].

6.8. Eczema and Atopic Dermatitis

Studies by Keemss et al. [105] and Becker et al. [111] determined that using non-UV blue light with a peak emission of 453 nm was both safe and effective in reducing eczema and atopic dermatitis lesions. The authors also saw improvements in patients' sleep and quality of life.

6.9. Acne

A rising number of research studies suggest that blue light can help treat moderate acne vulgaris [15]. According to Bonnans et al., exposure to a mixture of 415 nm and 470 nm LEDs had a long-lasting antibacterial effect, almost eliminating inflammatory nodules, pustules, and microcysts [8]. Furthermore, a study by Jung et al. demonstrated that red and blue light can interfere with sebocytes, preventing the production of sebum and enhancing the clinical appearance of acne [112].

6.10. Photodynamic Therapy (PDT)

The PDT method, which includes blue light, is mostly used to treat non-melanoma skin malignancies and actinic keratosis. It is important to apply a photosensitizer, followed by light radiation [106]. Target cells are destroyed when the photosensitizer and light react, producing cytotoxic reactive oxygen species (ROS) [113]. The most often employed topical compounds are 5-aminolevulinic acid (5-ALA) and methyl-aminolevulinate (MAL), which are then converted into protoporphyrin IX (PPIX) [114].

7. Negative Effects of Blue Light Radiation

Exposure to blue light radiation has been shown to improve a variety of skin problems. However, research has shown that extended exposure to high-energy blue light can increase the amount of DNA damage, cell and tissue death and injury, eye damage, skin barrier damage, and photoaging [115–119]. Liebmann et al. found that blue light radiation of 500 J/cm^2 at 453 nm or above had no adverse impact on human skin keratinocytes and endothelial cells [115]. Similarly, Opländer et al. demonstrated that blue light radiation at 453 and 480 nm was not harmful to human fibroblasts. However, blue light exposure at 410 nm or 420 nm induced intracellular oxidative stress and harmful consequences in a dose- and wavelength-dependent manner [116]. In addition, other studies [13,120] have found that blue light radiation, like UVA radiation, contributes to skin aging. According to the aforementioned data, blue light radiation at different wavelengths can generate varied degrees of intracellular oxidative stress with diverse physiological effects, thereby contributing to premature skin photoaging.

7.1. Risks to Eyes and Oral Mucosa

Research on the toxic and genotoxic consequences of blue light has mostly focused on the therapeutic and ophthalmic risks. It was reported that blue light damages the retina, induces cell dysfunction and mortality in gingival and lens epithelial fibroblasts, and causes apoptosis in retinal pigment epithelium (RPE) cells [1,121].

7.2. Production of ROS

It has been observed that exposing human keratinocytes to 200 J/cm^2 for 66 min results in a 147% increase in the formation of ROS [74,122]. Furthermore, dermal carotenoids are significantly reduced by blue/violet light radiation of 100 J/cm^2 at 380–395 nm in human skin, as shown by Raman spectroscopy, indicating the generation of free radicals [7,123], leading to cell death [124].

7.3. Damage to Cellular DNA

Blue light radiation has a dose-dependent genotoxic effect on skin cells, as demonstrated by the study of Chamayou-Robert et al. [123]. Specifically, blue light radiation could interact with intracellular porphyrins and flavoproteins, leading to the production of ROS

such as peroxide, nitric oxide, and peroxynitrite, causing damage to cellular DNA [1,117]. Furthermore, other research has shown that visible light damages DNA, induces oxidative stress, and alters the molecular structure of the skin, all of which have genotoxic effects on human keratinocytes that are comparable to those of UV radiation [119,125]. Also, it was reported that the reaction of nitric oxide (NO) induced by blue light produces peroxynitrites, which may be responsible for damaging cellular DNA [126].

7.4. Photoaging

HEV has been demonstrated to cause skin photoaging in vitro, ex vivo, and in vivo with respect to the photoaging effect of blue light radiation [119].

Specifically, it has been observed that keratinocytes exposed to UV and visible radiation contain carbonylated proteins (CPs), which are formed through lipid peroxidation and cause damage to DNA. When CPs are exposed to blue light radiation, they produce additional oxygen free radicals (O^{2-}) in the skin, which use lipid peroxidation to resynthesize more CPs [127].

7.5. Oxidative Stress and Hyperpigmentation

According to Mamalis, blue light radiation at 30, 45, and 80 J/cm^2 greatly boosts ROS production [128]. In Mahmoud's study, people with darker skin types exposed to daily amounts of visible light developed more continuous dark staining than those with lighter skin types [129]. The lowest PPD dose in participants with Fitzpatrick skin types V and VI was 80–120 J/cm^2, and induced pigmentation was visible for at least two weeks. When exposed to blue light radiation, darker skin showed greater molecular-size protein complexes, but types I and II did not [119]. This multimeric protein complex associated with tyrosinase is primarily generated in melanocytes in dark skin and generates sustained tyrosinase activity, explaining the long-term hyperpigmentation found only in skin types III and higher following exposure to blue light [26].

However, a recent study by Duteil et al. revealed that utilizing a computer screen for 8 h per day for 5 days at a distance of 20 cm (about 8 inches) did not aggravate melasma lesions [130]. In addition, subjecting HaCaT cells to blue light radiation at 41.35 J/cm^2 and 453 nm caused a rapid increase in ROS after 1 h via photoreduction of intracellular flavins in normal human keratinocytes [131]. This oxidative stress leads to immediate and persistent hyperpigmentation.

7.6. Effect on Fibroblasts

Human dermal fibroblasts exposed to blue light at 450 nm at low intensities (<30 J/cm^2) revealed inhibitory effects on metabolic activity and procollagen I synthesis, with cytotoxicity at higher intensities (>30 J/cm^2) [73]. Furthermore, Austin et al.'s study found that merely one hour of exposure at a distance of 1 cm can promote the formation of ROS in fibroblasts, leading to premature aging of the skin [90]. Furthermore, it has been observed that fibroblasts are more sensitive to blue light radiation exposure (410–453 nm) at non-toxic dosages [116]. Furthermore, a recent study demonstrated that ROS production was identified between 400 and 500 nm but not at 582 nm in dermal fibroblasts treated with 150 J/cm^2 at various wavelengths [98].

7.7. Effect on the Epidermal Barrier

Human skin exposure to blue light induces changes in stratum corneum lipids, causing damage to the skin barrier and delaying repair after injury [132], as demonstrated by tape stripping.

7.8. Effect on Antioxidants

ROS production harms healthy skin cells and alters the amounts of endogenous antioxidants, essential for skin protection, which are depleted after exposure to blue light radiation [6]. Endogenous recovery may take up to 24 h [99].

Researchers discovered that exposing the skin to blue light between 380 and 495 nm (with a maximum at 440 nm and 100 mW/cm^2) reduced carotenoid levels. These antioxidants dropped by up to 20% with the highest dose but reverted to baseline levels after 1 h and 24 h with the half and high doses, respectively [74]. The degradation of dermal carotenoids shows the number of free radicals produced, particularly reactive oxygen radicals in the skin [74,100].

7.9. Effect on Collagen

The synthesis of collagen and elastin is negatively impacted by blue light exposure. This occurs when blue light or UV radiation is directly exposed to the skin because free radicals are produced. Blue light causes skin cells to produce matrix metalloproteinases (MMPs), which have been demonstrated to break down collagen and cause photoaging [133]. These MMPs destroy the existing collagen and impede new production [134]. Blue light radiation at different wavelengths can generate varied degrees of intracellular oxidative stress in dermal fibroblasts and decreased proliferation, resulting in premature skin photoaging [116]. A comparable study indicated that blue/violet light at lower wavelengths (410, 420 nm) demonstrated substantial toxicity to human skin fibroblasts, although blue light radiation at longer wavelengths (453, 480 nm) did not impair cell viability [135]. According to Solano, ROS caused inflammation and accelerated cellular aging by causing the breakdown of the main components of the dermis scaffold.

7.10. Changes in Pigmentation

According to recent studies, blue light irradiation produces skin pigmentation in the same way that UVA radiation does. This can cause hyperpigmentation, a visible symptom of photoaging, or age spots [3,85]. Blue light-induced skin pigmentation is more durable than UVA-induced pigmentation and is evident in skin phototypes IV–VI, but not phototype II [129].

7.11. Changes in Circadian Rhythm and Delay in Damage Repair

Blue light emission at 460 nm strongly inhibits melatonin, affecting circadian cycles and damaging skin cells [1]. Blue light radiation at 410 nm inhibits the transcription of the clock gene PER1 in keratinocytes, which is involved in the circadian rhythm. Skin cells can modulate PER1 expression based on their sensitivity to light. This excites the cells, especially their nocturnal cycle, which is crucial for regeneration and repair [1].

7.12. Effect on the Endoplasmic Reticulum and Autophagy

Lee et al. investigated the link between oxidative stress, endoplasmic reticulum (ER) stress, autophagy, and apoptosis in blue light-induced skin damage [136]. ER stress and autophagy are two systems that allow cells to withstand external interfering influences, preserve cell homeostasis, and maintain proper function, ultimately affecting apoptosis [136]. In addition, blue light radiation at 435–445 nm for 6–24 h induces autophagy in skin cells, hastening cell death. These findings show that blue light radiation activates the ROS-ER stress–autophagy–apoptosis axis signaling pathway, leading to skin damage and apoptosis.

7.13. Effect on the Structure and Elasticity of the Skin

Exposure to 415 nm for 18 min at 5 mW/cm^2 produces fiber fragmentation in the upper dermis [8]. This result confirmed that blue light radiation at 415 nm penetrates the dermis and can harm its structure and flexibility, highlighting the need to create skin protection techniques, including shielding from visible light [74]. Furthermore, in another study, a light dose of blue light radiation between 180 and 269 J/cm^2 was employed, corresponding to 38 days of exposure to indoor blue light, which confirmed skin change under these settings [20].

8. Comparison of Different Light Spectra

In general, visible radiation acts deeper in the dermis compared to ultraviolet radiation, while it is more superficial compared to infrared radiation. The maximum penetration of blue light radiation into the epidermis is 0.07–1 mm, while UVB shows low penetration in the skin, mainly in the epidermis [3]. In addition, the quanta of radiation in the visible spectrum carry more energy than infrared radiation, showing its thermal and chemical effects. Furthermore, the ratio of free radical formation in the skin during exposure to UV and visible light ranges from 67% to 33%, respectively [107]. Violet/blue light (400–500 nm), also known as HEV, has the capacity to penetrate deeper into the skin compared to UVA and UVB rays [126]. In fact, pigmentation generated by visible light is more durable than pigmentation caused by ultraviolet A radiation [129].

UV radiation is the principal cause of sun-exposed skin aging, but more emphasis is devoted to the detrimental effects of HEV light, as it has been demonstrated that up to half of the free radicals created in the skin are due to the visible parts of the spectrum [87]. In a study by Zastrow et al. [137], the free radical action spectrum encompassing UV and visible light (280–700 nm) was calculated for the first time, and it revealed that visible light accounted for 50% of the skin's total oxidant burden. It has been shown in *f* skin graft investigations that visible light exposure accounts for half of the formation of reactive oxygen species (ROS), whereas UV-B exposure accounts for 4% and UV-A exposure for 46% [3]. Since UVB causes DNA damage, HEV's effect on skin aging is similar to UVA's, such as in the case of hyperpigmentation, because both contribute to the mechanical production of free radicals [19]. Table 2 depicts the most significant beneficial and harmful effects of different types of sunlight radiation.

Table 2. Negative and positive effects of different types of sunlight radiation.

Wavelength	Negative Effects	Positive Effects	References
Ultraviolet (<400 nm)	Vitamin D synthesis, vitiligo, sterilization, atopic dermatitis	Photoaging, skin cancer, inflammation, sunburn	[42,46,48,50,51,54]
Violet light (400–450 nm)	Down-regulation of keratinocyte differentiation, inhibition of innate immunity-related responses	Early keloid	[72,77]
Blue light (450–490 nm)	Photoaging, hyperpigmentation, skin barrier damage, fibrobast function, collagen production, elasticity of skin	Acne, eczema, psoriasis, atopic dermatitis	[8,109,118–120]
Green light (450–490 nm)	Not determined	Recovery for third-degree burns	[75]
Orange light (560–630 nm)	Not determined	Reduction in UVA-induced ROS, up-regulation of collagen, reduction in triglycerides	[77,78]
Red light (630–700 nm)	Not determined	Up-regulation of collagen, barrier recovery, wound healing, DNA excision repair	[75,77]
Near-infrared light (700–3000 nm)	Generation of ROS, down-regulation of collagen	Skin tone, up-regulation of collagen, wound healing	[138]

9. Conclusions

Due to its advantages and disadvantages, light of different wavelengths has both positive and negative effects on our daily lives (Table 1). Some studies suggest UV radiation is a major cause of skin aging but is effective in promoting vitamin D synthesis and

alleviating skin diseases such as psoriasis. Blue light also causes oxidative stress, slows down the recovery of skin barriers, and, ultimately, has a negative effect on the skin; however, it also contributes positively to the elimination of *P. acne*. Therefore, in this review, we have provided an overview of the effects of different levels of solar radiation on human skin, as it is valuable to exploit the beneficial effects of each wavelength of light under the appropriate conditions (information that is currently missing in the literature). Additionally, many people continue to seek non-invasive procedures to improve medical and aesthetic skin conditions. Phototherapy is the use of non-thermal or non-invasive light to achieve therapeutic effects. Therefore, it is important to identify photoreceptors and elucidate their underlying mechanisms in the skin.

In addition, in combination with systems biology, we can gain insights into which wavelengths are most effective for specific skincare or the treatment of skin-related diseases. Strategies to detect each wavelength of light at a specific intensity can help improve skin health.

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Effect of Fermentation and Extraction Techniques on the Physicochemical Composition of Copoazú Butter (*Theobroma grandiflorum*) as an Ingredient for the Cosmetic Industry

Luisa L. Orduz-Díaz, Kimberly Lozano-Garzón, Willian Quintero-Mendoza, Raquel Díaz, Juliana E. C. Cardona-Jaramillo *, Marcela P. Carrillo, Diana Carolina Guerrero and Maria Soledad Hernández

Instituto Amazónico de Investigaciones Científicas Sinchi, Calle 20 # 5-44, Bogotá 110311, Colombia; lorduz@sinchi.org.co (L.L.O.-D.); wiquintero@sinchi.org.co (W.Q.-M.); rdiaz@sinchi.org.co (R.D.); mcarrillo@sinchi.org.co (M.P.C.); dguerrero@sinchi.org.co (D.C.G.); shernandez@sinchi.org.co (M.S.H.)
* Correspondence: jcardona@sinchi.org.co

Abstract: The Copoazú is a *Theobroma* species of Amazonian origin, and its derived products have a high content of lipids valuable for both the cosmetic and food industries. The composition of the butter extracted from its seeds can vary depending on the postharvest process and the diverse extraction techniques employed. In this study, the composition of this butter processed with and without seed fermentation was analyzed using two extraction techniques: expeller pressing and hydraulic pressing. Parameters such as lipid profile, quality indexes, melting point, and the content of phytosterols and glyceric compounds were compared with a highly sought-after commercial raw material assessed through standardized volumetric and spectroscopic methodologies. The results showed that non-fermentation and cold-pressing conditions preserved the properties of the butter. This analysis is the first step in a standardized process for developing high-quality cosmetic ingredients derived from Copoazú butter.

Keywords: copoazú; copoazú butter; fermentation; cosmetics; pressing

1. Introduction

The utilization of natural ingredients (NIs) derived from diverse plant sources is emerging as a strategic approach for creating value during the shift towards a sustainable biobased economy, especially in biodiversity-rich countries. In these transitional models of the bioeconomy, Colombia has made efforts in various regions of the country through the implementation of agroforestry crops that provide both commodity and non-commodity benefits. Additionally, these models offer environmental advantages contributing to deforestation prevention, soil restoration, and socio-economic improvements for the local population [1,2].

Copoazú (*Theobroma grandiflorum*), also known as white cocoa or Amazonian cocoa, is a tropical tree from the Malvaceae family distributed across Brazil, Colombia, Peru, and Venezuela [3]. This widely distributed genus throughout the Amazon basin and the Orinoco region is one of the oldest with significant potential for local, regional, and global use. Cocoa (*Theobroma cacao*), Copoazú (*Theobroma grandiflorum*), and maraco (*Theobroma bicolor*) are the economically most-utilized species within the Theobroma genus. Unlike cocoa, all parts of the Copoazú fruit are utilized, either for direct consumption or for the extraction of natural ingredients and derivatives [4].

Cultivating Copoazú is integral to agroforestry systems in the Colombian Amazon, guided by a comprehensive management plan as a non-timber forest product (NTFP). This strategic approach not only supports the substitution of illicit crops but also contributes to the mitigation of deforestation and addresses the adverse effects of climate change. Additionally, Copoazú plays a role in the country's economic transition by strengthening rural

development and paving the way for the implementation of value chains for biodiversity products with high added value [1,5].

Copoazú seeds contain approximately 50% lipids, polar compounds with antioxidant properties, and emollients that are found attractive in the cosmetic and food industry, mainly due to their similarity with cocoa butter. This is why copoazú butter has been used as an alternative NI by several companies in the development of new products, primarily in the cosmetic field [6]. Regarding the cosmetic properties of the fat, this butter serves primarily as a natural emollient, featuring softening and moisturizing attributes. Its composition makes it suitable for use in emulsions, such as hydrating creams and lotions, as well as in soaps and other bioproducts with a high level of complexity [7]. For fat extraction, many mechanical and chemical techniques have been developed to preserve sample integrity and enhance yields. Since solvent-assisted and mechanical techniques are widely adopted for their simplicity and cost-effectiveness, newer approaches such as Supercritical Fluid Extraction (SFE), Ultrasound-Assisted Extraction (UAE), Enzyme-Assisted Extraction (EAE), and Microwave-Assisted Extraction (MAE) have shown considerable potential, albeit requiring more advanced technological infrastructure [8].

In the Colombian Amazon region, diverse communities and organizations of copoazú producers and processors possess the production capacity to potentially fulfill the increasing demand for copoazú butter [9]. Accordingly, the comprehensive utilization of Copoazú has become a tool for generating resources that enable the improvement of the quality of life of the inhabitants of the Amazon, and it is crucial to comprehend the implementation process required to meet market demands effectively. The value chain of Copoazú currently involves two production hubs (one well-established and expanding and one in development), four local grassroots organizations that bring together over 500 families, 240 hectares of sustainable production systems (agroforestry arrangements), and 4000 hectares of conserved forest designated as such via agreements [10].

To potentially contribute to a real sustainable use of nature and promote Natural Ingredient Value Chains (NIVCs), efforts need to be directed towards research and strengthening the knowledge base of NIs in accordance with a value chain (VC) approach [1,11]. There is a gap in terms of strengthening the technical and technological capacities of these NIs, as well as scientific research based on the processes of obtaining them, to ensure they meet the required quality standards and are truly competitive in the market [1,5].

This study aims to evaluate various copoazú butter extraction treatments (including both fermented and non-fermented processes, using an expeller or hydraulic pressing) conducted by an association of producers and processors in the Caquetá region of the Colombian Amazon. This assessment focuses on their impact on physicochemical composition, comparing the results with a sample of commercially sought-after copoazú butter available on the international market. The objective is to provide information supported by scientific evidence to inform decision-making in processes that enhance the value chain of this country's copoazú butter. To achieve this, this evaluation involves the quality parameters of the butter (quality indices), its fatty acid profile, infrared spectroscopy (IR) findings, phytosterol analysis, triglyceride analysis, and the color of each extraction treatment.

2. Materials and Methods

2.1. Copoazú Butter Extraction

Copoazú seeds obtained from pulping, containing approximately 30% natural pulp, were placed in a fermentation box and covered with clean cloth or canvas to prevent insect infestation. Manual turning was performed every two days, stirring the fermenting mass from the bottom of the container. Fermentation was evidenced by the seeds heating up (45 to 50 °C) in the initial days and subsequently exhibiting acidity. Cutting tests were also conducted to monitor the fermentation process, which lasted a total of 10 days.

After fermentation, the seeds were dried in a solar drying system with temperatures ranging between 37 and 60 °C for 10 days until reaching a moisture content of 6 to 10%. Non-fermented seeds were also dried under the same conditions.

Once dried, the seeds were subjected to mechanical extraction processes using two types of presses: an expeller press and a hydraulic press. The extraction technologies evaluated were selected not only because they are clean technologies but also because of their ease of operation and the availability of machinery in the processing plants of the community organizations with which this study was conducted. The operating parameters for both pressing methods are not described as they are part of proprietary development. Temperature ranges between 40 and 60 °C were utilized.

For our research purposes, four treatments using Copoazú butter from the Caquetá department of Colombia were conducted, namely, extracting non-fermented butter via expeller pressing (WF_EXP), extracting fermented butter using expeller pressing (FER_EXP), extracting non-fermented butter via hydraulic pressing (WF_HP), and extracting fermented butter using hydraulic pressing (FER_HP), while a trading pattern was used as control (COM). Butter extraction was conducted using the same batch of copoazú from the same producer to ensure the reliability of this study.

2.2. List of Applied Reagents

Glacial acetic acid, potassium iodine, sodium thiosulfate, sodium hydroxide, soluble starch, analytical-grade chlorophorm, pyridine, isoctone, THF, and methanol were purchased from Merck (Darmstadt, Germany). Methanolic boron trifluoride (BF_3) (10%), Fatty Acid Methyl Esters (Standard FAME 37), N, and O-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSFTA + TMCS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The external phytosterols standards (cholesterol, stigmasterol, campesterol, brassicasterol, and β-sitosterol) were purchased from Dr. Ehrenstorfer (Augsburg, Germany).

2.3. Instrumental Color Determination

The instrumental color was determined using a HunterLab MiniScan™ XE Plus colorimeter (HunterLab, Reston, VA, USA) via the HunterLAB scale and by performing mathematical conversion via the CieLAB scale.

2.4. Evaluation of Butter Quality Indices and Physicochemical Properties

The acidity value, saponification value, and iodine value were determined, following the same methodology described by Lozano-Garzón et al. [12], wherein titration, according to the AOCS methods, was carried out. All the analyses were performed in triplicate.

The peroxide value was determined by modifying the protocol proposed in USP (2013) [13]. For this purpose, butter samples were dissolved in a mixture of chloroform and glacial acetic acid in a 2:3 ratio. Immediately, 0.5 mL of saturated potassium iodine solution was added and shaken vigorously for a minute; then, 30 mL of water was added. The mixture was titrated with 0.01 N sodium thiosulfate until the yellow color almost disappeared. Finally, 5 mL of saturated starch solution was added, and titration was continued until the blue color vanished. The peroxide value is expressed in mEq of active O_2 per kg of sample.

The melting point was determined using a Thiele tube containing mineral oil and a mercury thermometer; all samples were measured in triplicate.

2.5. Determination of Fatty Acid Profiles

To obtain fatty acid profiles, derivatization with methanolic boron trifluoride (BF_3) was carried out using the methodology reported by Lozano-Garzón and Cardona Jaramillo [12,14]. The analysis was conducted utilizing an Agilent 7890B Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a Flame Ionization Detector (FID). Chromatographic separation was achieved using an Agilent DB23 column (60 m × 0.25 μm × 250 μm) (Agilent Technologies, Santa Clara, CA, USA), with Helium employed as the carrier gas at a constant flow rate of 1.1 mL/min. The injector temperature was set to 270 °C in split mode with a split ratio of 30:1. The initial oven temperature was maintained at 60 °C for 1 min, followed by a programmed increase of 6 °C per minute until reaching

210 °C, and this temperature was maintained 24 min. The detector temperature was set to 310 °C. The air flow rate was maintained at 400 mL/min, hydrogen flow was maintained at 40 mL/min, and makeup gas (helium) was maintained at 30 mL/min, compensated for with column flow. Additionally, a solvent delay of 9 min was implemented.

2.6. Determination of Phytosterol Profiles

An analysis of phytosterols was carried out according to the methodology outlined by Lozano-Garzón [12]. This involved obtaining the unsaponifiable fraction, followed by derivatization using N, O-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSFTA + TMCS) and pyridine. The analysis was conducted using an Agilent 7890B Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled with a Flame Ionization Detector (FID). Separation of compounds was achieved using an Agilent HP5-MS column (30 m × 0.25 μm × 250 μm) (Agilent Technologies, Santa Clara, CA, USA), with helium serving as the carrier gas at a constant flow rate of 1 mL/min. The injector temperature was set to 270 °C in split mode with a split ratio of 15:1. Initially, the oven temperature was maintained at 250 °C for 1 min, followed by a programmed increase of 2 °C per minute until reaching 300 °C, holding this temperature for 7 min. The detector temperature was maintained at 300 °C. The air flow rate was set to 400 mL/min, with hydrogen flow set to 40 mL/min and makeup gas (Helium) set to 30 mL/min. Additionally, a solvent delay of 9 min was incorporated into the method [15].

2.7. Evaluation of Triglyceride Profiles

Triglyceride analysis was performed via GC-FID, employing samples prepared in THF at a concentration of 5 mg/mL. The analysis was performed using an Agilent 7890B Gas Chromatograph coupled with an FID detector under the following conditions using an Agilent DB17 HT column (15 m × 0.25 μm × 150 μm) and helium as a carrier gas at 1.02 mL/min: injector temperature, 360 °C (20:1); initial oven temperature, 250 °C; 5 °C/min increase up to 350 °C (20 min); detector temperature, 310 °C; air flow, 400 mL/min; hydrogen flow, 40 mL/min; makeup (helium), 30 mL/min (compensated for with column flow); solvent delay, 1 min [16].

2.8. Attenuated Total Reflectance Fourier Transform Infrared (FTIR-ATR) Analysis

FTIR-ATR spectra of all samples were acquired with a Jasco 4600 FT/IR spectrometer (Jasco, Tokyo, Japan) with 4 cm^{-1} resolution in the 3500–400 cm^{-1} wavelength region using an attenuated total reflectance device (ATR) with single-diamond reflection and a zero-fill factor of 2. The spectra were obtained with 32 scans, and the average of each sample was calculated automatically using Jasco Spectra Manager software version 2.15.01.

2.9. Statistical Analysis

ANOVA was performed to analyze variables, namely, quality values, instrumental color, and melting point, followed by multiple comparisons with the Tukey test (maximum significant difference) using Statistix9 v8.1 software and a significance of 5%. Principal component analyses (PCAs) were performed using Simca 14.1 developed by Umetrics, using the lipid profile data and the percentage of the areas of each signal, along with the TAG profile (each retention time was an input variable), as input variables.

3. Results

Color is a significant attribute that not only serves as an initial indicator of product quality but also has implications for the formulation of food or cosmetic products. L*, a*, b* notation is used to represent different aspects of color. L* measures the difference between brightness and darkness, a* indicates the difference between red and green, and b* signifies the difference between yellow and blue. This notation was employed to evaluate the color of the butter. According to the results presented in Table 1 and Figure 1, a notable difference was observed between the various treatments evaluated and the control sample.

Table 1. Instrumental color on copoazú butter.

Sample	L*	a*	b*
COM	78.79 ± 5.80 A	2.13 ± 0.26 B	17.74 ± 0.91 A
FER EXP	69.22 ± 1.76 B	3.20 ± 0.19 B	7.78 ± 0.69 C
FER HP	75.43 ± 1.31 AB	5.78 ± 0.48 A	11.61 ± 0.09 B
WF EXP	70.10 ± 2.94 AB	4.90 ± 0.30 A	10.96 ± 1.38 B
WF HP	77.19 ± 2.28 AB	5.18 ± 0.63 A	10.15 ± 0.97 BC
p-Value	0.0154	0.0000	0.0000

Rows with the same letter (A, B, C, AB, BC) do not show a significant difference (n = 3, α= 0.05).



Figure 1. Color of the control sample (commercial) and the four evaluated treatments. WF EXP: extracted non-fermented butter via expeller pressing; FER EXP: extracted fermented butter using expeller pressing; WF HP: extracted non-fermented butter via hydraulic pressing; FER HP: extracted fermented butter using hydraulic pressing; COM: trading pattern used as control.

Regarding the quality indexes, in all four treatments, the most significant difference was the saponification value (Table 2). This value is a measure of the average molecular weight of the fatty acids in a fat or an oil. It is based on saponification, a chemical reaction that involves breaking the ester bonds present in a sample.

Table 2. Quality indexes and melting points of control and evaluated treatments.

Sample	Acidity Index (mg KOH/g Sample)	Iodine Value (g I_2/100 g Sample)	Saponification Index (mg KOH/g Sample)	Peroxide Index meq of Active O_2/kg Sample	Melting Point (°C)
COM	4.83 ± 0.12 C	48.83 ± 0.42 A	86.50 ± 4.69 B	2.67 ± 0.23 B	34.33 ± 1.21 AB
FER EXP	8.37 ± 0.31 A	43.13 ± 2.82 B	59.80 ± 5.79 C	3.27 ± 0.12 B	32.33 ± 1.86 B
FER HP	8.30 ± 0.00 A	40.30 ± 2.44 B	99.77 ± 6.81 AB	3.50 ± 0.10 AB	32.50 ± 1.05 B
WF EXP	6.17 ± 0.12 B	41.60 ± 0.62 B	103.93 ± 9.87 AB	4.33 ± 0.31 A	34.33 ± 1.21 AB
WF HP	4.87 ± 0.29 C	41.07 ± 1.10 B	117.93 ± 10.24 A	3.47 ± 0.61 AB	35.33 ± 1.51 A
p-Value	0.0000	0.0010	0.0000	0.0017	0.0035

Rows with the same letter (A, B, C, AB) do not show a significant difference (n = 3, α= 0.05).

The variations in iodine values were non-significant; nevertheless, COM exhibited a slightly higher value, implying a potentially greater amount of unsaturated fatty acids. For the acidity index, only WF_HP was comparable with the commercial sample; fermentation processes produce free organic acids, which could cause a decrease in pH.

On the other hand, fatty acids were identified via comparison with a fatty acid methyl ester (FAME) Sigma Supelco 37 standard, and the relative composition was determined by integrating the obtained peaks (Table 3).

Table 3. Fatty acid relative compositions.

ID	COM (%)	WF EXP (%)	FER HP (%)	FER EXP (%)	WF HP (%)
Palmitic (C16)	9.72	9.62	9.90	9.72	10.39
Palmitoleic (C16:1)	0.21	0.20	0.19	0.00	0.21
Stearic (C18)	34.74	32.77	32.67	34.50	32.33
Oleic (C18:1)	40.17	41.37	39.64	40.57	40.51
Linoleaidic (C18:2)	0.53	0.52	0.55	0.50	0.57
Linoleic (C18:2)	3.52	4.45	6.80	4.54	4.86
Arachidic (C20)	9.72	9.65	9.07	9.15	9.75
Gadoleic (C20:1)	0.27	0.31	0.21	0.17	0.27
Behenic (C22)	1.13	1.11	0.97	0.86	1.12

On the other hand, quantitative data were submitted to multivariate analysis, and a principal component analysis (PCA) (Figure 2a) was performed, finding that the main differences between the samples were due to the concentrations of linoleic and stearic acid (Figure 2b). This analysis also showed that there is a greater resemblance between COM and the non-fermented samples.

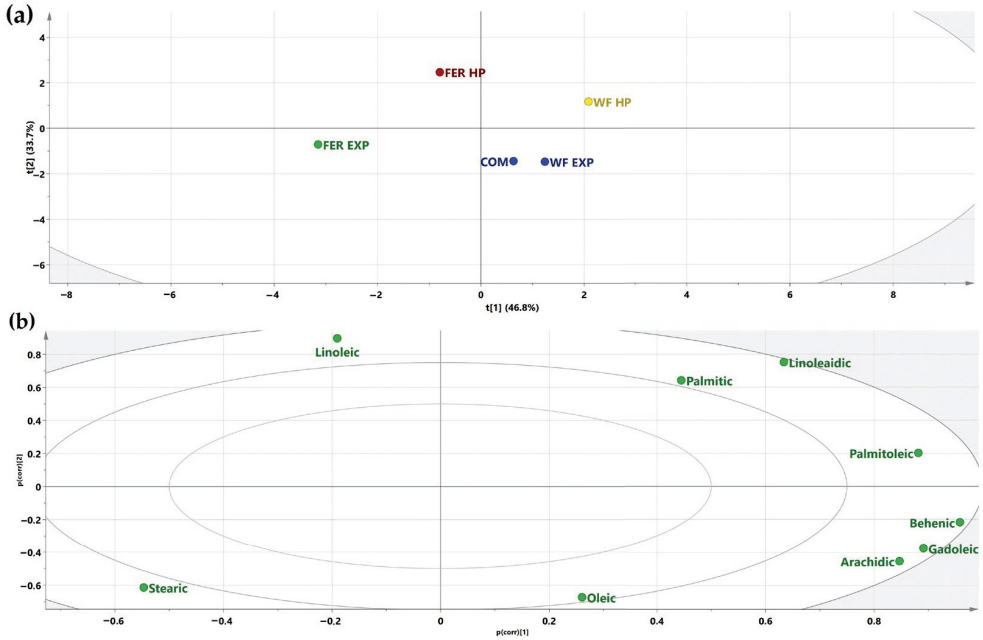


Figure 2. (**a**) Score plot based on the composition of fatty acids in the treated copoazú butter. The x-axis (T [1]) explains 46.8% of the total variance, while the y-axis (T [2]) explains 33.7%, for a total variance of 70%. (**b**) The axes represent Pearson correlation coefficients in the loading plot based on the composition of fatty acids in the treated copoazú butter.

Triglycerides showed a high complexity pattern but remained very similar (Figure 3) in all cases. All the profiles presented signals between 20 and 40 min, with WF EXP presenting the lowest intensities. This uniformity in the observed patterns suggests a notable consistency in triglyceride composition, while the subtle variations in signal intensities among cases provide valuable insights into the nuanced distinctions within these lipid profiles.

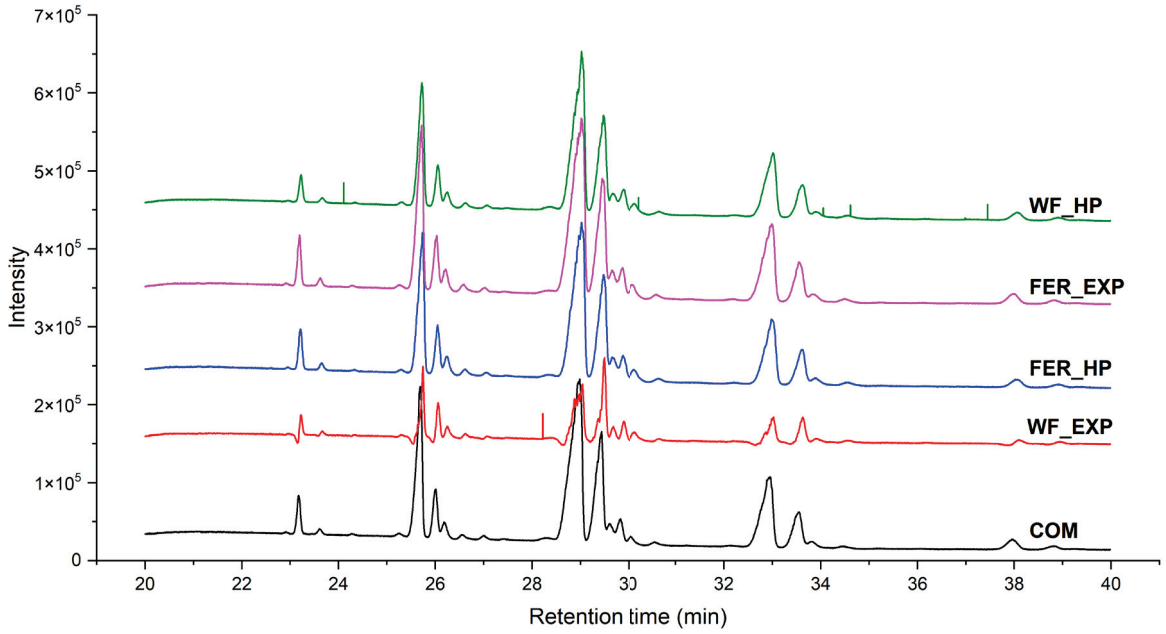


Figure 3. Chromatographic profile of triacylglycerols in copoazú butter samples obtained via GC-FID-HT analysis.

Despite the chromatograms exhibiting low quality, characterized by broad signals with low resolution (due to the non-volatile nature of triglyceride molecules), a principal component analysis (PCA) was conducted to assess the similarities among the samples. The results shown in Figure 4 corroborate the differences observed regarding WF EXP and the similarity between COM and FER HP.

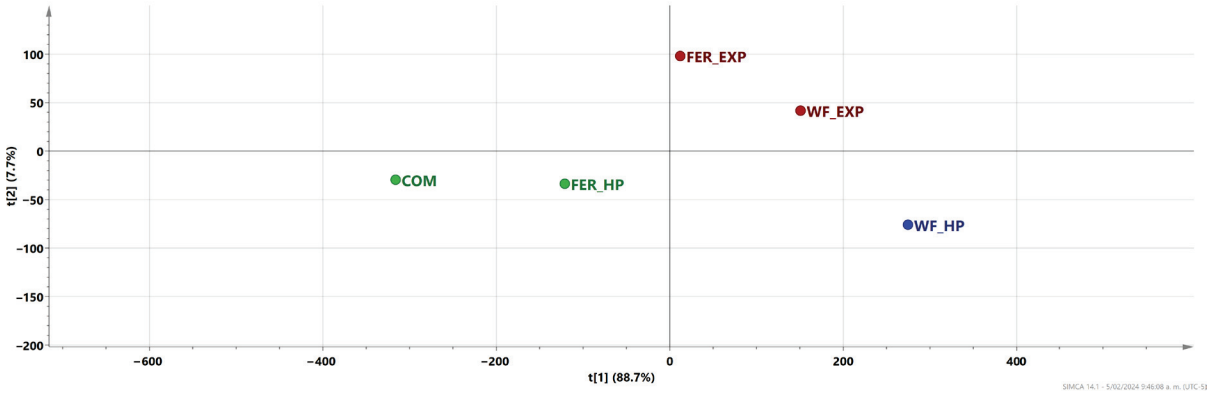


Figure 4. Multivariate analysis based on the chromatographic data of the triacylglycerol profiles. Here, 95% of the total variance of the data is presented in the first two components.

Phytosterols stand out as remarkable molecules within the cosmetics industry, primarily because of their well-documented photoprotective capabilities. These compounds function as an effective barrier shielding the skin from dehydration. Notably, when compared to cholesterol, phytosterols have a lower probability of inducing acne when included in topical products [17,18], which can be considered a comparative advantage in the formulation of cosmetics. Table 4 shows the results of the composition of phytosterols present in the unsaponifiable fraction of copoazú butter.

Table 4. Phytosterols profile in the unsaponifiable fraction of copoazú butter.

	Campesterol (µg/g)	Stigmaterol (µg/g)	β-Sitosterol (µg/g)
COM	-	680 ± 5	5244 ± 10
WF_EXP	-	632 ± 5	3532 ± 10
FER_HP	322 ± 2	812 ± 7	6006 ± 10
FER_EXP	300 ± 3	860 ± 5	6326 ± 11
WF_HP	-	880 ± 7	6180 ± 10

Complementarily, an IR analysis was carried out to establish differences between treatments and obtain a profile that allows quick a diagnosis of the samples. As can be observed in Figure 5, the spectral comparison revealed minimal differences between samples, suggesting that this technique may not be conducive to the intended quality control procedures.

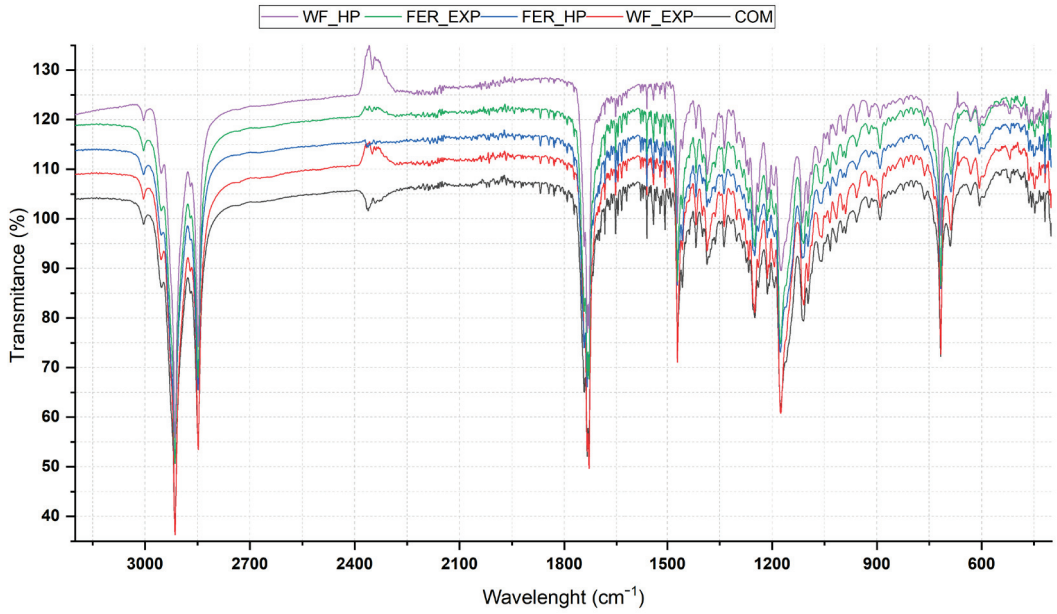


Figure 5. Average FTIR-ATR spectrum of copoazú butter samples.

4. Discussion

When evaluating color, a noticeable change in the b* parameter was observed, signifying a more pronounced intensity of the yellow color in COM. This resulted in stronger tones significantly differing from the other samples with a lighter coloration, particularly FER_EXP. This may be due to the pH and temperature changes occurring during pressing that could induce the degradation of the triglycerides present in the butter, as suggested by the decreased luminosity. In contrast, FER_HP and WF_HP showed an increase in a*, indicating a slightly redder tone, which might be attributed to preserved carotenoids in the cold-pressed samples.

The copoazú butter obtained through hydraulic pressing exhibited higher levels of brightness, possibly due to a process conducted at lower temperatures, resulting in a butter with a distinct crystalline structure and, consequently, varying opacity compared to that obtained through expeller pressing [19]. On the other hand, the darker coloration observed in the four treatments compared to the commercial sample may be linked to factors related to the soil conditions of copoazú growth. However, it is more likely associated with the fermentation process, as noted in a study on *Theobroma cacao*, where it was shown that fermentation significantly influences butter coloration [20,21]. This difference is also influenced by the unknown fermentation process of the commercial sample. As extensively researched, the fermentation of *Theobroma* grains leads to changes in both physical and

chemical properties. Various enzymatic reactions alter components such as polyphenols, proteins, and carbohydrates, among others [20,22].

Saponification value evaluation is crucial as it provides insight into the number of bonds per gram of a sample, offering a detailed reflection of the chemical composition and structure under examination. A lower saponification index usually hints at a reduced number of triglycerides in both fermented samples. The most significant contrast was noted in FER_EXP. The fermentation process induces acidification of the matrix, potentially causing partial triglyceride hydrolysis. During expeller pressing, temperatures of 80 to 100 °C can be reached, caused either by friction or the temperature ramp programed. Hydraulic pressing is performed with lower temperatures (approximately 40 °C), which are just enough for melting the butter. Higher temperatures during extractions may result in partial hydrolysis too. This divergence underscores the distinct impact of the fermentation process on the chemical dynamics and potential transformations within the matrix.

A high acidity index usually indicates poor quality since it is related to compounds such as lactic acid, citric acid, and other derivatives of fermentative processes that affect pH and some organoleptic properties [18,23]. Lipid peroxidation derived from fermentation can be ruled out since there were no significant differences in the values of the peroxide index. The results exhibit similarities with those reported for Copoazú butter by Carrillo and Moreno [7,24]. The acidity levels range from 1.0 to 15 mg of KOH/g sample; additionally, the values of the iodine and saponification indices align with the fatty acid profiles of the evaluated samples, showcasing a predominant presence of oleic and stearic acids. Furthermore, the low peroxide index values indicate that Copoazú butter has reduced susceptibility to oxidation.

The primary difference in fatty acid composition among the samples was attributed to the content of linoleic acid, with COM exhibiting a lower amount of this substance. Polyunsaturated fatty acids (PUFAs) applied to the skin help to restore the lipid barrier, integrating into the lipid layers and interfering in inflammatory processes related to prostaglandin synthesis, for which a higher proportion of PUFAs can improve the properties of the final product [25]. The technical data sheet of the commercial sample provides detailed ranges for each of the fatty acids: when compared with the expected values, FER HP and FER EXP were 0.14% lower for behenic acid (1–2.5%), and FER HP also showed a 0.36% lower amount of oleic acid, while the other samples presented a higher content (5–9%) of palmitic acid than expected.

The fatty acid profiles exhibit similarities with those reported in Copoazú butter studies [7,24,26], identifying oleic acid as the predominant component, followed by stearic acid. This composition has a direct effect on the melting point of butter due to the presence of unsaturated fatty acids [27]. Moreover, fatty acids have a determining role in cellular functions and influence proliferation processes. Essential unsaturated fatty acids (linoleic and arachidonic), which can be found in Copoazú butter, are highly recognized for their impact on inflammation and tissue-healing processes [28–30]. The presence of oleic acid further accelerates healing and scar tissue formation, making it a versatile matrix with applications in cosmeceuticals [26].

For *Theobroma grandiflorum*, the following triglyceride values were reported per kilogram of sample: PLiP, 6 (Palmitic Linoleic Palmitic triglyceride, 6); OOO 20, (Oleic Oleic Oleic triglyceride, 20); POO, 43, (Palmitic Oleic Oleic triglyceride, 43); PliS, 11 (Palmitic Linoleic Stearic triglyceride, 11); POP, 11 (Palmitic Oleic Palmitic triglyceride, 11); SOO, 161 (Stearic Oleic Oleic triglyceride, 161); SLiS, 18 (Stearic Linoleic Stearic triglyceride, 18); POS, 120 (Palmitic Oleic Stearic triglyceride, 120); OOA, 78 (Oleic Oleic Arachidic triglyceride, 78); SOS, 3.14 (Stearic Oleic Stearic triglyceride, 3.14); SOA, 181 (Stearic Oleic Arachidic triglyceride, 181) [31]. In this study, the signals were neither identified nor quantified as the analysis was conducted solely for comparative purposes. However, this TAG profile is correlated with the fatty acid profile presented in Table 3.

The fermented samples contained campesterol (Table 4), which could be a result of microbial development, such as the formation of cell membranes. Phytosterols support

microbial growth by acting as photoprotectors and maintaining cell integrity regardless of the pH and temperature changes associated with the fermentation processes [18]. During the extraction of the fermented material, the compounds resulting from the metabolism of microorganisms are also extracted.

The IR spectra (Figure 5) show C-H stretching bands in aliphatic chains for methyl and methylene close to 2900 and 2850 cm^{-1}, as well as an intense band of C=O stretching characteristic of carboxylic acids and asymmetric C-H-C methylene bending at 1471 cm^{-1}. The spectra obtained (Figure 5) for the samples mainly present characteristic fatty acid bands. When comparing the spectra of each sample, slight differences were observed, both in frequency and intensity, showing that this technique is not helpful for the desired quality control process [17].

5. Conclusions

To obtain a product with similar characteristics to the commercial sample, it is advisable to avoid fermentation and to conduct copoazú butter extraction using hydraulic pressing. The standardization of the copoazú butter extraction process can contribute to the development of local industry by strengthening the capabilities of local producers and enhancing the quality of the natural ingredient obtained, aiming to make it competitive in both national and international markets. This, in turn, encourages processes that have the potential to contribute to a genuinely sustainable use of nature and promote Natural Ingredient Value Chains (NIVCs).

On the other hand, according to the physicochemical results, the presence of campesterol could be used as a diagnostic parameter to determine whether fermentation was carried out prior to the extraction of copoazú butter from the seeds.

Although heating during the extraction step can help improve yields, it can also lead to the degradation of some compounds of interest, such as carotenoids and acylglycerols.

For future work, we recommend assessing the physicochemical composition concerning pressing yield, as this factor plays a crucial role when considering scaling up for industrial processes.

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Review

Adult Female Acne: Recent Advances in Pathophysiology and Therapeutic Approaches

Andreea Amuzescu [1,2], Mircea Tampa [1,2], Clara Matei [2,*] and Simona Roxana Georgescu [1,2]

[1] Department of Dermatology, "Victor Babes" Clinical Hospital of Infectious Diseases, 030303 Bucharest, Romania; amuzescuandreea@gmail.com (A.A.); dermatology.mt@gmail.com (M.T.); srg.dermatology@gmail.com (S.R.G.)
[2] Department of Dermatology, "Carol Davila" University of Medicine and Pharmacy, 020021 Bucharest, Romania
* Correspondence: dermatology.cm@yahoo.com

Abstract: Adult acne is a chronic inflammatory disease of the pilosebaceous unit characterized by the excessive production of abnormal sebum favoring an imbalance of the skin microbiota and the hyperproliferation of *Cutibacterium acnes* and other virulent microbial strains, leading to an inflammatory environment, innate immunity overactivation, and keratinocyte hyperproliferation in hair follicles pores. Degraded keratinocytes plug the pores, consequently forming microcomedons, which can later evolve to papules, nodules, pustules and scars. Distinct from juvenile acne, in adult female acne (AFA) the symptomatology occurs or persists in postadolescence (after age 25). Although hyperandrogenism or the excessive sensitivity of androgen receptors are the main causes, AFA can be triggered by multiple factors, either including or not including androgen disturbances. The prevalence in adult women is 15–20%. Hyperandrogenism is present in 50% of cases; 70% of hyperandrogenism cases feature polycystic ovary syndrome (PCOS), a complex endocrine and metabolic condition. Genetic susceptibility occurs in 80% of acne cases, often with familial inheritance. Beyond classical stepwise therapeutic protocols (topical agents, isotretinoin, antibiotics, hormonal therapy with estrogens, progestins, spironolactone), novel approaches include the highly effective topical antiandrogen clascoterone, the management of insulin resistance by diet, exercise, stress avoidance, and adjuvant therapies such as berberine. Vaccines against the pathogenic proinflammatory *C. acnes* hyaluronidase A are in development.

Keywords: adult female acne; hormonal acne; hyperandrogenism; polycystic ovary syndrome; insulin resistance; genetic studies; antiandrogens

1. Introduction

Considering the increasing number of acne cases after puberty, this pathology cannot be considered a disease of adolescence anymore, as it significantly affects sufferer's quality of life, possibly leading to depression and social anxiety [1].

The worldwide occurrence of acne among women across all age brackets stands at approximately 10% [2,3], with large variations across age groups [4]. Among adult women, the prevalence of acne ranges from 15 to 20% [5], with 20 to 30% of these cases exhibiting hirsutism [6], while a proportion varying between 18 and 88% demonstrate elevated androgen levels [7]. This suggests that the primary etiological factor lies in either heightened androgen secretion or the increased sensitivity of androgen receptors [8].

The majority of authors define acne as a chronic inflammatory illness affecting the pilosebaceous gland, resulting in increased sebum production. Although patients have a polymorphic clinical picture similar to *acne vulgaris*, adult female acne (AFA), also named postadolescent acne or hormonal acne, is defined as a chronic inflammatory disease of the pilo-sebaceous follicle predominantly distributed in the lower third of the face, appearing especially in adult women over 25 years of age [9].

So far, two clinical forms of AFA have been described: 1. the inflammatory form (accounting for 58% of cases), presenting papulopustular lesions and nodules that often result in residual hyperpigmentation; hyperseborrhea may not always be evident with this form, mainly located at the mandible level, and 2. the retention form, manifested by open comedones and microcystic lesions, with a reduced number of inflammatory lesions; hyperseborrhea is consistently present, affecting not only the jawline but also the upper facial areas, particularly the forehead [10]. In terms of evolutionary progression, AFA can be categorized into three groups: persistent acne (the most prevalent type), late-onset acne (developing for the first time after age 25), and recurrent acne (emerging during adolescence, disappearing for several years, then resurfacing in adulthood) [1,10].

Multiple factors contribute to the development of hormonal acne in adult women, including genetic predispositions, hormonal fluctuations, and external influences such as diet, smoking, heightened stress levels, skincare products, and use of medication. Chronic inflammation is particularly significant in this process. The scientific literature supports the hormonal hypothesis regarding the origin of AFA, which stems from the increased activity in androgen hormone metabolism and related enzymes like 5α-reductase. These enzymes are expressed in the skin hair follicles keratinocytes and sebaceous glands, as well as in the gonads (ovaries and testicles). They catalyze the conversion of testosterone to its active form, dihydrotestosterone (DHT), which exhibits a stronger binding affinity to human androgen receptors (AR) than testosterone. Consequently, the DHT-androgen receptor complex is considerably more stable [4,11].

Although the general pathophysiology of acne is exquisitely complex, most authors consider four fundamental processes at hair follicle level (Figure 1), interconnected by a large and variable network of feedback loops. The main triggers of the disease are androgens, particularly dehydroepiandrosterone sulfate (DHEAS) reconverted to active androgens like testosterone and DHT by steroid-metabolizing enzymes of follicular keratinocytes. Androgens stimulate keratinocyte proliferation and sebaceous glands secretion, producing the comedo plug, which is rapidly colonized by pathogenic *C. acnes* strains. These bacteria convert sebum lipids into free fatty acids, further promoting bacterial growth, while sebum lipoperoxides and bacterial factors trigger innate immunity and inflammatory mechanisms [12]. However, *C. acnes* is ubiquitous in sebaceous glands-containing skin after puberty, therefore the model stating its hyperproliferation in comedo-plugged follicles seems outdated; it seems rather that highly pathogenic and virulent variants contribute to acne dysbiosis [13–15].

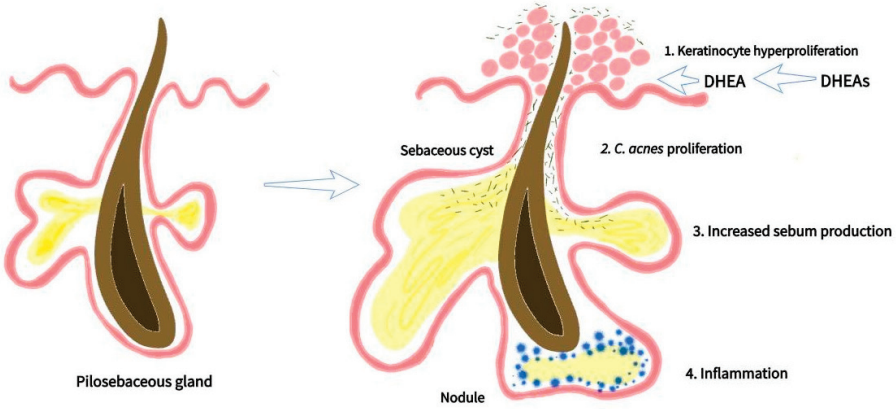


Figure 1. The main four pathophysiological processes involved in cutaneous acne lesions.

The distention of microcomedones eventually results in their rupture in the surrounding skin, inoculating the dermis with cell debris, keratin, sebum and bacteria, which trigger an acute inflammatory reaction [16]. Perifollicular CD4+ lymphocytes quickly invade the lesions during the first day, followed after 24 h by neutrophils, but the former play an essential role by activating specific pathways, such as the following: TLR2 and IFN-γ

signaling in Th1 cells with IL-12 secretion, TGF-β, IL-1β and IL-6 signaling in Th17 cells with the inhibition of Treg (Foxp3$^+$) cells [17], and TLR2,4 and NLRP3 inflammasome activation in macrophages with signal transmission along NF-κB, MAPK and caspase-1 pathways, resulting in increased IL-1β, TNF-α, IL-8 and IL-12 secretion [14] and many other molecular events. Both the innate and adaptive immune system play a role in acne, and there is a temporal and stage-dependent succession in perilesional cell infiltration and inflammatory signals [18]. The neurogenic component of inflammation is also important, and a remarkable electron microscopy study proved the existence of ultrastructural changes induced in sebaceous gland cells by neuropeptides such as substance P [19].

The aim of this study was to perform a systematic review focused on the pathophysiology of AFA and a related condition, polycystic ovary syndrome (PCOS), particularly on novel discoveries and genetic/epigenetic findings, as well as to highlight new therapeutic approaches. The relevant literature was retrieved by performing searches of the PubMed database with the keyword combination "adult female acne", "hormonal acne", and the same terms combined with "AND treatment".

2. New Insights into the Pathophysiology of AFA and PCOS

2.1. Hyperandrogenism and Increased AR Sensitivity

Hyperandrogenism was for a long time suspected to play a major role in the etiopathogenesis of AFA, hence the alternate name hormonal acne. The variation in acne prevalence with age is an indirect proof, with values ranging from 50 to 55% in high school or college students which are related to puberty-induced hormonal changes, to 40% in master/PhD students, and to negligible frequencies during menopause [4]. The overall prevalence in women of all ages amounts to 10–15% [2,3]. AFA prevalence in adult women varies between 15 and 20% [5]; within this patient group, approximately 20–30% also present hirsutism [6], and over 50% on the average feature high levels of circulating androgens [7]. Overall, these data suggest that the major cause of AFA is the increased secretion of androgens and/or an excessively high sensitivity to androgen receptors (AR) [8]. Thus, AFA pertains to the larger group of **hyperandrogenism disorders**, which can present as clinical features *acne*, *seborrhea*, hair loss on the scalp with a specific pattern—*androgenetic alopecia* (AGA)—*hirsutism* at face or body level, and *oligo/amenorrhea*. A total of 70% of hyperandrogenism states are caused by **polycystic ovary syndrome** (PCOS), and the rest by other conditions such as congenital adrenal hyperplasia (CAH), Cushing's syndrome, hyperprolactinemia, insulin resistance (IR), and certain malignant tumors or treatments [20]. The consensus document that established the Rotterdam PCOS diagnosis criteria defined hyperandrogenism based on *clinical criteria*, the most important one being hirsutism, as well as *biochemical criteria* such as circulating androgens levels [21]. Total and free testosterone levels are the most sensitive markers, but a few patients can feature isolated increases in DHEAS (dehydroepiandrosterone sulphate). Another useful marker is androstenedione, which can be moderately increased in non-classic adrenal hyperplasia (NCAH), an autosomal recessive disorder produced by a 21-hydroxylase deficit. Measuring matinal basal levels of 17-hydroxyprogesterone is useful to exclude this condition. Thyroid-stimulating hormone (TSH) levels measurement is of limited utility, while serum follicle-stimulating hormone (FSH) and estradiol (E2) levels are useful to exclude hypogonadotropic hypogonadism, and the endocrine disorder caused by central hypothalamo-hypophyseal conditions. PCOS was characterized as belonging to normoestrogenic normogonadotropic anovulatory states (group 2 according to the WHO classification). Also, serum prolactin level measurements are useful to exclude hyperprolactinemia, although many hyperandrogenism patients feature prolactin levels trending to the upper limit of normal or slightly increased.

The steroid hormones synthesis pathway (Figure 2) features several enzymes located in different organs and tissues, including gonads (ovary/testis), cortical adrenal glands and the epidermis (Figure 2). Starting from cholesterol, we can distinguish a **progestagens** (21 carbon atoms) **synthesis pathway**, continued with the **corticosteroid synthesis pathway**, which features two enzymes (11-β-hydroxylase and aldosterone synthase) and three prod-

ucts (cortisol, corticosterone and aldosterone). The prostagens pathway is coupled with the **androgens** (19 carbon atoms) **synthesis pathway** continued with the **estrogens** (18 carbon atoms) **synthesis pathway** by the enzyme 17–20 lyase acting on two progestagen intermediates, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, and transforming them into dehydroepiandrosterone (DHEA) and androstenedione, respectively. One enzyme is common for all pathways: 3-β-hydroxysteroid dehydrogenase (3β-HSD); 17α-hydroxylase and 21-hydroxylase are specific for the progestagens synthesis pathway, and 17β-HSD and 5α-reductase are specific for the androgens synthesis pathway. Aromatase converts androgens into estrogens: androstenedione into estrone, which is later converted into estriol (E3) by the liver and placenta enzymes, and testosterone into estradiol (E2), later also converted into E3. A majority of these enzymes are located in the smooth endoplasmic reticulum, except for 11β-hydroxylase (11β-HSD), aldosterone synthase and cholesterol side-chain cleavage enzymes, which are mitochondrial. In women, androgen- and estrogen-producing enzymes are located in the thecal and granulosa cells of maturating ovarian follicles, being controlled by the hypophyseal gonadotropic hormones FSH and LH. In the cortical adrenal glands, 17α-hydroxypregnenolone is converted into 17α-hydroxyprogesterone, and further into androgens (androstenedione and testosterone) and corticosteroids (11-deoxycortisol and cortisol); however, small amounts of 17α-hydroxypregnenolone are also converted into DHEA, which is transported at skin level as an inert sulphated derivative (DHEAS). This compound can be easily reconverted into DHEA and further processed into androstenedione, testosterone and dihydrotestosterone (DHT) by active hair follicle keratinocytes, which feature higher expression levels of 17β-HSD and 5α-reductase compared to epidermal keratinocytes [12]. This augmented androgen synthesis by follicular keratinocytes also increases their proliferation rate. Increased local androgen levels stimulate sebocyte activity, resulting in increased sebum secretion.

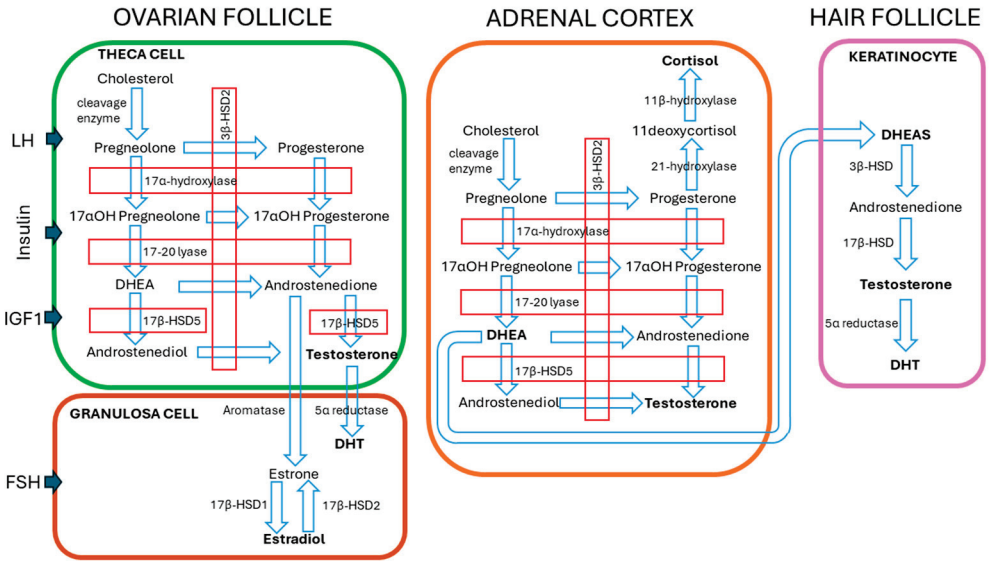


Figure 2. Steroid hormones synthesis pathways active in ovarian follicles (theca and granulosa cells), adrenal cortex and hair follicle keratinocytes, leading to androgen production.

2.2. Endocrine Disturbances Affecting Follicle Maturation and Ovulation

In humans, oocytes are formed during embryogenesis, when oogonia enter the gonadal ridge and proliferate by mitosis until the second or third trimester of pregnancy. They become surrounded by pregranulosa cells, forming primordial follicles. Thereafter, oogonia stop dividing and enter the prophase of the first meiotic division, remaining dormant in the dictyotene stage for decades, until follicular maturation and ovulation driven by the preovulatory LH peak. During each menstrual cycle, one or a few dominant follicles increase in size, become mature and undergo ovulation. Dominant follicles

receive a stronger FSH stimulus for maturation in their granulosa cells due to a larger size, better vascularization and a higher density of FSH receptors, and also start to express LH receptors; therefore they can survive falling FSH levels occurring by a feedback mechanism when granulosa aromatase activity increases, resulting in increased estrogen and inhibin secretion. Meanwhile, the FSH drop will drive the non-dominant follicles into an atretic state, with a regression in size followed by deterioration.

Some researchers hypothesized that PCOS is primarily the result of fetal exposure to increased levels of androgens that inhibit folliculogenesis, as suggested by finding polycystic ovaries in girls before puberty [22]. Fetal ovarian androgens may be produced in response to increased maternal human chorionic gonadotropin (HCG) in subjects with genetic susceptibility, and excessive testosterone secretion may be accompanied by pregnancy hyperglycemia and fetal hyperinsulinemia, resulting in epigenetic reprogramming disturbances in fetal primordial follicles leading to PCOS [23]. The progestagens and androgens synthesis pathways are primarily active in follicular thecal cells, under hormonal control exerted by LH, insulin and insulin-like growth factor (IGF) receptors, while dihydrotestosterone (DHT), estrone and estradiol (E2) are produced in granulosa cells by 5α-reductase, aromatase, and 17β-HSD following the activation of FSH receptors [11]. In PCOS thecal cells feature increased sensitivity to LH receptors due to the dysfunction of inhibitory feedback resulting in receptor downregulation, as well as IR and hyperinsulinemia. Increased androgen secretion by PCOS thecal cells results from an increased expression of 17α-hydroxylase, 17–20 lyase and cholesterol side-chain cleavage enzymes, as proved by in vitro experiments [24].

Altered follicular LH sensitivity in PCOS produces multiple endocrine feedback imbalances, resulting in GnRH (gonadotropin-releasing hormone) hyperpulsatility and a resistance to progesterone-induced negative feedback. Subsequently, the amplitude and frequency of preovulatory LH peaks increase, a phenomenon that can be alleviated by antiandrogens such as flutamide which can restore hypothalamic sensitivity to estrogens and progesterone [25]. In total, 55% to 75% of PCOS patients feature an excessive hypophyseal LH secretion and altered LH/FSH ratios upon hyperstimulation by GnRH, but it is not yet clear if the hypothalamus-hypophyseal dysfunction primarily appears or if it is subsequent to androgens/estrogens imbalance [26]. LH hypersecretion and/or increased LH receptor sensitivity may induce early granulosa cells' luteinization, stopping antral follicle growth, as well as activating premature oocyte progression through meiosis, resulting in aneuploidies [27].

Another factor playing a key role in ovarian folliculogenesis is the anti-müllerian hormone (AMH), a member of the GFRβ family (growth factor receptor), secreted by granulosa cells of growing preantral follicles smaller than 4 mm in diameter which inhibits granulosa cell sensitivity to FSH until follicles exceed 8 mm in diameter; after this stage, FSH produces accelerated follicle growth and estrogen secretion resulting in feedback inhibition, dominant follicle selection and ovulation. In PCOS patients, AMH secretion can exceed normal levels up to 75-fold, resulting in the increased recruitment of antral follicles featuring premature differentiation and growth stop [28]. These increased AMH levels are associated with increased LH or androgens levels, and may impede oocyte maturation, fertilization rates, as well as result in low embryo quality during in vitro fertilization procedures [29]. Other signaling factors that may impede follicle growth and maturation are TGF-β family members like bone morphogenetic (BMP) factors, activin, follistatin, β inhibins, the vascular endothelial growth factor (VEGF), other cytokines like interleukins (IL), tumor necrosis factor alpha (TNF-α), FS-7 associated surface antigen (FAS receptor), also known as TNF receptor superfamily member 6 (TNFRSF6) or CD95 and its ligand FASL, miRNAs, etc. [11,23].

Hyperinsulinemia can activate ovarian steroidogenesis by acting on both theca and granulosa cells, stimulating thecal cell proliferation, LH receptor-mediated androgens synthesis, and LH and IGF receptors expression [30], and similar effects can occur at the cortical adrenal level [31]. Insulin also promotes hyperandrogenism via inhibiting the syn-

thesis by hepatocytes of the sex hormone-binding globulin (SHBG) and insulin-like growth factor-binding protein 1 (IGFBP1), also known as placental protein 12 (PP12). Insulin action on follicular cells occurs via the inositol glycan system, which may explain the increased urinary inositol found in some PCOS patients [32]. A rare autosomal recessive disorder first described in the 1950s [33] is the Rabson–Mendenhall syndrome, caused by mutations in the insulin receptor gene leading to hyperinsulinemia, head, face (prognathism and senile-like face), teeth and nails abnormalities, *acanthosis nigricans*, enlarged genitalia and early puberty, as well as extreme hirsutism. The condition is commonly associated with Donohue syndrome (leprechaunism) [34]. It was proved that these diseases result from a combination of reduced insulin receptor sensitivity and a defective autophosphorylation [35]. The first proof of hyperinsulinemia and altered glucose tolerance in PCOS patients was brought in 1980, including the intriguing finding that some patients with classical PCOS featured *acanthosis nigricans* [36]. Insulin resistance in PCOS is not ubiquitous, occurring at muscle and adipose tissue level but not in ovarian follicles. Therefore, at this level, in theca cells, insulin signaling can activate androgen production by stimulating 17α-hydroxylase and 3β-HSD isoform 2 [37].

2.3. Genetic Insights in AFA and PCOS

Acne in general and particularly AFA have for a long time been considered diseases with associated genetic susceptibility. In an extensive review of acne histopathology, Albert Kligman stated that, in spite of the fact that the genetics of acne is poorly understood, it definitely presents features of a polygenic condition without a clear Mendelian inheritance pattern, whereby multiple susceptibility genes exert moderate but synergic predisposing effects, none of them playing a decisive role by itself [38]. Indeed, numerous epidemiology studies have confirmed the hypothesis of hereditary predisposition. A large cohort study on British subjects with "persistent acne" with ages between 26 and 78 years proved that a positive family history of acne increased the individual risk between 2.3 and 4.69-fold [39,40]. A retrospective study performed on British monozygote and dizygote twin pairs revealed that 41% of acne patients transmitted the disease to the progeny, 47% of them had affected first degree relatives, and for 25% of them their parents were affected by the same condition [41]. A very important study was performed in China on a group of 238 patients with *acne vulgaris* and 207 control subjects [42]. The authors found a variable number of CAG/GGN repeats in the N-terminal transcription activation domain (TAD) of the first exon of the AR gene, encoding polyglutamine/polyglycine sequences of variable length associated with different gene transcription transactivation efficiencies; they proved that acne patients presented shorter triplet repeats than controls (CAG<23/GGN $\leq$23, odds ratio OR = 3.33, $p < 0.05$), resulting in higher AR expression levels and increased androgen sensitivity. This finding explains acne susceptibility in subjects with an increased sensitivity of AR, who do not necessarily feature increased androgen levels, and can account for the approximately 50% of AFA cases not associated with increased androgen levels [7]. Other single-nucleotide polymorphysms (SNP) generally associated with *acne vulgaris* were identified in genes of innate immune system components: the 308 G/A polymorphism in the TNF gene [43], M196R in the TNFR2 gene and R753N in the TLR2 gene [44], 4845 G>T in the IL-1α gene [45], and several others; a comprehensive review on this topic is that of Zhang and Zhang 2023 [46]. Unsurprisingly, significant associations with acne were found for SNPs in genes of steroid synthesis pathways, such as 34 T/C in cytP450c17α (CYP17), encoding an enzyme with the function of both 17α-hydroxylase and 17–20 lyase [47]; T>C at −34 bp in the promoter of the same gene and T>C (W/R) in the codon 39 of CYP19A1 encoding aromatase (SNP code: rss2236722) [48]; several haplotypes of CYP21A2 encoding 21-hydroxylase [49]; as well as SNPs in CYP1A1, encoding aryl hydrocarbon hydroxylase (AHH), an enzyme converting polyunsaturated fatty acids into proinflammatory signaling molecules like epoxides [50]. We should add that gene mutations leading to 21-hydroxylase deficiency are the main cause of non-classic congenital adrenal hyperplasia (NCAH), a condition that has to be excluded in the differential diagnosis of hyperandrogenism [51].

Similar findings occurred in genetic susceptibility studies on patients with AFA and PCOS. For example, an epidemiology study on a group of 93 PCOS patients retrieved the disease in significant proportions of their first degree relatives: 35% of mothers prior to menopause and without hormone therapy, and 40% of sisters [52]. Another cohort study on 1332 monozygotic twin sisters and 1873 dizygotic twin sisters or singleton sisters of twins found PCOS occurrence correlation coefficients of 0.71 for monozygotic twin pairs and only 0.38 for dizygotic twins/singletons [53]. Some researchers consider that PCOS-associated mutations offered some selective advantages such as a decrease in the number of descendants, resulting in better maternal outcomes and children thriving, therefore they spread especially across Asian populations [23]. Hypophyseal tropic hormones, particularly the adrenocorticotropic hormone (ACTH), may produce an excessive stimulation of the inner *zona reticularis* of the adrenal cortex in PCOS patients, resulting in the increased conversion of 17α-hydroxyprogesterone into cortisol but also testosterone, and 17α-hydroxypregnenolone conversion into DHEA and further into DHEAS. However, certain gene variants of sulfotransferase 2A1 (SULT2A1) with augmented activity can easily reconvert DHEAS, an inert androgen, into the active DHEA [54]. Similarly, gene variants of cytP450c17α (CYP17) resulting in high enzyme activities can also increase adrenal androgen synthesis, a condition estimated to occur in up to 30% of PCOS patients [55]. Another interesting and complex gene disorder occurs via mutations in 11β-HSD1 and hexose-6-phosphate dehydrogenase, transmitted in a digenic triallelic manner, resulting in decreased cortisol levels and activation via the ACTH feedback of adrenal androgen steroidogenesis [56].

Folliculogenesis and its hormonal modulation are also the main factors of genetic susceptibility in AFA and PCOS. An interesting study performed on 80 PCOS subjects and 24 normal controls identified via whole-genome sequencing (WGS) 24 rare variants (allele frequency < 0.01) in the AMH gene, 18 of them in the PCOS group. When reproduced in pcDNA3.1 plasmids containing the AMH gene transfected into COS-7 simian fibroblasts, only the 18 PCOS-associated variants resulted in signaling deficiencies, as proved by a luciferin–luciferase assay [57]. A related study showed that AMH synthesis in the granulosa cells of PCOS patients with amenorrhea is increased by up to 75-fold [58]. Altered AMH signaling in PCOS may weaken its inhibitory effects on cytP450c17α activity and androgen synthesis, possibly leading to excessive follicular androgen secretion [57]. Many other genetic factors associated with ovarian folliculogenesis have been identified, for example the primordial follicles inhibitory transcriptional factors, such as liver kinase B1-serine/threonine kinase 11 (LKB1/STK11), the expression of which is inhibited by androgens like testosterone and DHT and activated by 17β-estradiol via the estrogen receptor α (ERα), proapoptotic FOX factors secreted by oocytes, some local follicular factors regulating follicle recruitment, growth and development, e.g., growth differentiation factor 9 (GDF9), and bone morphogenetic factors (BMP) 4, 6, 9 and 15, which are members of the transforming growth factor β (TGF-β) family, β inhibins, miRNAs, etc. [11]. Oocyte-secreted GDF9 is the main stimulus for early follicular development, activating the proliferation and differentiation of granulosa cells via a signaling chain represented by BMP receptor 2 followed by TGF-β receptor 1 (ALK-5), and the phosphorylation of SMAD2 and 3, which form a complex with SMAD4 that undergoes nuclear translocation, activating the transcription of specific gene sets [59].

The review by Han Zhao et al. 2016 [60] summarizes the results of several genome-wide association studies (GWAS) performed on groups of PCOS patients of different ethnicities. Thus, a study on a Chinese Han population [61] explored a group of 744 PCOS patients and 895 control healthy women; another study [62] explored an even larger Chinese Han population with 10480 cases in the PCOS group and 10,489 cases in the control group. Furthermore, a study was performed on the Korean population with a group of 976 PCOS cases and 946 control subjects [63], and two studies were performed on European Caucasian populations: one on 984 PCOS patients and 2964 control subjects [64], and another one on 5184 self-reported PCOS patients and 82,759 controls [65]. The results of these wide-scale

studies were significant and there were some overlapping results. Chen et al. 2011 [61] found several SNPs (rs13429458 and rs12478601) located close to the THADA gene (thyroid adenoma associated, or ARMC13—armadillo repeat containing), one SNP (rs13405728) associated with LHCGR (LH/choriogonadotropin receptor), and two SNPs (rs2479106 and rs10818854) close to the DENND1A gene (differentially expressed in normal and neoplastic development isoform A1), encoding a protein named connecdenn 1, expressed on chlatrin-coated membrane invaginations of theca cells where membrane receptors are located. Interestingly, the DENND1A SNP rs2479106 was also found by the other Chinese Han population study [62], as well as the THADA SNP rs13429458 and the LHCGR SNP rs13429458, while the European population study of Day et al. 2015 [65] retrieved the THADA SNP rs7563201 and the YAP1 (yes-associated protein 1, a transcription coregulator of cell proliferation/apoptosis genes) SNP rs11225154, and Shi et al. 2012 [62] retrieved another YAP1 SNP, rs1894116. The Korean population study of Lee et al. 2015 [63] validated one single SNP related to the KHDRBS3 gene. Other PCOS SNPs-related genes found in the European studies were c9orf3/FANCC (Fanconi anemia group C protein), GATA4/NEIL2, KCNA4/FSHB, ERBB4/HER4, FSHB, RAD50 and KRR1. The Chinese Han population study of Shi et al. 2012 [62] retrieved the most PCOS SNPs, related to genes RAB5B/SUOX, HMGA2, C9orf3, FSHR, TOX3, SUMO1P1, INSR, plus the above-mentioned ones. These findings are highly relevant, since DENND1A expression was found in the cortical adrenal gland *zona reticularis*, and other genes encode hormone receptors like the FSH receptor (FSHR), LH receptor (LHCGR), or the insulin receptor (INSR), associated with IR, THADA and HMGA2 involved in type 2 diabetes mellitus, and RAB5B and SUOX located in a susceptibility site for type 1 diabetes [11]. However, a search on the National Cancer Biology Institute (NCBI) gene database with the keyword combination "androgenic acne" retrieved only six entries, listed in Table 1, while a similar search in the OMIM (Online Mendelian Inheritance in Man) database retrieved 57 entries using the "Gene map" display mode; a NCBI Gene database search for "adult female acne" retrieved 52 entries, and a search of the same database for "hormonal acne" retrieved 19 entries (Figure 3). Similar findings are reported in the review by Heng et al. on genetic variability in *acne vulgaris* [66].

Table 1. Entries retrieved from the NCBI Gene database by search with keyword combination "androgenic acne". (MIM—gene identifier in the Online Mendelian Inheritance in Man (OMIM) database.)

Name	Gene ID	Description	Location	Aliases	MIM
FST	10468	Follistatin (*Homo sapiens*)	Chromosome 12, NC_000012.12 (102395874..102481839, complement)	IGF, IGF-I, IGFI, MGF	14,440
IL1B	3553	Interleukin 1 beta (*Homo sapiens*)	Chromosome 2, NC_000002.12 (112829751..112836779, complement)	IL-1, IL1-BETA, IL1F2, IL1beta	147,720
AR	367	Androgen receptor (*Homo sapiens*)	Chromosome X, NC_000023.11 (67544021..67730619)	AIS8, DHTR, HUMARA, HYSP1, KD, NR3C4, SBMA, SMAX1, TFM, AR	313,700
VDR	7421	Vitamin D receptor (*Homo sapiens*)	Chromosome 12, NC_000012.12 (47841537..47904994, complement)	NR1I1, PPP1R163	601,769
CYP17A1	1586	Cytochrome P450 family 17 subfamily A member 1 (*Homo sapiens*)	Chromosome 10, NC_000010.11 (102830531..102837413, complement)	CPT7, CYP17, P450C17, S17AH	609,300
IGF1	24482	Insulin-like growth factor 1 (*Rattus norvegicus*)	Chromosome 7, NC_086025.1 (24169608..24249446)	IGF	

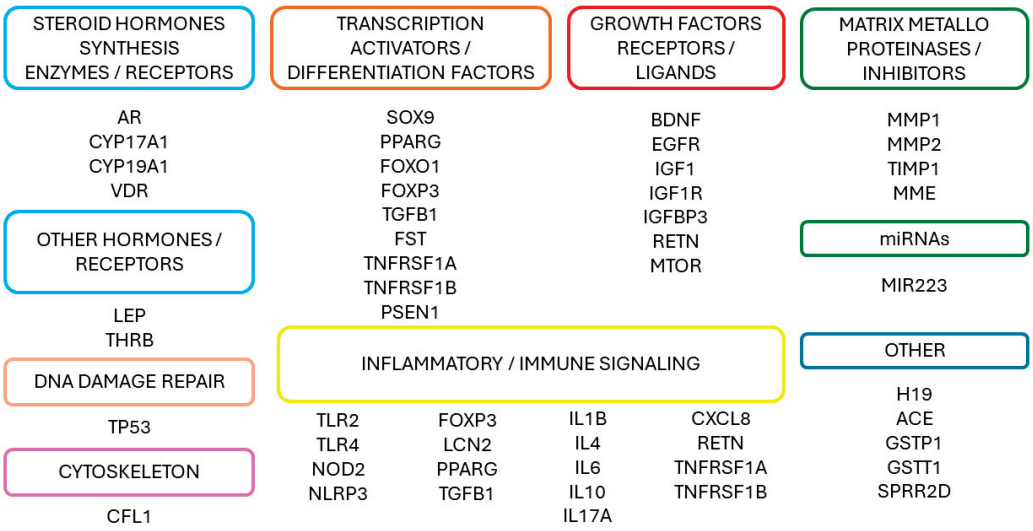


Figure 3. Functional groups of genes retrieved from the NCBI Gene database with the keyword combination "adult female acne".

Somatic mutations and segmental or nonsegmental somatic mosaicism have been involved in a variety of acne-related genetic syndromes such as the Apert syndrome (acrocephalosyndactyly) and *nevus comedonicus*, associated with FGFR2 (fibroblast growth factor receptor 2) mutations, and the autoinflammatory syndromes PAPA (pyogenic arthritis, *pyoderma gangrenosum*, acne), PASH (*pyoderma gangrenosum*, acne, suppurative hidradenitis), PAPASH (pyogenic arthritis, *pyoderma gangrenosum*, acne, suppurative hidradenitis), PsAPASH (pustular psoriasis, arthritis, *pyoderma gangrenosum*, synovitis, acne, suppurative hidradenitis), PASS (*pyoderma gangrenosum*, acne, suppurative hidradenitis, ankylosing spondylitis) and SAPHO (synovitis, acne, pustulosis, hyperostosis, osteitis), with most mutations associated with the IL-1β pathway and matrix metalloproteinases such as MMP 2 and 9 [67].

Recent studies have also documented the important roles of epigenetic changes in the etiopathogeny of PCOS. These mechanisms include DNA methylation, histone acetylation, protein phosphorylation, non-coding RNAs (ncRNA), and RNA processing (methylation, editing and splicing). Xu et al. 2010 found a decrease in DNA methylation in peripheral leukocytes in 20 PCOS patients relative to a control group [68]. Fetal exposure to testosterone is assumed to alter DNA methylation for the group of TGF-β regulatory genes [23]. Differentially methylated CpG islands of PPARG1 and NCOR1 genes were identified in granulosa cells, and they may lead to hyperandrogenism [69]. Decreased methylation levels of AMH receptor gene at follicular level and the increased methylation of INSR gene in the endometrium in PCOS patients have been associated with increased and decreased expression of these genes, respectively [70]. Even more complex transcriptomic changes associated with retinoids treatments in PCOS patients with acne have been described [71]. Thus, androgens and IGF-1 signaling in follicular keratinocytes activates via the AKT and mTOR phosphorylation of nuclear factors FoxO1 and FoxO3, enhancing the transactivation of proinflammatory and lipogenesis factors like AR, SREBF1, PPARγ, STAT3, while reducing the expression of GATA6, the main regulator of follicular keratinocytes homeostasis. The phosphorylation by AKT of the p53-binding factor MDM2 promotes p53 degradation, whereas isotretinoin treatment enhances the expression of p53, FoxO1 and FoxO3 in sebaceous glands of acne patients. A recent Chinese epigenetics study identified 23 differentially methylated sites related to severe acne, involving multiple genes such as PDGFD (platelet-derived growth factor D), ARHGEF10 (rho guanine nucleotide exchange factor 10), IL1R1 (IL-1 receptor), PARP8 (poly ADP-ribose polymerase 8), MAPKAPK2 (MAP kinase-activated protein kinase 2), MUC8 (mucin isoform secreted in the endometrium and endocervix), NAV1 (neuron navigator 1), KCNT2 (Na^+-activated K^+ channel T2 or slick

channel), and Wnt9A (an embryogenetic differentiation and patterning factor of the Wnt family) [72]. Another recent study performed a DNA methylation and multi-omics analysis of acne skin samples, finding 31,134 differentially methylated sites and 770 differentially methylated genes with changes in expression [73].

3. Classical and Novel Therapeutic Approaches in AFA

Adult female acne typically follows a chronic course, often marked by frequent relapses that necessitate ongoing maintenance therapy. Notably, postadolescent acne often proves challenging to effectively treat with all available medication options. For instance, Goulden et al. [74] found that up to 82% of postadolescent patients did not show improvement after undergoing multiple courses of oral antibiotics. Additionally, approximately 33% of patients experienced a recurrence of symptoms following one or more courses of oral isotretinoin. Many patients seem to endure unnecessary suffering because they are unaware of the alternative therapies that are available to them. Because adult female acne commonly presents with mild to moderate symptoms, it can typically be managed effectively with a well tolerated topical monotherapy, such as azelaic acid or retinoids [75], but in some cases topical treatments are not efficient and new strategies that approach the problem at molecular level are needed.

3.1. Topical Therapy

Azelaic acid (20% cream or 15% gel) has anti-inflammatory, antibacterial and comedolytic properties and is advantageous because it inhibits cellular protein synthesis in *C. acnes* without inducing bacterial resistance [76]. During two decades of clinical experience, no harmful effects on fetuses have been reported with the use of topical azelaic acid [77].

Retinoids for topical use are an essential component of acne therapy, due to their diverse beneficial effects, which include the control of keratinocyte maturation, a decrease in sebum secretion, comedolysis, anti-inflammatory actions, epithelial regeneration with scar and hyperpigmentation healing, and side effects such as dryness and skin irritation [78,79]. Commercial preparations include tretinoin gel 0.025%, deemed to provide similar efficiency to the third generation retinoid adaptalene 0.1% or 0.3%, alone or in combination with benzoyl peroxide 2.5% gel, but the latter is better tolerated [79]. Tazarotene 0.1% gel is even more efficient, and is the only topical retinoid completely forbidden during pregnancy or lactation, although in these conditions all retinoids should be avoided due to risk of teratogenic effects [75].

Benzoyl peroxide is widely used, alone or in combination, due to bactericidal, keratolytic and anti-inflammatory effects. The antimicrobial spectrum is large, because it acts by producing reactive oxygen species [79]. Side effects include dryness of skin, irritation, itching, photosensitivity, redness or peeling, and the bleaching of clothes, which limit applied concentrations to 5% [75,78].

Topical antibiotics include primarily erythromycin and clindamycin, both of them being effective in reducing skin and hair follicles colonization with *C. acnes* and related inflammation. They can be associated with topical retinoids and benzoyl peroxide, and combined therapy is recommended due to widespread microbial resistance [78,79].

Salicylic acid is a classical drug with anti-inflammatory and keratolytic effects, available in a variety of preparations for topical use such as solutions, lotions, gels, creams, shampoos, soaps, cotton pads and plasters. Its side effects include skin dryness, irritation and itching [78]. The related compound methyl salicylate should not be used for acne treatment.

Niacinamide (nicotinamide) may exert beneficial preventive and curative effects in acne, such as a reduction in sebum secretion, increased ceramides synthesis in keratinocytes, an increase in epidermal permeability [80], the prevention of *C. acnes*-induced TLR2 activation [81], and a decrease in risk of non-melanoma skin cancers [82]. It is present in a large number of cosmetic products such as creams, face sera, gels, lotions, etc., and has few side effects.

Dapsone was originally developed as an antibiotic for leprosy. However, it emerged as a useful first-line therapy in patients with inflammatory acne, particularly those with darker skin [79]. It can be applied as a 5% gel low-cost therapy [78], but it may produce irritation, dryness, an itching or burning sensation, yellow/orange skin discoloration, and sometimes severe hypersensitivity reactions [83].

Clascoterone is an AR inhibitor for topical use. The US Food and Drug Administration (FDA) has recently granted approval for clascoterone cream 1% for treating acne in individuals aged 12 and above. Clinical trials have demonstrated its superior effectiveness compared to a placebo, resulting in significant reductions in both non-inflammatory and inflammatory acne lesions after 12 weeks of treatment [84]. It has been demonstrated to specifically bind to ARs solely at the site of application on the skin, without inducing any systemic anti-androgenic effects. This targeted action makes it safe for use in both male and female patients. By binding to ARs in the sebaceous glands and hair follicles upon topical application, clascoterone effectively inhibits the binding of DHT [85].

3.2. Systemic Therapy

Systemic antibiotics include, according to the therapeutic guidelines and expert opinion, top choice compounds used for moderate-to-severe papulo-pustular or nodular/conglobate acne [76] such as tetracyclines (doxycycline, tetracycline, minocycline), macrolides (erythromycin, roxithromycin, clarithromycin, azithromycin), lincosamides (clindamycin), sulfonamides (co-trimoxazole) and fluoroquinolones (e.g., levofloxacin) [78]. Although tetracyclines are included in the first line of treatment, second-generation agents like limecycline and doxycycline are linked to fewer interactions with food and enhanced patient compliance, primarily because they are administered once daily [86]. Minocycline and doxycycline are superior to tetracycline due to anti-inflammatory and immunosuppressive effects [87]. Minocycline is advised as a secondary treatment option for females experiencing severe seborrhea and erythromycin should be given only during pregnancy for less than one month due to the frequent occurrence of *C. acnes* resistance [88]. Systemic antibiotic therapy duration should not be longer than 3 months [79], and they should be administered in combination with topical agents due to synergistic effects, not in monotherapy [89].

Isotretinoin is the preferred retinoid for oral administration in moderate-to-severe acne, when topical agents alone or other drugs are ineffective, or as first line treatment for nodular-cystic acne [90]. Isotretinoin is effective against all four pathophysiology mechanisms of acne: hyperseborrhea, *C. acnes* proliferation, inflammation and hyperkeratosis/comedogenesis [91]. Besides the above-mentioned teratogenic risk, the side effects of the drug include skin/mucosa dryness, biochemical disturbances (lipid profile, aminotransferases), an increased risk to develop inflammatory bowel diseases, depression and suicide [79]. A detailed description of the effects of isotretinoin on the complex molecular signaling pathways and gene regulation mechanisms in acne is provided by [71]. A consensus recommendation of the Global Alliance to Improve Outcomes in Acne is to administer oral isotretinoin as a first line therapy in nodular as well as cystic or conglobate acne (considered the most severe varieties), due to its high efficiency and lowest rate of recurrence. The recommended doses are 0.5–1 mg/kg body weight/day for 4 to 6 months, achieving a cumulative dose of 120–150 mg/kg. However, there is no consensus yet on the criteria and doses required to maintain remission [92].

Hormonal therapy can be applied using a number of compounds targeting mainly hyperandrogenism or increased AR sensitivity, such as AR inhibitors, oral contraceptives limiting ovarian androgen production, or glucocorticoids to inhibit adrenal androgen production. Hormonal therapy targets primarily sebum production, therefore it should be supplemented with other agents such as antibiotics or topical therapy [75]. Moreover, oral contraceptives are also the main therapy for PCOS, along with diet, exercise and drugs to limit IR, and pregnancy-inducing methods like laparoscopic ovarian drilling and assisted reproduction techniques [93].

Androgen receptor inhibitors (antiandrogens) include cyproterone acetate (CPA), spironolactone (SPL), its analog drospirenone, and flutamide. CPA (the first antiandrogen in medical use) and drospirenone were developed as progestatives with antiandrogen effects, combined with estrogens in birth control pills, while flutamide is a nonsteroidal antiandrogen. Flutamide use, even at the minimally active dose of 125 mg/day, alters liver enzyme levels [94]. SPL and drospirenone exert strong antialdosterone effects and act as K^+-sparing diuretics, requiring kalemia control. All AR inhibitors are prohibited during pregnancy due to the risk of feminization of male fetuses [79].

Spironolactone, which acts as an aldosterone antagonist with antiandrogenic properties, has emerged as a promising option for treating acne in females. In a randomized, double-blind, placebo-controlled trial involving sixty-three women, participants were equally divided into three groups: placebo, SPL25, and SPL50. The study found that SPL was effective in reducing both objective acne counts and improving subjective clinical grading and showed that utilizing a low dose of SPL (25–50 mg/day) in AFA led to a high success rate with minimal side effects such as menstrual irregularities in 13–33%, breast tenderness in 2–4%, and dizziness in 2–3%. The onset of spironolactone's action is typically slower compared to other systemic acne treatments, taking approximately 12 weeks (with a range of 8–20 weeks). However, a potential pharmacokinetic advantage is in that its effects last for at least 1 month after discontinuation, particularly when continuing topical medication maintenance [95]. Spironolactone may be combined with a third or fourth generation oral contraceptive to alleviate side effects.

Inhibitors of ovarian androgen production are oral contraceptive pills containing a combination of estradiol and a progestin with antiandrogen activity, such as CPA or drospirenone. These combinations seem to be more effective compared to those containing modern progestatives (levonorgestrel, desogestrel, norgestimate, gestodene) [96]. The medications are effective in both inflammatory and non-inflammatory acne, but the effects occur upon prolonged treatment, with a 62% reduction in inflammatory lesions at 6 months [97]. Paradoxically, in some cases acne can be triggered or aggravated by this treatment, requiring a substitution with a combination containing a less androgenic progestin or a lower estradiol dose [98]. Other compounds, such as 5α-reductase inhibitors finasteride and dutasteride, are not particularly effective in acne.

Zinc salts in oral preparations containing 200 mg elemental zinc/day may be effective in the treatment of acne-induced inflammatory lesions [99].

Probiotics. Some authors associated emotions like fear, anxiety, and depression with alterations in gut microorganisms, suggesting that these changes could lead to localized and systemic inflammation, known as *the brain–gut–skin theory.* Both animal and human studies have demonstrated that stress disrupts the balance of normal gut microflora, particularly affecting *Lactobacillus* and *Bifidobacterium* species [100]. Psychological stressors induce intestinal microbes to generate neurotransmitters (acetylcholine, serotonin, norepinephrine), that can traverse the intestinal mucosa and enter the bloodstream, ultimately leading to systemic inflammation [14]. Kazandjieva J et al. have shown that combining a prebiotic with an anti-inflammatory food supplement (prebiotic molecules: fructooligosaccharides—FOS and galactooligosaccharides—GOS, zinc, lactoferrin, and niacinamide) as part of the treatment regimen provides an additional clinical benefit, particularly in reducing inflammatory lesions and improving the severity of acne scores [101]. Clinical trials have investigated the impact of probiotics on acne. Kang et al. found that after 8 weeks of topical *Enterococcus faecalis* treatment, there was a statistically significant 50% reduction in inflammatory acne lesions count (specifically pustules) compared to placebo, as well as a decrease in non-inflammatory lesions such as comedones [102].

3.3. Therapeutic Guidelines for Acne

Although it is impossible to reach a universal agreement concerning the best methods and therapeutic protocols for acne in general (and for AFA in particular), the Global Alliance to Improve Outcomes in Acne formulated in 2018 ten consensus recommendations

based on a Delphi panel and questionnaire [92]. A major impediment in assessing effectiveness of different therapies is the lack of a standardized scale or grading system for acne severity. The high efficiency of modern topic treatments also tends to distort traditional classifications and therapy guidelines. For AFA some important principles are to apply early treatment in order to minimize acne scarring (consensus recommendation 10) and to use topical retionoids alone or in combination with benzoyl peroxide in AFA (consensus recommendation 9). Gollnick et al. 2016 established a number of specific therapeutic protocols for different clinical varieties of acne [76]. Thus, for *comedonal facial acne* the recommended therapy is topical (in the sequence topical retinoid > azelaic acid > salicylic acid) for 12 weeks, followed by topical maintenance therapy in case of success or more radical measures in case of no response or increase in severity. For *papulo-pustular facial acne,* the therapy is topic in mild cases (benzoyl peroxide, topical retinoid, azelaic acid or combination), while in moderate cases it is based on a fixed topical combination, and in moderate-to-severe cases this combination can be supplemented with oral isotretinoin, oral zinc, or oral antiandrogens. In the case of therapeutic success after 12 weeks, the maintenance therapy is based on topical retinoids > azelaic acid, while in the case of failure a careful analysis and more intensive therapies are required. For the most severe forms, *nodular/conglobate acne*, the treatment is different for males vs. females. The former group requires oral isotretinoin or fixed combination topic therapy plus high-dose oral antibiotics, while for females these therapies have to be supplemented or replaced with oral antiandrogens. In the case of success after 12 weeks, the maintenance therapy includes topic retinoids > azelaic acid or topic retinoid and benzoyl peroxide combinations, but never benzoyl peroxide or topic antibiotic monotherapy. In the case of a lack of response, updated and more aggressive therapies are required. It will be interesting to see how novel, deemed game-changing therapies, such as topic clascoterone, will impact these guidelines. However, alternative or adjunctive therapies and particularly lifestyle and diet changes are of utmost importance and may bring an essential contribution to success in controlling the disease.

3.4. Alternative Holistic Theraphy (Herbal Medicines)

Spearmint tea, a species of mint scientifically classified as *Mentha spicata*, also known as garden mint or common mint, is native to Europe and southern temperate Asia, and has confirmed antiandrogen properties. Peter Grant conducted a study with 41 patients completing the full 30-day treatment period. Following the 30-day treatment with herbal teas, the results showed significant reductions in both free and total testosterone levels in the spearmint tea group ($p < 0.05$). Additionally, LH and FSH levels increased ($p < 0.05$). Subjective assessments of hirsutism, as scored by the modified DQLI, showed significant reductions in the spearmint tea group ($p < 0.05$) [103]. Another study was made on 21 patients who consumed a cup of tea twice a day for 5 days during the follicular phase of their menstrual cycles. Following treatment with spearmint tea, there was a noteworthy reduction in free testosterone levels and an increase in luteinizing hormone, follicle-stimulating hormone, and estradiol levels. However, there were no significant decreases in total testosterone or DHEAS levels [104].

Fenugreek seeds. Trigonella foenum-graecum, commonly known as fenugreek, is an annual plant belonging to the family *Fabaceae*, originating from the Mediterranean region, southern Europe, and western Asia, with anti-diabetic and cholesterol-lowering properties. Additionally, it has been observed to reduce insulin resistance in women with PCOS. Hassanzadeh et al. conducted a study to examine the impact of fenugreek seed extract on insulin resistance among women with PCOS. The intervention group received three tablets of 500 mg metformin along with two tablets of 500 mg fenugreek, while the control group received three tablets of 500 mg metformin and two tablets of a placebo for a duration of 2 months. The study observed a significant reduction in ovarian cysts after the 2-month period. However, there were no notable changes observed in fasting glucose levels, insulin sensitivity, or hormonal concentrations between the intervention and control groups [105].

Berberine belongs to the protoberberine group of benzylisoquinoline alkaloids and is classified as a quaternary ammonium salt, and has been shown to modulate the diversity of gut microbes effectively at a daily dose of 500 mg. Moreover, it has been found to positively influence gene regulation related to cholesterol absorption when administered at a daily dose of 300 mg in humans. Additionally, it is an effective insulin sensitizer and improvements in glucose metabolism have been observed with a daily dose of 1.0 g, as outlined in a recent systematic review [106]. Also, a review study from 2023 indicated that berberine might enhance lipid concentrations [107]. Studies have indicated that berberine supplementation may have a positive role in reducing insulin resistance, acne, androgen, and inflammation, in regulating lipid metabolism, and in improving body composition, and therefore can represent a novel clinical supplementation strategy for PCOS, although the results are demonstrated only in a specific population, namely normal or overweight women with PCOS who exhibit normal menses [108].

Licorice, known as *Glycyrrhiza glabra* in botanical terms, is a flowering plant belonging to the bean family *Fabaceae*. It is commonly used for its sweet and aromatic flavoring, which is extracted from the plant's root. Yang et al. discovered that licorice extract mitigates the symptoms of PCOS by regulating serum FSH levels, LH/FSH ratio, and irregular ovarian [109]. Licorice inhibits the activity of 17-hydroxyl esterase dehydrogenase and 17,20-lyase activity, while stimulating aromatase activity, due to its estrogen-like effects [110].

Many other complementary and alternative medicines of the same type are discussed by Fox et al. 2016 [111].

3.5. Adjunctive Therapy

Adjunctive therapies are used less frequently compared to the main topic or systemic therapies; however, in selected cases they can provide supplementary effectiveness and boost healing or prevent relapse.

Light therapy with red or blue light could improve acne by reshaping the skin microbiota and lowering *C. acnes* counts within the lesions, as well as by limiting sebum secretion. Blue light should be used cautiously and with well-defined timing protocols due to higher energy of photons compared to red or near infrared light, to avoid skin irritation or lesions.

Photodynamic therapy (PDT) consists of a combination of phototherapy and a photosensitizing chemical agent such as aminolevulinic acid or its methyl derivative. Light can be produced by high-intensity light-emitting diodes (LED) or lasers, in a continuous or pulsed regime. Red light PDT can produce up to a 68% reduction in inflammatory acne lesions [112,113]. Caution in applying PDT is also recommended, to avoid frequent side effects such as pain and a flare-up of the acne when high doses are used.

Ultraviolet (UV) phototherapy is a widely recognized bactericidal therapy known to inhibit the release of lipotheichoic acid, lipopolysaccharides, and other bacterial metabolites associated with pro-inflammatory effects [14]. The time of application and intensity should be limited to avoid actinic lesions.

Chemical peels containing compounds such as glycolic acid (30–70%), salicylic acid (20–30%), Jessner's solution (a combination of salicylic acid, lactic acid and resorcinol) included by Dr. Max Jessner in his patented Jessner peels, or trichloroacetic acid (10–35% for superficial peels, 40–50% for medium peels, and >50% for deep peels) can be successfully applied in mild-to-moderate acne [75].

Microneedling with radiofrequency was successfully applied in four clinical trials, according to Pathmarajah et al. [114], resulting in a decrease in both inflammatory and non-inflammatory lesions counts (pustules, papules, comedones).

Physical therapies include mechanical procedures such as comedone extraction, microdermabrasion, electrocauterization, cryotherapy, cryoslush therapy, and intralesional injection of corticosteroids [75,111].

3.6. Lifestyle (Diet and Exercise)

Recently, there has been increased attention to the role of environmental factors, particularly the Western diet, in the development of acne. The Western diet typically comprises red meat, dairy products, high glycemic index foods such as refined carbohydrates, chocolate, and saturated fat, which have been suggested to exacerbate acne by triggering metabolic signals derived from nutrients. An optimal "anti-acne diet" would resemble a Paleolithic-style nutrition plan, emphasizing the consumption of vegetables and fruits with a low glycemic index, as well as sea fish rich in anti-inflammatory omega-3 fatty acids [115]. It was postulated that high-protein diets, based on meat, eggs or dairy products, predispose to acne due to their rich leucine content, resulting in the activation of mTOR (mechanistic target of rapamycin) complexes 1 or 2 with excessive lipogenesis and swelling of the pilosebaceous unit [116]. A good proof in this respect is the fact that populations that traditionally have diets low in meat, grains and dairy products never develop acne [117].

3.7. Novel Therapeutic Approaches

The progress in exploring the complex pathophysiology of acne, including a variety of molecular players, signaling pathways and genomic or epigenomic features, opened the perspective of new therapeutic approaches aiming to exploit these recently added elements of knowledge. A review of the advances in acne therapy identified several relevant topics and areas of interest in acne, such as the complex inflammatory and innate immunity pathways, peculiarities of the skin microbiome, genetic susceptibility, or diets and nutrition principles [78]. This study presents a relatively large list of compounds included in recent clinical trials, some of them completely new, e.g., B244 (nitric oxide-producing agent), S6G5T-3 (encapsulated benzoyl peroxide and tretinoin cream), GK530G (fixed-dose adaptalene-benzoyl peroxide combination gel, compared to adaptalene 0.1% gel-CD0271 and benzoyl peroxide 2.5% gel-CD1579), P005672-HCl (a novel tetracycline derivative for oral administration), CJM112 (an anti-IL-17A antibody), calcipotriene (a vitamin D3 analog for topical therapy), olumacostat glasaretil 5% gel (an acetyl-CoA carboxylase inhibitor of fatty acid synthesis), CD5789 (trifarotene—a fourth-generation selective retinoic acid receptor-γ agonist for topical use), cortexolone 17α-propionate (clascoterone), and afamelanotide (synthetic analog of α-melanocyte-stimulating hormone-αMSH), FMX-101 (4% minocycline foam). The list of recently available brand products for acne tratment presented in the same study includes Winlevi (Clascoterone topical cream), Solodyn (minocycline-tetracycline for oral use), Vibramycin (tetracyclines-antimalarials for oral use), Differin (adaptalene-retinoids topical gel), Absorica and Accutane (isotretinoin for oral use).

Karolina Chilicka et al. discuss the therapeutic potential for acne of cosmetic acids (such as pyruvic, lactic, glycolic, phytic, salicylic, azelaic, mandelic, ferulic acid), and of alkaline water with a pH of 8–10, produced by electrolysis in the cathode region and containing active molecular hydrogen, capable to neutralize free oxygen radicals with antioxidant, anti-inflammatory and antiapoptotic effects [118].

A recent study [119] describes the development of an in vivo acne model in double knockout C57BL/6 mice (TLR2$^{-/-}$ TLR4$^{-/-}$) injected intradermally with various *C. acnes* strains and treated topically with artificial sebum. The pathogenicity of each strain was estimated by collecting the inoculated skin after 48h, followed by homogenization, the seeding of bacterial cultures and counting the colony forming units (CFU). The most pathogenic acne-associated strain (HL043PA1, Clade IA-2, RT5) and the least pathogenic strain (HL110PA3, Clade II, RT6) expressed hyaluronidase A (proinflammatory acne-associated hylA) and hyaluronidase B (moderately anti-inflammatory hylB), respectively. Via in silico studies, a small peptide hylA inhibitor and a multiepitope hylA vaccine were produced and administered intraperitoneally to CD1 mice, showing the effectiveness against pathogenic *C. acnes* inoculated intradermally post-vaccination and the specificity of

effect for pathogenic strains. These pioneering studies open the way for creating efficient *C. acnes* hylA vaccines for clinical use.

Another recent review [120] explores the complex molecular pathways triggered in acne by pathogenic *C. acnes* strains via TLR and the activation by PAMPs and DAMPs (pattern/damage-associated molecular patterns) of PRRs (pattern recognition receptors) such as NOD (nucleotide-binding oligomerization domain) receptors and NLR (NOD-like receptors), with the subsequent assembly of the NLRP3 inflammasome, the activation of caspase-1 and the subsequent cascade of proinflammatory cytokines like IL-1β and IL-8. Some potentially useful inhibitors of this pathway, including NLRP3 inhibitors baicalein, eucalyptol extracted from *Laurus nobilis*, superoxide dismutases, auranofin (an antirheumatic colloidal gold compound), or ROS production inhibitor polyphyllin I may be successfully used in future therapeutic protocols for acne. Cong et al. [121] recapitulate the pathophysiology of acne and explore new emerging treatments. Thus, increased sebum secretion may be limited by melanocortin receptor antagonist JNJ-10229570 and peroxisome proliferator-activated receptor-γ (PPARγ) modulator N-acetyl-GED-0507-34-LEVO (NAC-GED). A multicentric randomized controlled clinical trial performed on 450 patients with moderate or severe facial acne tested the effects of topical NAC-GED applied at 2% or 5% concentration, concluding the product is effective and devoid of adverse effects [122]. IGF-induced lipogenesis and the AMPK-SREBP-1 signaling pathway are inhibited by epigallocatechin-3-gallate (EGCG); isotretinoin, metformin, olumacostat glasaretil and XEN103 exert similar effects. IL-1β-induced signaling and subsequent IL-8 release are inhibited by αMSH analogs afamelanotide and K_DPT (a tripeptide derivative of the αMSH C terminal), and by the phosphodiesterase 4 inhibitor apremilast. Other options are monoclonal antibodies against IL-1, IL-17, or IL-1β, for example gevokizumab (XOMA 052) [123]. Recently proposed non-conventional acne therapies are Rhizoma Paridis saponins (extracted from *Paris polyphylla*), which inhibit Nrf2 and MAPK inflammatory pathways via KEAP1 binding [124], the promotion of keratinocyte apoptosis via the PI3K/Akt pathway by bacteriophage φPaP11-13 [125], a reduction in *C. acnes*-triggered inflammation via the MAPK and NF-κB pathways by ethanol extracts of the harebell poppy (*Meconopsis quintuplinervia*) [126], or the inhibition of mechanisms associated with neutrophil extracellular traps by adipose-derived stem cells via Nrf2 signaling pathways [127].

4. Conclusions

AFA and PCOS as an underlying condition are diseases with a complex pathophysiology that is still incompletely understood, although during the last decades significant progress has occurred through establishing precise quantitative diagnosis criteria and protocols, and by exploring a large variety of signaling pathways and mechanisms with the methods of molecular biology and genomics. The treatment should be individualized, using an ever enlarging array of therapeutic options, and application of alternative or non-conventional methods such as dietary restrictions, exercise, stress avoidance, natural products, probiotics, etc. may bring important contributions to the induction and maintenance of remission in these difficult-to-treat diseases.

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Advancing Lip Augmentation: State-of-the-Art 2D and 3D Analysis for Assessing Volume Enhancement and Lip Line Redefinition

Ritamaria Di Lorenzo †, Lucia Ricci †, Eleonora Vardaro, Teresa Di Serio *, Elena Morelli and Sonia Laneri

Department of Pharmacy, University of Naples Federico II, Via Domenico Montesano 49, 80131 Naples, Italy; ritamaria.dilorenzo@unina.it (R.D.L.); lucia.ricci@unina.it (L.R.); eleonora.vardaro@unina.it (E.V.); elena.morelli@unina.it (E.M.); sonia.laneri@unina.it (S.L.)

* Correspondence: teresa.diserio@unina.it; Tel.: +39-081-678-601

† These authors equally contributed to this work.

Abstract: Over the preceding five decades, there has been a noticeable surge in the pursuit of achieving voluminous and well-defined lips. This trend has prompted an escalating number of individuals to undergo lip augmentation procedures, aiming for a natural three-dimensional enhancement of lip volume and distinct vermilion borders. Despite the proliferation of lip augmentation techniques, there remains a dearth of comprehensive investigations into their precise effects on the three-dimensional structural integrity of the lips. This research endeavors to address this gap by employing stereophotogrammetry as a quantitative tool to scrutinize lip augmentation outcomes and to appraise the efficacy of lip plumpers. The study methodology involves a comparative analysis of lip dimensions among subjects treated with a commercial lip plumper using multi-spectral imaging for lip dimension assessment, coupled with markerless tracking technology and 3D interpolating surface methodology to analyze lip volume and shape. Additionally, the study evaluated lip youth state, including moisture level, softness, firmness, and tissue density. The demand for lip augmentation procedures is driven by perceived advantages such as quick recovery and minimal risk. Therefore, it is crucial to substantiate their efficacy with robust findings. The investigation suggests that both 3D and 2D stereophotogrammetry techniques are reliable for evaluating lip size before and after augmentation, whether through cosmetic or aesthetic approaches. Overall, the study provides a comprehensive analysis of a lip treatment aimed at enhancing volume and redesigning lip lines. It demonstrates that stereophotogrammetry is effective for assessing 3D lip dimensions and their correlation with internal lip structure. This research could be particularly valuable for evaluating the efficacy and duration of various lip enhancement techniques, including dermal fillers, implants, and topical cosmetic formulations, offering quantitative and reproducible assessments over time.

Keywords: lip augmentation; volume; lip plumpers; stereophotogrammetry; 3D analysis; lip health

1. Introduction

The lips are muscular–membranous skin folds that surround the mouth opening and anteriorly delimit the buccal vestibule [1]. On the face, lips occupy the lower third and play a key role in defining one's overall aesthetic appearance [2]. Lips perform important functions, as they facilitate the articulation of words and sounds, and allow food intake; lips express one's emotional status by conveying facial emotions, during verbal and psychological communication. Moreover, they can serve as a tactile organ, and being rich in sensory nerve endings, they are a very important erogenous zone, making them pivotal elements of human relationship life [3,4]. Lips are one of the most alluring features on the face, and thus, their dimension and youthfulness take on an important symbolic role in determining the overall perception and attractiveness of the face [5–7].

Throughout the last half-century, it has become increasingly common to undergo cosmetic and aesthetic procedures to increase lip volume and plumpness [8,9]. An increasing number of people undergo procedures to obtain an attractive appearance with a three-dimensional enhancement of lip volume and well-defined vermilion borders. These customs are a consequence of the importance given to the lips, through the filter of social customs, as a means of expression of social status, sexual proclivity, and amorous attitude. Full and well-defined lips impart a sense of youth, attractiveness, and health [4,10].

Aging often creates undesirable changes in our bodies, not least in the lips. The upper lip undergoes adipose tissue atrophy, leading to a thinner vermilion [3]. Other expected changes include the filter's flattening, Cupid's bow widening, and loss of the vermilion's natural protrusion [5]. Furthermore, studies reported lower lip ptosis with age, with consequent elongation, which causes an unwanted increase in lip length and vermillion height decrease [11], modifications that are related to significant muscular atrophy together with subcutaneous changes (dermal thinning and subcutaneous fat increase) [12]. All these factors push patients to seek lip beautification and rejuvenation procedures.

From this perspective, the nasolabial and labiomental angles are two key landmarks not to be overlooked when considering aesthetic pleasantness in the profile view [5]. Ideally, the nasolabial angle should be 95.5–100.1° in women and 93.4–98.5° in men [13], while the labiomental angle is appealing at 117–188° [14]. Demand for lip augmentation is rising because of its quick recovery and low risk, and the solutions come not only from the aesthetics but also from the cosmetic world, the latter of which is proposed as more cost-effective and less invasive than the former.

Cosmetic products known as lip plumpers are topical formulations based on active ingredients designed to stimulate lip protrusion and size temporarily [15]. They were developed to obtain noninvasive, low-cost, and, above all, painless effective lip augmentation and lip line mouth-filling redesign solutions [16], acting through stimulation of the matrix and dermis, replenishing from within. Lip plumpers thicken, strengthen, hydrate, and elasticize the supporting structure of the dermis and dermal–epidermal junction, while also stimulating the synthesis of new connective tissue. Still, they can include ingredients that act by inducing vasodilation, potentially resulting in either reversible irritant contact dermatitis or urticaria [15].

While lip augmentation is rising and becoming increasingly popular, there is a lack of clinical studies that evaluate with instruments the effects of lip-plumping products or procedures. To this end, this study aims to establish a quantitative analysis for lip augmentation and assess the efficacy of lip plumping through an innovative 2D and 3D technique. The study compared the lips of subjects before and after treatment with a commercial lip plumper for 30 days, by assessing lip volume and protrusion, moisture, softness, firmness, and tissue density of the lips.

2. Materials and Methods

2.1. Study Design

This study intended to propose an innovative and a multi-factorial analysis to assess lip volume boosting and lip line mouth-filling redesign, related to lip plumper applications or lip augmentation techniques. Specifically, the method is, for the first time, applied to a commercial lip plumper in a panel of 50 healthy women that had to apply approximately 1 mL of the test product once daily for 30 days. The product is investigated to promote lip volume, hydration, and softness, and improve the internal and superficial lip texture with a visible impact on lip shape, resulting in plump lips with a visible cushion effect. The lip plumper is a pomade produced by NECOS Srl (Crema, Italy); the list of ingredients is reported in Table 1.

Table 1. Lip pomade ingredient list.

Name	Ingredients (INCI Name)
Lip pomade	HYDROGENATED POLYDECENE, CAPRYLIC/CAPRIC TRIGLYCERIDE, C21-28 ALKANE, DIISOSTEARYL MALATE, STEARALKONIUM HECTORITE, HYDROGENATED STYRENE/BUTADIENE COPOLYMER, PHYTOSTERYL/OCTYLDODECYL LAUROYL GLUTAMATE, ETHYLHEXYL PALMITATE, PROPYLENE CARBONATE, CETEARYL ETHYLHEXANOATE, PARFUM, CI 77891, MICA, DECYLENE GLYCOL, PENTAERYTHRITYL TETRA-DI-T-BUTYL HYDROXYHYDROCINNAMATE, 1,2-HEXANEDIOL, CAPRYLYL GLYCOL, DIETHYLHEXYL SYRINGYLIDENEMALONATE, ECLIPTA PROSTRATA EXTRACT, SORBITAN ISOSTEARATE, TRIHYDROXYSTEARIN, LECITHIN, PORTULACA PILOSA EXTRACT, MELIA AZADIRACHTA LEAF EXTRACT, TIN OXIDE, TOCOPHEROL, ASCORBYL PALMITATE, SUCROSE COCOATE, SODIUM HYALURONATE, MORINGA OLEIFERA SEED OIL, PALMITOYL TRIPEPTIDE-38, CITRIC ACID, GLUCOMANNAN

The study was run from March 2022 to October 2022 at the RD Cosmetics laboratory, Pharmacy Department—University of Naples Federico II. The study protocol was reviewed and approved by qualified clinical, toxicology, and regulatory personnel of the university. The instrumental trial was conducted in accordance with Good Clinical Practices conforming to the ethical guidelines of the Helsinki Declaration.

Participants: Eligible subjects were women 20–45 years old with normal or thin lips and a desire to increase them. Written informed consent and release for audio–video–photo content was obtained from all participants before enrolling. Exclusion criteria included subjects with a history of sensitivity or allergy to any component of the topical lip care product and subjects with previous lip augmentation, lip surgery, or clinical evidence of orthognathic deformity. Only general health subjects were enrolled. At each check-up, subjects were asked if they complied with all pre-visit instructions and restrictions to determine whether they were qualified to continue the study. Protocol deviations were recorded to determine which subjects, endpoints, and time points should be excluded from subsequent data analysis.

Baseline measurements of the established lip parameters were carried out after enrollment (T_0). The test site identified is the test subjects' lips. Subjects were instructed verbally and in writing regarding the proper use of the test product, with instructions reinforced during each control time. The product application was conducted at the research lab to ensure all volunteers applied the same quantity of the test product.

Lip parameters were re-measured reliably after 30 days of once-daily 1 mL application (T_f). The comparative assessment of the cosmetic efficacy was based on the data obtained by instrumental measurements. Lip volumes and shapes were analyzed using VECTRA H2 (Canfield Scientific Inc., Parsippany, NJ, USA), which is a quantitative approach to soft tissue assessment that characterizes the degree of stretch, compression, lift, and volumization. Dense image-to-image correspondence enables fine-scale 3D biomechanical analysis; skin surfaces are automatically aligned, tracked, and mapped, while vector arrows provide a precise indication of the direction and magnitude of skin movement, which helps demonstrate cosmetic treatments. The Lip Index was assessed through VISIA® (Canfield Scientific Inc., Parsippany, NJ, USA), which works by using multi-spectral imaging and analysis to analyze data. Full-face photos were made under standardized illumination, focusing on the lip area. Lip youth states were determined through evaluation of the moisture level, softness, firmness, and tissue density, employing worldwide recognized tools for cosmetic efficacy evaluation, like Corneometer® CM 825, Cutometer® Dual MPA 580 and Indentometer® IDM 800 (C + K electronic GmbH, Köln, Germany), and Dermascan®

C (Cortex technology ApS, Aalborg, Denmark). The analysis compared before-and-after lip dimensions and status in treated subjects, setting the following primary endpoints. Lip augmentation is demonstrated by upper and lower lip dimension and surface increase (e.g., upper, lower, and overall lip), volume enhancement (total lip volume), and upper lip protusion and eversion. Furthermore, rejuvenating and beautification efficacy is defined by moisturizing, softening, firming, and dermal densifying action. Finally, lips had to appear visibly hydrated, soothed, plumped, filled, and lifted, pointing to improving the subcutaneous structures and their principal components.

2.1.1. Lip Analysis

The instrumental measurements were carried out in an air-conditioned room at T = 20 ± 2 °C and at controlled humidity (50 ± 5% RH) after a conditioning time of about 30 min. The detected lip parameters useful for assessing the product's ability in plumping, filling, and reshaping lip lines, extinguishing aging-related lip volume reduction, and satisfying the newest general demand for more extensive and fuller lips is reported as follows:

- 3D and 2D Lip dimension analysis:

Each subject was imaged using the VECTRA H2 and VISIA 7th (Canfield Scientific, Inc., Fairfield, NJ, USA), and analyzed with stereophotogrammetry manually and digitally, employing a 3D generated facial model through VAM® software version 7.4.6. To ensure more accurate results, the subjects were imaged according to Patient Preparation and Positioning methods for Capture Standardization described by Sawyer et al. [17]. All recordings were completed by a single investigator to ensure reliability. The following 3D analysis was performed after positioning lip landmarks according to those described by Farkas [11]: cheilion (Ch), Cupid's bow (Cb), labiale inferius (li), subnasale (Sn), sublabiale (sl), stomion (sto), alar (al), christa philtri (chp) (Figure 1).

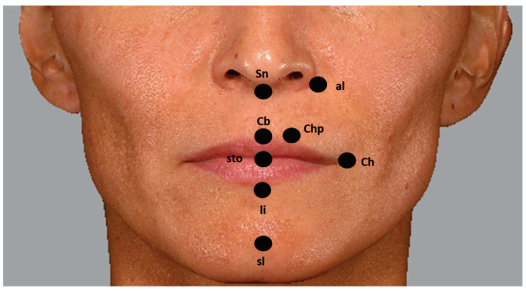


Figure 1. Facial and lip landmarks set with VAM® software for 3D lip dimensional analysis.

Furthermore, the Interpolating Surface method allows 3D surface volume analysis; the software first uses the area surrounding the bump to create an approximation of the base surface (interpolating surface). This surface will be shaped to match the shape of the face wall surrounding the lips. It cannot see what is under the lip mound and, therefore, will assume that the surface curve is consistent under the entire mouth (Figure 2).

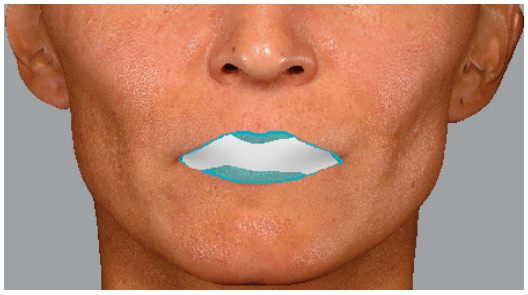


Figure 2. VECTRA H2 Interpolating Surface Volume (cc) measurement.

The angle that originates from the columella and a line intersecting the Sn and the most anterior point of the upper lips is called the nasolabial angle (NlA). It is visible laterally (Figure 3), and it is associated with the upper lip and can promote disharmony in the adult face if not balanced. NlA is reduced by practicing lip eversion, resulting in a mouth with a more pronounced and protruding contour.

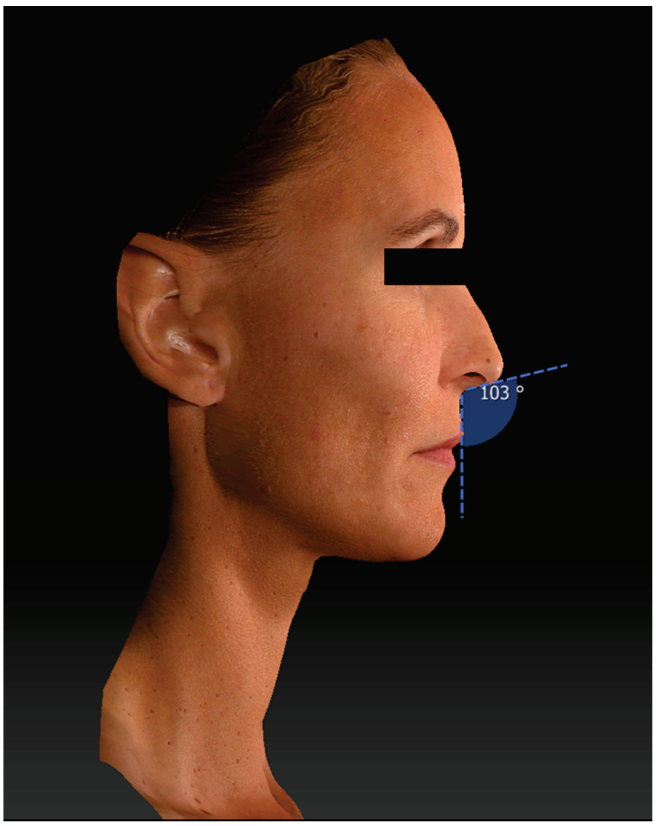


Figure 3. VECTRA H2 nasolabial angle detection.

Vectra H2 allows lip protrusion and eversion determination by calculating the distance (mm) from the lips to the nasomental angle (formed from the intersection of the line of the back of the nose and the nasomental line, also known as Ricketts line) [18], as shown in Figure 4a, and the average distance (mm) between Cupid's bow (Cb) and stomion (sto) (Figure 4b). If there is a lip size increase in terms of three-dimensionality, this can result in more significant protrusion and eversion of the lip itself. A greater protrusion can be indicated by the approach of the lip to the nasomental line, in which case a lesser distance has to be measured. Conversely, regarding eversion, the increase in the Cb-sto distance will indicate a greater torsion of the lip and thus an increase in its prominence. Still, as a further confirmation of eversion, the Upper Vermillion Height (UVH) was measured in mm. UVH is the distance (mm) from subnasale to Cupid's bow (Sn-Cb) [19].

Two-dimensional measurements were taken directly from standardized patient photos with the device designed by Lemperle [20], which allows reliable lip size analysis. The ruler enables sizing (in mm) of the vermilion height, indicated by Lemperle et al. as Upper Lip height (ULH) and Lower Lip height (LLH). Then, by lateral patient photos, as per Figure 5, the point of maximum protrusion was calculated (Upper Lip Protrusion [ULP] and Lower Lip Protrusion [LLP]).

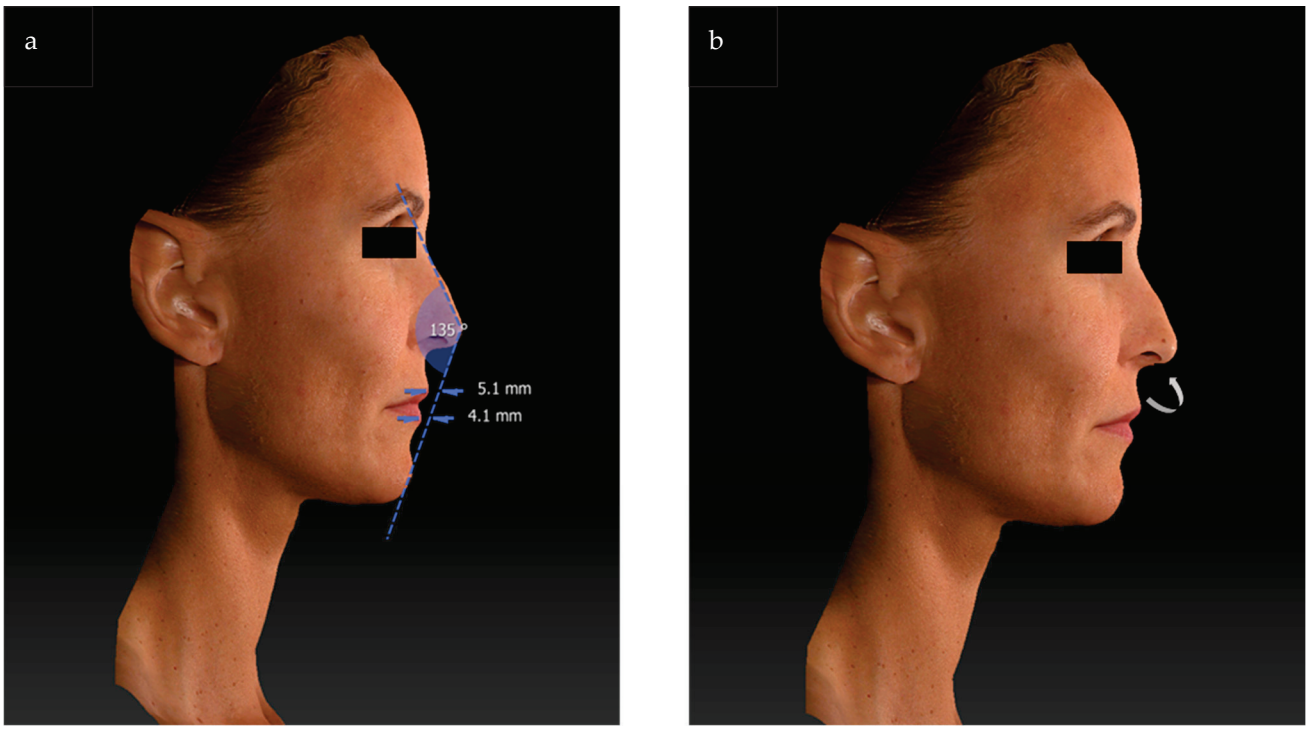


Figure 4. (**a**) Distance (mm) from the lips to the nasomental angle (lip protrusion). (**b**) Distance (mm) between Cupid's bow (Cb) and stomion (Sto) (lip eversion).

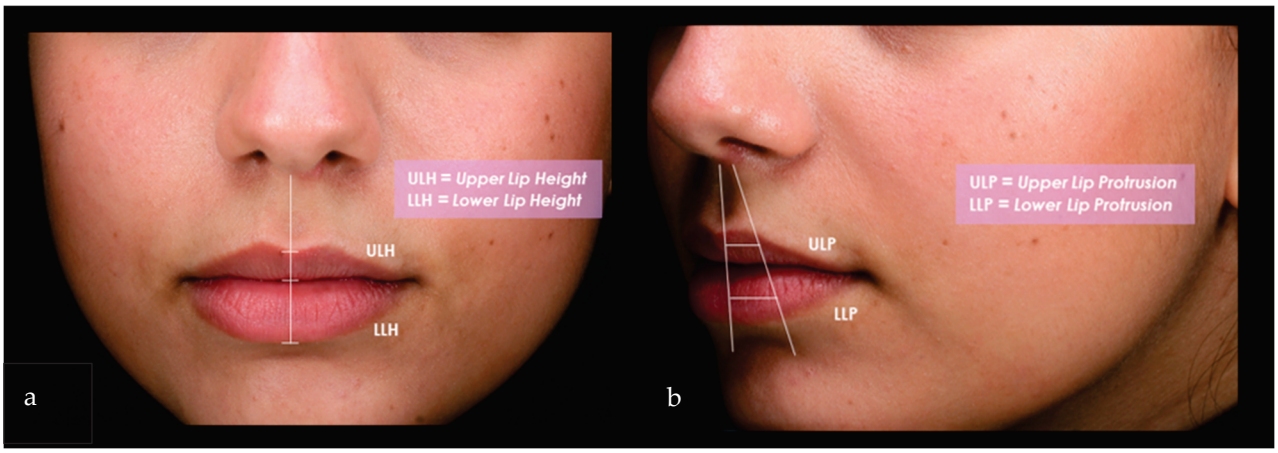


Figure 5. (**a**) Upper Lip Height [ULH] and Lower Lip Height [LLH] measured (in mm) frontally. (**b**) A line is drawn from the sub-nasale to the horizontal chin folds, and the lip protrusion is measured perpendicular to this line (ULP and LLP). The ratio was 1:1.

Finally, as suggested by Lemperle, the multiplication of the calculated ULH and ULP directly correlates with the upper lip area in mm^2. The same operation is performed for the lower lip by obtaining the patient's Upper Lip Index (ULI) and Lower Lip Index (LLI) in mm^2, respectively, whereas ULI and LLI addition results in the Overall Lip Index (mm^2).

ULI, LLI, and OLI became the baseline for comparative purposes following after-treatment augmentation.

- Lip youth state assessment:

Ultrasound imaging was performed with the subjects in an upright (90°) seated position employing a 20 MHz linear transducer (Dermascan®, Cortex Technology Aps, Aalborg, Denmark). Measurements were performed in the midline of the lower lip, without

applying pressure, allowing us to confirm the product's ability to plump lips by stimulating dermal and matrix modelling. Furthermore, the vermilion border is a body site particularly susceptible to water loss, with consequences in the surface smoothness, shape, tactile sensitivity, and healthy appearance [21]. Capacitance is a method recognized worldwide for establishing skin hydration [22–26]; therefore, since the skin is covered by an, albeit thinner, epithelial layer, it is possible to employ the same tool for assessing lip water content, as also shown in previous studies [27–29].

Lip fullness is ideally associated with softness and silky touch [30]. For the first time, the Indentometer IDM 800 was used to assess lip features, by measuring how the probe indenter displaces lips. The measurement principle is based on the force used by the tool to deform lips. The penetration depth of the pin (displacement) is measured in mm (0–3 mm). The softer the lips, the deeper the pin penetration.

2.1.2. Statistics

The recorded values were compared with intra-group analysis (T_0 vs. T_f) using Student's *t*-test, performed using SPSS software 15.0 for Windows (SPSS Science, Chicago, IL, USA). A *p*-value < 0.05 was considered significant.

3. Results

Lip dimension recorded values (mean ± SD and percentage variation) are shown in Table 2. The volume, ULI, LLI, OLI, area and NlA differences before and after treatment are highly significant ($p < 0.01$). Each patient's clinical response to treatment was assessed by comparing digital photographs taken before (baseline) and after treatment. The greater the values recorded, the higher the product-induced plump. So, a percentage increase is desirable to assess plumped, bigger, and filled lips. Figure 6 illustrates images of subjects treated with the tested lip plumper for 30 days.

Table 2. The trend of the lip dimension average values ± SD before and after the 30-day treatment with the commercial lip plumper (intra-group difference *t*-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Parameter	T_0	T_f	Δ ($T_f - T_0$) %
Volume (cc)	72.3 ± 3.2	76.7 ± 5.1	6.5% ***
Upper Lip Index (cm^2)	31 ± 5	35 ± 5	15.0% ***
Lower Lip Index (cm^2)	64 ± 16	66 ± 15	12.0% ***
Overall Lip Index (cm^2)	95 ± 28	101 ± 20	13.0% ***
Area (cm^2)	8.7 ± 0.3	8.9 ± 0.3	5.3% ***
Eversion (mm)	9.1 ± 0.4	9.3 ± 0.4	2.8% *
Protrusion (mm)	7.1 ± 0.2	6.9 ± 0.3	−3.3% **
Upper Vermillion Height (mm)	19.7 ± 0.8	19.0 ± 0.9	−3.8% *
Nasolabial Angle (°)	115 ± 3	112 ± 2	−3° ***

The results reported in Table 3 show that after treatment for 30 days with the test product, the lips are not only more plumped, lifted, and defined, but also more moisturized and with a more turgid and denser appearance. As a consequence, corneometry, skin density, softness, and firmness were statistically increased by treatment with the lip plumper ($p < 0.05$). Hydration strongly influences lip health appearance. During the study, panelists experienced an increase in their lip hydration, partly due to the film-forming action of the lipgloss, which thus manages to maintain a good water content on the lips.

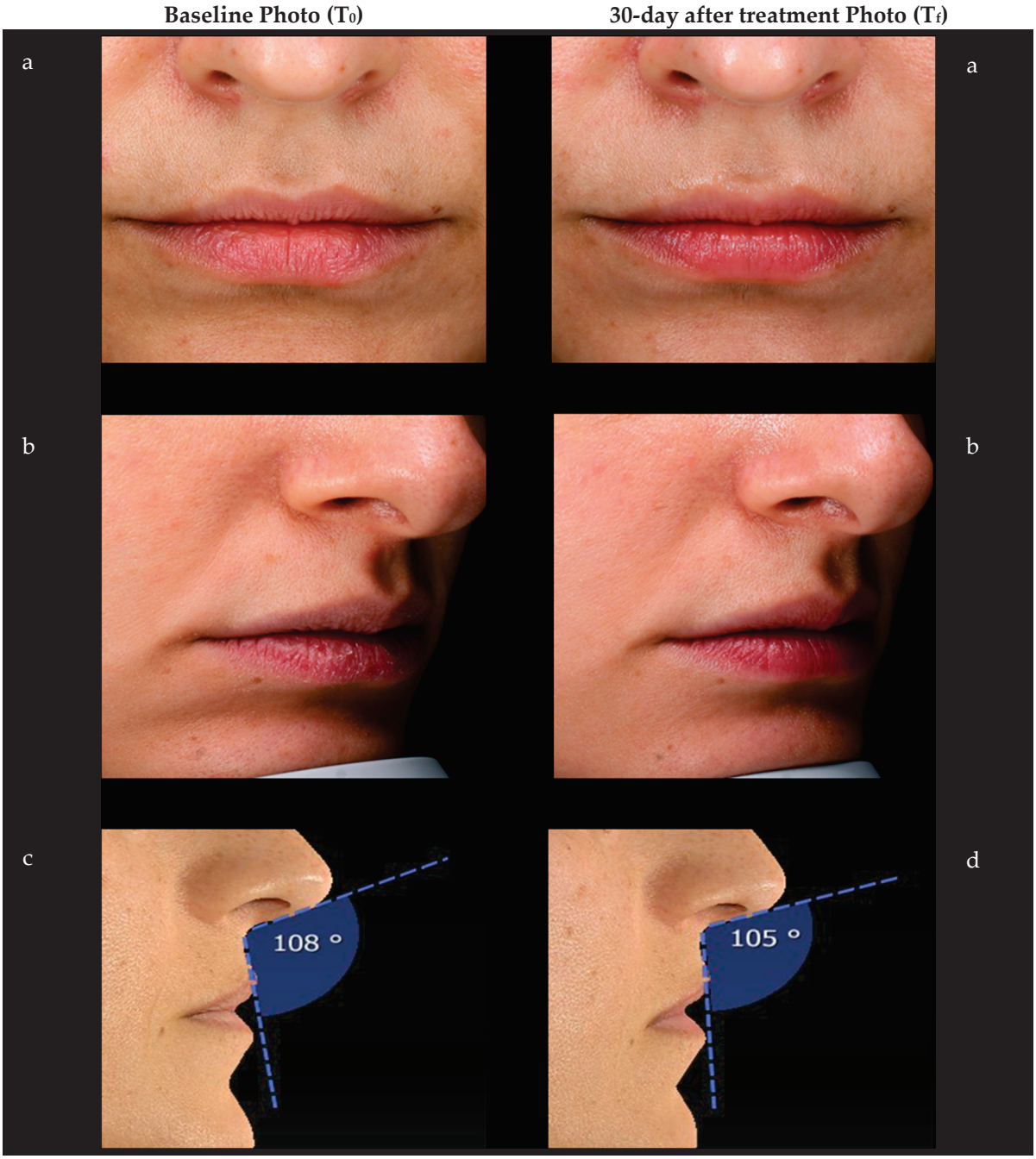


Figure 6. (**a**) Frontal and (**b**) lateral 2D panelist lip photos before (T_0) and after 30-day treatment (T_f) with the lip plumper; (**c**) 3D panelist lip photos and columella angle before (T_0) and (**d**) after 30-day treatment (T_f) with the lip plumper.

Table 3. The trend of the lip youth parameter average values ± *DS* before and after the 30-day treatment with the commercial lip plumper (intra-group difference *t*-test *** $p < 0.001$).

Skin Youth Parameter	T_0	T_f	Δ ($T_f - T_0$) %
Corneometry (A.U.)	44.1 ± 2.4	57.3 ± 5.9	30.4% ***
Lip Softness (mm)	2.832 ± 0.091	2.974 ± 0.052	5.0% ***
Lip firmness—R_0 (μm)	0.341 ± 0.067	0.275 ± 0.108	−17.0% ***
Collagen Index	34.0 ± 12.2	36.7 ± 12.5	11.0% ***

4. Discussion

In the 21st century, lips are protagonists of beauty that teach us how to transform it into a tool for empowerment. Over the past fifty years, full, plump, and pouty lips have been considered a desirable trait [6]. More and more young people undergo aesthetic surgical procedures for lip augmentation to obtain the commonly sought-after appearance [31–34]. In addition, aging creates a spectrum of undesirable changes in the lips, as for the rest of the face [11,12,35]. The upper lip undergoes dermal atrophy and stretches vertically, leading to a thinner and less visible vermilion border [18]. Other expected changes include flattening of the filter, a widening of the philtrum, and the loss of the natural lip protrusion [6,10–12,18]. Furthermore, the lower lip can also develop ptosis with age, resulting in an unattractive lower dental show [20,36]. All these factors contribute to pushing patients to seek lip augmentation. Lips become thin and stiff, and collagen and elastic fiber fragmentation occurs [12]. What is more, hyaluronic acid supply runs low, with the consequent appearance of wrinkles and fine lines on the lip surface [37]. In addition, excessive heat, pollution, and stress can affect the health of the skin and, thus, also lips [23,24]. Therefore, the goal of lip beautification and rejuvenation is a natural three-dimensional enhancement of lip volume with a well-defined vermilion border, accompanied by soft and nourished lips. Lip augmentation and rehydration is used to enhance the appearance of thin, aged, or asymmetrical lips, contributing to the ideal facial beauty. Therefore, good lip performance, whether induced by cosmetic or aesthetic treatment, requires a multifactorial approach involving all the elements that constitute the lips, which this method proposes to investigate.

Lip hydration is a technique used to improve the appearance of thin, aged, or asymmetrical lips. The health and size of lips largely depend on their hydration levels, which makes it crucial to use an intense moisturizing action while administering filler treatment. In our study, panelists observed an increase in their lip hydration, which could be attributed to the film-forming effect of the lip pomade as well as the presence of moisturizing ingredients such as Eclipta prostrata extract, Portulaca pilosa extract, Melia azadirachta leaf extract, Tocopherol, Sodium hyaluronate, Moringa oleifera seed oil, and Palmitoyl tripeptide-38. The intense moisturizing action is further related to the higher softness registered in the study population. Finally, the stimulation of the dermal elements was indicated by the collagen index and firmness increase. All these features were evaluated throughout the study to guarantee a complete understanding of the mechanism responsible for the recorded plumping effect. The NlA was monitored as confirmation of the recorded volumization treatment, because it is associated with the upper lip and can promote disharmony in the adult face if not balanced. While UVH is very useful in evaluating facial symmetries, in general, following lip-filling treatments, there is upper lip height increase, with a consequent reduction in the Cb distance from the Sn landmark. In this study, since it is a cosmetic treatment, the decrease recorded is far less than that obtainable through surgery, but not undetectable.

Despite several studies showed lip augmentation procedures [2,4,10,38–41] or investigated age-related lip disorders [3,11,12], there is a shortage of literature on the available noninvasive devices that can be employed for assessing the efficacy of anti-aging cosmetic or aesthetic lip treatment [14,37,42]. Many studies on lip filler injection efficacy and longevity have relied on subjective evaluation methods like patient satisfaction surveys and physician observation [43,44]. Some have used validated photonumeric lip fullness scales to compare augmentation results objectively [16,45,46]. However, only a handful have employed quantitative measurements to assess volume enhancement effects [19,47,48], and no one has related the volume enhancement to lip health amelioration. Our study lays the foundation for this kind of analysis, serving as a pioneer in determining the mechanisms of action underlying lip rejuvenation and beautification processes in accordance with the overwhelming desire to have more prominent and protruding lips. The main objective of this investigation was to provide a comprehensive analysis to assess lip treatment in order to gain valuable insights into the potential use of these innovative 2D and 3D techniques

for cosmetic, aesthetic surgical, and nonsurgical applications, through an observational in vivo study on healthy subjects treated for one month with a commercial topical cosmetic formulation with a plumping and filling action. The investigated lip pomade enhanced the volume, protrusion, and eversion of the lips by moisturizing them (+30.4% vs. T_0, $p < 0.001$) and inducing a dermis re-organization, as suggested by the increased density, softness, and firmness (collagen index: +11%, softness: +5% and R_0: −17% vs. T_0, $p < 0.001$). Nevertheless, our study presents some limitations. The short-term follow-up period (4 weeks) hinders our ability to assess long-term outcomes. The relatively small sample size reduces the statistical power of our results. Furthermore, the absence of a control group limits our ability to attribute observed changes solely to the tested product. Additionally, the study population comprised exclusively Italian females within a relatively narrow age range. Future research with larger, more diverse participant groups, encompassing various ages, ethnicities, and genders, would be recommended to corroborate our findings effectively.

5. Conclusions

In conclusion, the present investigation provided a comprehensive analysis for lip treatment designed to provide volume boosting and lip line mouth-filling redesign after cosmetic lip plumper applications or aesthetic lip augmentation techniques. Data showed that stereophotogrammetry produces a good approximation of 3D lip dimension analysis, and how it is correlated with the internal lip structure. This study may be particularly important in lip augmentation procedures, to provide a reliable method for the efficacy evaluation of the clinical effects or duration of lip enhancement dermal filler and implants, or topical cosmetic formulation. Moreover, quantitative and reproducible assessments over time are easy to calculate, with the most common worldwide skin testing devices.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: The data presented in this study are available from the corresponding author upon request. The data are not publicly available due to privacy.

Conflicts of Interest: The authors declare no conflicts of interest.

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Potential Benefits of a Cosmetic Ingredient Combining Thermal Spring Water and Diatom Algae Extract

Maria Lourdes Mourelle [1,*], Jordi Segura de Yebra [2], Jordi Ayats [2], Maria Vitale [3] and Ana López Sánchez [4,*]

[1] FA2 Research Group, Department of Applied Physics, University of Vigo, Campus Lagoas-Marcosende s/n, 36310 Vigo, Spain
[2] ALGAKTIV S.L., Baldiri Reixac 4, 08028 Barcelona, Spain; jsegura@algaktiv.com (J.S.d.Y.); jayats@algaktiv.com (J.A.)
[3] Medical Affairs, Cantabria Labs, 28043 Madrid, Spain; maria.vitale@cantabrialabs.es
[4] R&D, Cantabria Labs, 28043 Madrid, Spain
* Correspondence: lmourelle@uvigo.es (M.L.M.); ana.lopezsanchez@cantabrialabs.es (A.L.S.)

Abstract: The development of cosmetic ingredients improving the management of skin with irritation proclivity is an actual need. However, medical recommendations in specific cases, such as sensitive skin, include the use of as few and low-reactive ingredients as possible. In this context, we here describe the development of a new ingredient consisting of a mixture of thermal water and a diatom algae extract. First, we characterized a thermal spring water (La Solia, LS-TSW). Attending to its chemical composition, LS-TSW displayed an exclusive combination of different inorganic elements, with interesting potential properties when compared with other commercial spring waters. Then, LS-TSW hydrobiome was studied, and after finding specimens of *Phaeodactylum*-like sp., we proposed potentiating LS-TSW benefits with its combination with an oil extract of *Phaeodactylum tricornutum* (*Pt*OE). Finally, we assessed the potential of the mixture during pro-inflammatory stimulation, in the first instance using an immune cell model, and then in an in vitro system mimicking keratinocytes under skin irritation. In the last-mentioned model, the ingredient of interest effectively attenuated the induced levels of different pro-inflammatory mediators (*IL-6*, *IL-1*, *TNFα*, *NF-κB*, and *CCL1*), at the level of gene expression. Thus, our results highlight the potential benefits of this combination in the context of skin irritation, opening roads for its use in new skincare regimens, and addressing an important dermatological concern.

Keywords: thermal water; diatom algae extract; *phaeodactylum*; sensitive skin; skin irritation; inflammation

1. Introduction

The daily use of skincare products can provide the protection, maintenance, and improvement of skin health [1]. This is of special interest to individuals with skin irritation tendencies [2]. Skin irritation can originate from a wide range of pathological and non-pathological conditions. In fact, the induction of inflammation in the skin is a fundamental defense mechanism against external aggressions [3]. However, chronic inflammation of skin tissues is a very common and detrimental feature for different pathologies such as psoriasis, rosacea, acne, dermatitis; even photoaging and sensitive skin. In all these individuals, the use of cosmetic products has been demonstrated to have tremendous potential [2,4,5].

Specifically, the skincare recommendations for individuals suffering from what is defined as sensitive skin, appear as certainly complex. The International Forum for the Study of Itch (IFSI) defines sensitive skin as: "Characterized by the occurrence of unpleasant sensations (stinging, burning, pain, pruritus and tingling sensations) in response to stimuli that normally do not provoke such sensations". The skin can appear normal or be accompanied by erythema in most patients suffering from itch [6]. Thus, for people who suffer from sensitive skin, it is important to have dermocosmetic products that complement or can be an alternative to medications in periods of high irritation, dryness, or skin itching,

which can provide relief from their illness; and also to maintain skin hydration, a proper skin barrier function, and comfort during periods of remission. According to IFSI, sensitive skin affects approximately half of the population, is more frequent in women than in men, courses chronically in cycles of relapsing–remitting, and can have a significant impact on quality of life [7,8]. Experts considered that it is beyond doubt that holistic skin care should focus on therapeutic treatment first, but there are uncertainties about practical guidance on skincare routine [9,10], as the use of cosmetics itself has been identified as one of the most important elements in the exposome triggering sensitive skin [11]. Therefore, skincare routines for sensitive skin should consider individual skin differences, and dermatologists and other health professionals should provide the correct dermocosmetic customized advice, depending on the symptomatology and according to the severity of this condition. In terms of cosmetics formulation, experts advise the use of fewer ingredients and water-soluble cosmetics with inert, hypoallergic, fragrance-free, pure ingredients that are specially formulated for sensitive skin [10,12]. Additionally, those cosmetic ingredients that are able to repair and protect the skin barrier should be taken into consideration [2]. Under these premises, it is understandable that there is an increasing interest in the search for new ingredients meeting those multiple requirements.

Thermal spring waters (TSW) emerge as an excellent candidate in the treatment of sensitive skin since they have demonstrated their anti-inflammatory capacity, suppressing pro-inflammatory cytokines production [13]. TSW are mineral-rich waters with specific properties linked to their content of minerals and trace elements, which have been recognized by the competent authorities, and their health benefits have been proven. According to the mineral composition, TSW can be classified as chloride, bicarbonate, sulfide, carbon dioxide, and weakly mineralized waters. They can also contain soluble minerals such as Mg^{+2}, Ca^{+2}, Na^{+}, silicates, SiO_2, iron compounds, etc., and trace minerals, such as manganese, selenium, zinc, boron, etc. The role of TWS in the treatment of dermatological illness has been described by several authors. Caccipuoti et al. [14] described recent evidence of major dermatologic diseases that are frequently treated by balneotherapy with a remarkable rate of success. Authors considered that balneotherapy offers several advantages: no chemicals or potentially harmful drugs are needed; there are almost no side effects during and after treatment, and there is a low risk to the patient's general health and well-being [15]. Thus, TWS are of special interest for their use in the chronic inflammatory diseases of the skin.

On the other hand, the study of the biological communities, the so-called hydrobiome, of TWS has aroused great interest in the last decade, and several cosmetic companies investigated the potential of extracts, lysates, and ferments obtained from them as active cosmetic ingredients [16]. One of these aquatic microorganisms of interest is *Phaeodactylum tricornutum*, whose extracts are rich in omega-3 fatty acids and fucoxanthin with documented benefits in sensitive skin treatments [17], due to their anti-inflammatory activities [18,19].

In this scenario, the present work aimed to develop and characterize a new cosmetic ingredient with potential benefits for skin problems associated with irritation proclivity. Under this goal, we first characterized La Solia thermal spring water (LS-TSW), which possesses a unique combination of different inorganic elements, and explored its compared composition relative to other commercial spring waters in the market. The LS-TSW hydrobiome was also analysed, unveiling a characteristic diversity, including representation from the groups of Cyanophyta, Chlorophyta, and Heterokontophyta, among which specimens of *Phaeodactylum*-like sp. were isolated. Accounting for the mentioned benefits of *Phaeodactylum tricornutum*, we next proposed using oil extracts of *P. tricornutum* (*Pt*OE) to potentiate the natural benefits of LS-TSW. Under this aim, we developed a new ingredient combining LS-TSW and *Pt*OE, named Aquammunist. Keeping in mind the plausible emerging properties from the LS-TSW composition, the ones previously described for *P. tricornutum* extracts, and keeping a focus on skin irritation dermatological concerns, Aquammunist was tested for immunomodulatory activities. After a general anti-inflammatory character observed in monocytes, we addressed the effects of Aquammunist

treatment in human keratinocytes (HaCaT cells) subjected to a stimulus-mimicking skin irritation. In the selected model, Aquammunist showed a clear control of different immune factors, which are key for different skin inflammatory alterations, including skin reactivity.

Thus, our results highlight the potential benefits of this combination (Aquammunist), laying a scientific foundation for its use in new skincare regimens, and addressing an important dermatological concern.

2. Materials and Methods

2.1. Physicochemical Analysis

Thermal water samples were collected according to the "Standard Methods for the Examination of Water and Wastewater" (23rd edition https://www.standardmethods.org, accessed on 5 March 2024) in clean polythene and sterilized plastic bottles of 2 L and kept at 4 °C. The sampling point was taken at Cantabria Labs facilities, in the north region of Spain, where the natural emergence of the spring's water occurs, and with the specific collection point at the UTM ETRS89 coordinates X = 430.592 and Y = 4.803.85. Anions analysis was performed with an 850 Professional IC Ion chromatograph equipped with a chemical suppressor module (MSM II) and a digital conductivity detector coupled to a Metrohm 858 Professional Sample Processor with inline ultrafiltration (Metrohm, Herisau, Switzerland). Chromatographic separation was carried out with a Metrosep A Supp 7 (250 × 4 mm; 5 µm; Metrohm, Herisau, Switzerland). A 3.6 mM sodium carbonate in water solution was used as a mobile phase running in isocratic mode at 0.8 mL/min flow. The column temperature was set at 45 °C, the injection volume was 20 uL, and the run time was set at 32 min. The concentrations of metal and metalloid elements were determined by inductively coupled plasma mass spectrometry (ICP-MS) with an X-SERIES 2 Quadrupole ICP-MS with high-performance liquid chromatography (HPLC) coupled to a DIONEX model DGP-3600 A HPLC system (Thermo Scientific, Waltham, MA, USA). The water samples were filtered with syringe filters (0.45 µm) and subsequent acidification in 1% HNO_3. Samples are kept in the refrigerator and analyzed with adequate dilution as needed. The silica (SiO_2) has been obtained by calculation.

2.2. LS-TSW Microbiome Analysis

Water samples were collected from three distinct water points within LS-TSW. The sampling sites included the natural pond of thermal water, the thermal water source at Cantabria Labs facilities, and the point where thermal water returns to the river. Samples were acquired using sterile bottles, ensuring the preservation of the original microbial community. For microalgae collection from surfaces, sterile brooms were employed. All samples, suspended in thermal spring water, were promptly transported to the laboratory at room temperature, maintaining the integrity of the collected microbiota.

Upon arrival at the laboratory, the collected samples underwent an initial microscopic examination to assess their diversity. Subsequently, samples were subjected to a dilution of 1:10 in various culture media, namely BBM, BG-11 and f2/2 media enriched with nitrate, phosphate, and silicates. Specifically BBM (bold basal media): 0.25 g $NaNO_3$; 0.075 g $MgSO_4{\cdot}7H_2O$; 0.025 g NaCl; 0.075 g K_2HPO_4; 0.025 g $CaCl_2{\cdot}2H_2O$; 0.0057 g H_3BO_3; 50 mg $EDTANa_2$; 31 mg KOH; 49.8 mg $FeSO_4{\cdot}7H2O$; 8.82 mg $ZnSO_4{\cdot}7H_2O$; 1.44 mg $MnCl_2{\cdot}4H_2O$; 1.57 mg $CuSO_4{\cdot}5H_2O$, 0.49 mg $Co(NO_3)_2{\cdot}6H_2O$; 0.71 mg MoO_3. BG-11: 1.5 g $NaNO_3$; 0.04 g K_2HPO_4; 0.075 g $MgSO_4{\cdot}7H_2O$; 0.036 g $CaCl_2{\cdot}2H_2O$; 0.006 g citric acid; 0.006 g ferric ammonium citrate; 0.001 g EDTA (disodium salt); 0.02 g Na_2CO_3; 2.86 mg H_3BO_3; 1.81 mg $MnCl_2{\cdot}4H_2O$; 0.222 mg $ZnSO_4{\cdot}7H_2O$; 0.39 mg $NaMoO_4{\cdot}2H_2O$; 0.079 mg $CuSO_4{\cdot}5H_2O$; 0.0494 mg $Co(NO_3)_2{\cdot}6H_2O$. And f2/2: 0.075 g NaNO3; 5 mg $NaH_2PO_4{\cdot}H2O$; 0,03 g $Na_2SiO_3{\cdot}9H_2O$; 4,4 mg $Na_2EDTA{\cdot}2H_2O$; 4,6 mg $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$; 6.8 µg $CuCl_2{\cdot}2H_2O$; 152 µg $MnSO_4{\cdot}H_2O$; 23 µg $ZnSO_4{\cdot}7H_2O$; 7,3 µg $Na_2MoO_4{\cdot}2H_2O$; 14 µg $CoSO_4{\cdot}7H_2O$. All compounds came from Merck Group, Darmstadt, Germany. This strategic approach aimed to isolate Cyanobacteria, Chlorophyta, and Diatom species, respectively. Once the cultures were established, species were further isolated on enriched agar plates to facilitate

the isolation of pure axenic clonal cultures. Colonies originating from single cells were transferred to wells containing enriched isolation growth media. The cultivation conditions were meticulously controlled, maintaining a temperature of 25 ± 1 °C, light intensity at 60 μE.m².s¹, and a continuous 24-h light cycle. The atmosphere within the cultivation environment was enriched with 2% CO_2, ensuring optimal growth conditions for the isolated microorganisms (walk-in cabinet, Ibercex, Arganda del Rey, Madrid, n). Microscopic identification of microalgae was conducted using an optical microscope MOTIC BA310 (Motic, Xiamen, China).

2.3. Cell Culture

HaCaT cells were obtained from Eucellbank (Celltech, University of Barcelona, Spain). The cells were grown in Dulbecco's minimal essential medium high glucose (DMEM-Hi glucose; Gibco; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin streptomycin (Gibco; Thermo Fisher Scientific, Waltham, MA, USA). Thereafter, the cells were incubated at 37 °C in a humid atmosphere of 5% CO_2. THP1 cells were obtained from ATCC (ATCC:TIB-202™). The cells were grown in RPMI 1640 (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, USA) and 1% penicillin streptomycin (Gibco; Thermo Fisher Scientific, Waltham, MA, USA). Thereafter, cells were incubated at 37 °C in a humid atmosphere of 5% CO_2.

2.4. Induction of Inflammation Using LPS

THP-1 cells were seeded at 2.5 × 10^4 cells/well in 24-well culture plates (6 wells per experimental condition for a total of 1.5 × 10^6 cells/condition). After 24 h medium was refreshed, and cells were incubated for 1 h with different concentrations of *P. tricornutum* oil extract and/or La Solia thermal water (1% PtOE, 1% LS-TW, 10% LS-TSW, 1% LS TSW + 1% PtOE, or 10% LS-TSW + 1% PtOE). Inflammation was afterward induced with 1 μg/mL of LPS (O111:B4, Merck Group, Darmstadt, Germany). After 24 h, all supernatants were collected and centrifuged for 5 min at 4 °C. The different treatment conditions were pooled (n = 6) before subsequent analysis using cytokine arrays.

2.5. Cytokine Arrays

Cytokine arrays were performed and developed in accordance with the manufacturer's protocol (Proteome Profiler Array: Human Cytokine Array Kit, R&D Systems, Minneapolis, MN, USA). Membranes were scanned using ImageQuant LASS 4000 Mini (GE Healthcare Life Sciences, Chicago, IL, USA) and images were analyzed using the MultiGauge V3.0 software (Fujifilm Life Sciences, Tokyo, Japan).

2.6. Induction of Inflammation Using TNF-α and IFN-γ

HaCaT cells were cultured at a concentration of 2.5 × 10^4 cells/well in 6-well culture plates and incubated for 24 h at 37 °C in a humid atmosphere of 5% CO_2. Thereafter, they were treated with different concentrations of *P. tricornutum* oil extract and/or La Solia thermal water (1% LS TSW + 0.25 *Pt*OE, or 0.5% LS-TSW + 0.5 *Pt*OE). After 24 h of incubation, they were treated simultaneously with TNF-α/IFN-γ mixture (20 ng/mL each, #10291-TA-050, #285-IF-100, R&D Systems, Minneapolis, MN, USA) to induce inflammation. The experiment was repeated twice with similar results.

2.7. Total RNA Isolation and Real-Time quantitative Polymerase Chain Reaction (RT-qPCR)

After 24 h of inflammation induction, the total RNA of the HaCaT cells was extracted using the RNeasy kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Thereafter, complementary DNA (cDNA) was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, USA) from 1 μg of purified total RNA. Next, cDNA was amplified with a Light Cycler® 480 with 40 cycles of denat-

uration (95 °C for 15 s), annealing (60 °C for 60 s), and extension (60 °C for 60 s), using TaqMan probes: TNF-α (ID#: Hs00174128_m1); IL-6 (ID#: Hs00174131_m1); IL-1β (ID#: Hs01555410_m1); NF-κB (ID#: Hs00765730_m1); CCL1 (ID#: Hs00171072_m1). The relative quantification of gene expression was calculated according to the $\Delta\Delta C_t$ method, relative to a calibrator sample [20], and using the 18S (ID#: Hs03003631_g1) as a reference gene.

3. Results

3.1. La Solia, Thermal Spring Water Chemical Characterization

Thermal spring waters (also called medicinal mineral waters or natural mineral waters in some countries; TWS) are very well-known for their beneficial effects on different illnesses, mainly rheumatological and respiratory ailments, but also dermatological disorders. Additionally, thermal spring waters are used as raw materials and active ingredients in dermocosmetics to achieve balance, hydration, and the wellbeing of the skin [21]. Given this knowledge, a comprehensive analysis for the characterization of La Solia, a thermal spring water licensed to Cantabria Labs which emerges in the north region of Spain (LS-TSW), was performed.

The composition analysis showed that LS-TSW is an isotonic water particularly rich in sodium, calcium, magnesium, and silica and with trace elements such as zinc, copper, manganese, boron, and selenium (Table 1), which are of interest in skin care. With some of these features, it has been declared as medicinal mineral water by the IGME (Instituto Geológico y Minero de España) and the Department of Industry (Autonomous Government of Cantabria). According to the mineral content, La Solia TSW is classified as chloride, sodium, sulphate, bicarbonate, calcium, and magnesium mineral water [15,21].

Table 1. La Solia thermal spring water composition.

Anions/Cations/Trace Elements and Other Compounds	
Chloride (mg/L)	3200
Sulphate (mg/L)	300
Bicarbonate (mg/L)	210
Nitrate (mg/L)	2
Fluoride (mg/L)	<0.5
Phosphate (mg/L)	<0.04
Silica SiO_2 (mg/L)	27.1
Calcium (mg/L)	184
Magnesium (mg/L)	36
Potassium (mg/L)	19
Sodium (mg/L)	1956
Iron (mg/L)	<0.1
Manganese (μg/L)	67
Boron (μg/L)	214
Cadmium (μg/L)	6.57
Zinc (μg/L)	174.0
Copper (μg/L)	427.0
Selenium (μg/L)	11.97
Barium (μg/L)	96

Many beneficial properties have been assigned to different spring waters. With the aim of searching for plausible benefits derived by its composition, we performed a comparative analysis of LS-TSW and other commercial spring waters used in the market (Table 2).

Table 2. Comparison between La Solia TSW composition and other commercial TSW.

A/C/T/Other	La Solia (Cantabria Labs)	Uriage	Avène	La Roche-Posay	Vichy	Saint-Gervais
Chloride (mg/L)	3200	3500	5.4	22.6	--	530
Sulphate (mg/L)	300	2860	13.1	56.1	182.39	1812
Bicarbonate (mg/L)	210	390	226.7	387	4818.63	247
Nitrate (mg/L)	2	--	1.4	1.6	--	--
Fluoride (mg/L)	<0.5	--	0.1	0.2	7.67	--
Phosphate (mg/L)	<0.04	--	0.3	<0.1	0.210	--
Silica SiO_2 (mg/L)	27.1	42	14	31.6	11.78 *	--
Calcium (mg/L)	184	600	42.7	149.0	165.61	234
Magnesium (mg/L)	36	125	21.2	4.4	12.08	26.8
Potassium (mg/L)	19	45.5	0.8	1.9	103.56	29
Sodium (mg/L)	1956	2360	4.8	1.3	1862.88	944

Table 2. *Cont.*

A/C/T/Other	La Solia (Cantabria Labs)	Uriage	Avène	La Roche-Posay	Vichy	Saint-Gervais
Iron (mg/L)	<0.1	--	<0.1	<0.005	0.810	<30
Manganese (µg/L)	67	0.154	<0.1	<0.003	0.208	0.327
Boron (µg/L)	214	--	220	--	970	5030
Cadmium (µg/L)	6.57	--	2	--	--	--
Zinc (µg/L)	174.0	160	20	<5	--	57
Copper (µg/L)	427.0	75	<5	<5	--	<2
Selenium (µg/L)	11.97	--	<5	53	--	--
Barium (µg/L)	96	--	220	--	--	17

A: anions; C: cations; T: trace elements; Other: other compounds; --: no data available. * Expressed in silicon (Si).

Comparing with other thermal spring waters used in dermocosmetics and skin care, its chloride and sodium content is similar to Uriage TSW; its calcium content is similar to La Roche-Posay and Vichy TSW; its magnesium content is similar to Saint-Gervais Mont Blanc TSW; its SiO_2 content is similar to La Roche-Posay TSW and Uriage TSW; its boron content is similar to Avène TSW; and its zinc and copper is much higher than any other, though its selenium content is lower than La Roche-Posay TSW [21].

3.2. La Solia Microbiological Characterization

The microbiota of thermal spring waters (hydrobiome) and its relationship with cosmetics formulations has recently gained attention. Thus, we next investigated LS-TSW microbial communities.

As it can be seen in Figure 1, microscopic observations of freshly isolated samples revealed the presence of three main phylogenetic groups commonly named as microalgae: Cyanophyta (blue-green algae, Figure 1a), Chlorophyta (green algae, Figure 1b), and Heterokontophyta (diatoms, Figure 1c).

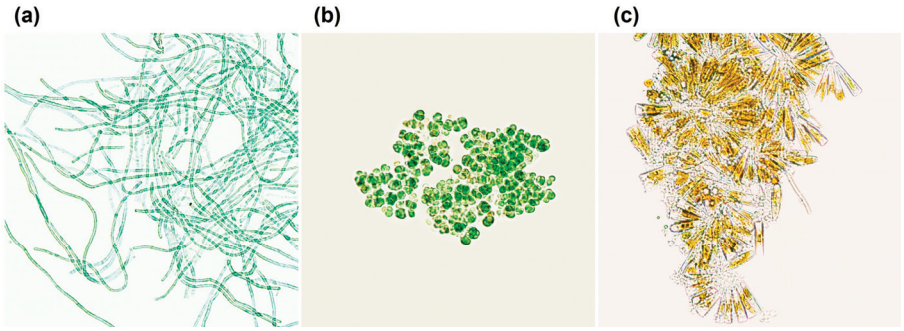


Figure 1. La Solia thermal spring water hydrobiome characterization. Representative pictures of the different microorganisms isolated from LS-TSW. All pictures were taken at 40× amplification. (**a**) Cyanobacteria; (**b**) chlorophyta; (**c**) diatoms.

3.2.1. Cyanobacteria

The Cyanophyta phylum, comprising ancient prokaryotic microalgae, showed the presence of different genera in LS-TSW samples: (a) *Spirulina*, known for its spiral filaments, a cosmopolitan species with thermophilic and alkaline characteristics, making it biologically significant. (b) *Oscillatoria*, a filamentous Cyanobacteria, often forming mats together with other identified filamentous Cyanobacteria, such as *Leptolyngbia*, *Geitlerinema*, or *Phormidium* [22]. These mats serve as a substrate for other organisms, being of key biological relevance in the ecosystem of TSW. And, (c) *Chroococcus*, a non-filamentous cyanobacteria with spherical cells [23].

3.2.2. Chlorophyta

A single chlorophyte from the *Chlorellaceae* family was isolated in the natural thermal water source of LS-TSW (Figure 1b). This species, potentially belonging to the *Chlorella* genus, represents the Chlorophyta phylum, known for its diversity and unicellular nature.

3.2.3. Diatoms

Diatoms, classified within the Heterokonta phylum, were observed in our isolation efforts from LS-TSW. The identified diatom phylum included individualized genera like *Navicula*, *Phaeodactylum*, and *Diatoma*, each contributing to the rich microbial diversity of the thermal water [24]. Diatoms are sensitive to environmental conditions, and their presence, abundance, and diversity can be used as indicators of water quality, and serve as markers reflecting the unique and ecologically significant water properties of the region [25,26].

Notably, specimens from *Phaeodactylum*-like sp. were specifically identified in our isolation efforts from the thermal spring. *Phaeodactylum* is recognized for its adaptability, thriving in both marine and brackish water strains [27], but it has also been reported to grow in freshwater [28], showing a remarkable versatility in aquatic environments.

3.3. Immunomodulatory Activity of a Combination of La Solia Thermal Water and Phaeodactylum Tricornutum Oil Extract: Aquammunist

Many beneficial properties have been previously described for *Phaeodactylum tricornutum*, being commonly used in the pharmaceutical [29] and cosmetic industries [18]. *P. tricornutum* extracts are used for their anti-inflammatory and barrier function repair activities [18,19]. Its bioactivities have been associated with its composition, rich in omega-3 fatty acids [30], and the xanthophyl fucoxanthin. Specifically, fucoxanthin exhibits anti-inflammatory properties by controlling the levels of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α [19]. Therefore, *Phaeodactylum tricornutum* extracts seem to be promising ingredients for dermocosmetic products, particularly in the context of reactive skin treatments.

Having in mind the presence of *Phaeodactylum* in LS-TSW, we proposed the development of a new cosmetic ingredient potentiating the natural composition of LS-TSW with a lipophilic extract of *P. tricornutum*, rich in omega-3 fatty acids, standardized to fucoxanthin levels and encapsulated in liposomes. Based on the plausible anti-inflammatory character suggested by the properties of the individual compounds described above, the combination in the form of a single ingredient, subsequently named Aquammunist, was tested.

3.3.1. Aquammunist Showed Potential Anti-Inflammatory Properties in In Vitro Conditions

First, we tested the activity of these two components (LS-TSW and *Pt*OE) for the control of pro-inflammatory factors produced during induced inflammation. To this purpose, we used THP-1 cells (monocytes from peripheral blood) as a common cellular model to address immune modulation [31]. Monocytes were first pre-treated with *Pt*OE extract, LS-TSW, or a combination of both at different proportions (1% PtOE, 1% LS-TW, 10% LS-TSW, 1% LS TSW + 1% PtOE, or 10% LS-TSW + 1% PtOE); and then stimulated by LPS as a general pro-inflammatory stimulus. In this experimental setup, the level of pro-inflammatory molecules was detected by a commercial protein microarray. At it is shown in Figure 2 (normalized data relative to LPS control treatment), the main part of these pro-inflammatory factors seems to reach lower levels (appeared as downregulated) in pre-treated samples, indicating a general anti-inflammatory character of the treatments. Both *Pt*OE and LS-TSW displayed a discrete control over the pro-inflammatory cyto/chemokines analyzed when tested at 1% (a reasonable proportion to be included in cosmetic formulas), and the strongest effect was observed with LS-TSW at 10%. However, the combination of 10% LS-TSW and 1% *Pt*OE did not show a clear benefit over 10% LS-TSW, and the inclusion of 10% LS-TSW in cosmetic formulations is a major challenge due to formula instabilities. Remarkably, the combination of both components in the mixture at 1% each, seemed to have a stronger anti-inflammatory character than both ingredients tested separately, supporting the use of the combination (Figure 2, 4th column vs. columns 1st and 2nd). Thus, this preliminary evidence supported further exploring the immunomodulatory properties of the proposed ingredient, preferably at similar proportions.

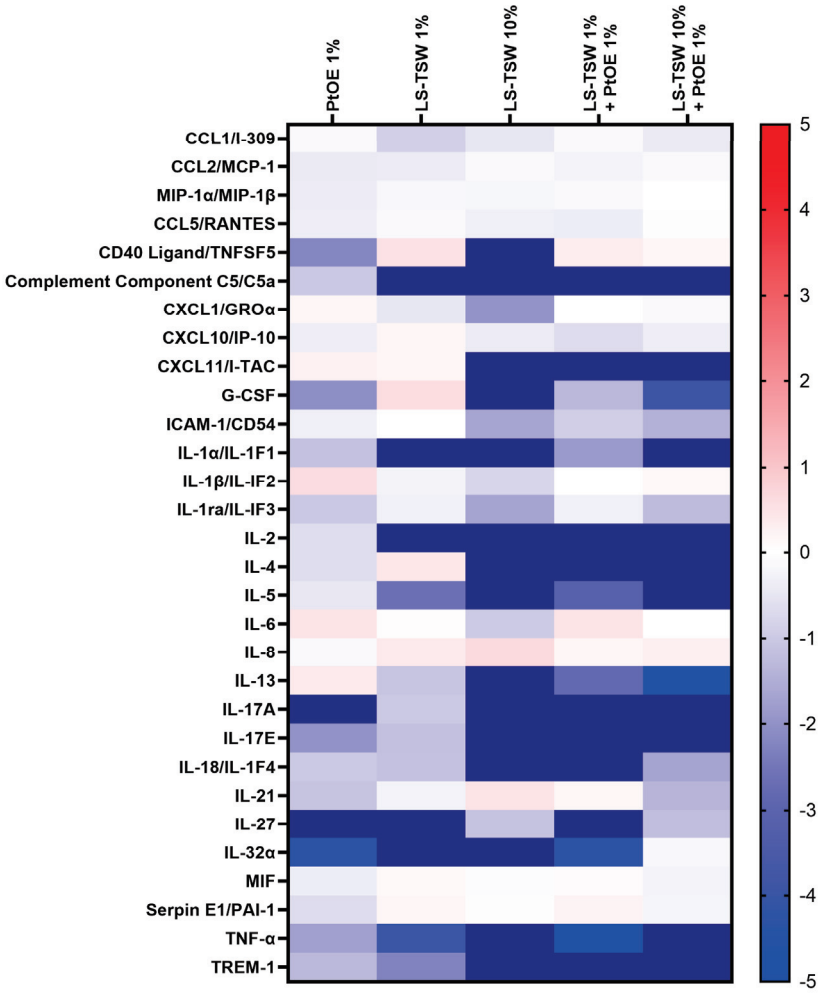


Figure 2. *Pt*OE and LS-TSW downregulate pro-inflammatory signals induced by LPS. Cell supernatants of THP-1 cells were assayed using a proteome profiler cytokine array. Samples treated with 1 µg/mL of LPS were used as positive control samples (irritated). The samples of interest were first subjected to the different proportions of the ingredients (1% PtOE, 1% LS-TW, 10% LS-TSW, 1% LS TSW + 1% PtOE, or 10% LS-TSW + 1% PtOE) for 1 h and then stimulated with 1 µg/mL of LPS for 23 h additional hours. Results are presented as a heatmap, representing the log2 fold change of treatment vs. positive control, for the 30 most downregulated pro-inflammatory cyto/chemokines.

3.3.2. Aquammunist Displays an Effective Control of Skin Inflammation in In Vitro Conditions

After the prospective results obtained in THP-1 cells, we next addressed the bioactivity of the proposed combination in skin cells, by using HaCaT cells as a cellular model of human keratinocytes [32]. Under this aim, a gene expression analysis was performed mimicking skin irritation by TNF-α/IFN-γ treatments [33]. HaCaT cell cultures were first pre-treated with Aquammunist. Due to cellular model specificities when compared to monocyte cultures, lower concentrations of Aquammunist were tested. Moreover, to confirm the results of the previous assay, we used two different Aquammunist pre-treatments, one including different proportions of LS-TSW and *Pt*OE (1% LS-TSW:0.25% *Pt*OE), and the other with the ingredients at the same proportions (0.5% LS-TSW:0.5% *Pt*OE). Then, 24 h after pre-treatment, the cell cultures were treated with TNF-α/IFN-γ to induce inflammation. Mock-treated cell cultures (untreated) were used as negative control (non-irritated samples). Non-pre-treated samples but stimulated with TNF-α/IFN-γ were used as a positive intraexperimental control for the induction of inflammation. As pro-inflammatory factors of interest, the gene expression levels of the interleukins *IL-1β* and *IL-6*, the transcription factor *NF-ƘB*, the chemokine *CCL1*, and the cytokine master regulator *TNF-α* were analyzed.

As it is shown in Figure 3, a clear induction of all pro-inflammatory factors analyzed was observed in TNF-α/IFN-γ treatments (orange boxes), validating the experimental set up (positive control of induced inflammation). Pre-treatments with the ingredient of interest showed a lower induction of all those pro-inflammatory factors (blue and turquoise boxes). Interestingly, culture cells pre-treated with a combination of LS-TSW/*Pt*OE at the same proportion (0.5% LS-TSW:0.5% *Pt*OE) showed the lowest induction of pro-inflammatory factors, reaching a statistically significant reduction for all tested genes with the exception of *IL-1β* (turquoise boxes). Specifically in that treatment, for the case of *IL-6* and *NF-ƙB*, the expression levels were not statistically distinguishable from mock samples, highlighting the potential of this combination in the control of induced-inflammation.

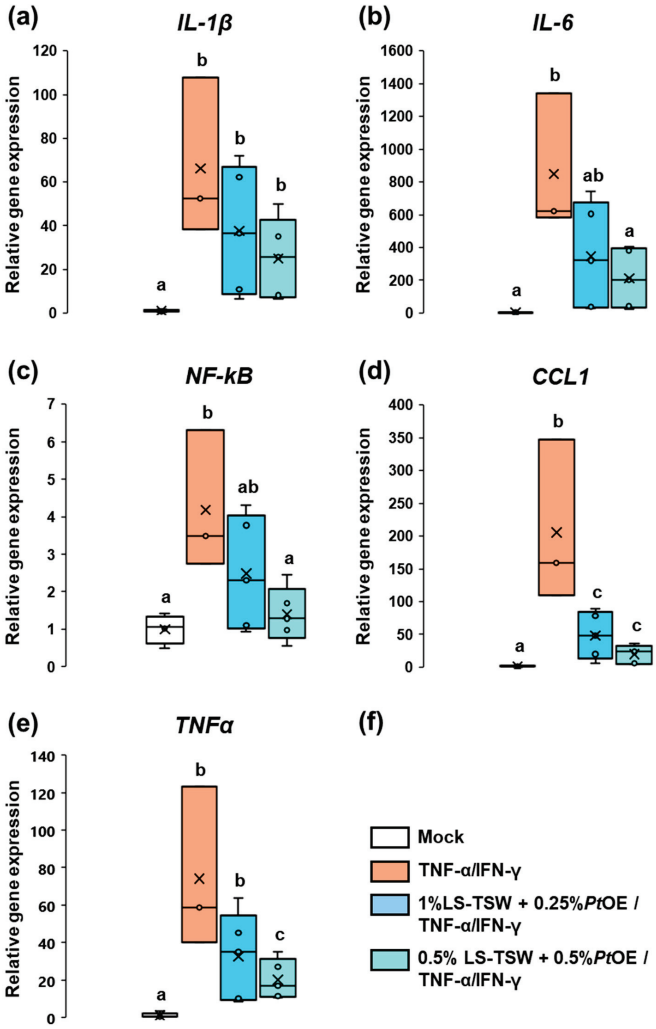


Figure 3. Aquammunist controls the induction of pro-inflammatory signals in HaCaT cells. The box and whisker plots represent gene expression analysis in human keratinocytes (HaCaT cells) under irritation simulated from TNF-α/INF-γ treatments. Boxes represent interquartile range (50% of the data around the median, Q1–Q3), median is marked as a line inside the boxes (Q2). Mean is expressed as "X". Whiskers extend to the maximum and minimum value of the dataset. Middle data points are overlapped on the boxes as opened circles. Mock samples were not treated and used as negative control. Samples treated with TNF-α and INF-γ (no-pre-treated) were used as positive control samples (irritated). The samples of interest were first subjected to Aquammunist at different proportions of the mixture components (1% LS TSW + 0.25 *Pt*OE, or 0.5% LS-TSW + 0.5 *Pt*OE) and then stimulated (irritated) by treatment with TNF-α and INF-γ. (**a**) *IL-1β*; (**b**) *IL-6*; (**c**) *NF-ƙB*; (**d**) *CCL1*; (**e**) *TNF-α*; (**f**) figure key. Letters above the boxes represent different statistical groups (Student's *t*-test *p*-value ≤ 0.05).

Therefore, the combination of the compounds here tested, seems to control skin-induced inflammation, supporting the use of Aquammunist in cosmetic formulations to treat skin conditions involving sensitivity or irritation.

4. Discussion

Individuals with a certain tendency to skin irritation can benefit from cosmetic treatments; however, in specific cases, such as sensitive skin, where the use of cosmetics itself has been demonstrated to trigger and/or worsen the skin condition, the experts find it difficult to agree on specific recommendations without scientific evidence [9–11]. This is the reason why the field is currently under an active search for alternative ingredients. In this context, the best candidate compounds should ideally control skin irritation, strengthen skin barrier function, and prevent skin reactivity. Under these premises, we propose the development of a new ingredient based on a thermal spring water and a lipidic extract from diatom algae.

Among the different options for the treatment of reactive skin, thermal spring waters have been recognized as one of the most effective dermocosmetic ingredients to improve sensitive skin condition. Several studies have been carried out in this field during the last decades. Avène thermal spring water (Avène TSW), is a bicarbonate mineral water rich in calcium, magnesium, and boron, and it has been found to exert anti-irritant and anti-inflammatory activity [34,35]; being able to decrease redness and reduce the overall sensitive scale after a dermatological chemical peeling [36]. Other bicarbonate thermal spring waters, such as São Pedro do Sul and Monforthinho, also showed anti-irritant and anti-inflammatory properties [37,38]. Sodium-rich TSW as Uriage TSW (UTSW) was suggested to effectively protect the skin from dehydration, through its effect on the expression of TauT and SVCT1 as it allows a more efficient delivery of taurine and vitamin C to the epidermis to protect it from other issues, such as oxidative stress [39]. Thermal spring waters rich in silica (SiO_2) are also of interest in the treatment of sensitive skin as they are able to improve the skin barrier, and have also shown anti-inflammatory properties [40,41]. Several research, both in vitro and clinical studies, have been conducted with La Roche-Posay thermal spring water. This bicarbonate calcium and magnesium water, which also contain strontium and selenium, showed anti-inflammatory and immunomodulatory properties and reduced redness and telangiectasia intensity in rosacea [42–46]. Finally, we must acknowledge hypersaline waters that are rich in magnesium, which have shown to have anti-inflammatory and antioxidant properties; the best-known is Dead Sea mineral water [47,48].

In this scenario, we presented the analysis of a spring water, LS-TSW, which emerges in the region of Cantabria, in the north of Spain. Under our analysis, LS-TSW is classified as chloride, sodium, sulphate, bicarbonate, calcium, and magnesium mineral water [15,21]. Comparing with other thermal spring waters used in dermocosmetics and skin care, due to its high chloride content, it could be of interest in the treatment of different skin disorders, such as psoriasis and sensitive skin. The anti-inflammatory potential of sodium-chlorinated waters has been demonstrated in different studies, with a decrease in PASI being observed in patients with psoriasis [49], and sulphated sodium-chlorinated waters have shown their anti-inflammatory potential in in vivo studies [50]. La Solia TSW is rich in SiO_2, which in its amorphous form, as found in thermal spring waters, has been shown to be anti-irritant and to repair the epidermal barrier [37]. In fact, emulsions made with water of very low mineralization but with a certain SiO_2 content (16 mg/L), such as Monfortinho, have been shown to be effective in hydration in cases of psoriasis and eczema [51]. Thus, putative anti-irritant properties can be expected in La Solia TSW. Despite the bicarbonate content in La Solia TSW being low (210 mg/L), it is similar to other thermal spring waters, such as Avène TSW, where its bicarbonate and calcium ions intervene in the inhibition of histamine release by mast cells [52]. Other studies showed the role of calcium from Uriage thermal waters in promoting eosinophil apoptosis in cases of hypereosinophilia and, therefore, exerting anti-inflammatory action as well [53]. Both bicarbonate and calcium are present in

La Solia TWS. The presence of trace elements such as selenium and zinc are also of great interest in skin care. Roche-Posay TSW and Avène TSW have also shown anti-inflammatory capacity on human keratinocytes, since both suppressed the induction of a prototypical inflammatory cytokine and the formation of ROS after UVB radiation [54]. It seems likely that, in the case of La Roche-Posay TSW, the observed effects are mediated by the high content of selenium (53 μg/L), which is a cofactor of glutathione peroxidase, a key enzyme in the removal of ROS. In the case of Avène TSW, authors considered that the relatively high zinc content (20 μg/L) seems responsible for the anti-inflammatory action. Considering the high zinc content of La Solia TSW and medium the level of selenium content (compared with other thermal spring waters), anti-inflammatory properties should be expected.

There is a rising interest of thermal spring waters microbiota, named as hydrobiome. Beyond the benefits of TSWs associated to its mineral composition, the contribution of the hydrobiome seems to be determinant for its bioactivities. Mourelle et al. (2023) revised the published studies related to the use of the microorganisms present in thermal spring waters in cosmetics, both as postbiotics and paraprobiotics [16]. They found 22 papers focused on extracts, lysates, or ferments of these microorganisms (mainly cyanobacteria and other microorganisms generally considered as microalgae), highlighting its potential as active ingredients in dermocosmetics. One of the most relevant examples is the *β-proteobacteria Aquaphilus dolomiae*, found in Avène TSW aquifer [16]. Among other evidence, a cosmetic prepared with the extracts from *Aquaphilus dolomiae* has shown to be effective in reducing pruritus and xerosis in a range of dermatologic and systemic diseases in an open-label, real-world study [55]. Furthermore, exopolysaccharides from *Cyanobacterium aponinum* (EPS-Ca), a cyanobacteria found in the Blue Lagoon silica-rich thermal water, stimulated dendritic cells to produce the immunosuppressive cytokine IL-10, increased the proportion of dendritic cells expressing CD141, and decreased T cell secretion of IL-17, IL-13, and IL-10 [56]. EPS-Ca. EPS-Ca also demonstrated to reduce keratinocyte secretion of chemokines CCL20 and CXCL10 that are involved in the recruitment of inflammatory cells [57]. In this scenario, this investigation delved into the microbiological profile of LS-TSW. Among the myriad microorganisms isolated, diatoms, took center stage and, specifically, specimens of *Phaeodactylum*-like sp. were isolated in LS-TSW. In general terms, diatoms are commonly used in the pharmaceutical [29] and cosmetic industries [18]. In particular, *Phaeodactylum tricornutum* extracts are used for their anti-inflammatory and barrier function repair activities [18,19]. The biomass of *P. tricornutum* is rich in proteins, carbohydrates, lipids, and essential mineral elements, and no significant amounts of toxic heavy metal elements [58], making it a valuable source for various applications [59]. Amid the spectrum of xanthophylls present in *P. tricornutum*, fucoxanthin, unique to brown algae and diatoms, has garnered significant attention for its therapeutic properties due to its anti-enzymatic, antimicrobial, photoprotective, and anti-inflammatory capacity; making it suitable for diverse dermocosmetic applications [60]. Additionally, the use of lipid vesicles as a carrier has been found to enhance the bioavailability of fucoxanthin [61]. Accounting on the isolation of *Phaeodactylum* sp. from LS-TSW, we wondered whether the addition of a *Pt*OE encapsulated in liposomes could potentiate the natural bioactivities of the TSW.

Keeping the focus on addressing the need of alternative ingredients in sensitive skin, we next studied the potential immunomodulatory benefits of both ingredients independently and combined in a single mixture, named Aquammunist. Using monocytes as a cellular model, and induction of unspecific inflammation by LPS, we observed how both ingredients displayed a general anti-inflammatory effect when tested independently. Surprisingly, specific combinations of the ingredients (at same proportions, each −1%), showed a more effective control of pro-inflammatory mediators, suggesting a certain synergistic effect. Going deeper into the characterization of this effect, we analyzed the induction of different pro-inflammatory molecules in cellular skin models. Our gene expression analysis confirmed the reduction in the induced-proinflammatory signals of interest when the keratinocytes were pre-treated with Aquammunist at different proportions of LS-TSW and *Pt*OE. The best results were obtained when both ingredients were mixed at equal

concentrations in accordance with the results obtained in monocytes. Consistently as well with the results observed in monocytes, all pro-inflammatory cytokines/chemokines analyzed showed lower levels in samples pre-treated with Aquammunist. These results highlight the potential of the proposed combination (LS-TSW and *Pt*OE), in the form of a single ingredient (Aquammunist) in controlling skin inflammation.

Under external aggression, skin cells are able to produce immune modulator factors to orchestrate the defense response and ensure the skin homeostasis [62]. However, misregulation of these molecules is common in the chronic inflammation of skin tissues, being considered part of the physiopathology for different skin alterations [63,64]. That is the reason why they are considered as molecular markers in diverse inflammatory skin diseases [65]. In fact, due to their key physiopathological role many of the newest treatments using biologics have these molecules as targets [63,64]. Anti-TNFα biologics is maybe the best-known example, being the most important contribution to the treatment of autoimmune skin diseases [66]. Nonetheless, the case of psoriasis treatment blocking TNFα is far from being an isolated case of success, and nowadays many biologics controlling the induction of different cytokines, such as IL-4/13, IL-12/23, IL-17, IL31, IL-36 etc., are being under investigation or already approved for the treatment of psoriasis, atopic dermatitis, chronic spontaneous urticaria, and hidradenitis suppurativa [64]. As exposed at the introduction, it is now clear that cosmetics could bring important benefits not just as adjuvant treatments in diagnosed pathologies, but as part of skin care routines in some conditions like sensitive skin [2,4,9]. Thus, considering that in our in vitro assays Aquammunist seems to control the overexpression of part of this immunomodulatory repertoire of interest, our next steps will focus on exploring the potential benefits of these ingredients in clinical studies. With regards to this, the first analysis addressing Aquammunist smoothing effect on skin discomfort induced by capsaicin have already brought positive results (manuscript under submission). Moreover, keeping in mind the SiO_2 levels of LS-TSW, which have been described to have positive effects on hydration [51] and the lipidic composition of *Pt*OE; the protection and improvement of skin barrier function would be another variable of interest to be investigated in the future. In any case, to translate the specific findings on the ingredient properties, the next steps forwards should also consider clinical trials including Aquammunist in complete cosmetic formulations.

5. Conclusions

Thermal spring waters and algae extracts are some of the most promising ingredients for cosmetic purposes, especially for the treatment of reactive skin and other skin alterations with a chronic inflammation component. The results presented here conform the first scientific evidence about the development of an alternative ingredient combining a specific thermal spring water and a diatom alga extract, grounding its following clinical research and dermocosmetological use.

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Eco-Designing Cosmetic Products while Preserving the Sensorial-Application Properties: An Instrumental Approach toward Sustainable Formulations

Giovanni Tafuro [1,*], Alessia Costantini [1], Mario Piatto [2], Silvia Lucchetti [1], Stefano Francescato [3], Laura Busata [3], Giovanni Baratto [3] and Alessandra Semenzato [2]

[1] Unired S.r.l., via Niccolò Tommaseo 69, 35131 Padova, PD, Italy; alessia.costantini@unired.it (A.C.); silvia.lucchetti@unired.it (S.L.)
[2] Department of Pharmaceutical and Pharmacological Sciences, University of Padova, via Marzolo 5, 35131 Padova, PD, Italy; alessandra.semenzato@unipd.it (A.S.)
[3] Unifarco S.p.A., via Cal Longa 62, 32035 Santa Giustina, BL, Italy; stefano.francescato@unifarco.it (S.F.); laura.busata@unifarco.it (L.B.)
* Correspondence: giovanni.tafuro@unired.it; Tel.: +39-334-6812046

Abstract: Driven by growing environmental concerns and regulations, cosmetic companies are seeking reliable methods to promptly assess the possibility of replacing high-impact ingredients with sustainable alternatives. In this work, we exploited rheological and texture analyses to evaluate the possibility of using natural and biodegradable raw materials for reformulating three commercial oil-in-water skin care emulsions from an eco-design perspective. Synthetic texturizers, like nylon-12 and PMMA, were replaced with starch, maltodextrin, and silica, while acrylic rheological modifiers were substituted with polysaccharide associations of sclerotium gum, xanthan gum, diutan gum, and carrageenan. Plant-based emollients and a biodegradable elastomer were used as alternatives to silicone oils. The flow and viscoelastic properties of the samples were characterized using rheological tests under continuous and oscillatory flow conditions. The immersion/de-immersion texture analysis allowed us to measure the mechanical properties of firmness, adhesiveness, and stringiness. A double-blind sensory test assessed the products' application and sensory characteristics. The results revealed that rheology and texture analysis are complementary and correlated techniques, useful for predicting cosmetics' sensory characteristics. While perfect replication of the original formulas might not be achievable, this protocol can aid formulators in selecting new eco-friendly ingredients ensuring the products' desired application and sensory properties without compromising consumer experience.

Keywords: eco-design; sustainability; rheology; texture analysis; polysaccharides

1. Introduction

The heightened focus on sustainability has become a central imperative within the global cosmetics industry, compelling companies to develop innovative products that could minimize environmental impact [1]. This transition represents a considerable challenge, demanding in-depth research into eco-friendly materials and the integration of life cycle thinking throughout every stage of the product development process. Emerging as a powerful tool for fostering innovation in sustainable product development, eco-design formulation offers a comprehensive and systematic approach that prioritizes sustainability throughout the entire product life cycle [2]. By meticulously integrating environmental considerations into every step of the design and development, this approach allows us to significantly reduce cosmetics' environmental impact without neglecting costs, processes, and regulatory aspects [3].

Cosmetics brands today face the dual challenge of meeting rising consumer expectations while acknowledging the complexities of sustainability, where "natural" or "green"

formulations do not automatically guarantee environmental responsibility [4]. At the same time, functionality, efficacy, and sensory appeal remain non-negotiable, posing distinct challenges for formulators seeking to align their products with these demands [5–7]. In this context, a holistic, eco-design approach to cosmetic development is crucial [8,9].

One critical area for optimization lies in the selection of raw materials as it significantly influences the environmental impacts [10]. The primary environmental impacts differ between rinse-off and leave-on products. With rinse-off cosmetics, water usage and the downstream effects on aquatic ecosystems are crucial concerns. On the other hand, for leave-on products, the upstream phase and raw material sourcing hold greater environmental significance, underscoring the importance of sustainable ingredient selection [9].

This point presents unique hurdles. Conventional high-performance ingredients such as synthetic acrylic polymers, texturizing microplastics, and silicone emollients, whose ecological impacts are not negligible, should be replaced by natural-origin and biodegradable ingredients or synthetic substitutes derived from green chemistry principles, while safeguarding product excellence. Moreover, recent developments such as the restrictions on microplastic usage in leave-on products and the expansion of the microplastic definition proposed by the European Chemicals Agency are propelling manufacturers toward reformulations in this direction [11–13]. However, these substitutes may lack extensive application performance data, exhibit instabilities, or introduce esthetic-sensorial limitations [14].

Rheological and texture analyses are powerful tools for the characterization of cosmetic formulations [15–17]. They can be used to quickly assess the impact of formulation changes, including the replacement of commonly used raw materials with more sustainable alternatives [18]. Rheological tests, performed under both continuous and oscillatory flow conditions, provide a wealth of information about the structure, flow behavior, and viscoelastic properties of cosmetic samples [19–21]. Texture analysis can be used to further investigate the mechanical properties of these products, such as firmness, consistency, adhesiveness, and stringiness [22–24]. These data are essential for the development and quality control of new cosmetic products and the optimization of existing formulations. They can also be used to support marketing claims of application and sensorial properties in an objective and scientific way.

Following an eco-design approach, in this study, we reformulated three cosmetic products sold in the pharmaceutical sales channel by replacing some high environmental impact ingredients with eco-friendly alternatives. Synthetic texturizers, such as PMMA and nylon-12, which are considered microplastics with a toxicity profile for the marine fauna [25], have been replaced with natural organic and inorganic raw materials. The non-biodegradable acrylic rheological modifiers, whose persistence in the environment is problematic, have been replaced with associations of polysaccharides. The silicon emollients and film-forming polymers, whose impact is determined by their chemical synthesis which requires the consumption of many energy resources and determines significative CO_2 emissions [26–28], have been replaced with plant-based emollients and a biodegradable elastomer.

After modifying the formulations as described above and after performing instrumental analyses to study their structural and application characteristics, a double-blind sensory test was conducted to verify whether these substitutions allowed us to preserve the sensory properties of the original products.

2. Materials and Methods

2.1. Materials

Three commercial skin care products provided by Unifarco S.p.A have been reformulated from an eco-design perspective: an acne-prone skin serum (F1), an eye contour gel-cream (F2), and a body butter (F3).

The product F1, whose formulative scheme is reported in Table 1, is a light-textured fluid emulsion, formulated with a lipidic complex with emulsifier properties and an anionic emulsifier. The structural properties are imparted by ammonium acryloyldimethyltau-

rate/VP copolymer as rheological modifiers. The lipidic phase is composed of fatty alcohols, esters, and dimethicone oil. Nylon-12 and PMMA have the function of texturizers and sensorial enhancers.

Table 1. Formulation scheme of product F1.

Phase	Ingredients	Composition % *w*/*w*
Aqueous Phase	Water	Add until reaching 100
	Chelating agent	0.1
	Humectants	5
	Preservatives	0.7
	Hydrophilic active ingredients	5
	Ammonium acryloyldimethyltaurate/VP copolymer	0.9
	Anionic emulsifier	0.3
	Nylon-12 Polymethylmethacrylate	2.5
Lipidic Phase	Oils	2
	Dimethicone	1
	Non-ionic emulsifier system	3.5
	Lipophilic active ingredients	0.2
	Antioxidant	0.1

The formula F2 is a gel/cream meant to uniform the under-eye area and ensure long-lasting hydration. The formulation scheme is shown in Table 2. The emulsifying system in this gel-cream is composed of a combination of two non-ionic emulsifiers. This formula is more structured than the previous one thanks to the presence of the cross-linked acrylic polymer carbomer. The silicones present in this formula are dimethicone, polysilicone-11, and dimethiconol, which work synergistically to form a film around the eye. Beyond the presence of silicone gums, the sensory and textural properties are provided by a combination of a synthetic esters, starch, and PMMA.

The formula F3 is a body butter; this O/W emulsion has a high percentage of oil phase consisting of fatty alcohols, waxes, and nourishing emollients in combination with non-ionic and anionic emulsifiers (Table 3). The synthetic polymers sodium polyacrylate and acrylates/beheneth-25 methacrylate copolymer work together with the lipids to build the body butter structure. PMMA is the synthetic texturizing agent.

The synthetic ingredients and the naturally derived alternatives chosen for the eco-design reformulation of the three commercial formulas are listed in Table 4. The synthetic texturizers of the formulas, i.e., nylon-12 (particle size of 20 μm) and polymethyl methacrylate (particle size of 6–10 μm) have been replaced using naturally derived organic or inorganic texturizers: Z (zea mays Starch, polyvinyl alcohol, and glycerin) whose particle size is 5–15 μm, M (maltodextrin) whose particle size is 2–20 μm, and S (sodium potassium aluminum silicate, titanium dioxide, and silica) whose particle size is 2–20 μm, H (Mica) whose particle size is 5–15 μm.

The acrylic rheological modifiers present in the formulas have been replaced using polysaccharidic raw materials in combination: t (sclerotium gum), v (sclerotium gum and xanthan gum), k (Sphingomonas Ferment Extract), r (succynoglycan), and c (carrageenan).

Dimethicone emollients have been replaced using plant-based emollients: CU (undecane and tridecane), EG (C15-19 alkanes), DSG (ethyl olivate and triheptanoin), EP (palm/kernel alkanes), and P (coco-caprylate/caprate, triheptanoin, C9-12 alkane, dilinoleic acid/butanediol copolymer, and castor oil/IPDI copolymer).

Table 2. Formulation scheme of product F2.

Phase	Ingredients	Composition % *w*/*w*
Aqueous Phase	Water	Add until reaching 100
	Chelating agent	0.1
	Humectants	3
	Preservatives	0.7
	Hydrophilic active ingredients	3
	Natural gums	1
	Carbomer	0.3
	Non-ionic emulsifier system	3
	Polymethylmethacrylate	1
	Buffering agent	0.5
Lipidic Phase	Coemulsifier	0.6
	Oils	5
	Dimethicone Polysilicone-11 Dimethiconol	5.5
	Antioxidant	0.1

Table 3. Formulation scheme of product F3.

Phase	Ingredients	Composition % *w*/*w*
Aqueous Phase	Water	Add until reaching 100
	Chelating agent	0.3
	Humectants	6
	Preservatives	0.6
	Hydrophilic active ingredients	0.1
	Sodium polyacrylate Acrylates/beheneth-25 methacrylate copolymer	1.3
	Anionic emulsifier	0.5
	Polymethylmethacrylate	3
	Buffering agent	0.03
Lipidic Phase	Non-ionic emulsifier system	4
	Emollients	36
	Fragrance	0.7

Table 4. Synthetic raw materials and natural alternatives chosen for eco-design reformulation of cosmetic products.

Category	Code	Name	Inci Name	Origin	Supplier
Texturizers		ORGASOL® 2002 D NAT COS	Nylon-12	synthetic	Arkema (Colombes, France)
		JURYMER MB-1	Polymethyl methacrylate	synthetic	Kowa (Dusseldorf, Germany)
	Z	ECOTOUCH® POWDER	Zea mays starch, polyvinyl alcohol, and glycerin	natural	Roelmi HPC (Origgio, VA, Italy)
	M	AGENAMALT 20.222	Maltodextrin	natural	Agrana (Vienna, Austria)
	S	RONAFLAIR® LDP WHITE	Sodium potassium aluminum silicate, titanium dioxide, and silica	natural	Merck (Darmstadt, Germany)
	H	SERICA 5	Mica	natural	Sensient (Saint-Ouen-l'Aumône, France)
Rheological modifiers		CARBOPOL® ULTREZ 10	Acrylates/C10-30 alkyl acrylate crosspolymer	synthetic	Lubrizol (Wickliffe, OH, USA)
		ARISTOFLEX® AVC	Ammonium acyloyldimethyltaurate/VP copolymer	synthetic	Clariant (Muttenz, Switzerland)
		COSMEDIA® SP	Sodium polyacrylate	synthetic	Eurotrading (Veggiano, PD, Italy)
		ACULYN™ 28 POLYMER	Acqua and acrylates/beheneth-25 methacrylate copolymer	synthetic	Dow Chemical (Midland, MI, USA)
	t	ACTIGUM™ CS 11 QD	Sclerotium gum	natural	Cargill (Paris, France)
	v	ACTIGUM™ VSX 20	Sclerotium gum and xanthan gum	natural	Cargill (Paris, France)
	k	KELCO CARE™ DIUTAN GUM	Sphingomonas Ferment Extract	natural	CP-kelco (Atlanta, GA, USA)
	r	RHEOZAN® SH	Succynoglycan	natural	Solvay (Bruxelles, Belgium)
	c	GENUVISCO® CG-131	Carrageenan	natural	CP-kelco (Atlanta, GA, USA)
Emollients		BRB™ DM 5	Dimethicone	synthetic	Lehvoss (Origgio, VA, Italy)
		X-21-5849	Dimethiconol	synthetic	Shin-etsu (Chiyoda, Tokyo, Japan)
		XIAMETER™ PMX-200	Dimethicone	synthetic	Dow Chemical (Midland, MI, USA)
		GRANSIL DMG-2	Dimethicone and polysilicone-11	synthetic	IMCD (Milan, MI, Italy)
	CU	CETIOL® ULTIMATE	Undecane and tridecane	natural	Eurotrading (Veggiano, PD, Italy)
	EG	EMOGREEN™ L15	C15-19 alkanes	natural	Seppic (Courbevoie, Francia)
	DSG	DUB™ SOGREEN	Ethyl olivate and triheptanoin	natural	Stearinerie Dubois (Boulogne-Billancourt, Francia)
	EP	EUTRADERM® PENTA	Palm/kernel alkanes	natural	Eurotrading (Veggiano, PD, Italy)
	P	GRANSENSE™ TC-18X	Coco-caprylate/caprate, triheptanoin, C9-12 alkane, dilinoleic acid/butanediol copolymer, and castor oil/IPDI copolymer	natural	Grant Industries (Elmwood Park, NJ, USA)

2.2. Preparation of Emulsions

All of the emulsions were prepared following the same formulation procedure. The lipidic phase was prepared by dispersing the emulsifiers and the lipophile molecules in a mixture of emollients, and then heated at 75 °C. The water phase was prepared by dispersing the hydrophilic molecules (actives, humectants, chelating agents, and polymers) in water and heating at 70 °C. The oil phase was added to the water phase using a Silverson L5T laboratory mixer (Ghiaroni & C srl., Buccinasco, MI, Italy) and homogenized for 5 min at 4500 RPM. The texturizing powders were added after emulsification stirring at 2500 rpm. The pH of the emulsions prepared was set between 5 and 5.5 with the addition of citric acid as a buffering agent and measured with a pH-meter Basic 20 (Crison Instruments, Alella Barcelona, Spain).

2.3. Stability Assessment

To predict physical instability phenomena, samples were subjected to strong mechanical stress conditions by using the centrifuge MPW-56 (MPW Med. Instruments, Warsaw, Poland) at 4800 RPM for 30 min.

2.4. Rheological Analysis

The rheological measurements conducted both in continuous and oscillatory flow conditions were performed using a Rheometer Physica MCR e302 (Anton Paar GmbH, Graz, Austria), maintaining a temperature of 23 °C ± 0.05 °C. A PP50-P2 sensor (parallel plates with serrated surfaces) with a fixed gap of 1 mm was used. Rheocompass^{TM} software (version 1.32.258, Anton Paar GmbH, Graz, Austria) was used to collect and analyze the data. A controlled shear rate test (CSR) was performed to obtain the samples' flow curves, which show the trend of viscosity (η) at an increasing shear rate, from 0.001 to 1000 s^{-1}. The zero-shear viscosity η_0, i.e., the viscosity of the material at rest, was calculated by fitting the flow curves with the Carreau−Yasuda mathematical model applicable to shear-thinning materials:

$$\tau = \eta_\infty \dot{\gamma} + \frac{(\eta_0 - \eta_\infty)\dot{\gamma}}{\left[1 + (\lambda\dot{\gamma})^2\right]^{\frac{1-n}{2}}} \quad (1)$$

where τ is the shear stress, $\dot{\gamma}$ is the shear rate, η_∞ is the infinite viscosity, i.e., asymptotic viscosity at a high shear rate, λ is the relaxation time, and n is the power law index [29].

An amplitude sweep test (AS) was used to evaluate the products' linear viscoelastic region (LVER) and the critical strain (γ G′ = G″). This test was performed at a fixed frequency of 1 Hz by increasing the amplitude strain (γ) from 0.01% to 1000%. A frequency sweep analysis allowed us to analyze the inner microstructure of the samples by considering the trend of the storage (G′) and the loss (G″) moduli. This test was conducted by fixing the oscillation strain within the LVER and decreasing at the same time the oscillation frequency from 10 Hz to 0.01 Hz.

2.5. Texture Analysis

A texture analysis was performed using a Texture Analyzer TMS-Pro (Food Technology Corporation, Sterling, VA, USA) equipped with a 10-Newton load cell at room temperature. A spherical nylon probe with a diameter of 2 cm moved vertically at a speed of 80 mm/min to a depth of 10 mm into the emulsions contained in 50 mL containers with a diameter of 5.3 cm. The probe then returned to its original position. Texture Lab Pro software (version 1.1) was used to collect and analyze the data, generating a curve depicting load (N) versus cumulative displacement (mm) (Figure 1). Firmness or hardness (N) was defined as the maximum force recorded, consistency (N.mm) as the area under the positive portion of the curve, cohesiveness (N) as the negative peak, adhesiveness (N.mm) as the area under the negative portion of the curve, and stringiness (mm) as the distance that the sample stretched during the probe de-immersion phase [30,31].

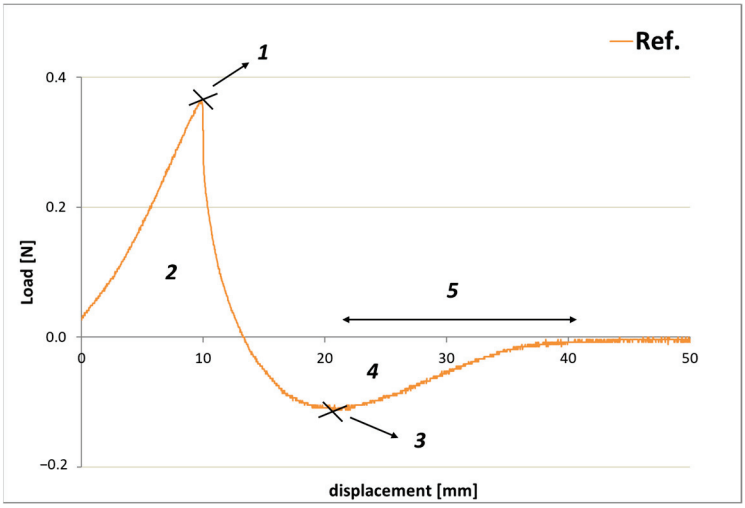


Figure 1. The curve obtained with an immersion/de-immersion test, performed with a texture analyzer, and the definitions of the calculated parameters are as follows: firmness (1), consistency (2), cohesiveness (3), adhesiveness (4), and stringiness (5). The data are plotted as load vs. cumulative displacement.

2.6. Sensory Analysis and Statistical Analysis

A double-blind sensory test was performed, where 33 healthy Caucasian volunteers of both sexes (age 23–45 years old) were asked to apply the final reformulated products and the original ones (1 mg cm^{-2}) on each forearm. The products were evaluated by panelists in the same room with a controlled temperature and relative humidity. Each participant was provided with paper and cleaning gel to clean their hands and forearms before starting the test and between each evaluation session. The products, randomly coded with a two-letter code, unknown to panelists and to test administrators, were presented to them in neutral 30 mL jars to minimize the influence of external factors on their responses. The order of presentation of the samples was changed every session to avoid a bias in rating score. Panelists were shown how to pick up and apply the samples through circular movements until they disappeared on the skin. One minute after application, they were asked to fill out a seven-question survey concerning sensory parameters in four different phases of the application: color for the appearance at first sight; consistency at first touch; spreadability and rate of absorption for the application phase; greasiness, stickiness, and softness of the skin for the after-feel phase. Each parameter was evaluated on a scale from 1 to 7, where 1 stood for the lowest rating and 7 stood for highest rating:

- Color: from 1 (yellowish) to 7 (greyish) through 4 (white);
- Consistency at first touch: from 1 (fluid) to 7 (very consistent);
- Spreadability: from 1 (not spreadable) to 7 (very spreadable);
- Rate of absorption: from 1 (very slow) to 7 (very fast);
- Greasiness: from 1 (not greasy) to 7 (very greasy);
- Stickiness: from 1 (not sticky) to 7 (very sticky);
- Softness of the skin after application: from (not soft) to 7 (very soft).

This study was conducted in accordance with the Declaration of Helsinki. All of the volunteers provided their informed consent for research analysis and the reporting of findings was anonymous, in accordance with Italian legal and ethical requirements. All participants were informed in detail about the aim of the study, the voluntary nature of their participation, and their right to withdraw from the study at any time without being penalized in any way.

The results and the data collected were elaborated with paired samples Student's t-test statistical analysis at a significance level of $p < 0.05$ to determine the statistical differences between the cream samples, based on the characteristics described by the panelists.

3. Results and Discussions

The aim of this research study was to reformulate three commercially available cosmetic skin care oil-in-water emulsions through the lens of eco-design formulation. These three formulations, each distinct in their technological approach, were F1, a fluid and light-textured oil-in-water emulsion; F2, a firm cream/gel; F3, a rich emulsion boasting a high content of lipidic phases. Our systematic approach involved the progressive substitution of the synthetic and more impactful ingredients within each formula, beginning from texturizers, followed by acrylic rheological modifiers, and culminating in the replacement of silicone-based materials, where applicable. This comprehensive strategy aimed to optimize the formulations while adhering to eco-design principles, without interfering with their sensorial profile.

3.1. Reformulation of Product F1

3.1.1. Substitution of Synthetic Texturizers

The product F1 is a fluid emulsion with high spreadability and a light evanescent texture. It contains nylon-12 at 1% *w*/*w* and PMMA at 1.5% *w*/*w* as texturizers which confer the mattifying and anti-shine effect. A copolymer derived from acryloyldimethyltaurate acts as a stabilizer and rheological modifier. The emollient portion is composed of fatty alcohols, esters, and dimethicone oil.

In the first phase of this work, the synthetic texturizers nylon-12 and PMMA, which falling into the category of microplastics could be harmful to the aquatic environment, have been replaced with naturally derived and biodegradable powders proposed as more eco-friendly alternatives: two organic polymers, Z (a combination of zea mays starch powder, glycerin from olive oil, and biodegradable polyvinyl alcohol) and M (maltodextrin), and two inorganic polymers, H (mica) and S (a mixture of aluminum silicate, titanium dioxide, and silica). Four formulations were prepared, each containing these different natural texturizers to replace the synthetic ones at the same total concentration of 2.5% *w*/*w*.

The texture curves in Figure 2 reveal that the parameters measured for the emulsions containing the organic polymers Z and M as texturizers (F1 Z and F1 M) did not show significant differences with those measured for the reference product F1 containing nylon-12 and PMMA. On the contrary, the samples formulated with the inorganic texturizers S and H (F1 S and F1 H) were characterized by higher values of firmness, consistency, cohesiveness, and adhesiveness (considering the absolute values), and lower values of stringiness. These two formulations, in fact, were less spreadable, since the force necessary to obtain a deformation was greater, and they left a stickier residue on the skin after the application phase [16].

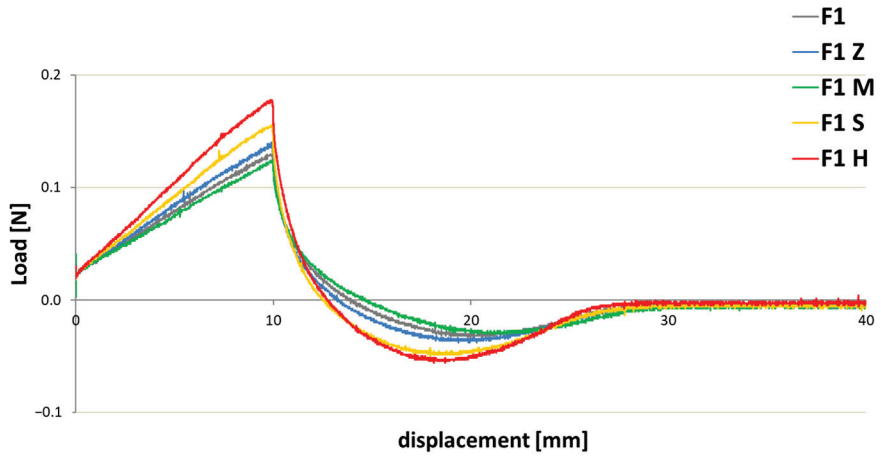


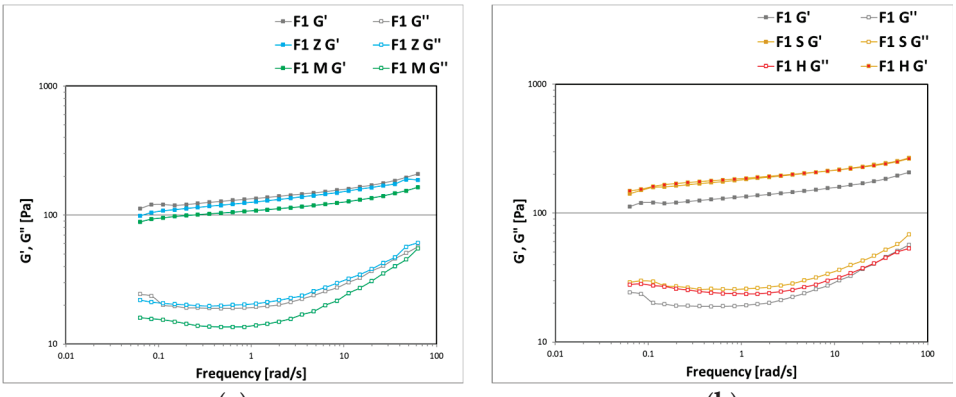
Figure 2. Texture curves obtained from an immersion/de-immersion test performed on the product F1 with the synthetic texturizers and the products F1 Z, F1 M, F1 S, and F1 H with natural ones.

From the flow curves obtained with a rheological controlled shear rate test, the zero-shear viscosity of the samples was calculated with the Carreau–Yasuda equation in the so-called Newtonian plateau region where the viscosity remains almost constant. The calculated values are listed in Table 5. In accordance with the firmness and consistency data recorded in the texture analysis, the samples formulated with the starch-derived texturizers (Z and M) showed lower viscosity values than the samples formulated with the inorganic texturizers (S and H). The substitution of the microplastics with the organic texturizers did not cause drastic changes in the viscosity of the product.

Table 5. The zero-shear viscosity (η0) values measured for the product F1 with the synthetic texturizers and the products F1 Z, F1 M, F1 S, and F1 H with natural ones.

Sample Name	Texturizer	% *w*/*w*	η0 (Pa·s)
F1	Nylon-12 and PMMA	2.5	6547
F1 Z	Zea mays starch, polyvinyl alcohol, and glycerin	2.5	6180
F1 M	Maltodextrin	2.5	5671
F1 S	Sodium potassium aluminum silicate, titanium dioxide, and silica	2.5	9610
F1 H	Mica	2.5	10,582

The rheological curves obtained from a frequency sweep test are shown in Figure 3. The samples showed a weak gel pattern of the viscoelastic moduli, in which the storage G′ modulus was always greater than the loss G″ modulus in the whole frequency range investigated. The inorganic polymers S and H caused a slight increase in the elastic component (Figure 3b), whereas the use of M caused a decrease in both G′ and G″ (Figure 3a). The moduli of the sample containing the texturizer Z were superimposed on those of the original product formulated with the synthetic polymers, demonstrating that the natural polymer Z can be considered a valid alternative to the synthetic texturizers in this product. In this formulation which contains a high percentage of aqueous phases (more than 70% *w*/*w*), the starch-derived polysaccharides gave more balanced viscoelastic properties compared to the inorganic powders, being able to interact and bind with water through the formation of hydrogen bonds, but also able to efficiently absorb the oil phase, without feeling sticky or greasy, as demonstrated in the study conducted by Marku et al. in which starch-stabilized surfactant-free emulsions with a high oil content were found to be stable and pleasant during application on the skin [32].



(**a**) (**b**)

Figure 3. Trends of the elastic G′ and viscous G″ moduli in the function of the oscillation frequency for the product F1 compared with the samples formulated with the organic texturizers F1 Z and F1 M (**a**) and the samples formulated with the inorganic ones, F1 S and F1 H (**b**).

3.1.2. Substitution of Acrylic Rheological Modifiers

The acne-prone serum with Z as a texturizer (F1 Z) contains ammonium acryloyldimethyltaurate/VP copolymer at 0.9% *w*/*w* as a rheological modifier and has been reformulated using the following polysaccharidic raw materials at different concentrations: t (sclerotium gum), v (a combination of sclerotium gum and xanthan gum), and r (succinoglycan). These natural ingredients are polysaccharides obtained from bacterial fermentation proposed as cosmetic rheological modifiers, since, according to their chemical structure characterized by the presence of several hydroxyl groups, they can bind with water molecules and modulate the viscosity and mechanical properties of the systems [33]. Since the aim of this study was to replace the acrylic polymer while maintaining the physical–mechanical properties of the formula, the four polysaccharides were chosen among those that were found to be able to form weak gel-type structures, according to the results of our previous studies characterizing the rheological and texture properties of aqueous dispersions of naturally derived rheological modifiers [34,35]. The samples containing the microbe-derived ingredients at a concentration of 0.75% *w*/*w* were not stable in the centrifuge test conducted at 4800 rpm for 30 min, showing phase separation (Table 6). The samples that were stable in the centrifuge test were subjected to a controlled shear rate rheological test. By increasing the concentration of the polysaccharides t and v at 1% *w*/*w*, we obtained stable emulsions, but we registered high values of viscosity at rest, firmness, and consistency, which are parameters correlated with poor spreadability (Figure 4). In their work, Kulawik-Piorò et al. stated that too much firmness, which is strictly correlated with viscosity, could cause difficulties in spreading the product on the skin, since higher forces are needed to deform it, which would discourage consumers from regular and frequent use [16]. We therefore maintained the concentration of the polysaccharides t and v at 0.75% *w*/*w* and we introduced 0.1% *w*/*w* and 0.2% *w*/*w* of the polysaccharide k (diutan gum) known for its stabilizing properties even at low concentrations.

Table 6. Mechanical stability test results for the formulation F1Z with the natural texturizer Z and different polysaccharides used at different concentrations to replace the synthetic acrylic rheological modifier. The samples that were stable in the centrifuge test were subjected to a controlled shear rate rheological test to measure the zero-shear viscosity (η0).

Sample Name	v % *w*/*w*	t % *w*/*w*	r % *w*/*w*	k % v	4800 rpm; 30′	η0 (Pa·s)
F1Z v075	0.75	-	-	-	separation	-
F1Z v1	1	-	-	-	stable	5609
F1Z t075	-	0.75	-	-	separation	-
F1Z t1	-	1	-	-	stable	5650
F1Z r075	-	-	0.75	-	separation	-
F1Z r1	-	-	1	-	separation	-
F1Z vk	0.75	-	-	0.1	stable	3852
F1Z tk	-	0.75	-	0.1	separation	-
F1Z tk2	-	0.75	-	0.2	stable	6929

To stabilize the formula containing the polysaccharide t at 0.75% *w*/*w*, it was necessary to use a concentration of k of 0.2% *w*/*w* (sample F1Z tk2), leading to an increase in firmness at higher values than the formulation F1Z t1 with t at 1% *w*/*w*. As shown in Figure 5, the association between v at 0.75% *w*/*w* and k at 0.1% *w*/*w* (F1Z vk) stabilized the emulsion, producing the lowest increase in firmness.

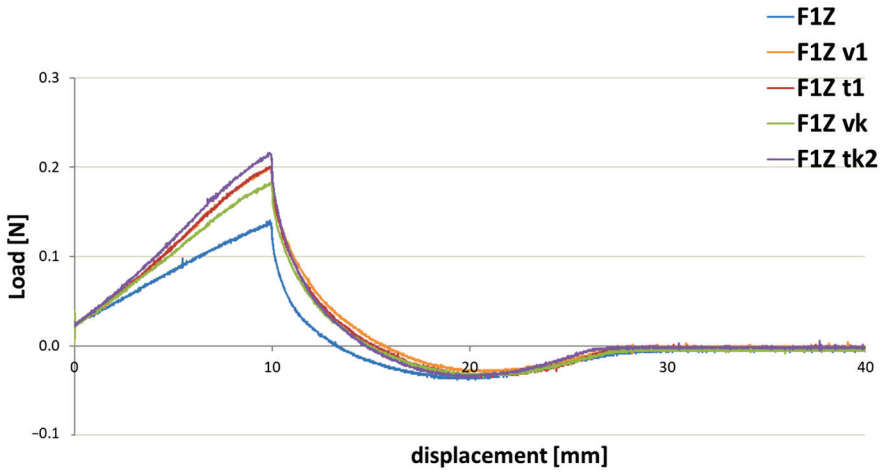


Figure 4. Texture curves obtained from an immersion/de-immersion test performed on the product F1Z with the synthetic texturizer Z and the acrylic rheological modifier and the samples with the polysaccharidic rheological modifier and F1Z v1, F1Z t1, F1Z vk, and F1Z tk2.

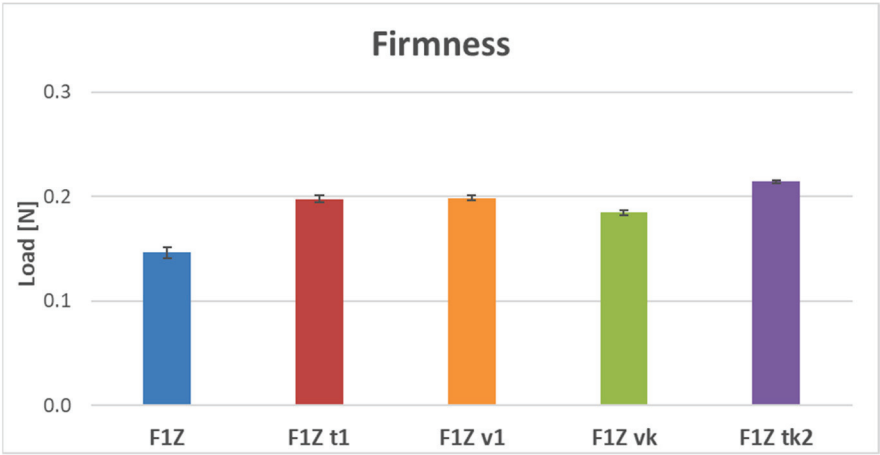
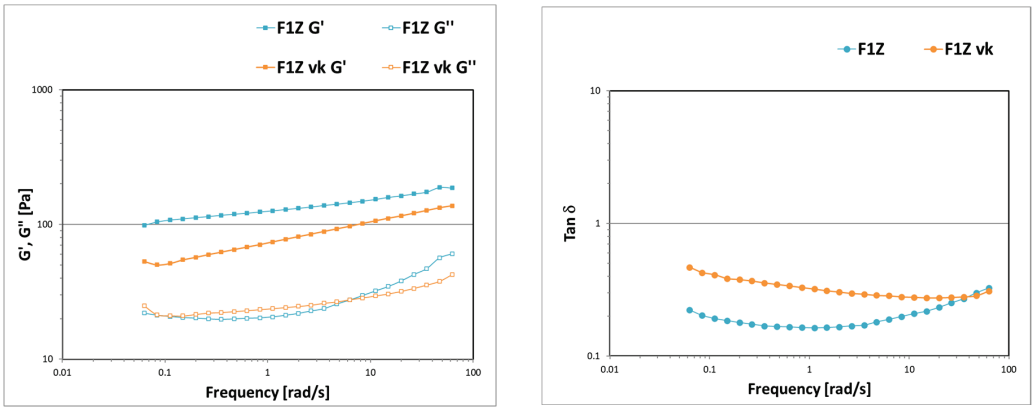


Figure 5. Firmness measured by means of an immersion/de-immersion test for the product F1Z by substituting the acrylic rheological modifier.

The frequency sweep rheological analyses showed that the substitution of the acrylic polymer with the polysaccharides determined a loss of the solid elastic component, since the formulation F1Z vk had a lower storage G′ modulus (Figure 6a) compared to the F1Z product and higher values of damping factor tanδ (Figure 6b), which is calculated from the ratio between the viscous modulus G″ and the elastic modulus G′.

Despite the differences found between the products after the replacement of the acrylic polymer with polysaccharides of microbial origin, by combining the polysaccharide raw material v e at 0.75% and the diutan gum k at 0.1%, we managed to obtain a fluid product stable against mechanical stress, with high spreadability, modules that fall between the second and third decade, and a viscosity similar to that of the original product (η at 0.01 s^{-1} for F1 was 1173.3 Pa·s; for F1Z vk, it was 1145.4 Pa·s). Compared to the raw material t composed of only sclerotium gum, the raw material v is composed of a mixture of sclerotium gum and xanthan gum, which act synergistically with diutan gum to form a structured network in the aqueous phase, giving greater stability to the emulsion and a better balance of texture properties.



(**a**) (**b**)

Figure 6. Trends of the elastic G′ and viscous G″ moduli (**a**) and the damping factor tanδ (**b**) in the function of the oscillation frequency for samples F1Z with the acrylic polymer and F1Z vk with a combination of polysaccharides.

3.1.3. Substitution of Silicone Emollients

In the formula F1Z vk, where the texturizers and rheological modifier were replaced, we proceeded by replacing the emollient dimethicone, which was present at 1% *w*/*w* and whose production process has a high expenditure in terms of carbon dioxide and is, therefore, highly impactful on the environment. Silicones are highly appreciated in cosmetics for their sensorial properties, as they give a light, smooth, and silky texture to the formula. The raw materials chosen for the replacement were plant-based emollients proposed on the market as sustainable alternatives to silicones as they create a skin-like feel and a dry touch like those given by silicones [8]. These emollients were used at the same concentration of dimethicone (1% *w*/*w*): CU (undecane and tridecane), EG (C15-19 alkanes), DSG (ethyl olivate and triheptanoin), and EP (coconut/palm kernel alkanes). As shown from the values of the damping factor (tanδ) in the function of frequency (Figure 7 and Table 7), the formulation which was found to have the same viscoelastic properties as the formula F1Z vk was F1Z DSG (containing ethyl olivate and triheptanoin as a dimethicone-substitute emollient). These data highlight how the qualitative composition of the internal phase can influence the rheological and application properties of the emulsion. Even if the differences were not so marked, the ester-based raw material compared to that based on alkanes allowed us to obtain a good compromise between the polarity, mechanical properties, and sensoriality, bieng similar to those imparted by dimethicone.

Table 7. Damping factor tanδ values registered at different frequencies (10 and 0.5 rad/s) and zero-shear viscosity values (η0) for samples F1Z v075 k01 with dimethicone and samples F1Z DSG, F1Z CU, F1Z EG, and F1Z EP with naturally derived emollients.

Sample Name	tanδ 10 rad/s	tanδ 0.5 rad/s	η0 (Pa·s)
F1Z vk	0.278	0.347	3852
F1Z CU	0.303	0.371	3151
F1Z DSG	0.282	0.342	4407
F1Z EG	0.302	0.374	3677
F1Z EP	0.290	0.321	2677

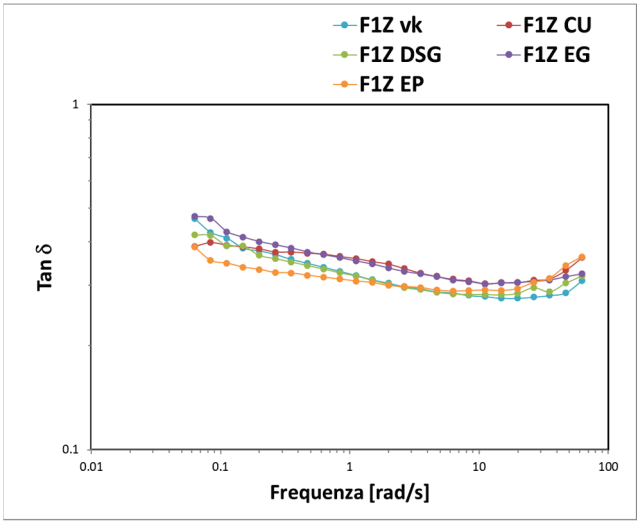


Figure 7. Trends of the damping factor tanδ in the function of the oscillation frequency for samples F1Z vk with dimethicone and samples F1Z DSG, F1Z CU, F1Z EG, and F1Z EP with naturally derived emollients.

The graph of texture parameters in Figure 8 shows the comparison between the original commercial formula F1 and the final formula F1Z DSG reformulated using an eco-design approach by substituting the synthetic texturizers nylon-12 and PMMA with the starch-based raw material Z, the acrylic rheological modifier with the combination of sclerotium, xanthan (v), and diutan (k) gums, and dimethicone with the natural emollients ethyl olivate and triheptanoin (DSG). The formulations had similar values of cohesiveness (F1: −0.036 N ± 0.003; F1Z DSG: −0.039 N ± 0.001) and adhesiveness (F1: 0.413 N.mm ± 0.029; F1Z DSG: 0.363 N.mm ± 0.018), as the negative part of the curves are almost superimposed. On the other hand, F1Z DSG showed slightly higher values of firmness (F1: 0.139 N ± 0.001; F1Z DSG: 0.205 N ± 0.003) and consistency (F1: 0.982 N.mm ± 0.053; F1Z DSG: 1.395 N.mm ± 0.030) and significantly lower values of stringiness (F1: 8.91 mm ± 1.05; F1Z DSG: 6.57 mm ± 0.521), indicating that the substitution made resulted in a more structured emulsion with lower pick-up properties. These differences are mainly attributable to the replacement of the acrylic polymer with polysaccharides, which, in order to guarantee the mechanical stability of the formulation, must be used at high concentrations, resulting in an increase in firmness and consistency.

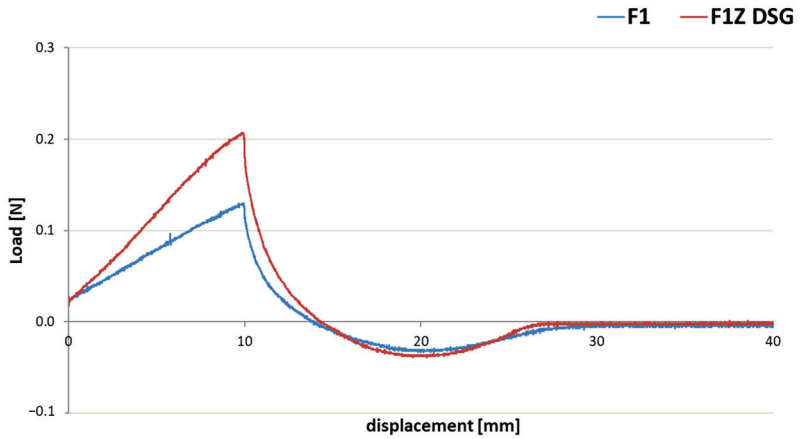


Figure 8. Texture curves obtained from an immersion/de-immersion test performed on the original product F1 and the sample F1Z DSG reformulated using an eco-design approach by replacing the synthetic texturizers, the acrylic rheological modifier, and dimethicone.

We performed a double-blind sensory test comparing the original formula F1 and the reformulated one F1Z DSG. Differences were detected just for one parameter, that is, the consistency at first touch, in agreement with the texture parameters measured in the texture analysis. On a scale of values where 1 is fluid and 7 is compact, F1Z DSG was evaluated on average as $\mu = 2.61$, while F1 was measured as $\mu = 1.73$ [t (32) = 3.98 $p < 0.01$]. No differences were detected in terms of the spreadability, absorption rate, and after-feel phase.

3.2. Reformulation of Product F2

3.2.1. Substitution of Synthetic Texturizers

The product F2 is a firm gel-cream to be applied on the under-eye area, whose emulsified system is composed of two non-ionic emulsifiers and carbomer acts as a stabilizer and rheological modifier. The film-forming properties are provided by PMMA and a mixture of dimethicone, polysilicone-11, and dimethiconol, which also provide sensorial and texturizing properties.

The synthetic texturizer present in the formula at a concentration of 1% *w*/*w* has been replaced by natural texturizers at the same concentration, namely, Z, M, S, and H. Four formulations, each containing these different natural texturizers at 1% *w*/*w*, were prepared and analyzed through texture analysis and rheology.

The texture curves in Figure 9 show that the incorporation of natural polymers instead of the synthetic ones caused a slight decrease in the emulsions' firmness, consistency, and cohesiveness. The most significant decrease occurred when the synthetic texturizer was replaced with polymer Z.

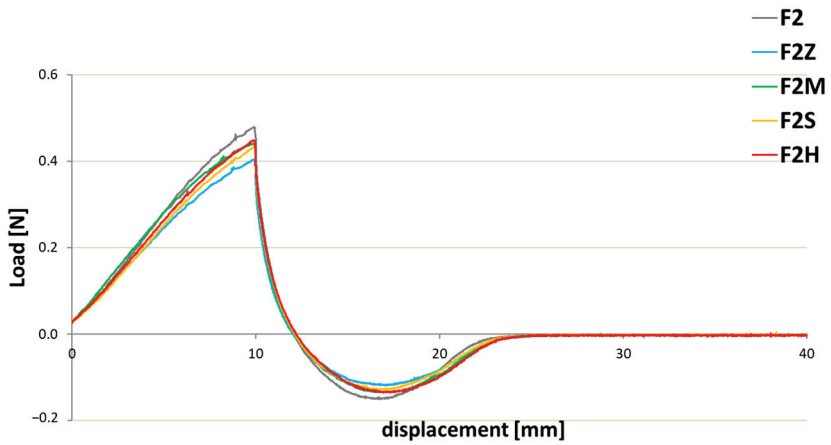


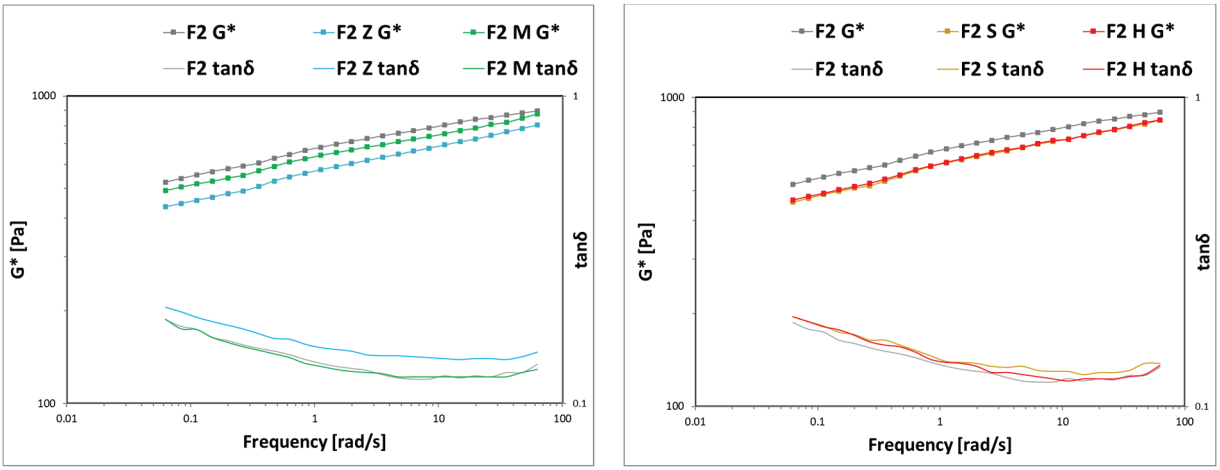
Figure 9. Texture curves obtained from an immersion/de-immersion test performed on the product F2 with the synthetic texturizers and the products F2 Z, F2 M, F2 S, and F2 H with the natural ones.

The values of zero-shear viscosity calculated with the Carreau–Yasuda model are listed in Table 8. The substitution of PMMA with the natural texturizers caused slight variation in the viscosity of the product. In accordance with the texture parameters, the sample containing Z had the lowest values of viscosity at rest.

The samples showed a weak gel rheological pattern, with G′ always dominating over G″ in the entire frequency range investigated, whose values were settled between the second and third decades. Polymer Z caused a decrease in the complex G* modulus and an increase in the damping factor tanδ, thus indicating a less structured system with a lower elastic component than the other samples (Figure 10). Polymer M turned out to be the texturizer that caused fewer variations in the rheological and textural properties of the product.

Table 8. Zero-shear viscosity (η0) values measured for the product F2 with the synthetic texturizers and the products F2 Z, F2 M, F2 S, and F2 H with the natural ones.

Sample Name	Texturizer	% *w*/*w*	η0 (Pa·s)
F2	PMMA	1	48,554
F2 Z	Zea mays starch, polyvinyl alcohol, and glycerin	1	36,186
F2 M	Maltodextrin	1	51,676
F2 S	Sodium potassium aluminum silicate, titanium dioxide, and silica	1	42,613
F2 H	Mica	1	48,589



(**a**) (**b**)

Figure 10. Trends of the complex G* modulus and of the damping factor tanδ in the function of the oscillation frequency for the product F2 compared with the samples formulated with the organic texturizers F2 Z and F2 M (**a**) and the samples formulated with the inorganic ones, F2 S and F2 H (**b**).

3.2.2. Substitution of Acrylic Rheological Modifiers

Once the synthetic texturizer was replaced with the natural texturizer M, we proceeded with the substitution of the acrylic polymer. This product contains carbomer at 0.35% *w*/*w* and has been reformulated using three raw materials alone and in combination, t (sclerotium gum), r (succinoglycan), and k (diutan gum), which are polysaccharides with high elastic and film-forming properties. The polysaccharide concentration used to prepare the samples and the results of the centrifuge test are reported in Table 9.

Table 9. Mechanical stability test results and zero-shear viscosity (η0) values for the formulation F2M with the natural texturizer M and different polysaccharides used at different concentrations to replace the synthetic acrylic rheological modifier.

Sample Name	t % *w*/*w*	r % *w*/*w*	k % *w*/*w*	4800 rpm; 30′	η0 (Pa·s)
F2M t	1.5	-	-	stable	18,434
F2M t2	1.75	-	-	stable	29,121
F2M t3	2	-	-	stable	20,052
F2M r	-	1.5	-	stable	12,576
F2M k	-	-	1.5	stable	9494
F2M tr	1.5	0.5	-	stable	17,331
F2M tk	1.5	-	0.5	stable	18,413

Even if the use of polysaccharide concentrations higher than 1.5% led to obtaining stable and structured systems, the emulsions were difficult to prepare and resulted in being non-homogeneous, sticky, and not very sensorial. It was therefore decided to compare the textural properties of the emulsions formulated with the different polysaccharides inserted in the water phase at 1.5% *w*/*w* (Figure 11). Compared to the F2M reference product, the F2M t, F2M r, and F2M k emulsions thus formulated showed lower firmness, consistency, cohesiveness, and adhesiveness values, demonstrating the difficulty of replicating the mechanical performance of the acrylic polymer through the use of a polysaccharide alone. Among the polysaccharides studied, t determined lower values of adhesiveness, r determined greater stringiness since the emulsion formed a longer filament during the de-immersion phase, which indicated greater pick-up properties, and k determined greater cohesiveness, which could reflect a more elastic and stiffer system. These different behaviors are linked to the different chemical structures of the polysaccharides which determine their application peculiarities. Sclerotium gum (t) is a non-ionic polysaccharide characterized by a linear chain of glucose with a single glucose branch at every third residue. Its molecular weight is about 1 million Dalton. In water, it assumes a triple-helix conformation which results in a rigid and pseudoplastic gel with a bouncy texture [36]. Succinoglycan (r) is an octasaccharide of 6 million Da consisting of seven glucose and one galactose with pyruvyl and succinyl substitutions. Its semi-flexible double-helix structure established in solution causes the formation of weak and soft gels [37]. Diutan gum (k) is a high-weight polysaccharide whose molecular weight is between 2.85 and 5.20 million Da. Its structure is characterized by a tetrasaccharide main chain with two rhamnose side chains, forming a rigid double-helix which enhances its stability in solution by forming a long-range ordered network [38].

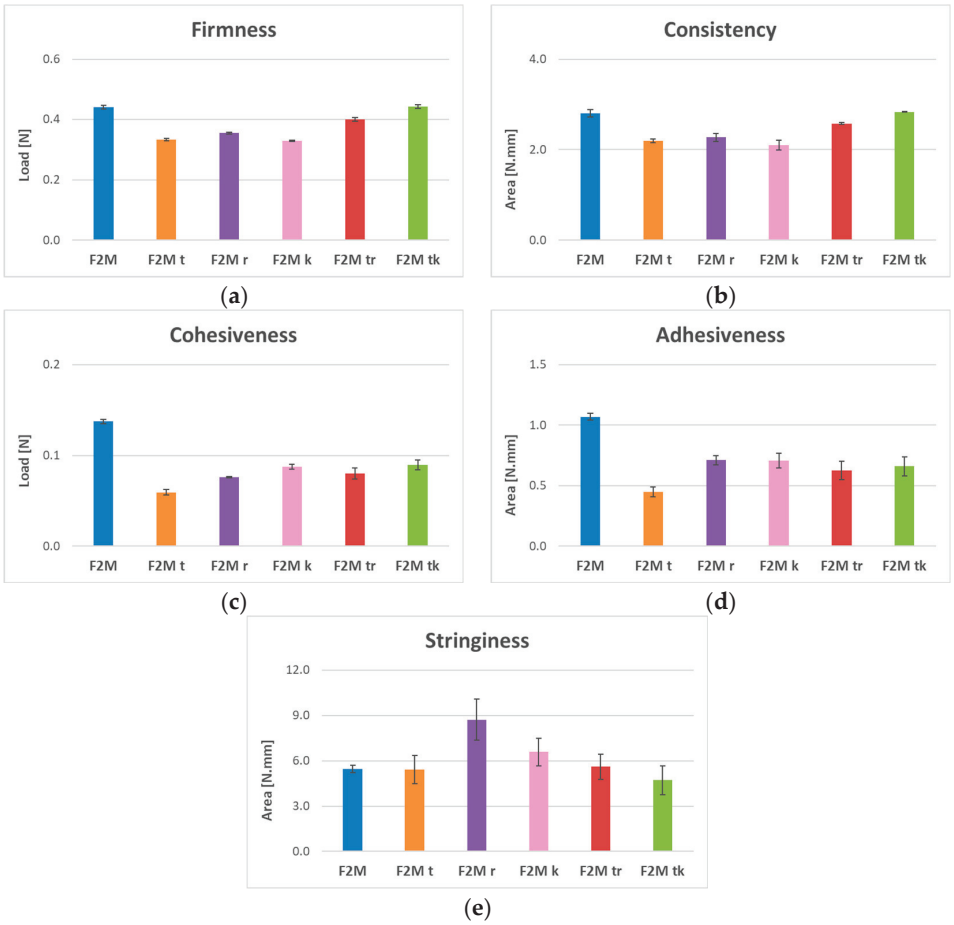


Figure 11. Firmness (**a**), consistency (**b**), cohesiveness (**c**), adhesiveness (**d**), and stringiness (**e**) measured by means of an immersion/de-immersion test for the product F2M by substituting the acrylic rheological modifier with polysaccharides t, r, and k alone and in combination.

By exploiting their different characteristics, it is possible to combine these polysaccharides together to obtain a wide range of textures and try to achieve the physical–mechanical properties imparted by acrylic polymers. Maintaining the total concentration of polysaccharides at 2% *w*/*w*, the textural properties of the associations between t at 1.5% *w*/*w* and r (formula F2M tr) or k (formula F2M tk) at 0.5% *w*/*w* were investigated (Figure 11). The formula F2M tk showed the texture parameters of firmness and consistency similar to those of the reference formula F2M. However, significant differences were found in terms of cohesiveness and adhesiveness, demonstrated by the negative part of the curve, which corresponds to the de-immersion phase, and could be related to the after-feel phase, i.e., the phase that follows the application of the product on the skin. Lower adhesiveness, in fact, corresponds to a smaller force needed to separate the product from the surface of the probe and could be attributable to lower film-forming properties.

The mechanical spectra investigated by frequency sweep analyses (Figure 12) reflect the results of the texture data. The samples presented a weak gel-type pattern where the elastic modulus G′ dominated over the viscous modulus G″, keeping a parallel trend with each other. For all of the samples, the moduli were located between the second and third decades, even though the acrylic polymer's replacement produced a significant reduction in their values.

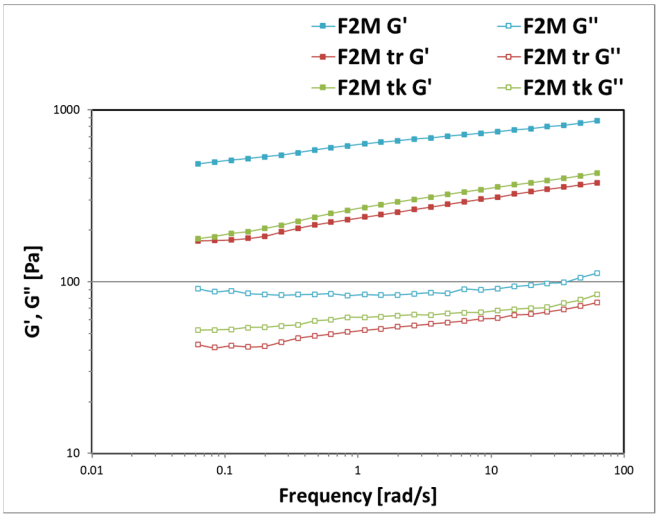


Figure 12. Trends of the elastic G′ and viscous G″ moduli in the function of the oscillation frequency for samples F2M with the acrylic polymer and F2M tr and F2M tk with combinations of polysaccharides.

3.2.3. Substitution of Silicone Emollients

The silicone ingredients were removed from the F2M tk formula: a five centistokes dimethicone oil at 3.5% *w*/*w*, dimethiconol at 0.5% *w*/*w*, and an elastomer composed of dimethicone and polysilicone-11 at 1.5% *w*/*w*. These raw materials were replaced by a natural ingredient (P), consisting of a polyurethane-based biodegradable elastomer dispersed in alkanes and esters of plant origin, used alone at 5.5% *w*/*w* in the formula F2M P or in combination with DSG (ethyl olivate and triheptanoin) at 1% in the formula F2M P DSG 1 and at 1.5% *w*/*w* in the formula F2M P DSG 1.5. All of the formulations were stable after the centrifuge test (Table 10).

From the rheological curves obtained in the frequency sweep test (Figure 13), there were no differences between F2M P DSG1.5 and the sample F2M tk in terms of viscoelastic properties, since the G* and tanδ curves were superimposed. The samples F2M P and F2M P DSG1, on the other hand, showed a higher G* value, which was related to more structured emulsions.

Table 10. Mechanical stability test results and zero-shear viscosity (η0) values for the formulations F2M tk with natural alternatives to silicones.

Sample Name	P % *w*/*w*	DSG % *w*/*w*	4800 rpm; 30′	η0 (Pa·s)
F2M P	5.5	-	stable	18,962
F2M P DSG 1	4.5	1	stable	19,501
F2M P DSG 1.5	4	1.5	stable	16,592

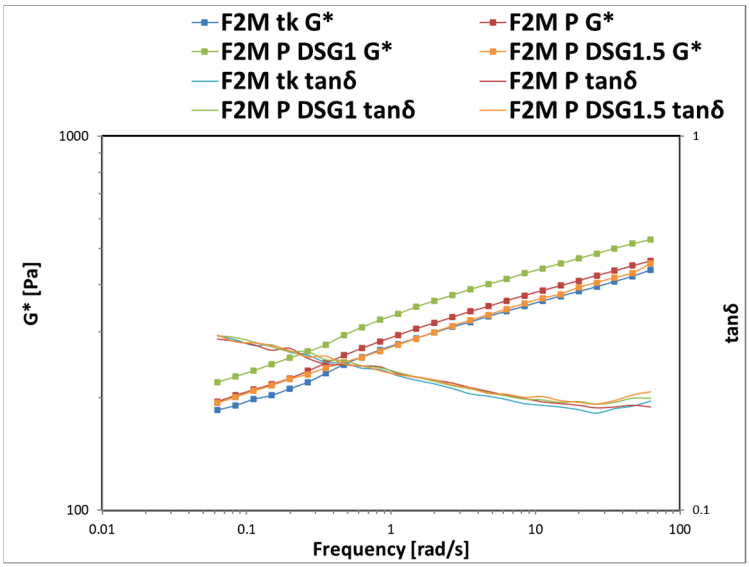


Figure 13. Trends of the complex G* modulus and of the damping factor tanδ in the function of the oscillation frequency for the product F2M tk formulated with silicones and the samples F2M P, F2M P DSG1, and F2M P DSG1.5 formulated with natural alternatives.

The texture curve in Figure 14 shows the comparison between the original commercial formula F2 and the final formula F2M P DSG1.5 reformulated using an eco-design approach by substituting the synthetic texturizer PMMA with the maltodextrin powder M, the acrylic rheological modifier with a combination of sclerotium (t) and diutan (k) gums, and the silicone ingredients with the biodegradable elastomer P and natural emollients ethyl olivate and triheptanoin (DSG). The formulations showed quite similar values of firmness (F2: 0.496 N ± 0.014; F2M P DSG1.5: 0.440 N ± 0.003), consistency (F2: 3.073 N.mm ± 0.066; F2M P DSG1.5: 2.746 N.mm ± 0.023), and stringiness (F2: 5.28 mm ± 0.32; F2M P DSG1.5: 5.43 mm ± 0.42). The main differences were found in the negative part of the curve: the new formula had lower values of cohesiveness (F2: −0.159 N ± 0.007; F2M P DSG1.5: −0.101 N ± 0.003) and adhesiveness (F2: 1.184 N.mm ± 0.094; F2M P DSG1.5: 0.768 N.mm ± 0.044). These differences indicated that the substitution made resulted in a more spreadable emulsion with lower film-forming properties. These differences were mainly attributable to the replacement of the acrylic polymer with polysaccharides, resulting in a decrease in the structure of the systems.

We conducted the consumer sensory test to compare the original formula F2 and the reformulated sample F2M P DSG1.5. Differences were detected for three parameters, even if the numerical data registered were limited and not so marked. For consistency at first touch, on a scale where 1 was fluid and 7 was very consistent, F2 had an average rating μ = 2.94, while F2M P DSG1.5 had an average rating μ = 4.39 [t (32) = 6.31 $p < 0.01$]. For spreadability, on a scale from 1—low to 7—high, F2 had an average rating μ = 5.91, while F2M P DSG1.5 had an average rating μ = 4.85 [t (32) = 4.79 $p < 0.01$]. For the rate of absorption, on a scale from 1—slow to 7—fast, F2 had an average rating μ = 4.85, while F2M P DSG1.5 had an average rating μ = 4.21 [t (32) = 2.18 $p < 0.04$].

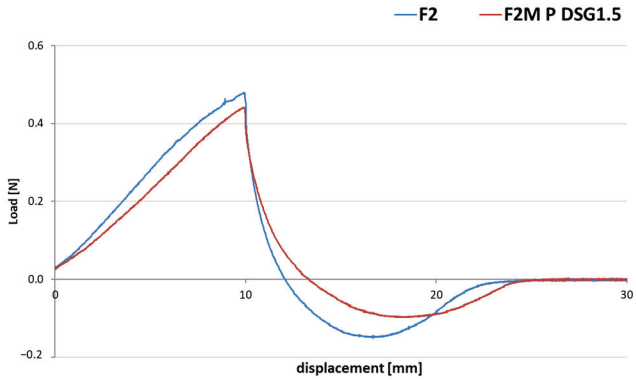


Figure 14. Texture curves obtained from an immersion/de-immersion test performed on the original product F2 and the sample F2M P DSG1.5 reformulated using an eco-design approach by replacing the synthetic texturizer, the acrylic rheological modifier, and the silicone ingredients.

3.3. Reformulation of Product F3

3.3.1. Substitution of Synthetic Texturizers

The product F3 is an O/W body butter characterized by a high percentage of lipidic phases composed of synthetic esters and triglycerides, natural waxes, and fatty alcohols. The emulsifying system is a combination of non-ionic and anionic emulsifiers. The semi-solid structure is held up by the synthetic polymers sodium polyacrylate and acrylates/beheneth-25 methacrylate copolymer. PMMA is the synthetic texturizing agent at 3% *w*/*w*, which has been replaced by natural ones at the same concentration: Z, M, S, and H. Four formulations, each containing these different natural texturizers at 1% *w*/*w*, were prepared and analyzed through texture analysis and rheology. From the texture curves, one can immediately see how the inorganic texturizers S and H increased the mechanical properties of the emulsion significantly (Figure 15a,b). The inorganic texturizers showed better performance in the formulations with a greater oil phase, thanks to their better wettability compared to organic texturizers. Reducing the silica concentration from 3% to 1.5% resulted in texture parameters similar to those of the reference product (Figure 15b).

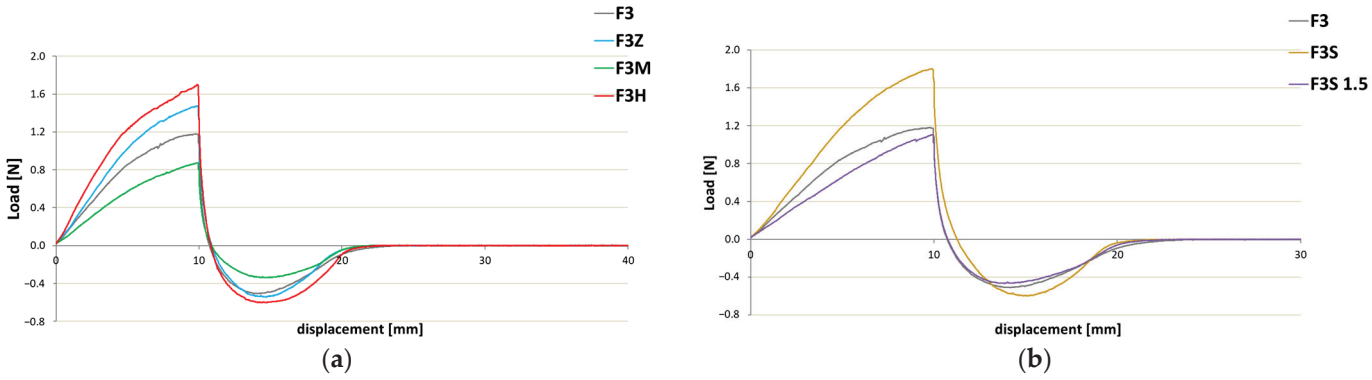


Figure 15. Texture curves obtained from an immersion/de-immersion test performed on the product F3 with the synthetic texturizer and the formula with the natural ones F3 Z, F3 M, F3 H (**a**), F3 S, and F3S 1.5 (**b**).

The rheological values obtained from the analysis conducted both in continuous and oscillatory flow conditions confirmed that the substitution of the synthetic texturizer with the inorganic polymer S at 1.5% *w*/*w* allowed us to obtain an emulsion with viscosity and elasticity parameters closer to those of the reference emulsion F3 (Table 11).

Table 11. Zero-shear viscosity η_0 values and damping factor tanδ values measured for the product F3 with the synthetic texturizers and the products F3 Z, F3 M, F3 H, and F3 S with the natural ones.

Name	Texturizer	% *w*/*w*	η0 (Pa·s)	tanδ (10 rad/s)	tanδ (0.5 rad/s)
F3	PMMA	3	210,510	0.187	0.291
F3 Z	Zea mays starch, polyvinyl alcohol, and glycerin	3	204,930	0.240	0.360
F3 M	Maltodextrin	3	111,900	0.223	0.333
F3 H	Mica	3	187,190	0.211	0.302
F3 S	Sodium potassium aluminum silicate, titanium dioxide, and silica	3	260,830	0.198	0.274
F3 S1.5		1.5	184,800	0.186	0.289

3.3.2. Substitution of Acrylic Rheological Modifiers

The body butter with silica at 1.5% *w*/*w* as natural texturizer, F3 S1.5, contained two synthetic rheological modifiers, sodium polyacrylate at 0.3% *w*/*w* and acrylates/beheneth-25 methacrylate copolymer at 1% *w*/*w*, which have been replaced with c (iota carrageenan) alone at increasing concentrations (1–1.5–2% *w*/*w*) or in a binary combination with t (sclerotium gum). All of the formulations were stable after the centrifuge test (Table 12).

Table 12. Mechanical stability test results and zero-shear viscosity (η0) values for the formulations F3S and polysaccharides used at different concentrations to replace the synthetic acrylic rheological modifiers.

Sample Name	c % *w*/*w*	t % *w*/*w*	4800 rpm; 30′	η0 (Pa·s)
F3S c1	1	-	stable	47,516
F3S c1.5	1.5	-	stable	100,150
F3S c2	2	-	stable	113,920
F3S ct	1.5	0.5	stable	126,410

Iota carrageenan, once dispersed in water, can form soft and elastic gels. This ability is due to its chemical structure characterized by galactose dimers with acidic sulfate groups that lead, in the presence of bivalent cations, to a double-helix-type conformation, stabilized by cationic bridges between the negatively charged sulfate groups [39]. For these reasons, this algae-derived polysaccharide was selected to replace the synthetic polymers and maintain the rich, consistent, and bouncy texture typical of a body butter. As the concentration of carrageenan increased, the texture parameters of firmness (Figure 16a), consistency (Figure 16b), and adhesiveness (Figure 16c) increased. However, the sample with the highest concentration (F3S c2), despite having texture parameters closer to the F3S sample, was not homogeneous from an organoleptic point of view. At this point, the carrageenan was associated with another structuring polysaccharide with a weak gel rheology, sclerotium gum (t) at 0.5% *w*/*w*. The sample thus formulated as F3S ct showed high firmness, consistency, and adhesiveness, similar to those of F3S.

The sample F3S ct containing a combination of c at 1.5% *w*/*w* and t at 0.5% *w*/*w* showed a weak gel rheological pattern, as did the sample F3 S1.5 with the acrylic polymers, but the mechanical spectra showed some qualitative and quantitative differences as the moduli values were lower (Figure 17a) and more dependent on the applied frequency, and the damping factor values were higher than those of F3 S1.5 (Figure 17b), indicating a decrease in the solid elastic properties.

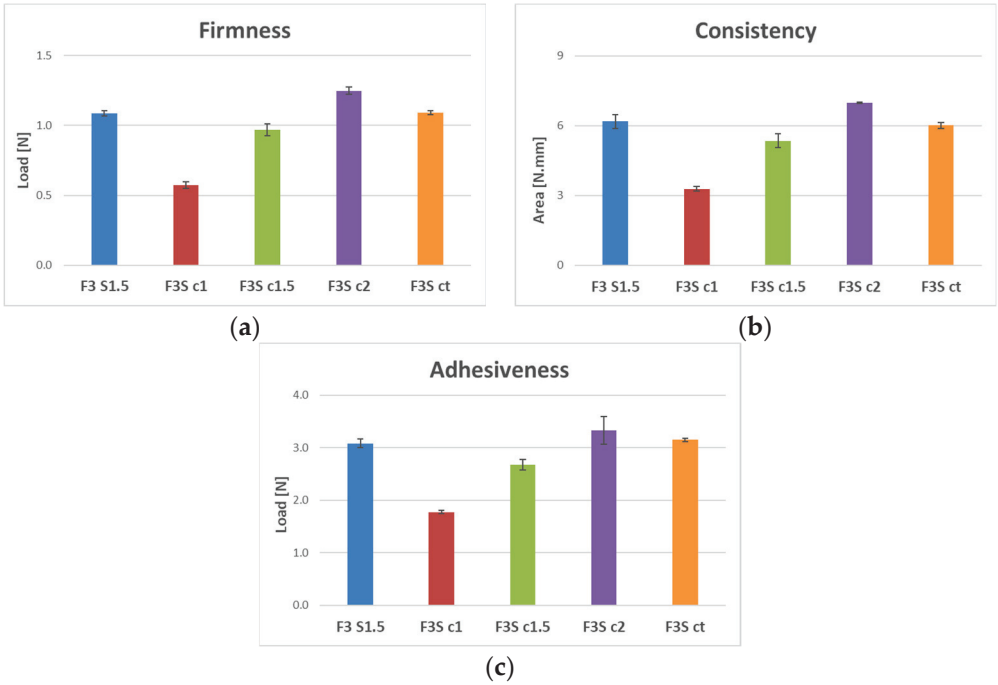


Figure 16. Firmness (**a**), consistency (**b**), and adhesiveness (**c**) measured by means of an immersion/de-immersion test for the product F3 S1.5 by substituting the acrylic rheological modifiers with polysaccharide c alone and in combination with t.

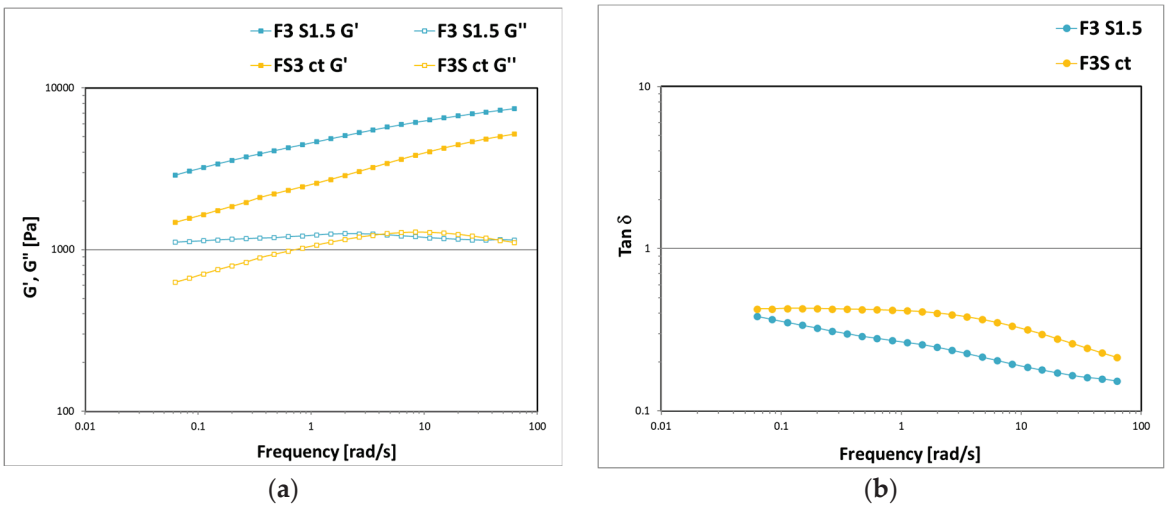


Figure 17. Trends of the elastic G′ and viscous G″ moduli (**a**) and the damping factor tanδ (**b**) in the function of the oscillation frequency for samples F3 S1.5 with the acrylic polymer and F3S ct with a combination of polysaccharides.

The texture curve in Figure 18 shows the comparison between the original commercial body butter F3 and the final formula F3S ct reformulated using an eco-design approach by substituting the synthetic texturizer PMMA with the silica powder S and the acrylic rheological modifiers with a combination of carrageenan (c) and sclerotium gum (t). The formulations showed close values of firmness (F3: 1.090 N ± 0.089; F3S ct: 1.091 N ± 0.014) and consistency (F3: 6.715 N.mm ± 0.951; F3S ct: 6.016 N.mm ± 0.132). The new formula had lower values of cohesiveness (F3: −0.484 N ± 0.025; F3S ct: −0.399 N ± 0.002) and adhesiveness (F3: 3.597 N.mm ± 0.171; F3S ct: 3.149 N.mm ± 0.031), and higher values of stringiness (F3: 4.73 mm ± 0.97; F3S ct: 5.31 mm ± 0.49). These differences indicated

that the substitution made resulted in a body butter with similar mechanical–structural properties to the original product, with lower adhesiveness and greater pick-up properties.

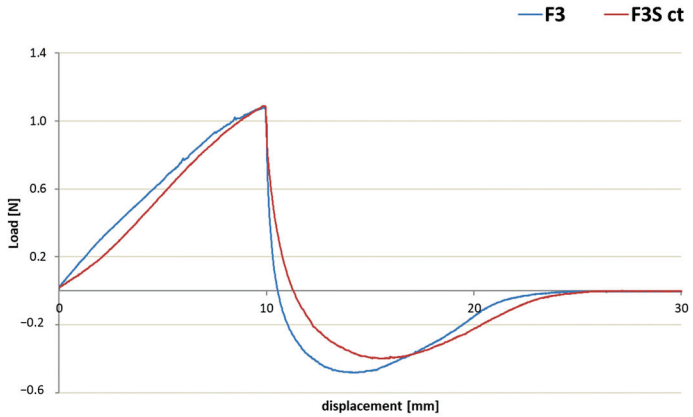


Figure 18. Texture curves obtained from an immersion/de-immersion test performed on the original product F3 and the sample F3S ct reformulated using an eco-design approach by replacing the synthetic texturizer, the acrylic rheological modifier, and the silicone ingredients.

In the double-blind sensory test performed on the F3 product and the formula F3S ct, differences were perceived for only two parameters. For consistency at first touch, on a scale of values where 1 was fluid and 7 was very consistent, F3 had an average rating $\mu = 5.33$, while F3S ct had an average rating $\mu = 6.24$ [$t(32) = 5.71$ $p < 0.01$]. For spreadability, on a scale where 1 was low spreadability and 7 was high spreadability, F3 had an average rating $\mu = 5.21$ and F3S ct had an average rating $\mu = 4.12$ [$t(32) = 3.31$ $p < 0.01$].

4. Conclusions

Replacing synthetic ingredients without affecting the applicative properties of a product is a hard challenge for the formulator. Synthetic materials like acrylic polymers and silicones are meticulously engineered to deliver specific functionalities—impacting the product's structure, application characteristics, and sensory experience. When replacing those ingredients with nature-derived raw materials, factors like choosing the right material, calibrating its concentration, and exploring synergistic combinations become crucial in order to achieve the desired performance. Eco-design focuses on selecting environmentally friendly raw materials without compromising the product's stability, ease of application, or the sensorial aspects to which consumers are accustomed. This study proposes a novel formulation approach that leverages combined rheology and texture analysis to identify the optimal concentration and ingredient combinations, facilitating effective comparisons between products. The studies present in the literature that include the combined use of rheology and texture analysis exploit instrumental techniques to comprehensively describe the physical–mechanical characteristics of raw materials and finished products in order to predict the sensorial characteristics perceived by consumers. Kulawik-Pióro et al. in their paper [16] investigated the relationship between rheological, textural, and sensorial properties to assess the effectiveness of barrier creams, finding good correlations between viscosity, firmness, and spreadability. Callixto and Maia Campos, analyzing the physical and textural properties of skin care products, found a strong correlation between the concentration of UV filters, spreadability, and cohesiveness. In the study by Gilbert et al. [19], they concluded that sensory attributes used to describe cosmetic products during the pick-up and rub-out phases can be foreseen through instrumental rheological and texture analyses. Terscensco et al. [21] set up an instrumental protocol to investigate the influence of emollients on the structural properties and the rheological behavior of emulsions, discovering that the formation of larger oil droplets led to enhanced elastic behavior and spreading properties. Correlations existing between the textural and sensory parameters determined by the use of different cosmetic ingredients, such as butters, oils, and starch, were highlighted by

Vieira et al., in which [27] fluidity and consistency were found to be inversely correlated. In this work, the instrumental protocol is incorporated into the concept of eco-design formulation, with the aim of identifying the correct combinations of natural ingredients that can effectively replace synthetic raw materials with a high environmental impact and ensuring that the final consumer does not notice these formulation changes.

The replacement of the rheological modifiers in the three formulas highlighted how the use of polysaccharides resulted in a decrease in the elastic properties together with a reduction in cohesiveness and adhesiveness. On the other hand, the replacement of silicone emollients, highly appreciated in terms of sensory properties as they give a light, flowing, and silky texture to products, with different naturally derived emollients caused different interactions with the skin, which are difficult to detect with instrumental characterization, having an impact on the after-feel phase of the product after application.

In F1, an acne-prone skin serum, the substitution of the microplastics with a starch-based texturizer, the substitution of the acrylic polymer with a combination of sclerotium gum, xanthan gum, and diutan gum, and the substitution of dimethicone with a mixture of ethyl olivate and triheptanoin allowed us to obtain a stable and fluid system with good pick-up properties, and with a skin feel almost equal to that of the original formula, according to the consumer sensory evaluation.

In F2, an eye-contour gel-cream, the substitution of PMMA with maltodextrin, the substitution of the acrylic polymers with a binary combination of sclerotium gum and diutan gum, and the substitution of the silicone ingredients with a raw material containing a biodegradable elastomer allowed us to produce a stable and smooth system without increasing the stiffness. However, in the final formula, we were not able to reach the same adhesiveness and cohesiveness of the original product, as shown by the different trend of viscoelastic moduli that reflected the differences detected during the sensory test.

In F3, a body butter, the substitution of the synthetic texturizer with silica and the substitution of the acylates with a combination of carrageenan and sclerotium gum allowed us to reproduce the rigid structure and the low pick-up value of the original formula. However, the reformulated emulsion was perceived to be less spreadable and to have a thicker consistency than the commercial one.

The differences recorded were limited and not so marked, being inevitably due to the specificity of the raw materials used and the intrinsic performances of the selected polysaccharides. Although perfectly replicating the structural and application properties of existing formulas with natural alternatives might be impractical, combining rheology and texture analyses allows for the selection of the most suitable natural ingredients that closely resemble the performance of their synthetic counterparts. This instrumental protocol offers a significant advantage over traditional trial-and-error methods, facilitating the development of viable solutions with reduced costs and development time. This proves particularly valuable when reformulating cosmetic products from a green and sustainable perspective.

The replacement of unsustainable ingredients and the reformulation of products represents the first step of the eco-design approach. Life cycle assessment studies are required to have objective data on the sustainability of cosmetic products. Future steps must consider the entire life cycle of the product, taking into account the emissions during the production processes and the choice of materials that will make up the packaging.

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Review

Innovative Strategies for Photoallergy Assessment: Breaking Free from Animal Models in Cosmetic Ingredient Development

Adriana Solange Maddaleno, Maria Pilar Vinardell and Montserrat Mitjans *

Department of Biochemistry and Physiology, Universitat de Barcelona, 08028 Barcelona, Spain; adrianamaddaleno@ub.edu (A.S.M.); mpvinardellmh@ub.edu (M.P.V.)
* Correspondence: montsemitjans@ub.edu

Abstract: Photoallergy, a unique form of skin sensitization induced by specific compounds under ultraviolet irradiation, has traditionally been investigated using animals. However, the prohibition of animal testing for the assessment of cosmetic ingredients in Europe and other countries underscores the necessity for in vitro or in silico alternative methods. Currently, there are no validated methods for assessing photoallergy or photosensitization, presenting a significant challenge in the development of new cosmetic ingredients. This review examines the landscape of alternative methods for detecting photosensitization, emphasizing recent publications, and considering the underlying principles of the different proposed assays.

Keywords: photoallergy; photosensitization; non-animal methods; alternative methods

1. Introduction

The exposure of the skin to sunlight can lead to adverse effects that are not specifically limited to the development of malignant alterations (cancer) or aging, but also involve photosensitivity reactions. The latter, though less studied, are also important and constitute a growing public health problem. Thus, using a patch test, ref. [1] revealed that almost 27% of the European population develops allergic reactions. Furthermore, this highlights a rise in allergic dermatitis in children [2], alongside instances of exogenous photodermatosis affecting the elderly and polypharmacy patients. Additionally, it underscores the exacerbation of skin conditions due to sunlight exposure, including transient photosensitivity [3,4].

Photosensitivity refers to a skin response to light, particularly ultraviolet light (UV), triggered by the presence of intrinsic or extrinsic chromophores in the epidermis or dermis. Common culprits for photosensitivity include photoactive molecules found in skin-applied products or systemic medications. Phototoxicity is defined as an acute reaction that develops after the application of a chemical, topically or systemically, along with exposure to ultraviolet light [5]. When it comes to topical administration, instead of phototoxicity, we can also refer to photoirritation, as the clinical manifestations are similar to contact dermatitis [6].

However, there is also the potential for the development of hypersensitivity reactions to the chemicals or their photoproducts, specifically mediated by T-cells, leading to what is termed photoallergy. Frequently, both phototoxicity and photoallergy mechanisms coexist, where non-specific phototoxic inflammation can contribute to sensitization to the photoactive product. This process may have implications such as the recognition of chemically related compounds, establishment of lifelong immunologic memory, and the potential progression to persistent light reactivity [7]. Photosensitivity arises when a chromophore, whether exogenous or endogenous, accumulates in the skin and undergoes selective activation upon exposure to radiation. This activation primarily occurs with UVA (320–400 nm) but can also involve UVB (290–320 nm), visible, or infrared light. The resultant photoactivation of the chromophore can lead to various outcomes: the induction of non-specific inflammation and aggressiveness within skin cells, referred to as photoirritation;

the initiation of a specific immune response known as photoallergy; the development of enduring sensitivity to UV light, recognized as chronic actinic dermatitis; or the induction of persistent DNA damage with delayed repercussions such as photocarcinogenesis and photoaging [8].

The pathophysiological response differs between photoirritation or photoallergy. Clinically, activation of a photosensitizer leads to an eczematous eruption, characterized by symptoms such as erythema (redness), papules/vesicles (bumps/blister-like lesions), and occasionally bullae (large fluid-filled blisters) [5]. In opposition to the photoirritant reactions, the presence of keratinocyte necrosis and hyperpigmentation are not observed in skin histological samples of photoallergic patients [9].

On the other hand, photoallergy is not common in the entire population; it is a delayed-type hypersensitivity reaction of type IV that manifests as allergic contact photodermatitis. This type of dermatitis occurs when a photoantigen is applied to the skin of individuals previously sensitized to that substance. In this adverse reaction, UV radiation, typically UVA, is necessary to form a complete antigen, and therefore, the inducing substance must be in contact with the skin while exposed to UVA rays [10,11]. Hence, dermal photosensitization reactions represent a special type of allergic contact dermatitis wherein the allergen is activated by electromagnetic radiation, such as ultraviolet light. Historically, photodermatitis reactions were reported to be linked to plant components or extracts [7], but in 1961, cases of photodermatitis were identified among factory workers in England who used tetrachlorosalicylanilide as a bactericidal agent in soaps [12,13].

In any case, the immune system becomes involved, leading to an allergic reaction upon re-exposure to the substance and sunlight. In a first exposure or sensitization phase (Figure 1), the photoallergen will form the complete hapten by binding to skin proteins. These events will trigger cellular responses at various levels involving the activation, maturation and release of humoral mediators that finally will promote naïve T cells into memory/effector T cells [9,13]. During later exposures, sensitized T cells elicit the clinical manifestations of photoallergic contact dermatitis.

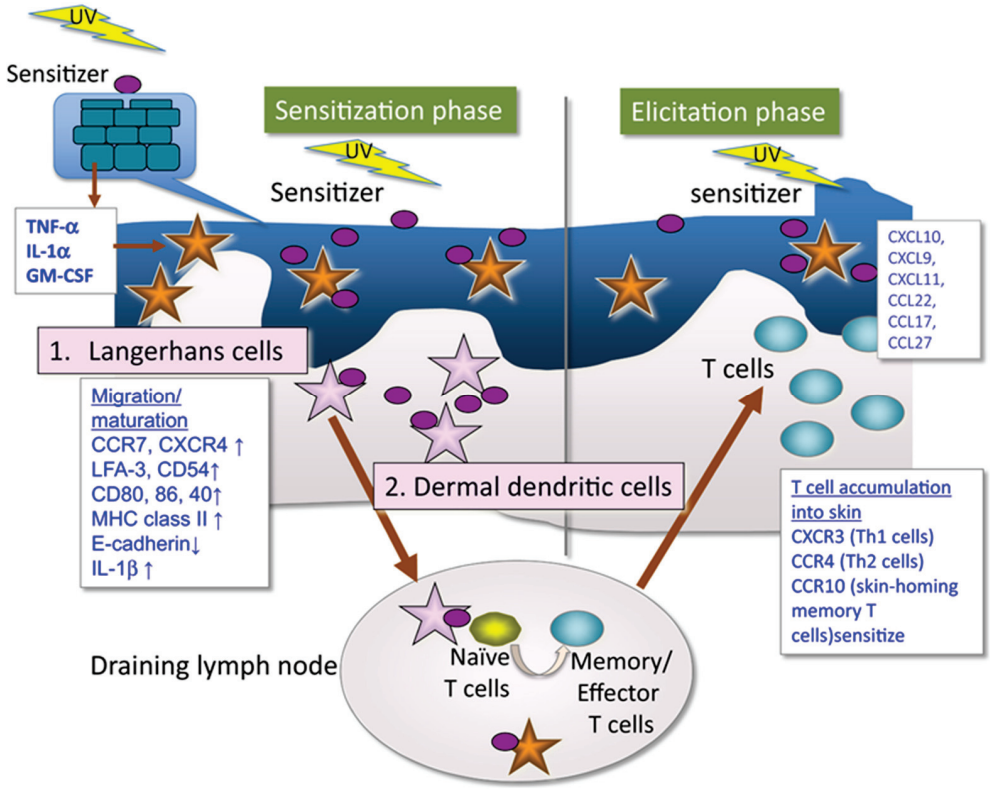


Figure 1. Mechanism of photoallergic contact dermatitis [13]. In a first exposure or sensitization phase, the photoallergen is converted to a complete hapten by UV irradiation that can bind to skin

proteins, leading to production proinflammatory cytokines that induce maturation of epidermal Langerhans cells and/or dermal dendritic cells that migrate to the draining lymph nodes where they sensitize naïve T cells to be memory/effector T cells. Subsequent exposure induces sensitized T cells elicit the clinical manifestations of photoallergic contact dermatitis (elicitation phase).

Currently, the growing prevalence of photoallergy is due to a combination of factors, including the continual introduction of new drugs, cosmetics, and chemicals each year, along with shifts in behaviors due to heightened exposure to natural sunlight or artificial lamps. These trends have contributed to greater concern about this type of response, both among patients and within dermatologists and the scientific community [6,14]. Dermatologists use the photo patch test, employing a battery of photoallergenic products, for diagnostic purposes. Over the years, changes in population habits have altered the products that cause photoallergic contact dermatitis. The decrease in the use of certain products and the introduction of new products have a significant impact on the prevalence of specific photoallergens that trigger photoallergic contact dermatitis. While in the 1960s, antimicrobial agents were mainly responsible for photoallergies, in the 1970s, fragrances such as 6-methylcoumarin were the culprits, and from the 1980s onwards, topical sunscreens and non-steroidal anti-inflammatories emerged as the leading causes [15]. At present, ketoprofen is recognized as an important photoallergen with high cross-reactivity with UV filters, especially benzophenones [16,17]. Etofenamate, which is particularly common in the Mediterranean area, and octocrylene are among the other compounds that often elicit positive reactions. However, the photoallergic reaction is attributed to photosensitization induced by ketoprofen [18]. Therefore, the potential phototoxic or photoallergic effects must be evaluated through appropriate and validated safety assays before they are launched into the market to avoid potential adverse effects on consumers [19]. These assays serve as preventive tools to mitigate adverse effects and prevent the development of a pathological process.

Traditionally, the phototoxic properties of chemicals are generally assessed using experimental animals, typically guinea pigs, rabbits, rats, and mice [13,20–22]. For pharmaceutical products, the European Medicines Agency mandates these assays, as well as photoallergy tests, to ensure their safety [23]. Nevertheless, the constraints of animal models, combined with recent regulations created by various international regulatory bodies [24–26] banning animal use in cosmetic safety assessments, and the tightening of ethical considerations demanding the reduction and refinement of such practices, highlight the urgent necessity for the development of in vitro assays as preventive tools to identify potential hazards for both safety and human health [6]. Our aim is to offer additional perspectives on the gradual incorporation of New-Approach Methodologies (NAMs) into chemical risk assessment protocols, with the goal of safeguarding human health. This transition is envisioned to culminate in the adoption of an animal-free approach termed "Next-Generation Risk Assessment" [27]. For this reason, there is an urgent demand for the development of feasible, reliable, and reproducible alternative methods.

Concerning mandatory phototoxicity studies for regulatory purposes, currently, the pharmaceutical and cosmetic industries must rely on the in vitro 3T3 Neutral Red Uptake phototoxicity assay (3T3 NRU PT) [28], alongside newer methods such as the ROS assay for photoreactivity [29], and the reconstructed human epidermis phototoxicity test method [30]. However, none of these methods discriminate between photoirritants and photoallergens, and therefore, novel animal-free proposals must be further explored. In this regard, the objective of this work is to investigate the latest advances in the development of methodological proposals as alternatives to traditional assays for identifying products with photoallergic potential (hazard identification). Specifically, we focus on assays that allow for the reduction or replacement of animal models by addressing the question: "What are the most recent developments in alternative methods to animal experimentation for identifying photoallergens?".

The beauty industry continually adapts to meet the diverse needs of consumers, evolving through innovation and a quest for perfection. Despite this drive, prioritizing the safety

of cosmetic products remains essential. Notably, the European Union and several other nations have made significant advancements in discontinuing animal testing for cosmetics. This progressive shift underscores a dedication to ethical standards, animal welfare, and the endorsement of alternative testing methods that are both humane and efficacious.

2. Materials and Methods

A search was conducted across different databases, following the recommendations of PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) statement [31] including Pubmed Central (National Institutes of Health's National Library of Medicine, Pubmed), Web of Science (Clarivate, WoS), Scopus (Elsevier), and Toxcenter (Toxicology Center Database). The search was performed by combining representative keywords for the adverse effect, namely photoallergy, photoallergen/ic, photoallergenicity, photoassay, photosafety, with those representation evaluation methods, alternative, in vitro, in chemico and in silico, and limited to the title, abstract or indexing words. The search was refined by including the documents published between 2013 and 2023.

The found documents have been classified in an Excel file where the following fields have been recorded: authors, title of the work, journal, year of publication, type of document (article, review, book chapter, conference abstract, etc.), database where it was found, and language. Each document has been identified by a reference number, and their appearance in the same or more than one database has been assessed to eliminate repetitions. Then, those records not written in English as well as those that were not original articles or reviews (conference abstracts, clinical studies, letters, book chapters, etc.) were discarded. Finally, the registers were assessed for eligibility by screening the title and abstract. Eligible documents were based on the mechanism of photoallergy.

3. Results

3.1. Study Selection

We found a total of 876 registers in the different databases selected (Figure 2): 201 in Pubmed, 249 in WoS, 213 in Scopus, and 215 in Toxcenter. According to the words used, photoassay provided very few results (5 documents), while photosafety, with 298 reviews, and photoallergy, with 213, retrieved the maximal number of registers. Regarding the words related to methods, in chemico (51) is the phrase that generated the fewest results and in vitro the one that generated the most (595).

Once the documents found in more than one database and different searches were eliminated, the number of studies was reduced to 338 (Figure 3), which represents 38% of the initial documents. An initial analysis of these 338 documents was performed to find out in which years they were published (Figure 3a), the type of document (Figure 3b), in which database they were found, as well as the language in which they were written. This previous analysis has allowed us to apply a pre-eligibility criterion to include or exclude the studies based on the type of document and language used.

The distribution of documents based on the year of publication (Figure 3a) indicates that 30% of the articles were published during 2020 and 2021, when most laboratories were closed due to COVID-19. However, the maximum number of reviews was found in 2013 (22), followed by 2021 (14), and 2020 (10).

Regarding the type of document found (Figure 3b), almost 50% were research articles. However, reviews, books, and book chapters, and even editorials and letters (included in other) have also been found.

Regarding the database, 117 of the 338 documents were found in PubMed, 146 in WoS, 149 in Scopus, and 76 in Toxcenter. Only 19 documents (6%) were found in the four databases, which represents the 35% of the documents found in Toxcenter, and only 13% of the registers were found in WoS and Scopus. Based on this, we could assume at first sight that these 19 documents are the ones of greatest interest for answering our question. If this is corroborated, the best database for our search would be Toxcenter, while WoS and Scopus would be the least accurate.

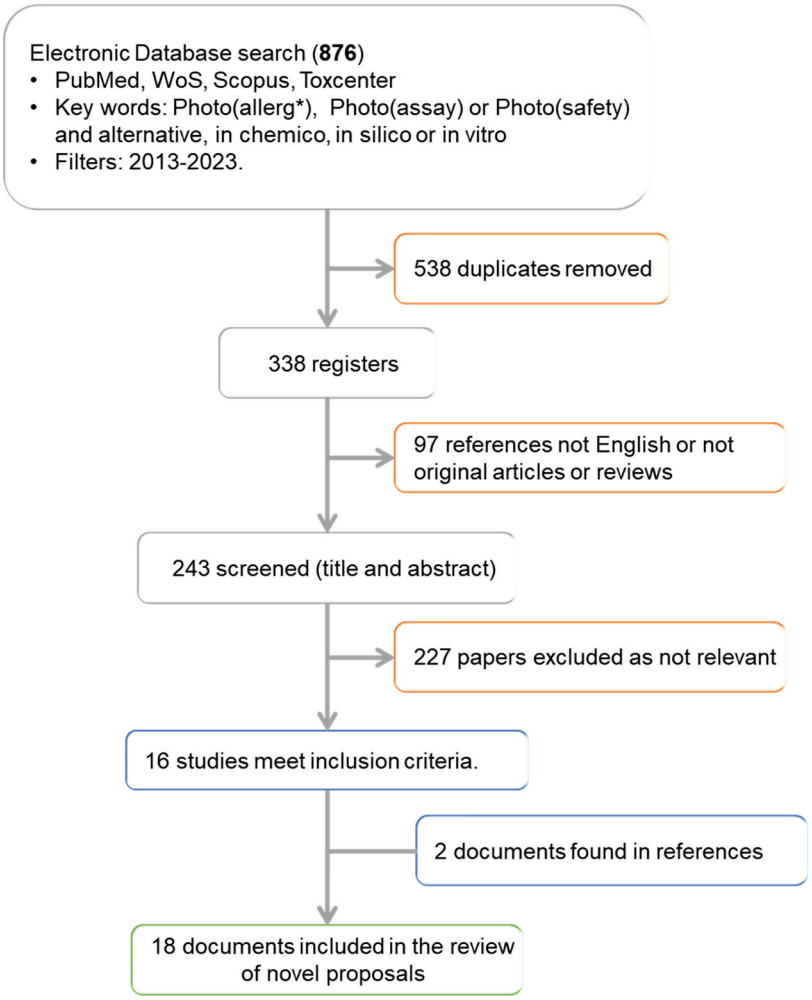
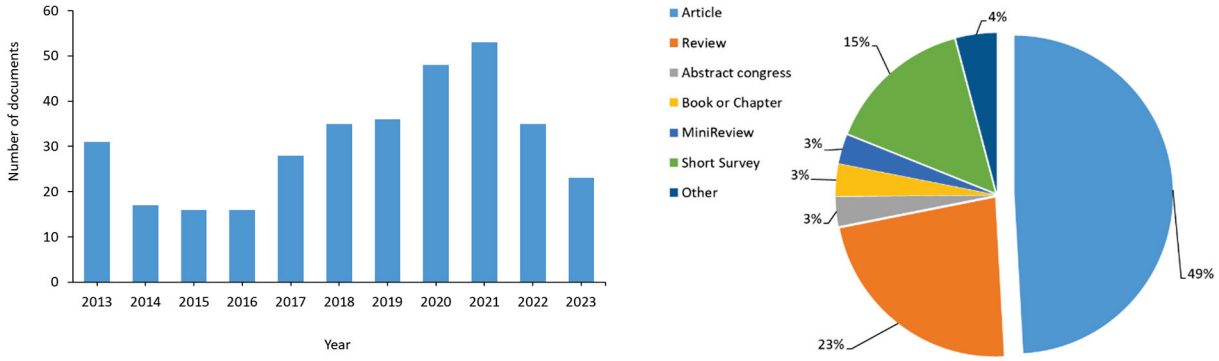


Figure 2. Study flowchart. * Photoallergy, photoallergen/ic or photoallergenicity.



(**a**) (**b**)

Figure 3. Analysis of the registers found: (**a**) Distribution of the number of documents according to the year of publication; (**b**) distribution of the number of documents according to type. Other document types include letters, conferences, short communications, editorials, short surveys, clinical trials, retrospective and comparatives studies, case reports, protocols, and guidelines.

Finally, in terms of language, less than 4% (13) of the documents were written in a language other than English. Thus, seven documents were written in Chinese, three in Japanese, and of the remaining three, one was written in German, another in Polish, and the last one in Turkish.

Based on this previous analysis, we excluded from subsequent analysis documents that did not fit into the categories of articles and reviews. Likewise, articles written in a language other than English were not considered either, although we may have lost some information as a result. With the application of these criteria, we evaluated the main objective of the remaining 243 registers (Figure 2), via analysis of the title and abstract. This analysis allowed us to classify the works in a very general way according to Table 1, allowing us to identify the works related to our final objective.

Table 1. Main objective of the 231 articles analyzed.

Objective	Number of Articles
Photoallergy	16
Photoprotection	13
Phototherapy	16
Phototoxicity [1]	50
Skin [2]	25
Cosmetics [3]	46
Miscellaneous [4]	78

[1] Includes studies of photomutagenesis or photogenotoxicity. [2] Skin biology, disorders, and treatments; photoaging. [3] Contains articles dealing with safety studies of ingredients or herbal extracts, as well as antiaging or photoaging activity. [4] Exploration of biological activity of extracts or active ingredients; analytical methods (stability, pharmacokinetics, etc.); diagnostic techniques; mechanisms of physiological disorders and diseases other than skin conditions.

As seen in Table 1, studies that contain the prefix "photo" within the title or description objectives deal with physiological changes or pathophysiological entities (photoaging, photoallergy, phototoxicity); protection against sunlight (photoprotection); therapeutic strategy (phototherapy) but also analytical and diagnostic techniques (photostability, photodegradation, photoreactivity) or safety studies of ingredients and formulations (photosafety). Therefore, there are a considerable number of studies that do not correspond to the objective of this review.

Finally, only 16 articles had the study of photoallergenic capacity or activity as their main objective (Table 2). Most of these studies were found in Scopus (13 of 16) and Pubmed (12 of 16), with 3 documents not identified in these databases but rather in WoS. This suggests that to obtain reliable results, at least two databases must be consulted in parallel [32]. In this scenario, Pubmed or Scopus together with WoS, are the preferred choices, as they enable the identification of all 16 registers. However, despite initially appearing as the top database choice, Toxcenter yielded the least accurate results, with less than 50% of records being localized.

Table 2. Documents included in our study. References are listed according to the year of publication.

Article	Authors	R. [1]
Toxicol In Vitro **2013**, *27(1)*, 103–110	Galbiati et al.	[33]
Toxicol In Vitro **2013**, *27(6)*, 1920–1927	Martínez et al.	[34]
Toxicol In Vitro **2014**, *28(1)*, 13–17	Galbiati et al.	[35]
Int J Cosmetic Sci **2014**, *36(6)*, 561–570	Stiefel and Schwack	[36]
J Toxicol Sci **2016**, *41(1)*, 129–142	Oeda et al.	[37]
Toxicol In Vitro **2016**, *33*, 147–152	Onoue et al.	[38]
J Appl Toxicol **2016**, *36(7)*, 956–968	Tsujita-Inoue et al.	[39]
Chem Res Toxicol **2016**, *29(1)*, 40–46	Vayá et al.	[40]
J Dermatol Sci **2017**, *85(1)*, 4–11	Onoue et al.	[9]
*Chemistry***2017**, *23(56)*, 13986–13994	Pérez-Ruíz et al.	[41]
Toxicol in Vitro **2017**, *45*, 386–392	de Àvila et al.	[42]
J Cutan Immunol Allergy **2018**, *1(2)*, 48–57	Tokura	[13]
J Toxicol Sci **2018**, *43(4)*, 247–256	Toyoda and Itagaki	[43]
ALTEX **2019**, *36(3)*, 373–387	Patel et al.	[44]
J Appl Toxicol **2020**, *40(5)*, 655–678	Yamamoto et al.	[45]

Table 2. *Cont.*

Article	Authors	R. [1]
Toxicol In Vitro **2021**, *77*, 105245	Nishida et al.	[46]
Toxicology **2023**, *495*, 153613	Nguyen et al.	[47]
Regul Toxicol Pharmacol **2023**, *145*, 105499	de Ávila et al.	[48]

[1] Article reference.

After analyzing these 16 documents, 2 additional studies were identified in the list of references of de Ávila et al. [48] and consequently were included in the final list of selected studies as depicted in Table 2. Our search strategy failed to identify the work of de Ávila et al. 2017 [41] and Nguyen et al. [47] because these two studies included photosensitization in the title, abstract or indexing keywords instead the search terms used here (photoallergy, photoallergen/ic, photoallergenicity, photoassay, photosafety).

Out of the 18 articles selected, 3 were reviews, 3 mechanistic studies, and 12 were innovative in vitro or in chemico proposals aimed at categorizing the assessed compounds as photoallergen, photoirritant, or non-phototoxic. Five of the eighteen studies were published in the last five years, suggesting the need for further investigation and exploration of this safety aspect.

3.2. Alternative Assays Proposed

Like the approach taken in the case of ACD, an adverse outcome pathway (AOP) has been proposed to guide the development of novel alternative methods [48,49]. This AOP includes four key events that will guide both the development of alternative methods to animal experimentation as well as the integrated test strategies (ITS) to assess the skin photosensitization hazard of chemicals.

The different assays proposed to identify photoallergens are briefly described in the following sections. A summary of the proposed methods according to adverse outcome pathway and key events described in [48] is shown in Table 3.

Table 3. Summary of the existent and novel assays proposed to identify photoallergens based on the adverse outcome pathway and key events described in [48].

Key Event	Assay Proposed	Prediction Model	References
Chemical structure and properties	UV/VIS spectra	MEC [1] < 1000 M^{-1} cm^{-1} for pharmaceutical substances	[38,46]
	ROS/mROS data	25 (ΔA_{440} nm·103) and 20 (ΔA_{560} nm·103)	[38,46]
Molecular initiating event (protein covalent binding)	Protein reactivity	Not defined	[36,40]
	Amide adduct formation	Not defined	[41]
	Photo-ADRA	ΔUV [2] $\geq$ 10	[44]
		ΔUV [2] $\geq$ 15% NAC and NAL or $\geq$10% in average	[45]
	Photo-DPRA	ΔUV [2] $\geq$ 10	[44]
		Mean $\Delta Cys/\Delta Lys$ [3] > 6.38% or ΔCys > 13.89%	[46]
	Photo-mDPRA	Not defined	[42]
Keratinocyte activation	NCTC 2455	IL18 SI [4] $\geq$ 1.3, CV [5] $\geq$ 80%	[33,35]
	PhotoSENSIL	IL18 SI [4] $\geq$ 1.5	[47]
	Photo-ARE	Keap1-Nrf2-ARE pathway induction	[39]
Dendritic cell activation	IL-8 release	SI [4] > 2.0, CV $\geq$ 75%	[34]
	Photo-SH/NH_2	CV [5] > 50%; ΔRFI [6] UVA $\geq$ 15%	[37]
	Intracellular ROS	CV [5] $\geq$ 80; 95% CI [7] lower limit value $UVA^+ \geq$ 2/upper limit value UVA^- < 2	[43]

[1] MEC: molar extinction coefficient; [2] ΔUV: difference between average % depletion values in the presence and absence of UV exposure. [3] $\Delta Cys/\Delta Lys$ difference in % depletion values with and without UV exposure for cysteine and lysine. [4] SI: stimulation index or ratio of IL-18 production in irradiated conditions versus non-irradiated conditions for a specific chemical. [5] CV: percentage of cell viability; [6] ΔRFI_{UVA}: change in relative fluorescence intensity (mean fluorescence intensity of sample-treated cells/vehicle-treated cells) before and after UV exposure; [7] CI: confidence interval.

3.2.1. Galbiati et al., 2013 and 2014 [33,35]

The research team proposed the use of keratinocytes (NCTC 2455) and the production of Interleukin-18 (IL-18) as a tool to discriminate among photoirritants and photoallergens. Using the NCTC 2455 cell line, the authors have determined the conditions of the assay [33] by using chlorpromazine (CPZ) as a well-known photoirritant and photoallergen chemical. Then, a panel of other 16 chemicals including photoirritants, photallergens, and negative compounds (Table 4) allowed the authors to propose a prediction model (SI) based on the relationship between IL-18 production when cells are exposed to subtoxic concentrations of chemicals and UVA light (I) respect to the non-irradiated conditions (NI). Thus, a potential photoallergen was identified when SI $\geq$ 1.3 and statistical differences were found in the amount of IL-18 with respect to untreated cells. The in vitro assay was considered a promising tool in photosafety assessment according to the reproducibility of data and accuracy of chemical classification. Key aspects of the assay were discussed in a subsequent article [35].

Table 4. Summary of the compounds assessed with the proposed photoassays reported in 13 of the selected articles. Classification among phototoxic, photoallergen or non-phototoxic agents was performed according to assumption provided by the authors. Activity of the compounds and relationship to cosmetic (C) industry is also presented. Only EU accepted ingredients are considered.

Classification	Compound	Activity *	References
Phototoxic and photoallergen	4-Aminobenzoic acid (PABA)	UV, C	[33,35,47]
	8-Methoxypsoralen (8-MOP)	PUVA	[46]
	6-Methylcoumarin (6-MC)	Fragrance, C	[37–39,46]
	3,3′,4′,5-Tetrachlorosalicylanilide	Antibacterial, C	[37,39,45,46]
	3,4′,5-Tribromosalicylanilide	Antibacterial, C	[37,39,45]
	Amiodarone HCl	Antiarrhythmic	[44]
	Anthracene	Dye production	[44]
	Benzophenone (BNZ)	UV, C	[46]
	Bithionol	Anti-infectious, C	[37–39,46]
	Chlorpromazine HCl (CPZ)	Antipsychotic	[33–35,37,39,43,46,47]
	Diclofenac sodium salt	NSAID	[37,39,45,46]
	Enoxacin	Antibacterial	[46]
	Fenticlor	Antimicrobial, C	[46]
	Furosemide	Diuretic	[46]
	Griseofulvin	Antimycotic	[46]
	Hexachlorophene	Antiseptic	[43]
	Hydrochlorothiazide	Diuretic	[46]
	Ketoprofen (KET)	NSAID	[46,47]
	Lomefloxacin HCl	Antibiotic	[46]
	Norfloxacin	Antibiotic	[44]
	Ofloxacin	Antibiotic	[46]
	Piroxicam	NSAID	[46]
	Protoporphyrin IX	Porphyrin, natural chromophore	[44]
	Promethazine	Antipsychotic	[33,35,37,46]
	Pyridoxine HCl	Vitamin B6, C	[46]
	Quinine	Antimalarial	[46]
	Tetracycline HCl	Tetracycline	[46]
Photoallergen	4-Aminobenzoic acid (PABA)	UV, C	[32]
	2-Ethylhexyl-4-methoxycinnamate	UV, C	[33–35,47]
	7-Methoxycoumarin	Fragrance, C	[45,46]
	8-Methoxypsoralen (8-MOP)	PUVA	[45]
	6-Methylcoumarin (6-MC)	Fragrance, C	[33–35,47]
	4-Methyl-7-ethoxycoumarin	Fragrance, C	[45,46]
	Anthracene	Dye production	[37,39]
	Acridine	Dye	[37,39]
	Amiodarone HCl	Antiarrhythmic	[37,39,42,45]
	Avobenzone (Butyl methoxydibenzoylmethane)	UV, C	[33–36]
	Benzophenone (BZN)	UV, C	[33–35,37–39,45]
	Benzophenone-3 (Oxybenzone)	UV, C	[36]
	Benzydamine	NSAID	[37,39]
	Bithionol	Anti-Infectious, C	[43,45]

Table 4. *Cont.*

Classification	Compound	Activity *	References
Photoallergen	Dibenzoylmethane	UV, C	[36]
	Dichlorophene	Antimicrobial, C	[38,45,46]
	Enoxacin	Antibacterial	[37–39,45]
	Fenofibrate	Fibrates	[45]
	Fenofibric acid	Fibrates	[40]
	Fenticlor	Antimicrobial, C	[38,45]
	Furosemide	Diuretic	[37,39]
	Glibenclamide	Antidiabetic	[38,45]
	Hexachlorophene	Antiseptic	[37,38,45,47]
	Hydrochlorothiazide	Diuretic	[38,45]
	Indomethacin	NSAID	[37–39,45]
	Isoniazid	Antituberculotic	[38,45,46]
	Ketoprofen (KET)	NSAID	[33–35,37–39,41,45]
	Mequitazine	Antihistamines	[46]
	Musk ambrette	Fragrance, C	[37–39,45–47]
	Musk xylene	Fragrance, C	[38,45–47]
	Octocrylene	UV, C	[36]
	Octyl dimethyl PABA	UV, C	[37,45,46]
	Omadine Na	Antibacterial/antifungal, C	[37,45,46]
	p-Phenylenediamine (PPD)	Dye, C	[37,39,45,46]
	Piroxicam	NSAID	[37–39,45]
	Protoporphyrin IX	Porphyrin, natural chromophore	[42]
	Pyridoxine HCl	Vitamin B6, C	[37,45]
	Quinine HCl ($2H_2O$)	Antimalarial	[45]
	Sulfanilamide	Antibacterial	[37–39,43,45,46]
	Sulfasalazine	Antirheumatic	[37,45,46]
	Tenoxicam	NSAID	[37,39]
	Tribromsalan	Antibacterial/antifungal	[37,45]
	Triclocarban	Antibacterial, C	[37–39,45]
Phototoxic	5-Methoxypsoralen	PUVA	[37,39,46]
	8-Methoxypsoralen (8-MOP)	PUVA	[33–35,44,47]
	Acridine	Dye	[33,35]
	Ibuprofen	NSAID	[33–35]
	Musk ketone	Fragrance, C	[47]
	Retinoic acid	Vitamin A1 metabolite, Skin affections	[33–35]
	Tetracycline HCl	Tetracyclines	[37]
Non-phototoxic	2-Aminophenol	Dye	[35]
	1,3-Butylene glycol	Antimicrobial, moisturizing, emollient, C	[45,46]
	2,4-Dinitrochlorobenzene (DNCB)	Developer, industrial intermediate	[47]
	5-Methoxypsoralen	PUVA	[45]
	4′-Methylbenzylidene camphor	UV, C	[38,45,46]
	2-Propanol	Industrial and household chemicals; antiseptics, disinfectants, hand sanitizer and detergents.	[45]
	Acridine	Dye	[45]
	Anthracene	Dye production	[45]
	Aspirin	NSAID	[38,45,46]
	Ascorbic acid	Vitamins	[37–39,45]
	Benzocaine	Local anesthetic	[38,45,46]
	Cetyl alcohol	Opacifier, emollient, emulsifier, thickening, C	[45]
	Chlorhexidine	Disinfectant/antiseptic	[42]
	Cinnamic acid	Flavor, C	[46]
	Cinnamaldehyde (Cinnamic aldehyde)	Flavor, C	[42,46]
	Dextran	Antithrombotic/volume expander	[37,39]
	Diethylmaleate	Pesticide	[34]
	Dimethyl sulfoxide (DMSO)	Solvent	[37,39,45]
	Erythromycin	Antibiotic	[38,45,46]
	Ethanol	Antiseptic/solvent, C	[45]
	Ethylhexyl methoxycinnamate	UV	[36]
	Glycerin	Antimicrobial/antiviral Smoothness/lubrication/humectant, C	[45,46]
	Glyphosate [N-(phosphonomethyl) glycine]	Herbicide	[42]
	Hexachlorophene	Antiseptic	[42,44]
	Isopropyl myristate	Texture enhancer/emollient, C	[45]
	Lactic acid	Acidifier/flavor enhancer. Antibacterial/moisturizing/exfoliating, C	[33,35,37,39,45–47]

Table 4. *Cont.*

Classification	Compound	Activity *	References
Non-phototoxic	Lauric acid	Emollient/cleansing, C	[45]
	L-histidine	Essential amino acid, C	[42,44,46]
	Methyl-N-methylanthranilate	Fragrance, C	[45]
	Methyl salicylate	Analgesic, fragrance, flavor, soothing, C	[37–39,43,45,46]
	Musk ketone	Fragrance, C	[45]
	Nickel sulfate	Dye, printing, coatings, ceramics.	[34]
	Octanoic acid	Ester production/dye, C	[34]
	Octyl salicylate	UV, C	[46]
	p-Phenylenediamine (PPD)	Dye, C	[33,35,47]
	Penicillin G	Antibiotic	[37–39,43,45,46]
	Phenytoin	Anticonvulsants/barbiturates	[38,45,46]
	Polyethylated tallow amine	Surfactant	[41]
	Propylene glycol	Polymer production, solvent, humectant, moisturizer, C	[45]
	Sodium dodecyl sulfate	Surfactant, C	[33,35,37,39,43,47]
	Sodium laurate	Soap, C	[45]
	Sulisobenzone (benzophenone-4)	UV, C	[45,46]
	Tetracycline HCl	Tetracyclines	[45]
Not specified	Ethylhexyl salicylate	UV, C	[36]
	Ethylhexyl triazone	UV, C	[36]

* NSAID: Non-steroidal anti-inflammatory drug; PUVA: photodynamic therapy including psoralen and ultraviolet; UV: UV absorber, used in sunscreens.

3.2.2. Martínez et al., 2013 [34]

Based on the capacity of the THP-1 cell line to secrete IL-8 when exposed to contact sensitizers [50], the authors explored the suitability of this model to identify photoallergens. They calculated a stimulation index based on the ratio of IL-8 measured in non-irradiated and irradiated conditions and established a cut-off of two to identify photoallergens. The chemical panel studied includes seven photoallergens, three photoirritants, and five non-phototoxic chemicals (three allergens and two irritants) as summarized in Table 4. The assays showed an accuracy of 93%.

3.2.3. Stiefel and Schwack, 2014 [36]

Protein adducts formation is pivotal in skin sensitization, and the authors have previously reported that some UV filters can react with primary amines under UV radiation [51]. Based on this observation, in this article, the reactivity of these UV filters towards skin proteins was explored by using more intricate models such as Boc-protected lysine, the tetrapeptide Boc-Gly-Phe-Gly-Lys-OH, bovine serum albumin, and porcine gelatin. A high reactivity was reported for the UV absorbers 4-t-Butyl-40-methoxydibenzoyl methane, octocrylene and benzophenone-3, which is attributed to their capacity to develop allergic or photoallergic reactions (Table 4). Although no prediction model was established, the authors determined a gradation of UV filter reactivity.

3.2.4. Oeda et al., 2016 [37]

The authors developed a cell-based in vitro photosensitization assay, examining the changes in cell-surface thiols and amines on human monocytic cell line THP-1 (photo-SH/NH_2 test) in a similar way as previously reported for contact sensitizers [52]. In this case, Ketoprofen (KET) was selected as a paradigm of photoallergic chemical. The final panel consists of 18 photoallergens, 8 photoirritants, and 7 non-photosensitizers (Table 4). The seven known non-photosensitizers, along with three photoallergens and one photoirritant, did not alter cell-surface thiols or amines. This overall result achieved an accuracy and sensitivity of 87.9% and 84.6%, respectively. However, the assay was much less precise when predicting photoallergy because accuracy was lower, at 69.7%. Photosafety information of the chemicals was obtained from in vivo assays and clinical data [53,54]. The discussion regarding false-negative prediction suggests that the exclusive

use of UVA, and not a solar simulator that includes UVB, could explain this phenomenon in the cases of furosemide, piroxicam, sulfanilamide, and tenoxicam [55,56].

3.2.5. Onoue et al., 2016 and 2017 [9,38]

In 2016, the research team evaluated the suitability of different assays to identify photoallergens. These assays included the ultraviolet/visible (UV/VIS) spectral analysis [57,58]; the reactive oxygen species (ROS)/micellar ROS (mROS) assays [29]; and the 3T3 NRU PT [28]. A battery of different phototoxins that included 23 photoallergens and 7 non-phototoxic/non-photoallergenic chemicals (Table 4) was employed to establish the ability of the strategy to predict the photoallergenicity. Finally, the ROS assay was found to be useful for predicting photoallergenic potential, although it presented some limitations regarding false-positive classification.

In 2017, the authors reviewed the mechanism of photoallergic reactions, including the clinical manifestations, the physicochemical characteristics of photoallergens, and the pathophysiology of photoallergic reactions. Descriptions of some known photoallergens previously used in pharmaceutic and cosmetic formulations and products are also included. Finally, a discussion of the potential use of current predictive tools of phototoxicity and the novel in vitro proposals regarding the identification of photoallergens was included. However, no specific integrated strategy or sequential test battery was suggested.

3.2.6. Tsujita-Inoue et al., 2016 [39]

The authors examined whether activation of the Keap1 (Kelch-like ECH-associated protein 1)–Nrf2 (nu-clear factor-erythroid 2-related factor 2)–ARE (antioxidant response element) pathway could be used to assess the photoallergenic potential of chemicals, using the reporter cell line AREc32 or KeratinoSens™ and concluded that activation of the Keap1-Nrf2-ARE pathway is an effective biomarker for evaluating both photoallergenic and phototoxic potentials. Twelve out of seventeen photoallergens (Table 4) were judged as positive.

3.2.7. Vayá et al., 2016 [40]

The research team explored the photoreactivity mechanism of fenofibric acid, a pharmacologically active metabolite of fenofibrate, by using human and bovine albumins and different light sources of photolysis. Fenobric acid was identified by the FDA as responsible for photosensitivity reactions [59]. The authors concluded that the primary photochemical process underlying photoallergy involved formal hydrogen atom transfer from an amino acid residue present in the binding site to the excited benzophenone chromophore. The authors' intentions did not extend to identifying photoallergens.

3.2.8. Pérez-Ruíz et al., 2017 [41]

The authors reported that the covalent binding of β-lactams to proteins upon photochemical activation leads to an amide adduct by a different protein haptenation pathway than that previously described [60]. The research team has proven that by an integrated approach that combines photochemical, proteomic, and computational studies, human serum albumin (HSA) was selected as a target protein and ezetimibe as a probe.

These results could allow the development of new in vitro methods, although further research in this area is still required.

3.2.9. de Àvila et al., 2017 and 2023 [42,48]

A modified Direct Peptide Reactivity Assay (DPRA) assay to minimize the reaction volume was explored alongside the introduction of an irradiation step (Photo-mDPRA) to assess the potential skin sensitization and photosensitization capacity of agricultural formulations and ingredients. The authors propose the assay as a first step of a battery of photosafety tests, emphasizing that further research is needed to determine the applicability domain to other products such as cosmetics and medicines. Moreover, they highlight

the importance of the environmentally friendly assay by reducing the organic waste and the economic cost. The OECD 442C guideline was followed to predict the photosensitive ability [61]. Finally, the authors reported a greater peptide depletion in the case of photosensitizers after irradiation, particularly in the case of cysteine.

Recently, de Ávila et al. [48] reviewed the mechanisms involved in the photosensitization process and discussed the different methodologies. While this review is compelling, there are several important papers that have not been analyzed. Other potential photoallergens are natural products which are more difficult to evaluate using in vitro methodologies. There are some studies involving volunteers [62], but this kind of study presents ethical concerns, as pointed by the Scientific Committee of Consumer Safety of the European Union [63]. Photopatch tests should be performed only for diagnostic purposes not for the safety evaluation of cosmetic ingredients.

3.2.10. Toyoda and Itagaki, 2018 [43]

The authors determined the intracellular ROS production of THP-1 A31 cells exposed to UV light as an endpoint of phototoxicity. The assay seemed to be suitable for identify photoreactivity but failed to discriminate between photoirritants and photoallergens, as no differences could be found in the production of ROS. Moreover, the number of chemicals was very poor (Table 4) and further studies are needed. The authors used 95% confidence intervals to calculate the maximal ROS production when the cell viability (CV) was $\geq$80% in both UVA- and non-UVA-exposed cells. The use of chemical quenchers demonstrated the participation of ROS in photoallergenic reactions. Interestingly, the assay identified methyl salicylate (MS) as a non-phototoxic chemical whereas it failed to identify sulfanilamide (SA) as a photoallergic one, probably because this chemical is activated by UVB [43]. Despite the limited number of chemicals used, the authors proposed this method due to the lack of in vitro methods.

3.2.11. Patel et al., 2019 [44]

The authors modified the DPRA (photo-DPRA) as well as the Amino acid Derivative Reactivity Assay (ADRA) (photo-ADRA) by introduction of a photoirradiation parameter. Analysis using photo-DPRA and photo-ADRA correctly distinguished known photoallergens from non-photoallergens. The authors suggested that photoallergens selectively showed higher depletion of model peptides or modified amino acids. Although no threshold was defined, assayed photoallergens showed an average depletion higher than 40 in both assays, while for non-photosensitizers, this percentage was nearly zero.

Thus, photo-DPRA and/or photo-ADRA can serve as non-animal in chemico methods for the identification and assessment of photoallergens/photosensitizers. The principal limitation of the method is that only nine chemicals were used (Table 4).

3.2.12. Yamamoto et al., 2020 [45]

In this study, the authors performed a more detailed analysis of the predictive capacity of the photo-ADRA assay. The information of the photoallergenic activity of the different chemicals has been obtained from in vivo animal experiments and from patch test studies in volunteers [64]. Criteria to classify the 60 chemicals and mixtures analyzed (Table 4) as contact photosensitizers or non-photosensitizers was based on the difference of percentage of N-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) and α-N-(2-(1-naphthyl)acetyl)-L-lysine (NAL) depletion after reaction in non-irradiated and irradiated conditions. When the threshold was set at a value $\geq$ 15% for each peptide or an average value of $\geq$10%, the authors correctly identified 48 of 60 or 48 of 59 chemicals, depending on the high-performance liquid chromatography analysis used.

3.2.13. Nishida et al., 2021 [46]

The authors developed the photo-DPRA based on the OECD guideline 442C [51] to be used in an in chemico sequential testing strategy that includes spectral UV-VIS analysis

and ROS/mROS assay [29]. A battery of products consisting of 34 photoallergens and 16 non-photoallergens was studied (Table 4). The authors proposed a sequential testing strategy (STS) starting with the determination of the molar extinction coefficient (MEC), followed by the ROS/mROS assay, and finally the photo-DPRA test. Guideline criteria were followed to classify chemicals as potential photoallergens [29,58].

3.2.14. Nguyen et al., 2021 [47]

The authors proposed the method PhotoSENSIL to identify photoallergens. In a similar way as in the work of Galbiati et al. [33,35], here, IL-18 was explored as an in vitro biomarker on the reconstructed human epidermis model EpiCS™ RHE. The model was assessed with sixteen chemicals (Table 4) with different photosensitizing capacities. The authors concluded that the best assay conditions were 1 h of preincubation followed by a recovery period of 23 h after the exposition to the potential photoallergen and UV light. The ratio of IL-18 production in non-irradiated and irradiated conditions was set at 1.5 because eight to nine photosensitizers were identified.

In total, 96 chemicals and compounds were tested, not including the six glyphosate-based herbicides assessed in [42]. Among them, about 50% were ingredients or actives in cosmetics formulations, with UV filters being the main group (14), followed by preservatives. The other 51 compounds were mainly drugs with medicinal uses (79%) as NSAIDS, diuretics, antibiotics, and others. The primary classification of products for establishing the predictive ability of assays was mainly based on in vivo and human reports, as summarized in the review of de Àvila et al., 2023 [48].

The main objective of the photoassays proposed was to identify photoallergens. In this sense, ten articles have previously classified the chemicals and compounds used to validate their potential predictive capacity into photoirritants, photoallergens (or photosensitizers) and non-phototoxic agents, and in three of them, the non-phototoxic agents were identified as irritants or allergens. In two cases, the authors only refer to phototoxicity because they used the information obtained by the NRU 3T3 PT that only allows classification among phototoxic and non-phototoxic agents [44] or because the phototoxic chemicals used are considered both phototoxic and photoallergenic [42]. Finally, Stieffel and Schwack [36] did not provide any classification at all.

BNZ, CPZ, and KET were used in thirteen studies and thus are referred to the most often. However, the authors classified BNZ and KET as only photoallergens in eight studies, while Nguyen [47] considered both phototoxic and photoallergic agents and finally, Nishida et al. [46] indicated that KET was both a photoirritant and a photoallergen (Table 4). Similarly, 6-MC, BIT, Enoxacin, Fenticlor, Furosemide, Hidrochlortiazide, Hexachlorophene, PABA, Piroxicam, and Pyridoxine were classified as phototoxic and photoallergenic or only photoallergenic. Other discrepancies were detected for Acridine considered either as phototoxic or only photoallergen or even completely non-phototoxic; PPD was either classified as photoallergenic and allergenic, while 5-MOP and Musk ketone were identified as phototoxic and, surprisingly, as non-phototoxic. These disagreements make it difficult either to establish adequately novel assays and to further validate them.

4. Discussion

The primary objective of the cosmetic industry is to develop, produce, and market products that enhance or ameliorate the appearance of the human body. The cosmetic industry encompasses a wide range of products, including skincare, hair care, makeup, perfumes, and personal care items. However, beauty products are formulated not just for aesthetic purposes but also to optimize the natural functions of the skin as enhancing hydration [65]. On the other hand, ensuring the safety of cosmetics involves navigating numerous challenges, including the complexity of formulations, potential adverse reactions, and long-term health effects. The last regulation of EU on cosmetic products banning the traditional animal testing [24] along with the REACH regulation [25] and other regulatory norms has served as a catalyst for the emerge of innovative NAMs and underscores the

necessity for new integrated approaches in testing and assessment besides using more human-relevant data [27]. In this sense, the International Cooperation on Cosmetics Regulation (ICCR) has studied the fundamentals that should be observed to incorporate such NAMs into an integrated strategy for risk assessment of cosmetics ingredients [66] and the Scientific Committee of Consumer Safety (SCCS) has included the requirements for evaluation of cosmetic ingredients [63].

Among the potential adverse effects that cosmetic ingredients or products can cause, photoinduced toxicity is described for different ingredients [4,67]. As explained previously, phototoxic reactions are caused when a compound or substance triggers harmful effects when exposed to light. UVA radiation is primarily responsible for such reactions, but adverse responses to certain drugs have also been documented upon exposure to UVB, visible light, or a combination of various wavelengths [68,69]. Phototoxic reactions encompass photoirritation and photoallergy, which differ from each other by their inherent pathophysiological mechanisms. Photoallergic reactions involve an immune response triggered by exposure to sunlight, as described elsewhere (Figure 3), and are regarded as T cell-mediated delayed type hypersensitivity [5,9,13,70]. In contrast, photoirritation induces cellular cytotoxicity, is a more common affection that can happen after the first and single contact with the photosensitizer, and is dose dependent [7,9,71–73].

There is extensive literature that explores the chemical and biological basis of drug-induced photosensitivity [72,74–78] but not much in the case of cosmetics. In this context, a recent examination by Hofmann et al. [69] identified a total of 393 distinct drugs or drug compounds reported as photosensitizers. However, the level of evidence varies across cases and may not be sufficient in all instances.

Personal care and cosmetic products contain a variety of compounds, including fragrances, plant extracts, polymers, detergents, and emulsifiers that sometimes make it difficult to use NAMs for safety assessment [79]. It has been reported that exposure to some of the constituents in personal care and cosmetic products could result in skin sensitization [5,80,81], but also other immune responses such as photoallergy. This is the case for fragrance mix I, a well-known contact sensitizer that contains a mixture of eight individual fragrances (cinnamic alcohol, cinnamic aldehyde, hydroxycitronellal, amylcinnamaldehyde, geraniol, euginol, isoeuginol, and oakmosse absolute) that also can cause photoallergy, as reported in a photopatch study [82].

UV filters and fragrances are the cosmetic ingredients considered to be the main culprits of photoallergic reactions [83–88]. In the case of sunscreens, allergic and photoallergic contact dermatitis were first reported over thirty years ago [89], and these adverse reactions were mainly attributed to organic filters, with oxybenzone (benzophenone-3) being the most frequently reported contact and photocontact allergen [88]. It is reported that patients photosensitized to KET develop photoallergy to octocrylene, a NSAID that contains a benzophenone moiety in its chemical structure. Synthesis of octocrylene implies the use of benzophenone that can remain as a residue, explaining such cross-reactivity [90]. Additionally, photoallergy from fragrances is observed in patients with drug allergies, such as ketoprofen [91].

Other cosmetic components that frequently hold photoallergenic capacity are preservatives like methylisothiazolinone, as demonstrated through photopatch tests [92]. Certain derivatives of cinnamaldehyde, cinnamyl alcohol, dihydrocinnamyl alcohol, and cinnamic acid, commonly used in cosmetics, face restrictions due to their photoallergenic effects [93].

Based on all of these considerations, to assay the potential photoallergy induced by UV filters, as well as other ingredients such as fragrances, presents a challenge for innovation in discovering and synthesizing novel components without adverse effects, optimizing sun-protection effects [94].

Assaying photoallergic reactions in the case of cosmetics ingredients and formulations is thus paramount for comprehensive safety assessments, requiring methods that accurately replicate human responses without relying on animal testing. Despite the rapid progress in

the development of skincare products, there is a scarcity of suitable models for assessing the efficacy and safety of new active ingredients and formulations.

The studies collected here propose different assays based on the mechanism of photoallergy according to the strategy proposed by OECD for contact sensitization [49] and in keeping with the key events (KE) described by de Ávila et al. [48]. Moreover, the methods described in the articles try to adapt the validated assays for contact sensitization by including the UV irradiation, and some of them propose a sequential test strategy combining the novel adopted proposals with the first steps described for phototoxicity assessment, in particular UV-VIS spectral analysis and ROS assay [29,58].

Regarding the KE1 or molecular initiating event, seven studies have explored the capacity to form the complete hapten–protein complex formation. However, three of them were more mechanistic and focused on a specific class of compounds as UV filters [36], fenofibrates [40], or β-lactams [41] rather than in hazard identification. These studies can be useful for understanding certain aspects of photobinding steps that will help researchers to design future potential in silico models, but do not provide real progress in the field of photosafety assessment. The other four adapt ADRA [44,45] or DPRA [42,44,46] assays to identify contact sensitizers by coupling UVA lamps. The assays proved to be transferable among laboratories experienced in conducting high-performance liquid chromatography analysis [61]. Taking into consideration that among the four studies, almost 70 different chemicals besides allergen mixtures and herbicide formulations had been assessed, it has been proven that the volume of reaction [42], irradiation and time of incubation [44] can be lowered, and therefore these photoassays are promising tools for photosafety assessment.

In the case of KE2, two endpoints were selected as a strategy to identify photoallergens. The first one is based on the production of IL-18 using a keratinocyte cell line [33,35] or reconstructed human epidermis (RhE) [47]. It is well known that the development of allergic contact dermatitis (ACD) relies significantly on keratinocytes produced by the secretion of different cytokines as IL-18, which is necessary for the maturation and migration of dendritic cells activated by hapten, from the skin to the draining lymph nodules where they will present the antigens to naïve T cells [95]. Moreover, this interleukin has shown its utility to identify allergens even with weak capacity using cell lines [96], evaluating hair-coloring products [97] and could be a useful tool for classifying photoallergens according to their potency, as described for allergens using RhE [98]. The second one is an adaptation of Keratinosens™ [99] that also explores the use of the AREc32 cell line, but the number of chemicals assessed is still very low and the accuracy needs to be improved.

The third key event in the process of photoallergy implies phenotypic and functional changes in dendritic cells (DCs) as well as the induction of cytokines to facilitate the antigen-presenting capacity of DCs. Related to ACD, changes in the expression of surface molecules CD54 and CD86 were described in OECD 442E [100] to identify allergens in addition to the changes in IL-8 expression. Thus, Martínez et al. [34] demonstrated that using IL-8 to discriminate photoirritants from photoallergens could be an interesting tool for hazard identification. However, more studies need to be performed regarding the applicability domain. In the case of the other two proposals, one centered in changes in cell-surface thiols [37] and the other in intracellular ROS production [43], only the photo-SH/NH_2 presented encouraging outcomes with an acceptable predictivity to identify photosensitizers but a less satisfactory performance regarding its ability to discriminate among photoirritants and photoallergens. More recently, Forreryd et al. [101] and Lindberg et al. [102] presented adaptations of the GARD™skin [100] using the SenzaCell cell line by analyzing changes in the genomic signature after exposure to chemicals. However, further research is still ongoing to improve their use for evaluating fragrances in the cosmetics industry.

Important aspects for the validation of any novel assay are the prediction model, as well as which chemicals and compounds with well-known toxicity patterns are used as positive controls. In this sense, some chemicals present different classifications among the studies, although most of them are recognized as phototoxic.

Moreover, to establish a reliable predictive capacity, the previous classification must be reliable and based on human data. Epidemiological studies indicate the difficulties of evaluating the prevalence of photoallergy, attributing such difficulty to different patch test protocols, among factors. However, it was reported that between 18 and 37% of the patients with a positive reaction were diagnosed with photoallergy [82,103,104], indicating that photoallergic reactions seem more frequent than previously believed. Moreover, it has been found that a narrow relationship exists among chronic actinic dermatitis (CAD), allergic contact dermatitis, and photoallergic contact dermatitis, although it is not clear whether CAD predisposes people to developing skin allergy or photoallergy or vice versa [7,82]. Similarly, an exacerbation of ACD manifestations with light has been described in a low percentage of patients [92,104]. In a recent case report, photoaggravation of ACD has been attributed to the UVB absorber isoamyl p-methoxycinnamate [105], which previous reports had identified as a photoallergic agent [106].

Identifying photoallergy presents challenges due to complex clinical presentations, broad differentials, and a scarcity of specialists conducting photo-testing [103]. Therefore, standardizing the panel agents as well as the patch-test procedure is crucial not only to facilitate comparisons among studies, but also to swiftly incorporate new agents when necessary [107,108]. Moreover, is important that physicians can recognize and manage drug-induced photosensitivity early by reviewing constantly renewed information [109].

Cosmetic ingredients in Europe are subject to two different regulations: the cosmetic regulation, which bans in vivo testing [24], and the REACH regulation [25], which allows animal testing for chemical safety assessment. However, there are still cosmetic ingredients registered under the REACH regulation that involve animal testing [110]. This situation requires more investment to accelerate the improvement of existing photoassays and the development of new NAMs. In this sense, the recent progress in microfluid technology as well as bioprinting offers us the opportunity to improve and refine all the photoassays described here. Examples of such improvements might include constructing three-dimensional skin models that include immunocompetent cells and melanocytes, or utilizing skin-on-a-chip platforms that mimic tissue microenvironments [111]. For these reasons, it is imperative to coordinate and collaborate among the industry, regulatory bodies, research institutions, academia, dermatologists, and administrative entities.

5. Conclusions

To address the complexities of photoallergic reactions and the challenges in discriminating them from phototoxic reactions, it is crucial to adopt a comprehensive approach to testing strategies. In silico, in chemico, and in vitro tests, including reconstructed human epidermis models, present promising alternatives. By integrating these methods into a sequential battery of tests, a thorough evaluation of cosmetic safety can be achieved, ensuring that products meet stringent standards without compromising ethical principles.

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Anti-Aging Properties of *Cannabis sativa* Leaf Extract against UVA Irradiation

Kunlathida Luangpraditkun [1], Preeyanuch Pimjuk [2], Preeyawass Phimnuan [3], Wisanee Wisanwattana [2], Chothip Wisespongpand [4], Neti Waranuch [2] and Jarupa Viyoch [3,*]

[1] Research Unit of Pharmaceutical Innovations of Natural Products (PhInNat), Burapha University, Chonburi 20131, Thailand; kunlathida.lu@go.buu.ac.th
[2] Cosmetics and Natural Products Research Center, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000, Thailand; preeyanuchp@nu.ac.th (P.P.); wisanee.wi@nu.ac.th (W.W.); netiw@nu.ac.th (N.W.)
[3] Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences and Center of Excellence for Innovation in Chemistry, Naresuan University, Phitsanulok 65000, Thailand; p.preeyawass@gmail.com
[4] Institute for Small and Medium Enterprise Development (ISMED), Pathum Thani 12120, Thailand; bpd.project030@gmail.com
* Correspondence: jarupav@nu.ac.th; Tel.: +66-55-96-18-75

Abstract: Hemp extract has garnered interest as a potential cosmeceutical agent with multifunctional activities, particularly in protecting against UV-induced skin cell aberrations and restoring aged skin cells. The ethanolic extract of *Cannabis sativa* leaves was prepared into an aqueous solution (CLES) to investigate its anti-photoaging ability. HPLC analysis revealed that the CLES contained 1.64 ± 0.01% *w*/*w* of cannabidiol and 0.11% *w*/*w* of Δ^9-tetrahydrocannabinol. Additionally, the total phenolic content was found to be 4.08 ± 0.30 mg gallic acid equivalent per g of solution using the Folin–Ciocalteu method. The CLES exhibited potent scavenging activity using a DPPH assay, with an EC_{50} value of 277.9 ± 2.41 µg/mL, comparable to L-ascorbic acid, with 2.19 ± 0.28 µg/mL. The anti-photoaging potential of the CLES was evaluated using UVA-irradiated and *in vitro*-aged fibroblasts as a model. Pre-treatment with 20 µg/mL CLES for 24 h significantly alleviated the reduction in type I procollagen and suppressed the overproduction of MMP-1 and IL-6 induced by UVA. Moreover, the percentage of senescence-associated β-galactosidase-expressing cells decreased significantly to 11.9 ± 0.5% in the aged cells treated with CLES compared with untreated cells (18.8 ± 3.8%). These results strongly indicate the cosmeceutical potential of the CLES as an effective active agent for the anti-photoaging prevention and/or treatment.

Keywords: *Cannabis sativa*; hemp leaves extract; UVA-irradiated fibroblasts; *in vitro*-aged fibroblasts; anti-photoaging

1. Introduction

The skin is an organ responsible for covering and protecting our body's internal environment from external factors, including UV light, heat, and pollution. It plays a crucial role in responding to external exposures, aiming to prevent further damage to itself. For instance, after a short duration of UV exposure, the skin may exhibit redness or erythema accompanied by a burning sensation as a warning sign of inflammation. The process of UV-induced inflammation is initiated by the presence of free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as oxidized molecules generated when the skin is exposed to UV rays. This oxidative stress triggers the activation of a transcriptional factor called NF-κB in skin fibroblasts and keratinocytes. As a result, several inflammatory cytokines, such as TNF-α, IL-1, and IL-6, are upregulated [1–3]. Ultraviolet (UV) exposure, especially UVA, generates free radicals that can penetrate the dermal layer of the skin. In response, skin fibroblasts are activated through the AP-1 and

MAPK signaling pathways, leading to a complex cascade of events affecting collagen metabolism. UV-induced signaling pathways influence the regulation of type I procollagen and collagenase (matrix metalloproteinase-1, MMP-1) release [4,5], with a particular focus on the role of autocrine cytokines such as IL-6 in mediating collagenase expression [6]. UV exposure induces ROS/RNS–cytokine–collagenase loop activation, leading to the upregulation of NF-κB/MAPK/AP-1 pathways and subsequent accumulation of aging or senescent cells in the skin. Senescence is characterized by reduced cell growth and increased SA-β-gal activity. However, promising research has shown that the aging phenotype of fibroblasts can be partially reversed by treating cells with plant extracts or extracellular matrices derived from young fibroblasts [7,8].

In general, endogenous antioxidants play a crucial role in neutralizing free radicals generated from exposure to UV radiation [9,10]. However, chronic UV exposure can disrupt the delicate balance between oxidation and antioxidation, leading to the activation of signaling pathways responsible for skin aging via ROS/RNS activity [11–13]. Therefore, targeted skin treatments utilizing antioxidant compounds capable of suppressing ROS/RNS–cytokine–collagenase activation hold great promise in reinforcing the skin's resistance against UV damage, thereby minimizing the accumulation of aged skin cells [14–16]. Furthermore, the potential of these antioxidant substances to reverse the senescent/aged skin cells to a more youthful state presents an exciting opportunity for preventing photoaged skin.

Recently, there has been growing interest in natural extracts with multifunctional activities, particularly in their ability to protect against UV-induced skin cell aberrations and restore aged skin cells, making them promising candidates for anti-photoaging treatments. Notably, there have been reports highlighting the cosmeceutical potential of products based on *Cannabis sativa* or hemp [17,18]. The dermopharmacological activities attributed to hemp, including antioxidation, anti-inflammation, and anti-wrinkle effects, have been closely linked to the presence of cannabinoids and phenolic compositions in hemp extract. In recent years, considerable research has been conducted on the biological activities of ethanolic extract [19] derived from hemp leaves. These activities include antioxidation, pro-MMP-2 inhibition, melanogenesis, 5α-reductase inhibition, anti-bacterial activity, and anti-proliferation. However, to date, there is no existing literature on the preventive effects of *C. sativa* leaves extract on UVA-induced aberrations in human skin fibroblasts. This study aims to investigate the potential of *C. sativa* leaf extract in preventing and/or treating photoaging by assessing its impact on type I procollagen, MMP-1, and IL-6 levels in UV-irradiated fibroblasts compared to non-pretreated UV-irradiated fibroblasts. Additionally, the restoration ability of *C. sativa* leaf extract in *in vitro*-aged fibroblasts is evaluated by observing the expression of SA-β-gal activity.

2. Materials and Methods

2.1. Preparation of the Extract Solution of Cannabis sativa Leaves (CLES)

The leaves of *C. sativa* (RPF3 variety) were collected from Wiang Pa Pao District, Chiang Rai Province, Thailand. The fresh leaves were thoroughly cleaned and dried at a temperature of 55–60 °C using a hot air oven. A reflux process using ethanol as the solvent was employed. The ratio of dried leaves to ethanol was maintained at 1:15 by weight at 80 °C for 2 cycles to obtain the ethanolic extract. The ethanolic extract was filtered to remove any residual particulates, and the ethanol was then evaporated using a vacuum evaporator. The evaporated paste was subsequently redissolved in the vehicle composed of a mixture of propylene glycol (food-grade, Dongying Hi-tech Spring Chemical Industry Co., Ltd., Dongying, China) and polysorbate 20 (cosmetic-grade, Croda Singapore Pte Ltd., Seraya Avenue, Singapore) in a ratio of 1:1 to obtain the *C. sativa* leaf extract solution (CLES). The obtained solution was stored in sealed packaging at 4 °C (light protection) for further investigation.

2.2. Determination of Cannabidiol (CBD) and Tetrahydrocannabinol (THC) Contents in the CLES

High-performance liquid chromatography (HPLC) was employed, with certain modifications from a previous study [20], to accurately determine the CBD and THC contents. The HPLC system was composed of a photodiode array detector (G7115A 1260 DAD WR detector, Agilent Technologies, Santa Clara, CA, USA) and a quaternary pump (G711A 1260 Quat pump VL, Agilent Technologies, Santa Clara, CA, USA). The standard delta-9-tetrahydrocannabinol (Δ^9-THC) and delta-8-tetrahydrocannabinol (Δ^8-THC) were ordered from Lipomed, Arlesheim, Switzerland and Cayman Chemical, Ann Arbor, Michigan, USA, respectively. The standard CBD was ordered from THC Pharm GmbH, Frankfurt, Germany. For the chromatographic separation, a Phenomenex C18 core–shell (150 mm × 4.6 mm, 2.6 µm) with a column guard (C18, 4.6 × 10 mm, 5.0 µm) was employed as the stationary phase. The mobile phase comprised a mixture of acetonitrile (HPLC grade, RCI Labscan, Bangkok, Thailand) and 20 mM ammonium formate (Analytical grade, Sigma Aldrich, Steinheim, Germany) (pH 3.60) in a ratio of 75: 25% *v*/*v*, respectively. The column oven temperature was carefully maintained at 40 °C throughout the analysis. The mobile phase was delivered at a flow rate of 0.8 mL/min to achieve optimal chromatographic conditions. The detection of analytes was performed at a wavelength of 220 nm. The quantification of CBD and THC was achieved by creating standard curves for CBD, Δ^9-THC, and Δ^8-THC. The study was performed in triplicate.

2.3. Determination of Total Phenolic Content (TPC) of the CLES

The TPC in the CLES was determined by using the Folin–Ciocalteu method [21]. Briefly, 20 µL of CLES was combined with 100 µL of Folin–Ciocalteu reagent (diluted in water at a 1:10 *v*/*v*) and 80 µL of sodium carbonate solution (7% *w*/*v* Na_2CO_3). The mixtures were incubated at room temperature for 30 min. Subsequently, the sample's absorbance was measured at 765 nm using a microplate reader (SpectraMax M3 UV spectrophotometer, Molecular Devices, San Jose, CA, USA). For the preparation of standards, gallic acid solution (Sigma-Aldrich, Steinheim, Germany) with a concentration in the range of 0.016–0.5 mg/mL was used. The TPC was expressed in gallic acid equivalents (mg GAE per g of CLES). All measurements were performed in triplicate.

2.4. Determination of Antioxidant Activity of the CLES by Using DPPH Assay

The free radical scavenging activity of the CLES was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Steinheim, Germany) assay [22], and the results were compared with a positive control, L-ascorbic acid (Chem-Supply Pty Ltd., Gillman, Australia). To conduct the assay, 75 µL of the CLES diluted in methanol or L-ascorbic acid solution was mixed with 150 µL of 0.2 mM DPPH (Sigma-Aldrich, Steinheim, Germany) methanolic solution in a 96-well plate. Subsequently, the mixture was incubated at room temperature in the dark for 30 min. The remaining DPPH's absorbance was then measured at a wavelength of 517 nm using a microplate reader (BioStack Ready, BioTek instruments, Winooski, VT, USA). The radical scavenging activity was determined as the percentage of DPPH decoloration and calculated using the following equation:

$$\% \text{ Radical scavenging activity} = [1 - (A_S/A_B)] \times 100$$

where A_S is the absorbance of the DPPH solution without any sample and A_B is the absorbance of DPPH solution after being treated with the samples. EC_{50}, which denotes the concentration at which 50% of the desired effect is achieved, was ascertained through a log-probit analysis employing nine distinct final concentrations of the samples, with each concentration tested in triplicate.

2.5. Determination of Cytotoxicity of the CLES on Human Skin Fibroblasts

Normal human dermal fibroblasts (NHDFs) were purchased from Promocell (Lot no. C-12302, Eppelheim, Germany). To increase the number of NHDFs, the cells were

cultured in DMEM-high glucose (Gibco™, New York, NY, USA) containing 10% fetal bovine serum (FBS, Gibco), 1% of a stock penicillin/streptomycin solution (Gibco), and 1 mg/mL amphotericin B (Gibco) at 37 °C with a humid atmosphere containing 5% CO_2. Subsequently, the cells (passage number, 5–8) were trypsinized and seeded in 96-well plates at a density of 1.0×10^4 cells/well, then cultured in DMEM with 10% FBS and antibiotics for 24 h. Following the removal of the old medium, the cells were washed with phosphate-buffered saline (PBS), pH 7.4, and cultured in serum-free medium with or without various concentrations of CLES (10, 20, 50, 100, 500, and 1000 μg/mL) for 24 h. Cell viability was assessed using an XTT assay, and a mixture of 50 μL of the serum free-medium and 50 μL of XTT solution was added for further incubation at 37 °C in 5% CO_2 for 4 h. The results were measured by absorbance at 490 nm with a microplate reader (BioStack Ready, BioTek instruments, Winooski, VT, USA) to quantify the viability of the cells [23]. The viability of the non-CLES-treated NHDFs was considered as 100% viability. All experiments were performed in triplicate.

2.6. Determination of Preventive Effect of the CLES on UVA-Induced NHDFs Alterations

2.6.1. UVA Irradiation and Cell Treatment

NHDFs (passage number, 5–8) were seeded into cell culture flasks (T25) at a density of 1×10^6 cells per flask. The cells were cultured in DMEM with 10% FBS and antibiotics at 37 °C, with a humid atmosphere containing 5% CO_2, for 24 h to allow for cell adherence and growth. The adhered cells were washed with PBS and then incubated with serum-free medium without or with 20 μg/mL CLES. Following 24 h of incubation, the tested cell samples were washed and covered with a thin layer of PBS. Uniform UVA irradiation was administered using a UV test chamber BS-04 (Opsytec Dr. Gröbel GmbH, Baden-Württemberg, Germany) with a wavelength of 352 nm. The UVA intensity at the level of the culture flasks was measured using a UVA meter (Solarmeter, Solartech, Glenside, PA, USA). The distance between the samples and the lamp was maintained at 25 cm. The UVA irradiation was applied at a dosage of 5 J/cm^2. After UVA irradiation, the samples were further incubated in the serum-free medium for 24 h. Subsequently, the cell-free media were then collected and stored at −80 °C for further studies. The control group consisted of the non-UVA-irradiated cells without CLES treatment. The study was performed in triplicate.

2.6.2. Determination of the Secreted Type I Pro-Collagen, Matrix Metalloproteinase-1 (MMP-1), and Interleukin-6 (IL-6) Levels

The commercial enzyme-linked immunosorbent assay (ELISA) kits (Human Pro-Collagen I alpha 1 ELISA Kit (ab229389, Abcam, Cambridge, UK)) and Human MMP-1 ELISA kit (ab215083, Abcam) were employed to quantify the concentration of pro-alpha1 chains of type I collagen and MMP-1 released by NHDFs into the culture medium. Furthermore, an ELISA kit (ab229434, Abcam) was employed to assess the concentration of interleukin-6 (IL-6) secreted into the culture medium by NHDFs.

2.7. Determination of Restoration of Aged NHDFs by the CLES

To investigate the senescence-associated β-galactosidase (SA-β-gal) activity of aged NHDFs (passage number 18–20) and NHDFs treated with CLES, a senescence detection kit (ab65351, Abcam, Cambridge, UK) was utilized. Briefly, the aged fibroblasts were seeded into a 96-well plate (1×10^4 cells/well) and grown in DMEM containing 10% FBS and antibiotics at 37 °C with a humid atmosphere containing 5% CO_2 for 24 h. The cultured cells were washed with PBS and cultured in the serum-free medium without or with 20 μg/mL CLES for 24 h. After the treatment, the tested samples were further incubated in the serum-free medium for 48 h. Following the incubation, the cells were washed with PBS and fixed with fixative solution for 15 min at room temperature, according to the manufacturer's instructions. After washing with 3X PBS, they were incubated with staining solution at 37 °C overnight. Subsequently, the stained samples were washed with

3X PBS, and the percentage of stained cells was observed under an inverted microscope. The senescent cells exhibited a characteristic blue color due to the enzymatic activity of SA-β-gal.

3. Results

3.1. The Appearance of the CLES and Its CBD, THC, and TPC

The ethanolic extract of *C. sativa* leaves exhibited the appearance of a green paste. For formulation, the extract was dissolved in a vehicle containing a mixture of propylene glycol and polysorbate 20 at a concentration of 19.5 g ethanolic extract per 100 g of the vehicle, resulting in a clear green solution. The HPLC assay was employed to determine the CBD and THC composition of the CLES. The analysis revealed that the CLES contained 1.64 ± 0.01% *w*/*w* of CBD and 0.11% *w*/*w* of Δ^9-THC. Notably, the content of Δ^8-THC was found to be below the limit of detection (LOD), which was 0.25 µg/mL, indicating its presence in negligible amounts. The chromatograms of the CBD and THC in the standard solution and the sample are presented in Figure 1A–C. Moreover, the TPC in the CLES was quantified using the Folin–Ciocalteu method. The results indicated a concentration of 4.08 ± 0.30 mg GAE per g of the CLES, signifying the presence of phenolic compounds in the extract.

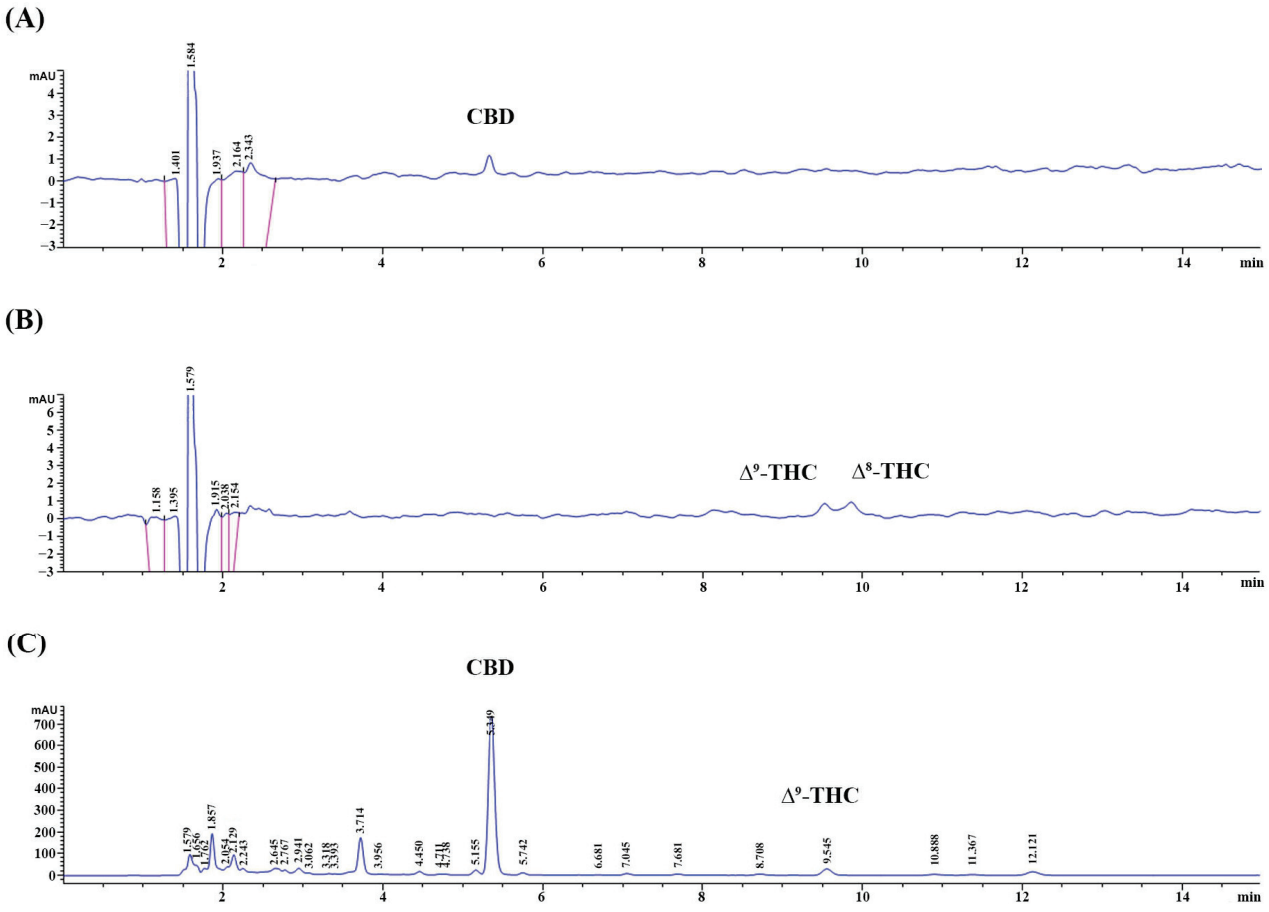


Figure 1. HPLC chromatograms of CBD (**A**) and THC (**B**) standard at a concentration of 0.25 µg/mL (limit of detection, LOD) and those of CBD and THC in the CLES at a concentration of 10 mg/mL (**C**).

3.2. Free Radical Scavenging Activity of the CLES

The DPPH assay was employed to assess the scavenging activity of the CLES and L -ascorbic acid (positive control) in a dose−dependent manner. The EC_{50} values were determined as 277.9 ± 2.41 µg/mL for the CLES and 2.19 ± 0.28 µg/mL for L-ascorbic acid.

3.3. Effect of the CLES on Viability of NHDFs

The cytotoxicity of CLES on NHDFs in a range of concentrations (10–1000 μg/mL) demonstrated that the viability of the cells, which was lower than 50%, was seen in the NHDFs treated with the CLES at 50 μg/mL or higher concentrations (Figure 2). However, no cytotoxicity was observed at concentrations of 20 μg/mL or lower. Furthermore, NHDFs treated with these concentrations maintained a characteristic spindle-shaped morphology typical of fibroblasts. Based on these findings, the CLES at the concentration of 20 μg/mL was selected for further studies.

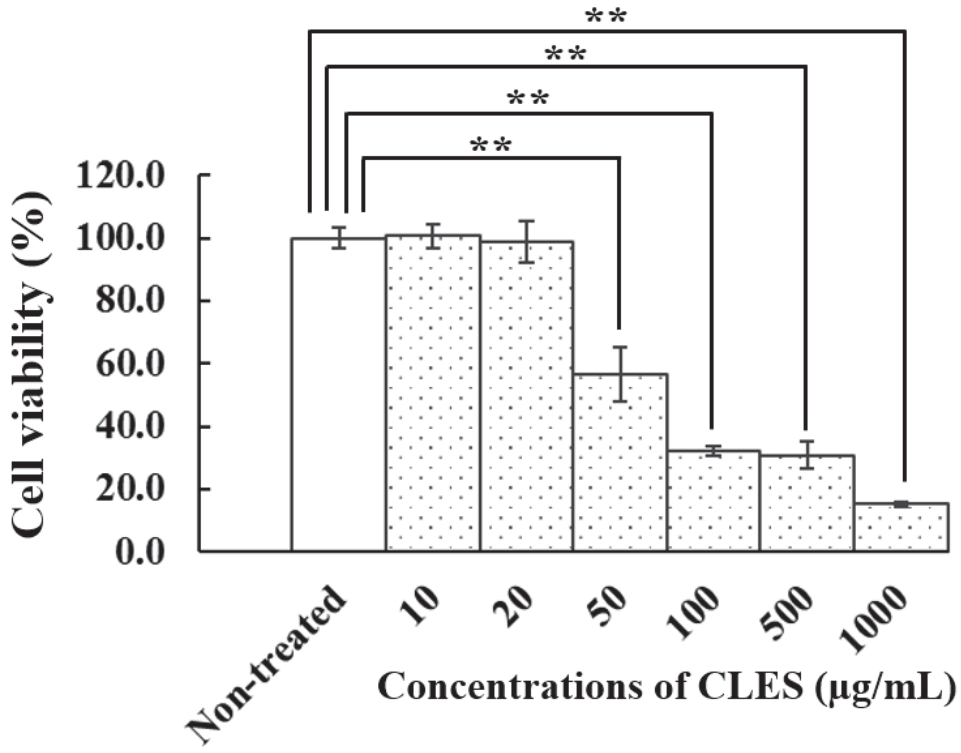


Figure 2. Effect of the CLES on viability of NHDFs. Cells were treated with the CLES at concentrations in the range of 10 to 1000 μg/mL for 24 h. Results are expressed as percentages of cell viability as compared to non-treated cells, for which the OD was adjusted to 100%. Each bar represents mean ± S.D. of triplicate study. ** $p < 0.01$, when compared between two groups using unpaired Student's *t*-test.

3.4. Preventive Effect of the CLES on UVA-Induced Alterations of NHDFs

Following UVA exposure, a marked reduction in type I procollagen ($p < 0.01$) (Figure 3A) and increased MMP-1 levels ($p < 0.01$) (Figure 3B) were observed in the NHDFs' media. However, pre-treatment with 20 μg/mL CLES for 24 h significantly prevented ($p < 0.05$) the reduction in type I procollagen and suppressed the overproduction of MMP-1 ($p < 0.01$). Notably, the levels of type I procollagen and MMP-1 in the pre-treated group did not reach those of the control group (non-UVA-irradiated cells without CLES treatment). Furthermore, UVA-irradiated NHDFs exhibited a marked increase in IL-6 secretion ($p < 0.01$) (Figure 4) compared with the control group. Nevertheless, pre-treatment with 20 μg/mL CLES significantly attenuated IL-6 overproduction ($p < 0.05$).

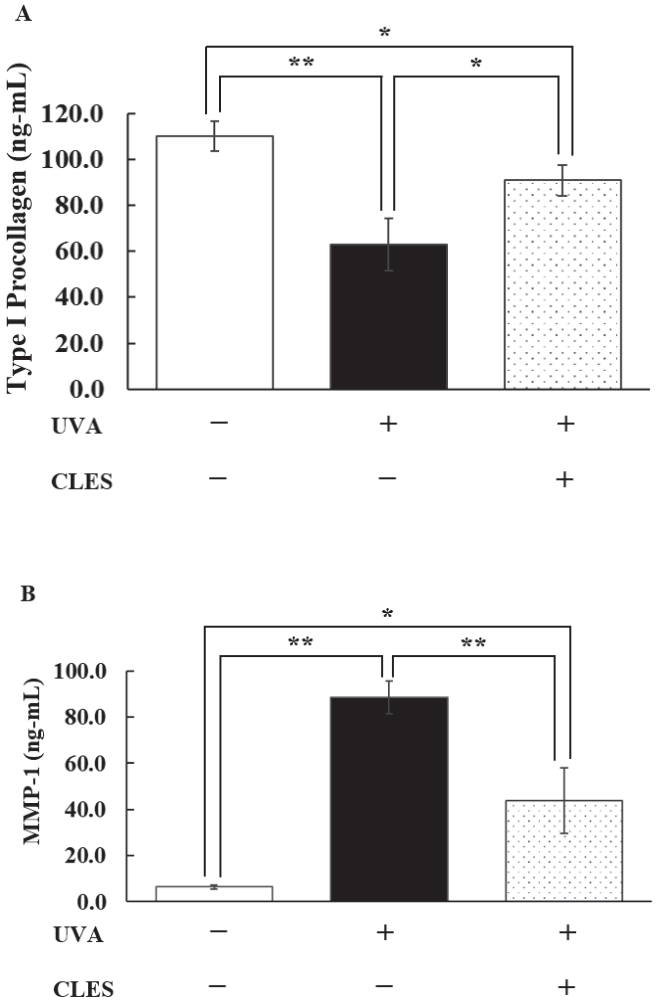


Figure 3. Effects of the CLES (20 μg/mL) on type I procollagen (**A**) and MMP-1 (**B**) secretions from UVA-irradiated NHDFs. Each bar represents mean ± S.D. of triplicate study. * $p < 0.05$ and ** $p < 0.01$ when compared between two groups (Student's *t*-test).

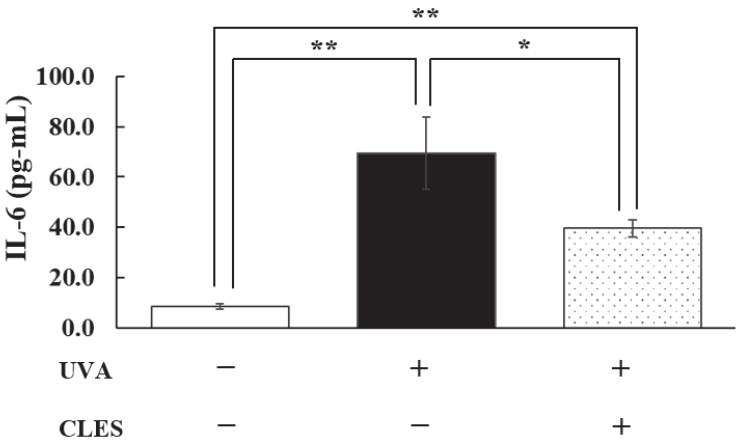


Figure 4. Effects of the CLES (20 μg/mL) on IL-6 secretion from UVA-irradiated NHDFs. Each bar represents mean ± S.D. of triplicate study. * $p < 0.05$ and ** $p < 0.01$ when compared between two groups (Student's *t*-test).

3.5. Restoration of Aged NHDFs by the CLES

Figure 5A illustrates the presence of SA-β-gal activity in aged NHDFS (passage number 18–20), with 18.8 ± 3.8% of the total number of cells showing positive staining. Remarkably, Figure 5B demonstrates a significant reduction ($p < 0.05$) in SA-β-gal-expressing cells (11.9 ± 0.5%) with CLES treatment after 24 h compared with untreated aged cells.

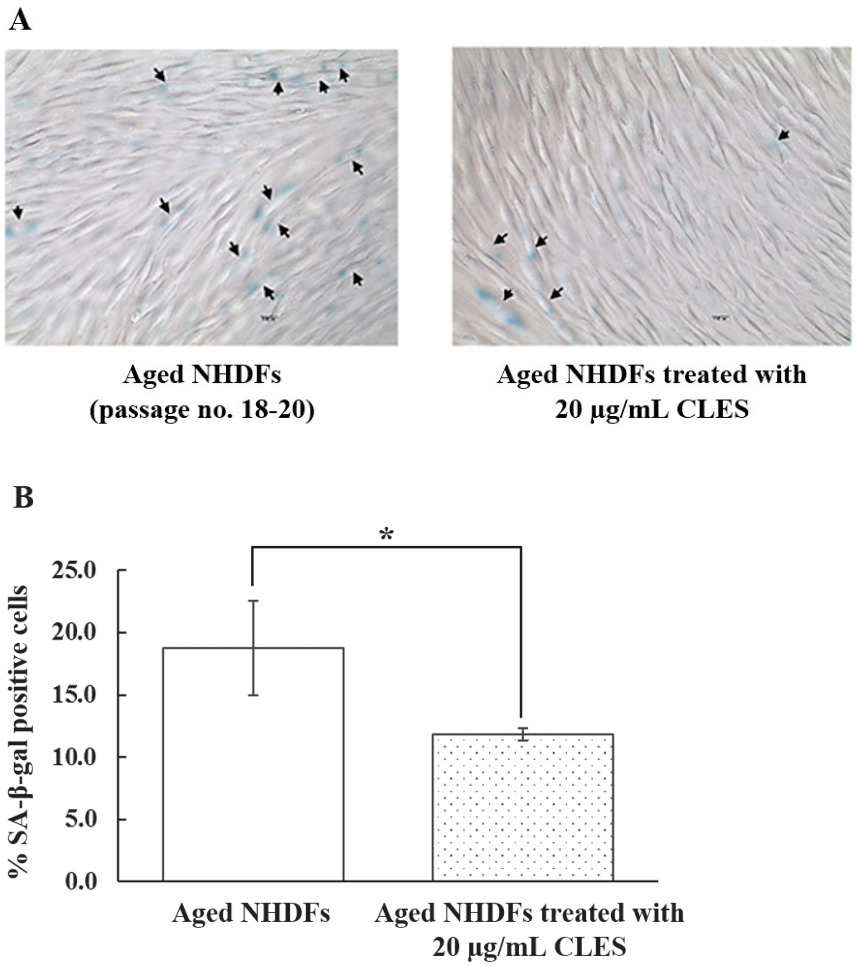


Figure 5. The presence of senescence-associated beta-galactosidase (SA-β-gal) (**A**) and percentage of SA-β-gal-expressing cells in the aged NHDFs and those treated with 20 µg/mL CLES (**B**) is percentage of SA-β-gal- expressing cells in the aged NHDFs and those treated with 20 µg/mL CLES. Each bar represents mean ± S.D. of triplicate study. * $p < 0.05$ when compared between two groups (Student's *t*-test). The arrows pointed out the SA-β-gal-expressing cells.

4. Discussion

Tetrahydrocannabinol (THC) and cannabidiol (CBD) are two prominent cannabinoids found in hemp [24–26]. These compounds exhibit a range of pharmacological activities, including pain relief and anti-inflammation, but they may also lead to undesirable effects, such as anxiety and immunosuppression [27]. In hemp, the THC content is typically low, usually below 0.2% dry weight, directing the focus of dermopharmaceutical research towards CBD and other secondary metabolites, such as phenolic compounds. The therapeutic potential of hemp-based dermopharmaceuticals primarily lies in CBD and its interaction with the endocannabinoid system, which plays a vital role in skin homeostasis and health. However, the penetration of CBD into the skin without the use of enhancers is challenging due to its lipophilic nature [28]. Consequently, researchers have turned to phenolic compounds as potential skin penetration enhancers and antioxidants, which have garnered significant attention in the cosmeceutical market, particularly in products for photoaging

prevention and treatment. Among the phenolic compounds found in hemp leaves, particularly in CBD-dominant strains, are flavonoids such as orientin, vitexin, and isovitexin [29]. These flavonoids have demonstrated promising properties as antioxidants and have shown potential in enhancing skin permeation, allowing for improved dermal delivery of CBD and other beneficial compounds [30,31].

This study investigated the biological activity and composition of the ethanolic extract of hemp leaves prepared in an aqueous solution known as CLES. The aqueous form of the extract holds potential in the cosmetic market for its ease of homogenous blending into conventional cosmetic formulations, including emulsions, solutions, and gels [17,31,32]. Additionally, the solubilized form of the extract is expected to enhance the skin penetration of its active compound(s). However, the composition and biological activity of the extract solution must be investigated to ensure its beneficial effects. The composition of CLES was found to contain a small amount of CBD and THC, while its TPC was determined to be 4.08 ± 0.30 mg GAE per g of CLES. The obtained contents of CBD and THC corresponded with another study [33] indicating a quite low content of THC and CBD in hemp leaves. For the content of phenols found in the CLES, this content was significant enough to exhibit *in vitro* antioxidant activity and other biological activities linked to photoaging prevention. The antioxidant activity of CLES was evaluated through DPPH assay, indicating that CLES exhibited the EC_{50} value with lower antioxidant activity (277.9 ± 2.41 μg/mL) than L-ascorbic acid (2.19 ± 0.28 μg/mL). It is well-established that L-ascorbic acid possesses remarkable antioxidant activity, which is primarily attributed to its rapid reaction with DPPH radicals. Conversely, most phenolic compounds exhibit intermediate or slow interactions with DPPH radicals. In the case of the compound CLES, its lower antioxidant activity can be attributed, at least in part, to its comparatively slow interaction with DPPH radicals [34].

For the evaluation of the biological activities of the CLES, its preventive effects on UVA-induced alterations in NHDFs were observed in cells pretreated with a concentration of 20 μg/mL CLES. UVA radiation can penetrate the upper dermal layers and cause damage to crucial extracellular matrix (ECM) components, including collagen, elastin, and proteoglycans [35–38]. Consequently, UVA is considered a significant contributor to photoaging. Upon exposure to UVA, the skin rapidly generates free radicals, such as ROS and RNS, which directly interact with dermal components. Additionally, these free radicals can modulate fibroblast functions by activating several signaling pathways, notably NF-κB, MAPK, and AP-1. As a result, the expression of cytokines and matrix metalloproteinases (MMPs) increases, while the expression of type I procollagen by fibroblasts decreases [39].

In our current investigation, we observed significant changes in the expression of type I procollagen and MMP-1 in UVA-irradiated NHDFs compared with non-irradiated cells. Specifically, we noticed a considerable decrease in type I procollagen and an increase in MMP-1 expression ($p < 0.01$) in the UVA-irradiated cells. These findings align with our previous study [20], providing further support to our research. Notably, when UVA-irradiated cells were pre-treated with 20 μg/mL CLES, we observed a slight decrease in type I procollagen levels compared to non-pretreated UVA-irradiated cells. The levels of type I procollagen in the pretreated UVA-irradiated cells were significantly higher than in the non-pretreated UVA-irradiated cells ($p < 0.05$). However, it is essential to highlight that the procollagen level in the pretreated UVA-irradiated cells did not reach the same level as that of non UVA-irradiated cells. Focusing on the MMP-1 and IL-6 levels, pretreatment with 20 μg/mL CLES could significantly suppress the elevated levels of MMP-1 ($p < 0.01$) and IL-6 ($p < 0.05$) in UVA-exposed cells. Given the pivotal role of MMP-1 in collagen degradation within the dermis, this intervention may hold promise for preventing skin aging and related disorders. Additionally, previous research has implicated the UVA-activated NF-κB pathway [40] in upregulating the release of IL-6 from fibroblasts [41]. This autocrine phenomenon is linked to inflammation and an upregulation of MMP-1 expression and activation, ultimately leading to the formation of wrinkles and manifestations of photoaging. The preventive effects of CLES on UVA-activated photoaging

pathways may be attributed to its potent antioxidant activity. By efficiently scavenging free radicals, the extract minimizes the activation of downstream signaling pathways, including MAPK/AP-1/NF-κB/IL-6-mediated inflammation-induced aging. Additionally, the presence of phenolic compounds in the extract might activate the nuclear factor erythroid-2-related factor 2 (NRF2) signaling pathway, thus promoting the production of endogenous antioxidants and enhancing the processes that counteract aging [41,42].

Aging is a complex biological process characterized by the dysregulation of various cellular functions. In aged fibroblasts, notable changes in gene expression patterns have been observed, including significant upregulation of MMP-1, inflammatory cytokines, and biomarkers associated with senescent phenotypes. Additionally, the presence of SA-β-gal activity is indicative of cellular aging. Interestingly, recent reports suggest the potential for restoring senescent or aged human diploid fibroblasts to a more youthful state through interaction with a young ECM [8]. Furthermore, the study observed the reversal of several senescence phenotypes, including the presence of SA-β-gal activity. These restorative effects were attributed to specific proteins, such as Ku and SIRT1, which play crucial roles in maintaining telomere structure. In the present study, it was demonstrated that CLES has the potential to partially reverse the expression of SA-β-gal activity in *in vitro*-aged fibroblasts. This beneficial effect of CLES may be linked to its ability to induce the expression and secretion of type I procollagen by fibroblasts. The interaction between the cells and the newly secreted ECM likely contributes to the reversal of the senescent/aged state. However, further investigations are warranted to elucidate the precise mechanisms behind the senescent reversibility of CLES. A deeper understanding of these mechanisms could have significant implications for the prevention and treatment of photoaging.

5. Conclusions

The extract derived from *C. sativa* leaves exhibits promising potential in preventing and/or treating photoaging. Its effects include the suppression of overproduction of MMP-1 and IL-6, while also mitigating the reduction in type I procollagen in UVA-irradiated fibroblasts. Additionally, CLES demonstrates the ability to partially reverse senescent fibroblasts by reducing the expression of SA-β-gal activity in *in vitro*-aged fibroblasts.

Author Contributions: K.L. performed the experiment using a cell-based assay and analyzed and interpreted the data. P.P. (Preeyanuch Pimjuk) performed the experiment using an *in vitro* assay and analyzed and interpreted the data. P.P. (Preeyawass Phimnuan) generated and revised the manuscript. W.W. performed the HPLC experiment and analyzed and interpreted the data. C.W. prepared the extract. N.W. and J.V. esigned the experiments; contributed reagents, materials, analysis tools, and data; and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The raw/processed data required to reproduce these findings cannot be shared at this time as the data also form part of an ongoing study.

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Conflicts of Interest: All authors have no conflicts of interest, and they agree to submit the manuscript to "cosmetics" as an original research article. This manuscript has not been published elsewhere and is not currently under consideration by another journal.

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Olive Leaves and Citrus Peels: From Waste to Potential Resource for Cosmetic Products

Nicola d'Avanzo [1,2,†], Antonia Mancuso [1,2,†], Rosario Mare [3], Antonio Silletta [1], Samantha Maurotti [1], Ortensia Ilaria Parisi [4,5], Maria Chiara Cristiano [3,*] and Donatella Paolino [1,2,*]

[1] Department of Experimental and Clinical Medicine, "Magna Graecia" University of Catanzaro, Campus Universitario "S. Venuta"—Building of BioSciences, Viale S. Venuta, 88100 Catanzaro, Italy; nicola.davanzo@unicz.it (N.d.); antonia.mancuso@unicz.it (A.M.); antonio.silletta@unicz.it (A.S.); smaurotti@unicz.it (S.M.)

[2] Research Center "ProHealth Translational Hub", Department of Experimental and Clinical Medicine, "Magna Graecia" University of Catanzaro, Campus Universitario "S. Venuta"—Building of BioSciences, Viale S. Venuta, 88100 Catanzaro, Italy

[3] Department of Medical and Surgical Sciences, University "Magna Græcia" of Catanzaro, Campus Universitario "S. Venuta"—Building of BioSciences, Viale S. Venuta, 88100 Catanzaro, Italy; mare@unicz.it

[4] Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, 87036 Rende, CS, Italy; ortensiailaria.parisi@unical.it

[5] Macrofarm s.r.l., Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, 87036 Rende, CS, Italy

* Correspondence: mchiara.cristiano@unicz.it (M.C.C.); paolino@unicz.it (D.P.)

† These two authors equally contributed, and an alphabetic order is followed.

Abstract: Resource depletion and food waste accumulation represent a tremendous socio-economic and environmental problem. One promising strategy involves the use of byproducts derived from food waste as ingredients for cosmetic products. The aim of this work is to propose clementine peels and olive leaf extracts as value-added bioproducts for a cosmetic cream. Extracts were obtained by super critical extraction showing an antioxidant activity of ca. 25%. No cytotoxic effects of the extracts were recorded on keratinocyte cells up to a concentration of 4% v/v ratio within 24 h. The incorporation of clementine peels and olive leaf extracts into creams did not compromise their stability, as demonstrated by Turbiscan analyses at room and extreme (40 °C) storage conditions. The safety profiles of the final cosmetic formulations were further in vivo demonstrated on human volunteers. We analyzed the trans-epidermal water loss and variation of the skin's erythematous index, which showed profiles that almost overlapped with the negative control. Moreover, rheological analysis of the resulting creams evidences their suitable spreadability with similar pseudoplastic profiles, although a slight reduction of viscosity was recorded by improving the extracts' concentrations. The proposed approach highlights the advantage of combining byproduct resources and supercritical fluid extraction to obtain a safe and eco-friendly face cream.

Keywords: waste production; agri-food production; clementine peels extract; olive leaves extract; emulsions

1. Introduction

In 2023, it was estimated that more than 58 million Kg of food waste was produced in the European Union, which corresponds to 131 Kg/inhabitant per year (Eurostat, 2023, https://food.ec.europa.eu/index_en, accessed on 14 February 2024), thus representing a critical socio-economic and environmental problem [1,2]. The development of new approaches to obtaining biomaterials from food waste is an urgent priority, and several strategies have been put in place to develop a circular economy able to reduce environmental degradation and resource depletion [3–5]. In this context, the use of byproduct-derived compounds for the cosmetic industry represents a promising alternative to conventional

approaches [6,7]. In line with this approach, this study evaluates the use of clementine peels and olive leaf extract (CPE and OLE, respectively) for cosmetic purposes, emphasizing their role as ingredients for the production of face cream.

Clementine peels and olive leaf extracts offer multiple beneficial features, thus making them suitable raw materials for use in the cosmetic industry [8–11]. The main bioactive compounds of these byproducts' extracts are polyphenols, which serve as forefront bioactive molecules for plants defending themselves against pathogens and stress conditions [12]. The inclusion of polyphenols derived from clementine peel and olive leaf extracts in cosmetic products offers several advantages for skincare. These compounds, renowned for their potent antioxidant properties, effectively neutralize free radicals that can cause premature aging and skin damage [13,14]. Additionally, their anti-inflammatory properties make them valuable compounds to treat irritated skin, thus reducing redness and promoting a more even complexion [15–18]. Polyphenols also exhibit remarkable skin-brightening effects, helping to reduce the appearance of dark spots and hyperpigmentation [19,20] and support collagen production, thus improving elasticity and contributing to a tighter and more youthful-looking skin texture [21]. With their multifaceted benefits, clementine peel and olive leaf extracts are suitable ingredients for cosmetic products, offering natural solutions to several skin concerns. Apart from their skin benefits, the inclusion of citrus peels and olive leaf extracts in cosmetic products also represents a valuable approach to obtain sustainable cosmetic formulations. In fact, the many potentials in polyphenols make these extracts proper candidates as natural components, thus reducing the side effects associated with synthetic ingredients [10,22,23].

The integration of clementine peels and olive leaf extracts aligns with the goals of sustainable cosmetic manufacturing, thus minimizing the environmental impact of this ever-growing industry. Moreover, their abundance as byproduct of the food industries highlights their potential contribution to the circular economy, creating value-added bioactive ingredients [24,25].

Another interesting, but still not much explored, re-use of waste concerns the water used in the process of obtaining fibers from citrus or other fruits, or from washing processes, or from other passages of the food production chain. This water waste contains many compounds potentially beneficial both from a technological and health-related point of view, as well as being able to be used as an added value for a cosmetic formulation, for example providing a light and pleasant fragrance similar to the fruit from which it is obtained [26].

Extraction procedures can be also optimized to maximize the resource's efficacy and minimize its environmental impact. In this attempt, supercritical fluid extraction processes represent a valid alternative to the conventional ones [27]. Supercritical carbon dioxide (CO_2) extraction is a highly efficient and environmentally friendly technique that uses CO_2 in its supercritical state [28]. One significant advantage of this technique is the ability to modify the nature and the percentage of the co-solvent, thus obtaining a purer and more concentrated target molecules extract, while leaving behind unwanted impurities [29]. Moreover, supercritical CO_2 extraction operates at lower temperatures compared to conventional methods, minimizing the risk of thermal degradation and preserving the integrity of sensitive bioactive compounds [27]. Additionally, CO_2 is non-toxic and readily available, making it a safer and more sustainable alternative to the chemical solvents commonly used in extraction processes. Overall, supercritical CO_2 extraction presents a superior approach to obtaining high-quality extracts from clementine peels and olive leaves to be used to develop products with enhanced efficacy and sustainability.

The main goal of this study is to elucidate the multifaceted benefits of incorporating clementine peels and olive leaf extracts into face cream, emphasizing their pivotal role in advancing the principles of a circulating economy in the cosmetic field. To further minimize the environmental impact of the realized product, a supercritical fluid extraction technique was used, thus reducing the environmental footprint, and preserving at the same time the integrity of thermo-sensitive compounds. For making the final cosmetic

product more appealing to a hypothetical consumer, the cream was naturally perfumed using bergamot water waste. The described advantages underscore the significance of combining byproducts as a resource and eco-friendly supercritical fluid extraction processes in cosmetic products development.

2. Materials and Methods

2.1. Materials

Glutamate Emulsifier Olivoil was kindly provided by Kalichem (Brescia, Italy). Sweet Almond Oil USP, Ceteareth 25, Xantan Gum was purchased by A.C.E.F. A supply of dl-α-Tocopheryl Acetate was obtained from Roche (Milan, Italy). Rutin, oleuropein, hesperidin, naringin, apigenin, sodium nitrite, aluminum chloride, sodium hydroxide, gallic acid (GA), L-ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) for chemical analyses, sodium dimethyl sulfoxide (DMSO), trypan Bleu dye solution (0.4% *v*/*v*), 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT), and glycerol were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Clementine peels taken from industrial processing waste were kindly offered by the Company Medi Mais Calabra of Corigliano-Rossano (Cosenza, Italy). Olive leaves from *Olea europea* and bergamot wastewater were provided by a local factory. Dulbecco's Modified Eagle Medium, fetal bovine serum, and penicillin (100 UI/mL)-streptomycin (100 µg/mL) solution (1% *v*/*v*) were purchased from S.I.A.L. Group (Rome, Italy). Trypsin/EDTA 1× was purchased from GIBCO (Invitrogen Corporation, Giuliano Milanese, Milan, Italy). NCTC244 cells were provided by "Istituto Zooprofilattico di Modena e Reggio Emilia" (Modena, Italy). All materials and solvents used for the study were of analytical grade and do not require further purification processes.

2.2. Extracts Preparation and Characterization

Before performing the extractive procedures, the olive leaves were individually removed from the wooden twigs, washed, dried, and ground with a mortar, while the clementine waste material was used as it was supplied.

The extracts were obtained using a supercritical CO_2 extractor (SFE Process—SFE 100 mL—Tomblaine, France) using 90 g of raw material for every extraction. The chiller was set at a temperature between 0 °C and 5 °C, while the extraction vial was set at 40 °C. In each extraction, 600–800 g of CO_2 was consumed, with the CO_2 pressure set at ~250 bar and ethanol used as the co-solvent with a flow rate of 20 mL/min for extracts collection. Each extractive procedure required approximately 20% *w*/*v* of ethanol compared to the carbon dioxide consumed. Subsequently, the volume of ethanol was reduced by a rotary evaporator (Rotavapor Buchi, Flawil, Switzerland) equipped with a vacuum pump, with the aim of increasing the concentration of bioactive molecules. The obtained samples were centrifuged (2000× *g*—10 min—4 °C), filtered through 0.22 µm cellulose filters, and stored in the freezer at −20 °C before all analyses.

All extracts were characterized in terms of total phenolic content (TPC), total flavonoids content (TFC), and antioxidant activity (DPPH assay) using a UV-Vis spectrophotometer (ThermoScientific, Rosano, Milan, Italy—Genesys® 150), while the analytical profile of the molecules contained in the extracts was obtained by high pressure liquid chromatography (HPLC), as better described below.

2.3. Total Phenolic Content

The polyphenolic profile of the extracts, including hydrolysable tannins, was obtained as previously described in the literature with slight modifications [30]. Briefly, 100 µL of extract was mixed with 500 µL of Folin-Ciocalteu's phenol reagent and 1 mL of sodium carbonate (5% *w*/*v*). The obtained mixtures were vortexed, incubated 25 min in the dark, and their absorbance (Abs) acquired at wavelength (λ) of 760 nm. Gallic acid was used as the reference molecule, with a concentration between 0.1 and 0.5 mg/mL. The total phenolic content of the extract was finally described as gallic acid equivalent (GAE mg/mL).

2.4. Total Flavonoid Content

The TFC in the extracts was quantified according to a colorimetric approach recently described [30]. Briefly, 1 mL of the sample, properly diluted, was incubated with 60 µL of sodium nitrite solution (5% *w*/*v*) and 120 µL aluminum chloride (10% *w*/*v*). After 5 min of incubation, 0.40 mL of sodium hydroxide (1 M) were added to the sample to neutralize its pH. The final volume of each sample was 2 mL. A suitable calibration curve was obtained using a mixture of flavonoids standard molecules in equal weight ratio.

2.5. Antioxidant Activity of the Extracts

The antioxidant activity of our extracts was estimated using a DPPH assay and described as the inhibition percentage of free radicals (I%) as commonly described [31]. DPPH solution and L-ascorbic acid solution (5 mg/mL) were used as the negative and positive controls, respectively. Methanol was used as a blank for the analyses. The percentage of radical scavenging activity was normalized as a function of the extracts' concentrations and finally calculated using the following equation:

$$\mathrm{I}(\%) = ((\mathrm{A0} - \mathrm{A1})/\mathrm{A0}) \times 100 \quad (1)$$

where A0 = Absorbance of negative control and A1 = Absorbance of extracts/standards.

2.6. HPLC Analyses

The HPLC apparatus consisted of a ThermoFisher Scientific Vanquish System Base equipped with a quaternary pump and a UV/VIS variable wavelength detector (ThermoFisher Scientific—Rosano, MI, Italy). The mixtures were separated by an Acclaim® 120 reverse phase C18 column (100 mm–4.6 mm–5 µm particle size).

The mobile phase consisted of different ratios of acetonitrile (ACN) and phosphoric acid (10 nM) or formic acid (0.1% *v*/*v*) aqueous solutions for flavonoids or oleuropein quantification, respectively. The column temperature was 25 °C and the absorbance acquired at wavelength 260–270 nm or 254–280 nm, respectively, for flavonoids or oleuropein detection. Chromatograms were compared with retention times of rutin, naringin, hesperidin, oleuropein, and apigenin standard solutions. Suitable calibration curves ($r^2 > 0.98$ in all cases) were used to identify the molecules contained in the samples. The extracts (20 µL) were injected into an HPLC apparatus. The total acquisition time never exceeded 30 min.

2.7. In Vitro Studies on NCTC2544 Cells

The cytotoxicity of clementine peels and olive leaf extract (CPE and OLE, respectively), was evaluated on human keratinocytes (NCTC2544), considered as a model of a cutaneous cell line. The results of cell viability were determined using the MTT assay [32]. Cells were seeded at a density of 7×10^3 cells/well in 96-well plates and incubated for 24 h. Subsequently, the cells were treated with 100 µL of fresh media with OLE or CPE added at different concentrations (0.5%, 1%, 2% *v*/*v*) or combined (1%, 2%, 4% *v*/*v*, final concentration). Ethanol was used as the control and tested at several concentrations equivalent to those used for extracts during the study (0.5–4% *v*/*v*). After treatment, 10 µL of the MTT solution (5 mg/mL dissolved in PBS solution) were added to each well, and the plates were incubated for 3 h. Ethanol-DMSO solution (50:50 *v*/*v*, 100 µL/well) was used to dissolve precipitated formazan salts using an orbital shaker (IKA® KS 130 Control, IKA® WERKE GMBH & Co., Staufen, Germany). Cell viability was quantified using a Varioskan™ LUX microplate reader (Thermo Fischer Scientific, Waltham, MA, USA), according to the following equation:

$$\text{Cell viability } (\%) = \frac{\text{Abs T}}{\text{Abs C}} \times 100 \quad (2)$$

where Abs T and Abs C represent the absorbance values of a treated and untreated cell, respectively.

2.8. Preparation of Proposed Face Creams

The compositions of empty and citrus and olive leaf extract-loaded face creams are reported in Table 1.

Table 1. Quali-quantitative composition of prepared formulations.

Formulation	Phase	INCI Name	Concentration % (*w*/*w*)
Empty	A	Prunus Amygdalus Dulcis (Sweet Almond) Oil	10.5
		Olivoil Glutamate Emulsifier G-PF	15
		Tocopheril Acetate	1
		Cetyl Alcohol	2
	B	Bergamot wastewater	67.5
		Xantan Gum	0.2
		Glycerin	3.6
	C	Imidazolidinyl Urea	0.2
2% CPE-OLE	A	Prunus Amygdalus Dulcis (Sweet Almond) Oil	9.5
		Olivoil Glutamate Emulsifier G-PF	15
		Tocopheril Acetate	1
		Cetyl Alcohol	2
	B	Bergamot wastewater	66.5
		Xantan Gum	0.2
		Glycerin	3.6
	C	Imidazolidinyl Urea	0.2
		Olive leaf extract	0.5
		Clementine peel extract	1.5
3% CPE-OLE	A	Prunus Amygdalus Dulcis (Sweet Almond) Oil	9
		Olivoil Glutamate Emulsifier G-PF	15
		Tocopheril Acetate	1
		Cetyl Alcohol	2
	B	Bergamot wastewater	66
		Xantan Gum	0.2
		Glycerin	3.6
	C	Imidazolidinyl Urea	0.2
		Olive leaf extract	0.5
		Clementine peel extract	2.5
4% CPE-OLE	A	Prunus Amygdalus Dulcis (Sweet Almond) Oil	9
		Olivoil Glutamate Emulsifier G-PF	15
		Tocopheril Acetate	1
		Cetyl Alcohol	2
	B	Bergamot wastewater	65
		Xantan Gum	0.2
		Glycerin	3.6
	C	Imidazolidinyl Urea	0.2
		Olive leaf extract	0.5
		Clementine peel extract	3.5

All ingredients of phase A and phase B were collected in two different beakers and heated to a temperature of 72 ± 1 °C using a multi-point heating magnetic stirrer (AM4 Digital PRO, VELP Scientifica, Usmate, MB, Italy). When the required temperature was-reached, phase B was added to the phase A under homogenization at 2000 rpm using a high shear mixer Silverson® L4TR. The mixing was continued until a homogeneous emulsion was obtained and the temperature of emulsion had reduced to around 40 °C. Finally, phase C, containing the preservative Kemipur 100 and OLE and CPE (in the case of the 2%, 3%, and 4% OLE-CPE Formulations) were added to the cooled emulsion. The same protocol

was performed to prepare emulsions in the presence of natural extracts. The formulations were kept at rest for 24 h at room temperature to ensure a suitable equilibration of the emulsions, and then all characterization studies were carried out as described below [33].

2.9. Microrheological Evaluation of Empty and Citrus and Olive Leaf Extracts-Loaded Face Creams Using Diffusing Wave Spectroscopy (DWS)

Microrheological characterization of all formulations was carried out using diffusing wave spectroscopy and the Rheolaser Master™ (Formulaction, Alfatest, Milano, MI, Italy). This technique exploits the Brownian motion of particles in the sample to define their viscoelastic features. The samples were loaded into suitable glass vials (20 mL for each sample) and the light intensity was detected for all durations of analysis (1 h). The software RheoSoft Master 1.4.0.0. permitted us to obtain some microrheological parameters of the analysed samples such as the mean square displacement (MSD), elasticity index, and solid liquid balance (SLB).

2.10. Dynamic Rheological Characterization of Empty and Citrus and Olive Leaf Extracts-Loaded Face Creams Using Kinexus Rotational Rheometer

A Kinexus Pro+ Rotational Rheometer (Malvern Panalytical Ltd., Spectris plc, Malvern, UK) was used for in-depth characterizing of the formulations from the rheological point of view. Knowing the rheological behavior of semisolid formulations when they are solicited is of fundamental importance, above all for cosmetic purposes. The tool is equipped with cone-plate geometries (40 mm diameter; 2° angle) [34]. For all analyses, a fixed gap between the upper and lower geometries was pre-set at 1 mm, and the temperature was maintained constant at pre-fixed values: 25.00 ± 0.01 °C and 40.00 ± 0.01 °C, as a function of experimental design. The functionality of the tool was guaranteed by a compressed air flow (2 bar) that was pre-filtered through fine and superfine Clearpoint filters (Beko, Atlanta, GA, USA), which permitted us to obtain the pressure needed to perform the analysis. For the rheological investigation, each sample was carefully and gently loaded on the lower measuring geometry, and then the upper geometry was lowered at a very slow speed. Preliminary tests of sweep strain tests (0.01–100%, and 1 Hz of frequency) were carried out to define the suitable experimental conditions and to maintain the subsequent rheological measurements within the linear viscoelasticity region (LVR).

Two different types of rheological analysis were carried out on empty and citrus and olive leaf extract-loaded face creams: (i) viscosity studies as a function of shear rate (from 0.1 s^{-1} to 100 s^{-1}), and (ii) oscillatory tests with a frequency sweep (from 0.1 Hz to 10 Hz) at controlled shear stress equal to 1 Pa.

2.11. Stability Studies

The long-term stability of emulsions was investigated using a Turbiscan Lab® (Formulaction, L'Union, France) [35]. Cylindrical glass vials were filled with samples and any variations in transmission (ΔT) and backscattering (ΔBS) profiles were recorded over time. Measurements were collected up to 1 h at 25 ± 1 °C and 40 ± 1 °C. Values of ΔT and ΔBS were plotted as mean values ± standard deviation. The diameter kinetics profiles of the formulations were also investigated using Turbiscan Lab® and reported as a function of time. The destabilization kinetic profiles of emulsions were determined using Turbiscan Stability Index (TSI) values, and different formulations were compared each other [36].

2.12. Microscopy Studies

A Morphologi G3-S microscope equipped with a Nikon® CFI 60 Brightfield/Darkfield optical system was used to investigate the emulsions' structure. Each emulsion was diluted with water (~5 mg/mL) and a drop of the diluted sample was placed on a slide and covered using a cover slip (20 × 20 mm, Syntesys, Padova, Italy). Microscopies were performed at 20× magnification and images were exported as TIFF using the Morphologi software v. 8.30 package (Malvern Panalytical, Worcestershire, UK).

2.13. In Vivo Studies on Healthy Human Volunteers

2.13.1. Transepidermal Water Loss (TEWL)

The skin tolerability of the formulations was evaluated on 16 healthy human volunteers (mean age 27 ± 9) using a C+ K Multi Probe Adapter equipped with a Tewameter® TM300 probe (Courage & Khazak, Cologne, Germany). This probe uses two pairs of sensors to measure temperature and humidity to assess transepidermal water loss (TEWL), which is a worldwide-accepted measurement to recognize any damage to the skin water barrier [37]. Volunteers were accommodated for 20 min in a day surgery room (24 ± 1 °C, 40–50% RH) and informed about the experimental procedures. After giving their written consent, volunteers were enrolled in the study and five sites were marked on their forearms using a circular template (1 cm^2). The sites were traced on skin regions lacking discolorations and comedones, and sites were separated by at least 2 cm to avoid any interference between formulations. The first site was treated with 200 µL of saline solution, used as a negative control. The second site was treated with 200 mg of empty formulation. The last three sites were treated with 200 mg of formulations containing both OLE and CPE (2%, 3%, and 4% OLE-CPE Formulations). TEWL values were recorded 0, 1, 2, 4, 6, and 8 h after application and expressed as $g/h \cdot m^2$.

2.13.2. X-Rite

The X-Rite Ci62 (X-Rite Incorporated, Grandville, MI, USA) was used to detect any variations of skin color following the cutaneous application of emulsions [32]. Since melanin and hemoglobin play a key role in defining skin color, these two chromophores were monitored during the study [38]. Baseline values of erythema index (E.I.) were collected from each site demarcated on the forearm of each volunteer according to the scheme previously reported (Section 2.13.1. After 0, 1, 2, 4, 6, and 8 h had passed since the application of the samples, any variation in E.I. values was recorded according to the following equation:

$$\text{E.I.} = 100\left[\log\frac{1}{\text{R560}} + 1.5\left(\log\frac{1}{\text{R540}} + \log\frac{1}{\text{R580}}\right) - 2\left(\log\frac{1}{\text{R510}} + \log\frac{1}{\text{R610}}\right)\right] \quad (3)$$

where 1/R is the inverse value of reflectance measured at different wavelengths (510, 540, 560, 580, 610 nm) linked to hemoglobin and melanin.

Data are reported as ΔE.I. and were obtained by subtracting the E.I. value recorded at each time point from that of the baseline at the same site.

2.14. Skin Feeling

After testing the skin tolerability, volunteers were divided into two groups (8 for each group) and were asked to apply two different formulations and to express their primary and secondary skin feelings [39]. A single blind procedure was used for the study, and only the operator was aware what formulations the volunteer was applying. Participants filled out an anonymous questionnaire evaluating primary and secondary skin feelings from −5 to +5, indicating a "very low/absent" to "very nice/excellent" feeling, respectively [40]. All the studies performed on human volunteers were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Committee of the University of Catanzaro "Magna Graecia" (Approval number: 392/2019).

2.15. Statistical Analysis

Statistical analyses were performed using a one-way ANOVA test. A Bonferroni t-test was used to confirm the results, and the probability was set at * $p < 0.05$. The analysis was carried out using GraphPad Prism v8 software (San Diego, CA, USA) and Excel (Office 2010).

3. Result and Discussion

3.1. Characterization of the Extracts

The use of different analytical methods made it possible to characterize the chemical profile of the extracts obtained via supercritical CO_2 as well as their antioxidant activity against free radicals. The results obtained in every single analysis are listed and summarized in Table 2.

Table 2. Chemical characterization of extracts.

Sample	UV-Vis Spectrophotometer		
	TPC (mg/mL GAE)	**TFC (mg/mL)**	**Antioxidant Activity (%I)**
Olive leaves extract	0.0903 ± 0.0035	0.2164 ± 0.762	23.23 ± 1.47
Clementine peels extract	0.330 ± 0.017	0.8844 ± 0.0796	25.86 ± 3.07
Sample	**Liquid Chromatography (HPLC)**		
	Rutin (ppm)	**Naringin (ppm)**	**Hesperidin (ppm)**	**Oleuropein (ppm)**
Olive leaves extract	n.a.	n.a.	n.a.	7.44 ± 0.52
Clementine waste extract	25.97 ± 1.10	28.16 ± 2.13	679.88 ± 59.29	n.a

GAE = Gallic Acid Equivalent; TPC = Total Phenolic Content; TFC = Total Flavonoid Content; I% = Inhibition percentage; ppm = part per million; n.a. = not available.

The olive leaf extracts showed a total phenolic content of 0.0903 ± 0.0035 mg/mL (GAE) and a flavonoids concentration of 0.2164 ± 0.762 mg/mL, which gave them an antioxidant power of 23.23 ± 1.47%I. The clementine waste extracts, on the other hand, contained gallic acid-like polyphenols equal to 0.330 ± 0.017 mg/mL GAE and a flavonoids concentration of 0.8844 ± 0.0796 mg/mL, showing an antioxidant activity of 25.86 ± 3.07%I (Table 2).

Interestingly, despite the phenolic and flavonoid contents of the CPE being three and four times higher, respectively, than the OLE, the antioxidant activity levels of the two extracts were almost overlapping. The results obtained can be explained by the differences in chemical structure of the molecules contained in the extracts. In fact, antioxidant activity is a unique and characteristic property of each molecule, affected by the insaturations and functional groups contained in the molecules as well as by their spatial location, factors that have an overall impact on their chemical reactivity and therefore the tendency to interact with other circulating molecules [41,42].

In agreement with our hypothesis, the HPLC analyses showed the presence of different molecules in the extracts. Indeed, in decreasing order, the CPE contained hesperidin (679.88 ± 59.29 ppm), naringin (28.16 ± 2.13 ppm), and rutin (25.97 ± 1.10 ppm), while the OLE contained only 7.44 ± 0.52 ppm of oleuropein (Table 2). Interestingly, in contrast to the scientific literature, our OLE did not contain rutin, a flavanol that is generally detectable in olive leaf extract [43]. However, the presence and concentration of bioactive molecules in plants is susceptible to numerous variations. In fact, various external and seasonal parameters influence the concentration of the molecules, as well as the genetic and geographical profile of the species. These parameters are known to significantly influence the vegetative activity and metabolic processes of plants, as previously assumed by Al Foraih M. and Williams C.A. and their respective co-workers [44,45].

3.2. In Vitro Cytotoxic Effects

The MTT cell proliferation assay allowed us to detect cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity [46]. Figure 1A,B shows that CPE and OLE as single agents do not induce any relevant variation in cell viability percentages of normal keratinocytes after up to 48 h, even at the highest concentration tested (2% *w*/*v*). Indeed, cell viability values higher than 80% were recorded for all the samples tested. A slight pro-proliferative effect was also shown by CPE at the different concentrations tested up to 24 h after treatment. These data are in very good agreement with the pro-proliferative

effect previously reported from Chung J.H. et al. and Gunes S. et al. for green tea and spirulina platensis extracts, respectively, on human keratinocytes [47,48].

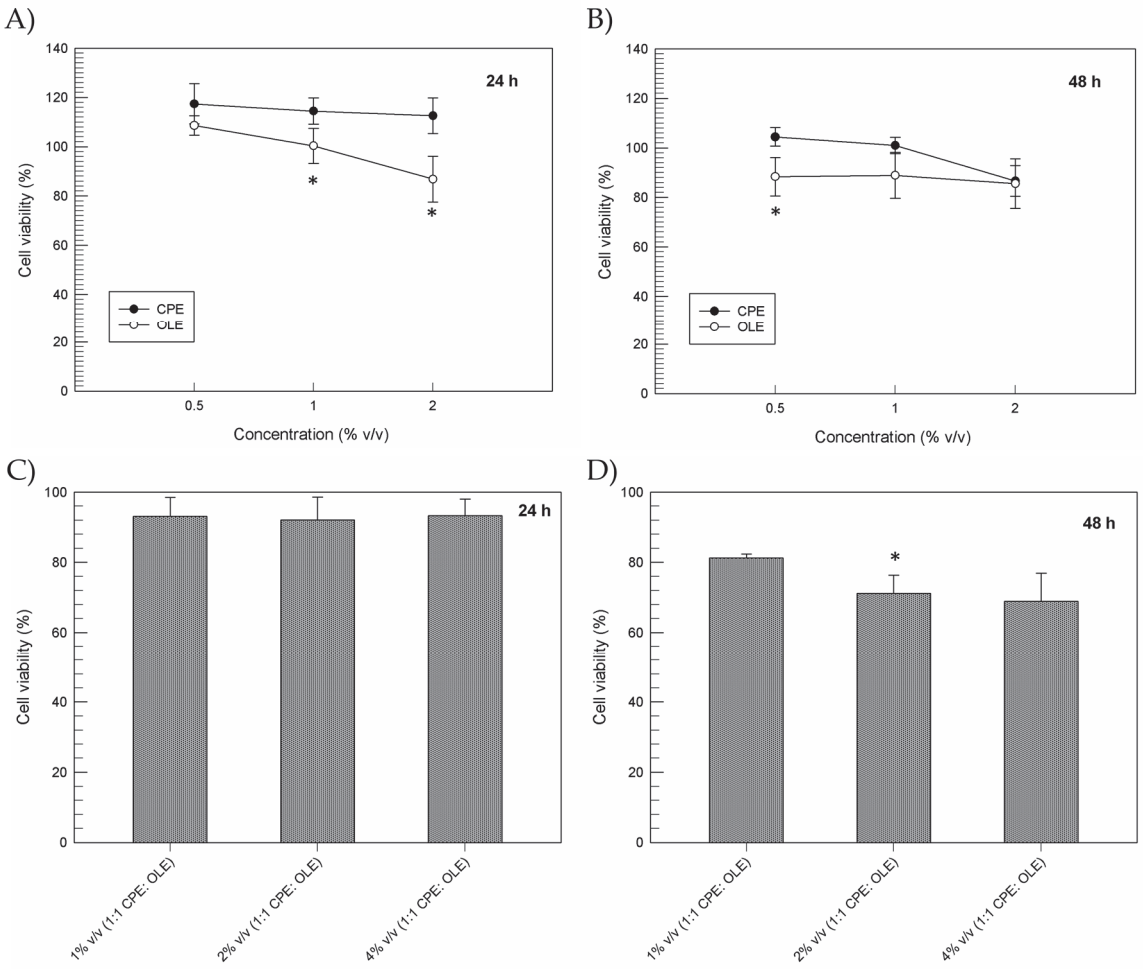


Figure 1. In vitro cytotoxic effect of CPE and OLE as single agents (**A**,**B**) or combined (**C**,**D**) on human keratinocytes. Results are reported as a function of incubation time and extract concentration. Data are normalized with respect to the cytotoxic effect of ethanol at the same concentrations used for the extracts. Results are the average of three independent experiments ± SD. * $p < 0.05$.

Figure 1C,D show the results obtained from a combined treatment using CPE and OLE at an increasing final concentration (1–4% v/v, 1:1 ratio). As can be seen, the combination of both extracts did not induce relevant cytotoxic effects on human keratinocytes, keeping values above 90% up to 24 h after treatment and proving the great safety of the extracts tested (Figure 1C). Formulation 1% v/v (1:1 CPE:OLE) guaranteed a high safety profile even up to 48 h after exposure to the cells, while increasing concentrations of the extracts (final concentrations 2% or 4%) reported lower cell viability percentages (~70%). This effect could be due to some compounds present in olive leaf extracts, such as oleuropein, hydroxytyrosol, and oleocanthal, which can interfere with cell proliferation processes, blocking cell division or inducing apoptosis through different mechanisms [49–51]. The simultaneous presence of the organic acids contained in CPE, i.e., citric acid, could alter the stability of these compounds as well as causing combined effects on oxidative stress and increased cytotoxic effects. For this reason, OLE was kept at 0.5% v/v in all the realized emulsions in order to exploit beneficial effects, thus preventing cytotoxic effects.

3.3. In-Depth Characterization of Empty and Citrus and Olive Leaf Extracts-Loaded Face Creams

For the cosmetic application of natural extracts obtained from clementine peel from industrial processing waste and olive leaves as waste material, an optimized face cream formulation was chosen. The empty formulation, and consequently the citrus and olive leaf extracts (CPE-OLE)-loaded formulations, were prepared with an easy laboratory process based on the homogenization and emulsification of their components. The composition of formulations permitted us to obtain O/W emulsions that were apparently stable, pleasant in appearance and smell, and offered a good apparent spreadability. To better and more scientifically characterize all of the prepared emulsions, a deep analysis was carried out in terms of stability, microrheological, and dynamic rheological investigation. Another aim was to evaluate how the presence of increasing percentages of natural extracts could influence and modify the physico-chemical properties of emulsions.

3.3.1. Microrheological Investigation of Empty and Clementine Peels and Olive Leaf Extracts-Loaded Face Creams

The comparison between different formulations can be carried out through a microrheological investigation using a Rheolaser Master™, which permits the researcher to characterize the formulations at rest, without inducing a solicitation and without disrupting or forcing the sample to flow. This technique is called diffusing wave spectroscopy (DWS), similar to dynamic light scattering, and provides information about the internal structure of analyzed samples based on the Brownian movements of emulsion droplets, taking into account that internal structure can limit this movement [52]. Starting from these purposes, the microrheological profiles of the four prepared formulations were analyzed as a function of temperature; 25 °C for room conditions and 40 °C as an example of extreme storage conditions.

The first parameter obtained from the Rheolaser Master™ was a Mean Square Displacement (MSD) curve of each formulation. The slope of an MSD curve is related to the elasticity and viscosity of the sample. In detail, the movement of particles (in this case of droplets) within samples can induce a deformation of speckle images, and the entity of the induced deformation is recorded from a specific detector [53]. When the sample can be considered soft or fluid, the particles within its structure are characterized by more freedom of movement. In this case, the slope of the resulting MSD curve is directly proportional to the viscosity of the sample. In other cases, when the sample is characterized by a hard structure, the Brownian movement of the particles is strongly limited, and the MSD curves tend to decrease until a plateau is reached [54].

Figure 2 shows the MSD curves of all of the formulations analyzed both at 25 °C and 40 °C at different aging times (0 min, 20 min, 40 min, and 60 min). Comparing them, it is possible to note that no marked differences between the samples' structures and behavior are present when the samples are analyzed at room temperature. In fact, the MSD curves of all of the samples tended to decrease during the analysis time. The progressive reduction of MSD curves, until the plateau was reached, highlights the observation that the droplets within samples have a certain freedom of movement. We can assume that the three-dimensional internal structures of the prepared emulsions are sufficiently soft to permits it, at least at rest. Comparing the microrheological profiles of each sample analyzed at different temperatures, it is possible to note that the increased temperature induced a slowing down of plateau achievement, showing a greater freedom of movement of droplets within emulsions at 40 °C with respect to the same samples analyzed at 25 °C.

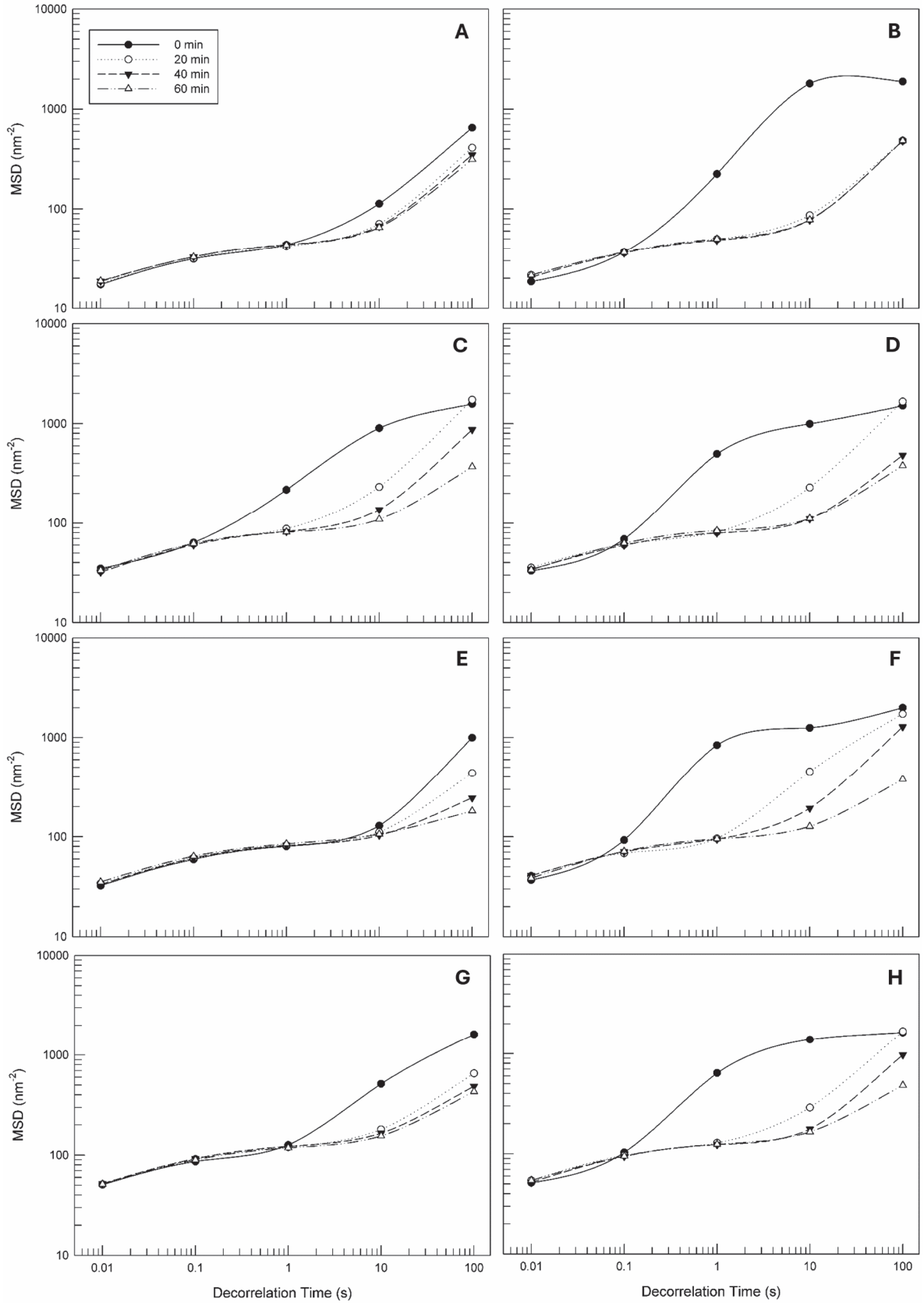


Figure 2. Mean square displacement (MSD) curves as a function of decorrelation time (s) of empty and OLE-CPE formulations. The illustrated results are representative of three independent experiments. Legend: Empty Formulation at 25 °C (**A**) and 40 °C (**B**); 2% OLE-CPE Formulation at 25 °C (**C**) and 40 °C (**D**); 3% OLE-CPE Formulation at 25 °C (**E**) and 40 °C (**F**); 4% OLE-CPE Formulation at 25 °C (**G**) and 40 °C (**H**).

Starting from the MSD curves, other microrheological parameters can be obtained thanks to the software package RheoSoft Master 1.4.0.0. These parameters are the elasticity index (nm^{-2}) and SLB. The Elasticity Index curves reported in Figure 3A,B permit us to appreciate the small differences that are difficult to find in the MSD curves (Figure 2). Generally, an increase in elasticity index values corresponds to a reduction in the particles' movement within emulsion structures [55]. From the data reported in Figure 3A, it can be noted that the empty formulation was characterized by greater values of Elasticity Index (mean Elasticity Index value equal to 0.035 ± 0.00 nm^{-2}) with respect to formulations prepared with increasing concentrations of natural extracts when analyzed at room temperature. Moreover, there do not seem to be any differences between the Elasticity Index profiles of 2% and 3% OLE-CPE formulations. On the contrary, the presence of the highest natural extract concentrations in a formulation resulted in the lowest elasticity values. Interpreting these data, we can assume that the presence of the extracts induces a reduction in the hardness of the internal structure of the formulations and, consequently, the oily droplets could better move within the three-dimensional network of the emulsion. In fact, as the concentration of the extracts increases, the movement of the droplets appears to be freer, resulting in a mean elasticity index value equal to 0.013 ± 0.00 nm^{-2} in the 4% OLE-CPE formulation.

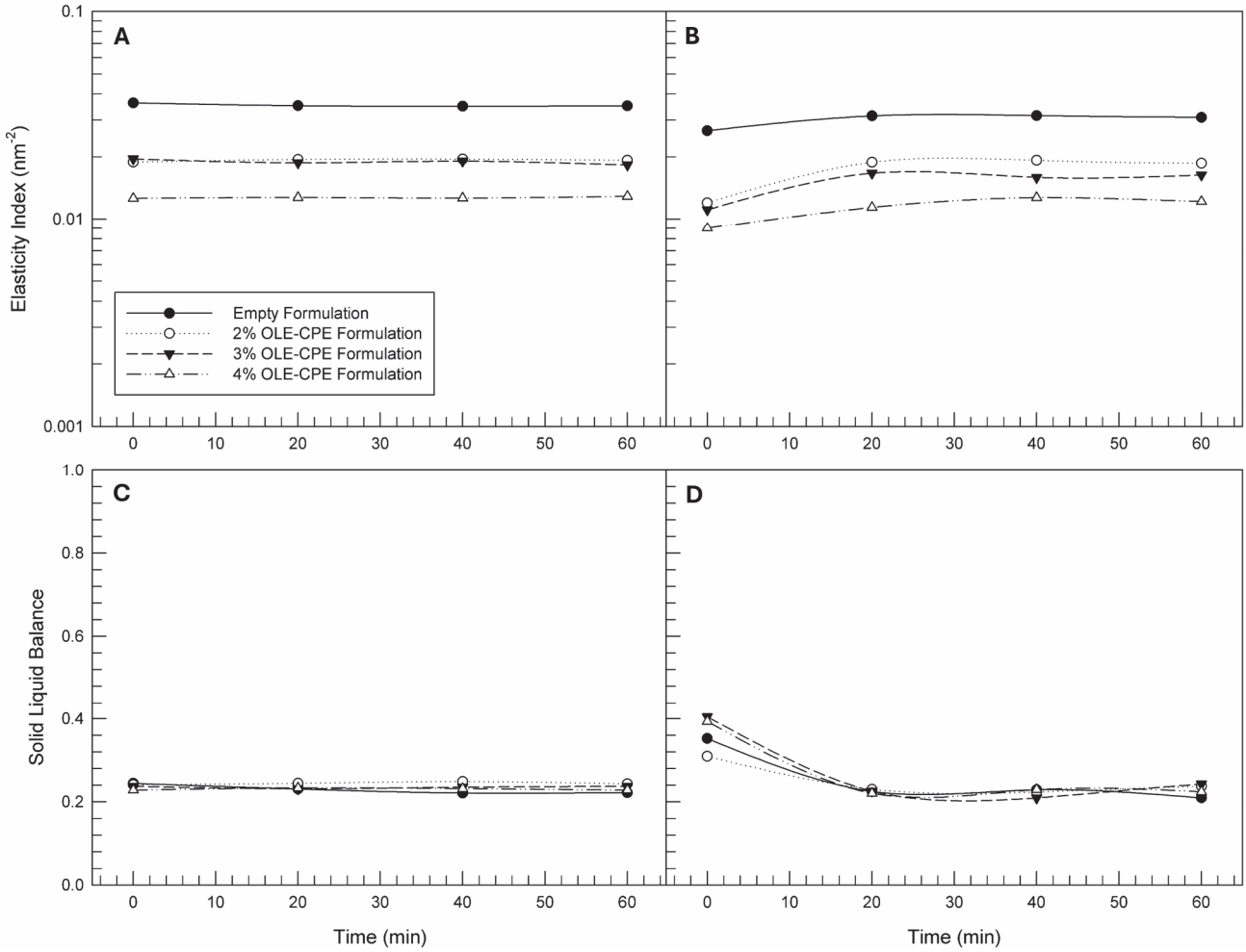


Figure 3. Elasticity Index and Solid Liquid Balance curves as a function time of formulations analyzed at different temperatures. The illustrated results were representative of three independent experiments. Legend: Elasticity profiles at 25 °C (**A**) and 40 °C (**B**); Solid Liquid Balance profiles at 25 °C (**C**) and 40 °C (**D**).

The other mentioned microrheological parameter is the SLB, which corresponds to the ratio between the liquid-like and solid-like behavior of the analyzed sample [56]. For some types of samples, SLB can indicate the gelling point, the adhesiveness, and spreadability. SLB is a dimensionless parameter that can have values between 0 and 1; for $0 < SLB < 0.5$, the sample is characterized by predominantly solid behavior, while for $0.5 < SLB < 1$ the sample is characterized by predominantly liquid behavior. Crucial is a value of SLB equal to 0.5, because it indicates that a transformation from a liquid to a solid (or vice versa) occurred. In our study, no formulation underwent a transition, because the SLB values all remained below the value of 0.5, as shown in Figure 3C,D. Regardless of the presence or absence of extracts within the formulations, all of the samples showed a predominantly solid behavior, with SLB values between 0 and 0.5.

The increase in the analysis temperature (up to 40 °C) did not induce an alteration in either the Elasticity Index or the SLB profiles, confirming that the prepared emulsions are able to resist at temperature changes when they are maintained at rest and analyzed from a microrheological point of view.

3.3.2. Dynamic Rheological Characterization of Empty and Citrus and Olive Leaf Extracts-Loaded Face Creams

The previously mentioned microrheological characterization permitted us to observe the behavior of the samples at rest, without any solicitation and without inducing a structural modification. When a formulation is designed for topical application and above all for being spread on skin, a crucial aspect to consider is its dynamic rheological behavior and its responsiveness to specific solicitation [57]. For this purpose, two different rheological analysis were carried out on empty and OLE-CPE extracts-loaded formulations, i.e., viscosity evaluation in rotational mode and oscillatory test with frequency sweep.

In the Figure 4 the rheological profiles as shear viscosity versus shear rate are reported for all of the characterized formulations. As we can see, regardless of the analysis temperature, all samples showed a non-Newtonian behavior, modifying their structures and adapting them to the induced flow. In fact, the samples' viscosity decreases when the shear rate is increased. This pseudoplastic flow behavior is important because it is correlated with the spreadability of cosmetic products [58]. Moreover, observing Figure 4, the slope of the viscosity curves seems to be comparable for all of the analyzed samples. Comparing instead Figure 4A,B, it can be noted that the rheological behavior of formulations with and without extracts did not change when the temperature was increased, but a certain reduction in shear viscosity values occurred (Table 3), confirming a fluidizing effect of the temperature on the prepared emulsions.

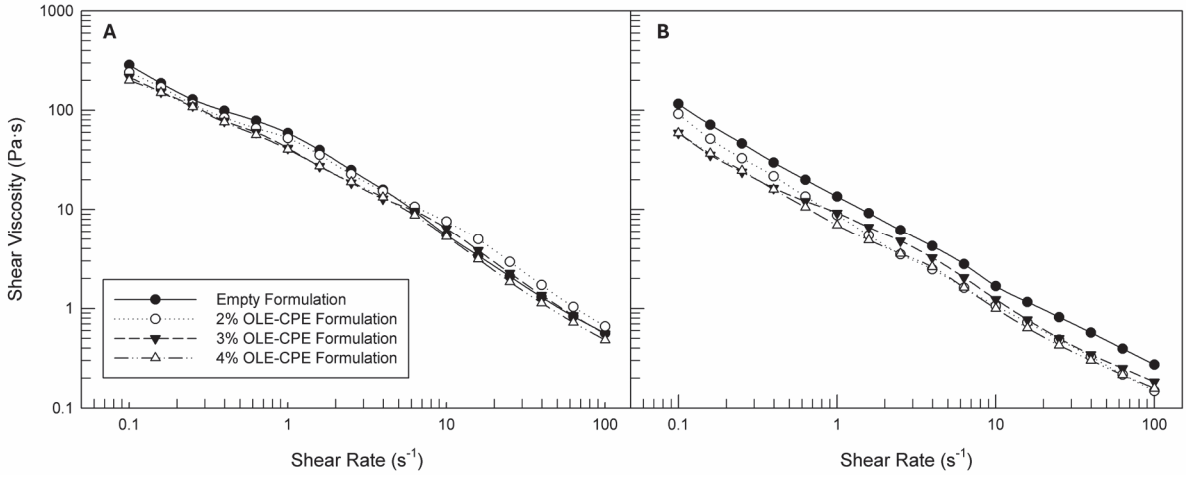


Figure 4. Rheological profiles (shear viscosity versus shear rate) of empty and OLE-CPE formulations analyzed at 25 °C (**A**) and 40 °C (**B**). The illustrated results are representative of three independent experiments.

Table 3. Shear rate-dependent viscosity (Pa·s) for empty and OLE-CPE Formulations at different shear rates. Values are reported as the average of three independent experiments ± standard deviation.

Formulation	Shear Viscosity (Pa·s) at Different Shear Rate (s^{-1})			
	0.1 s^{-1}	**1 s^{-1}**	**10 s^{-1}**	**100 s^{-1}**
T = 25.00 ± 0.01 °C				
Empty Formulation	285.40 ± 9.21	58.96 ± 6.54	5.51 ± 0.86	0.56 ± 0.02
2% OLE-CPE Formulation	240.10 ± 6.28 *	52.51 ± 4.36	7.56 ± 1.04	0.66 ± 0.04 *
3% OLE-CPE Formulation	215.20 ± 5.98 **	41.58 ± 3.05 *	6.41 ± 1.58	0.55 ± 0.00
4% OLE-CPE Formulation	199.90 ± 7.02 **	40.40 ± 4.35 *	5.29 ± 0.99	0.48 ± 0.07
T = 40.00 ± 0.01 °C				
Empty Formulation	115.60 ± 11.21	13.50 ± 2.45	1.67 ± 0.58	0.27 ± 0.05
2% OLE-CPE Formulation	91.55 ± 8.24 *	8.76 ± 1.98	1.06 ± 0.78	0.15 ± 0.02 *
3% OLE-CPE Formulation	58.99 ± 8.25 *	9.23 ± 1.14	1.23 ± 0.45	0.18 ± 0.02 *
4% OLE-CPE Formulation	59.05 ± 6.25 *	6.92 ± 1.87 *	0.99 ± 0.05	0.16 ± 0.00 *

* $p < 0.05$ and ** $p < 0.001$ with respect to empty formulation at the same shear rate values and temperature.

Observing Table 3, it is also possible to note that the presence of increasing percentages of natural extracts in the formulations induced a statistically significant reduction in viscosity values, above all at the lowest shear rate, while not modifying the rheological behavior of the formulations.

Finally, rheological oscillation tests were carried out on all formulationd to determine the G′ and G″ values as a function of frequency and temperature, inducing sinusoidal periodic deformations on the material. G′ is defined as the elastic or storage modulus, while G″ is the viscous or loss modulus; when G′ is greater than G″, the induced deformation is elastic and recoverable, and the sample appears as a solid-like material. On the other hand, when G′ is greater than G″ the sample shows a predominantly solid-like behavior [59]. In Figure 5A,B, the values of G′ and G″ were reported. For all samples, the values of G′ were greater than G″, indicating that the elastic behavior of the formulations was maintained in the frequency range of the analysis and confirming the resistance of the emulsions' structure to the oscillatory solicitation. This is an important result, because it permits us to assume that the formulations will resist and maintain their structure during storage, commercial distribution, and use. In this case, the effects of temperature increase are more evident when compared to shear viscosity curves (Figure 4). When the temperature was increased from 25 °C to 40 °C, both the G′ and G″ moduli increased. It is probable that the increased fluidity of empty and OLE-CPE formulations due to heating could allow better mobility of the droplets in the material, resulting in an increase in both moduli. Moreover, the reduction in viscosity could promote the formation or reorientation of internal structures, which would contribute to the material's resistance to deformation. As a result, the values of G′ and G″ increase. Considering the obtained rheological data, we can conclude that the prepared formulations were suitable for skin spreading due to their responses to the induced flow. They are characterized by an internal structure able to resist induced deformation.

3.4. Stability Studies on Emulsions

The long-term stability of the emulsions was studied using the multiple light scattering technology of Turbiscan Lab®. All the formulations displayed great stability profiles, which were not compromised by the addition of OLE and CPE during the preparation phase. Values of ΔT and ΔBS were between ±2% regardless of the amount of extracts used (Figure S1), thus indicating the absence of any creaming, flocculation, or sedimentation phenomena [60]. This result was further confirmed by TSI analyses that showed the absence of relevant variations in TSI values between the empty formulation and the formulations containing OLE and CPE (Figure 6). In detail, all of the formulations displayed overlapping profiles at 25 °C, thus confirming the lack of destabilizing phenomena

induced by the addition of the extracts during preparation (Figure 6A). Contrarily, a slight increase in TSI values was reported at 40 °C for the formulation containing the smallest amount or no extracts (Figure 6B), thus indicating a lower stability compared to the other formulations tested [61].

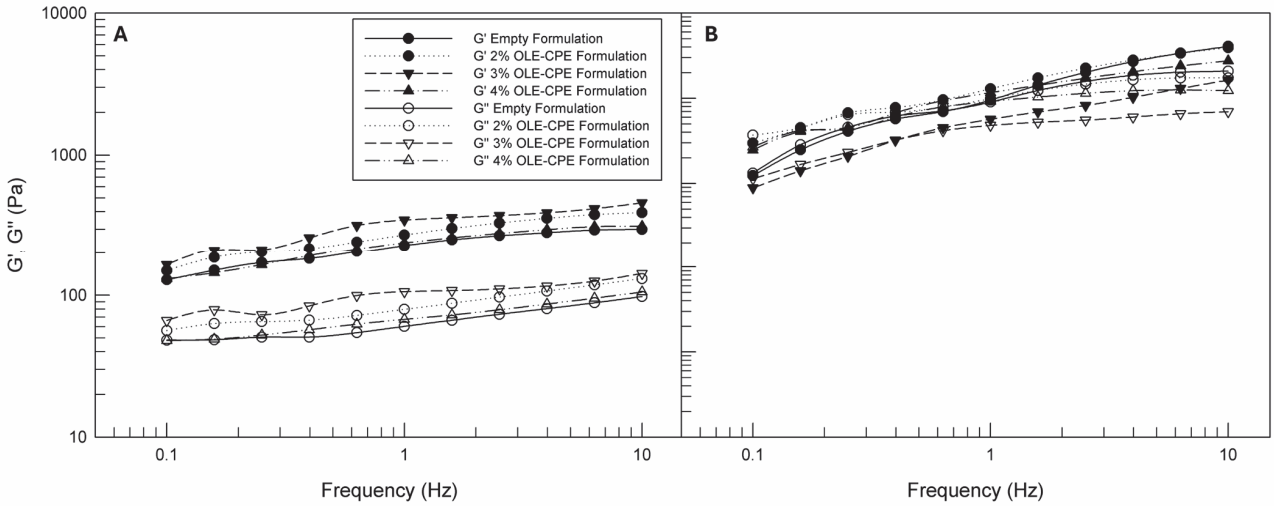


Figure 5. G′ and G″ moduli (Pa) curves as a function of frequency (Hz) of empty and OLE-CPE formulations analyzed at 25 °C (**A**) and 40 °C (**B**). The illustrated results were representative of three independent experiments.

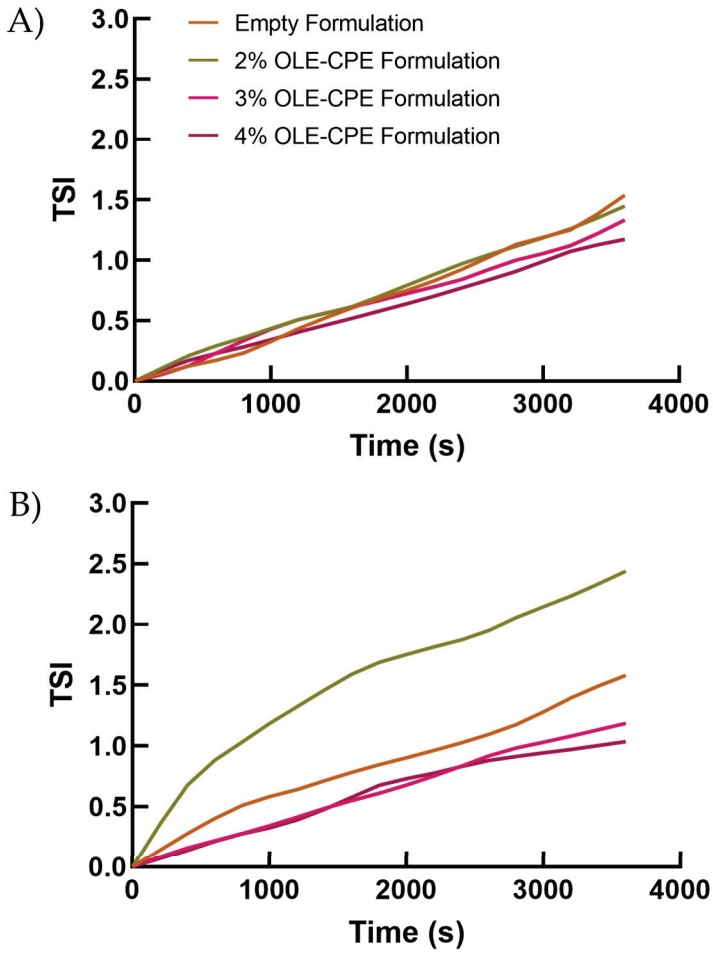


Figure 6. *Cont.*

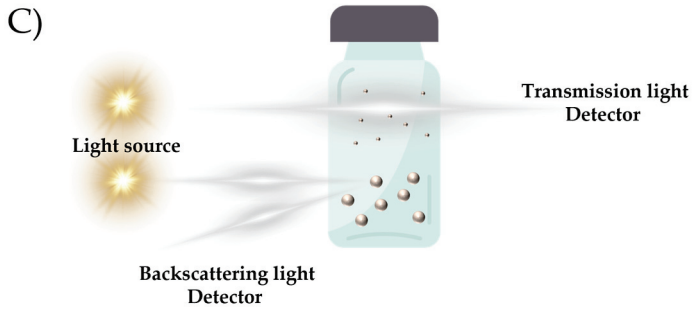


Figure 6. Turbiscan analysis of emulsions. The kinetic destabilization profiles are reported as a function of time (0–60 min) and temperature (25 °C and 40 °C for Panel (**A**,**B**), respectively). The results are representative of three analyses carried out on three different batches of each formulation. Panel (**C**) is a graphic representation of the working principles of the Turbiscan Lab analyzer. Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License.

Turbiscan Lab® also provided information on the diameter kinetic profiles of the emulsions, demonstrating the maintenance of globule size (5.5–6.5 µm) for the entire duration of the analysis performed at 25 °C, which is an indicator of high stability (Figure S2A). Contrarily, a slight increase in diameter of both the empty formulation and that containing 2% CPE-OLE was obtained during the first 20 min of the analysis performed at 40 °C (Figure S2B), subsequently reaching a dimensional plateau. These effects confirmed the results of TSI, leading to the hypothesis of a stabilizing effect of the extracts. The sizes of the emulsion globules were also confirmed by microscopy studies, reporting diameters lower than 10 µm for all the analyzed samples regardless of their composition (Figure S3).

3.5. In Vivo Evaluation of Cutaneous Tolerability and Feelings Induced by Emulsions

Cutaneous tolerability represents a fundamental requirement for the appeal of the face cream. The monitoring of the TEWL and increase in erythematous index after application of the cream represent two non-invasive strategies that allow the evaluation of the safety profile of the proposed formulations. TEWL quantifies the water loss from the outermost skin layer. Higher TEWL values can indicate a disruption in the skin's protective barrier [37]. Figure 7A shows the skin tolerability of emulsions expressed as TEWL values over varying exposure times (0–8 h). Saline solution (0.9% NaCl w/v) was used as a control during the study. Neither the empty formulations nor those containing extracts caused increases in TEWL values, which is a very good marker of an intact skin barrier function. TEWL values below 11 were reported for all of the formulations tested and were in very good agreement with those normally recorded for healthy skin [62]. Rather, a slight decrease in TEWL values was recorded in the results obtained from emulsions in comparison to those obtained from the saline solution. This effect could be due to a film-forming potential of the cream, creating a protective layer on the skin, as well as a hydrating effect on the part of the oils contained in the emulsion, which overall reduce the loss of transepidermal water [63].

The results of skin tolerability were further confirmed by reflectance spectroscopy studies using X-Rite Ci62 to assess any occurrence of cutaneous erythema following the application of the emulsions. Figure 7B shows the results of skin tolerance as variations of erythema index values (ΔE.I.) compared to baseline values. All of the samples tested did not lead to relevant changes in erythema index values, remaining in the same range of variation as the sites treated with saline solution alone. The data confirmed the great safety of the tested formulations, regardless of the quantity of extracts loaded in the formulation. The lack of relevant changes in the erythematous index toward more negative values was also a sign of a lack of opacification after application of the cream, with an attractive potential for the user.

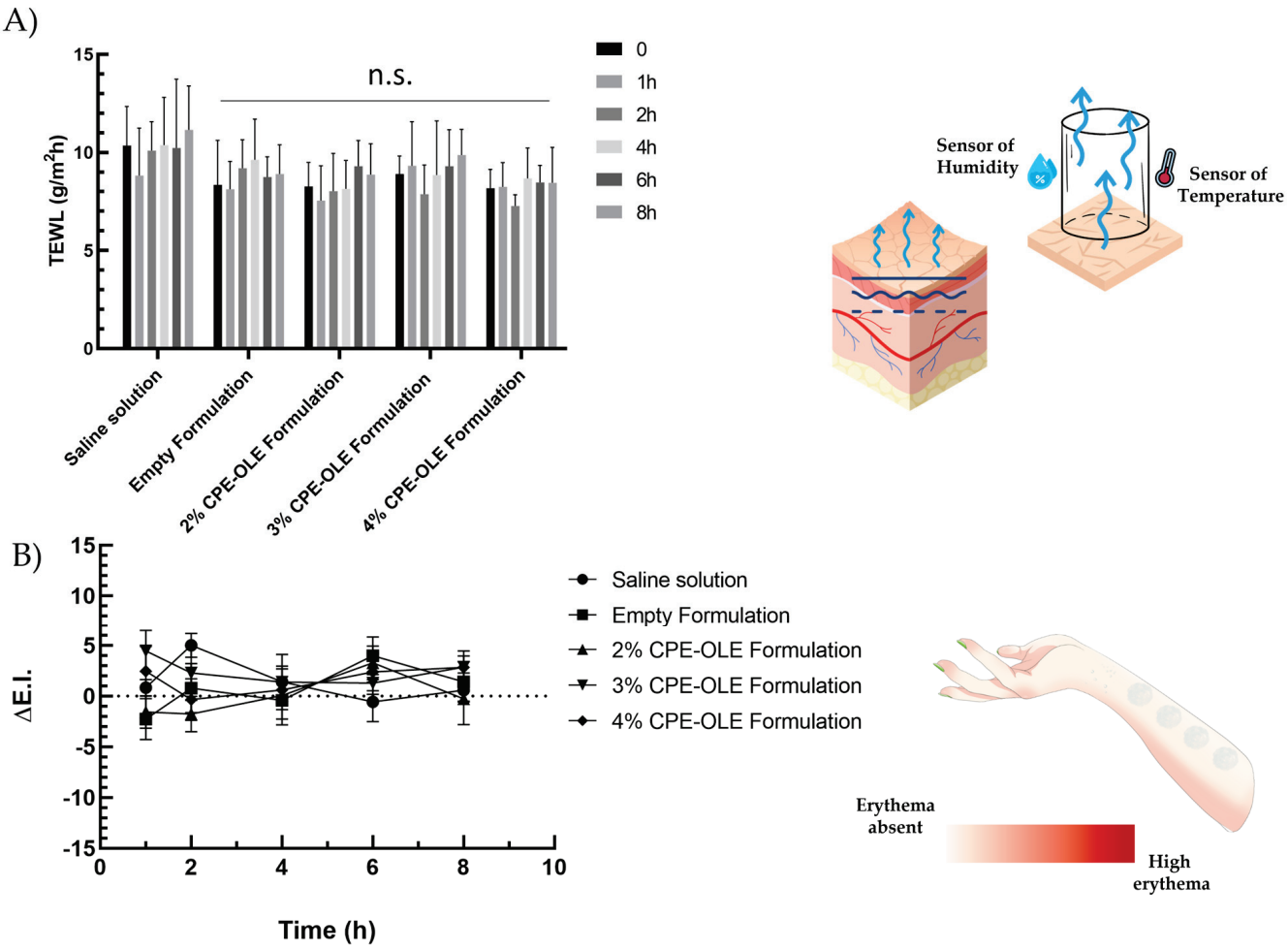


Figure 7. Studies of skin tolerability on human volunteers. Panel (**A**) shows the values of TEWL after topical administration of the empty formulation or formulations containing 2%, 3%, or 4% CPE-OLE. Panel (**B**) shows the variation of erythematous index values after the administration of empty formulations or formulations containing CPE-OLE. Saline solution (NaCl 0.9% *w*/*v*) was used as a control for both studies. n.s. = not significant. Parts of the figure were drawn using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License.

The volunteers enrolled in the in vivo studies were invited to complete a confidential survey on their primary (softness, heaviness, spreadability, freshness) and secondary (moisturizing sense, silkiness, greasiness) sensations experienced during and after applying the empty formulations or those that contained 2–4% of CPE-OLE [40]. Volunteers gave a rating between +5 and −5 to each formulation, and the results were collected into a radar diagram (Figure 8). Overall, the formulations were highly appreciated by all candidates, and primary odors appeared pleasant for all samples due to the presence of bergamot water and extracts in the formulations. However, some parameters were very different between the investigated emulsions. In detail, spreadability and silkiness were more appreciated, with increased amounts of OLE and CPE in the preparation, which agrees with the results reported in our rheological studies.

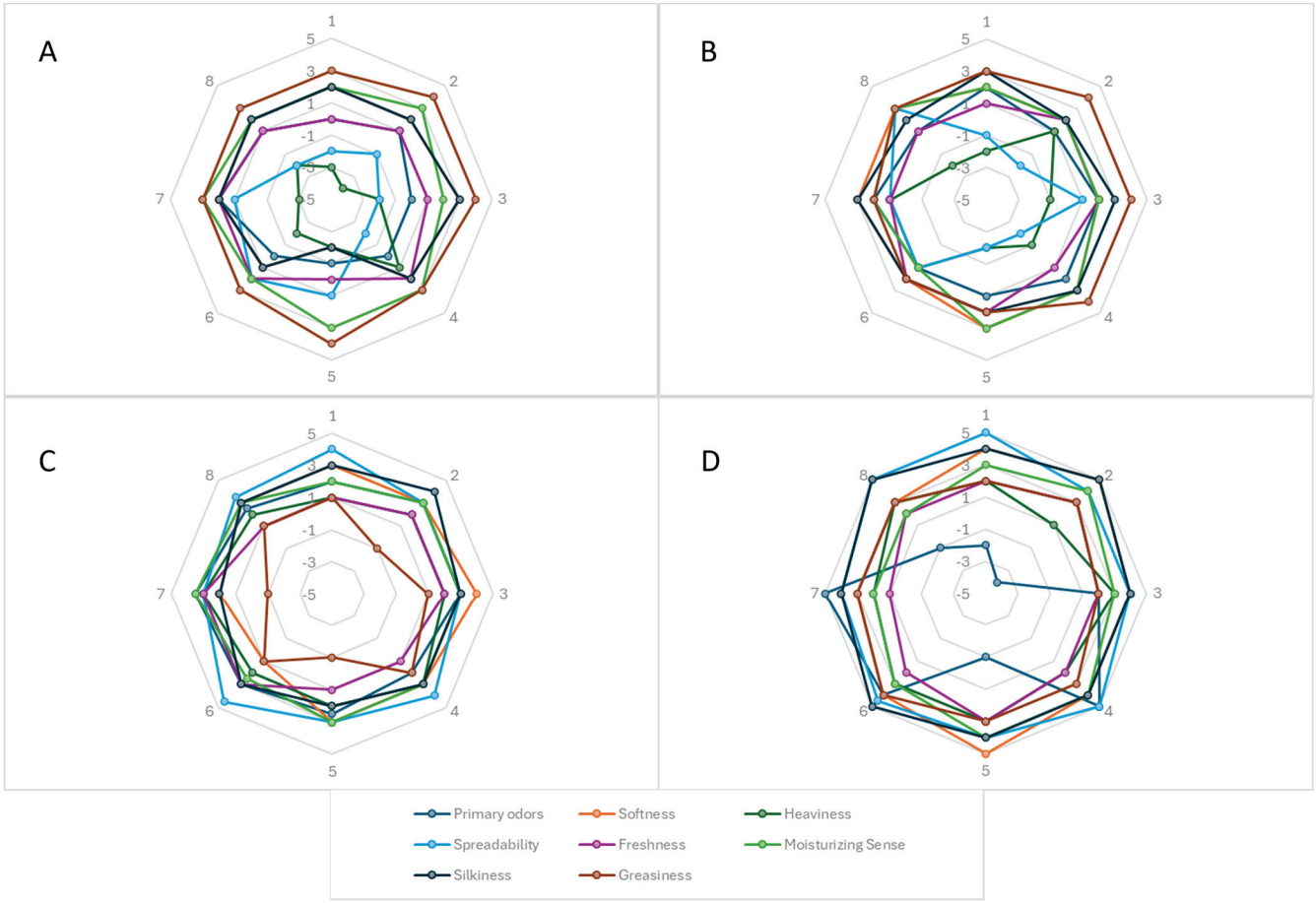


Figure 8. Radar diagram of skin feelings after cutaneous application of emulsions. Human volunteers were asked to rate primary and secondary skin feelings through an anonymous questionnaire. Panel (**A**) refers to the empty formulation; panels (**B**–**D**) refer to the formulations containing 2%, 3%, and 4% CPE-OLE, respectively.

4. Conclusions

In this study, we proposed the use of clementine peels and olive leaf extracts as value-added food-waste-derived ingredients for face cream. Extracts were obtained using a carbon dioxide supercritical fluid extraction technique and showed suitable antioxidant features, with an antioxidant activity of ca. 25%. The inclusion of these ingredients in the proposed cosmetic results in stable creams, as demonstrated by Turbiscan analyses at room temperature and extreme storage conditions, i.e., 25 and 40 °C, respectively. Both extracts demonstrated safe profiles in vitro on NCTC human keratinocytes, without any significant cytotoxic effect after up to 48 h of incubation at extract concentrations ranging between 1 and 2% v/v. The resulting face creams embedded with clementine peel and olive leaf extracts showed proper spreadability and demonstrated pseudoplastic behavior under dynamic rheological analysis. Similar trends were recorded for the investigated creams regardless of the extract concentrations added, although a slight progressive reduction of overall viscosity was found by increasing their concentration. The safety profiles of the creams were investigated in vivo, showing no significant variations of TEWL and erythematous index in healthy volunteers with respect to a negative control. The positive feedback received from healthy human volunteers confirmed the potential use of these cosmetic formulations, since the OLE-CPE formulations were described as suitably spreadable, with a good texture and a pleasant fragrance provided by bergamot wastewater. The proposed approach highlights the re-use of food waste byproducts as ingredients in the cosmetic field

in order to develop a circular economy able to provide value-added biomaterials while at the same time reducing environmental impacts and resource depletion.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cosmetics11020041/s1, Figure S1: Turbiscan analysis of emulsions. Transmitted and backscattered signals recorded at 25 °C (A,B) or 40 °C (C,D) are reported as a function of sample height (mm). Figure S2: Diameter kinetic profiles of emulsions. Data of average diameter are reported as a function of time (0–1 h) and temperature (25 °C and 40 °C for Panel (A) and (B), respectively). Figure S3: Microscopies of emulsions. (A) refers to Empty formulation; (B–D) refer to Formulations containing 2%, 3% and 4% CPE-OLE, respectively.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Committee of University of Catanzaro "Magna Graecia" (Approval number: 392/2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available from the corresponding author upon request. The data are not publicly available due to privacy.

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A *Hylocereus undatus* Extract Enhances Skin Microbiota Balance and Delivers In-Vivo Improvements in Skin Health and Beauty

Fabien Havas [1], Shlomo Krispin [1], Moshe Cohen [1] and Joan Attia-Vigneau [2,*]

[1] Lucas Meyer Cosmetics Israel, Yavne 8122503, Israel
[2] Lucas Meyer Cosmetics, 31036 Toulouse, France
* Correspondence: joan.attia@lucasmeyercosmetics.com

Abstract: Skin microbiota, and its diversity and balance, play a key role in skin health and beauty, influencing skin moisture, barrier function, and radiance. A healthy skin microbiota limits the growth of detrimental species, protecting the skin from pathologies. Prebiotics can support beneficial populations in outcompeting detrimental ones. Dragon fruit (*Hylocereus undatus*) contains prebiotic polysaccharides effective on gut bacteria. Its extract was tested in vitro, in a coculture model including representative beneficial and detrimental species, and in double-blind, placebo-controlled clinical trials. Effects on the skin microbiota were measured via 16S rDNA sequencing, and skin health and beauty benefits were evaluated through image analysis, TEWL measurement, and chromametry. Doppler flowmetry measured skin resilience. The extract supported *S. epidermidis* and *S. hominis* (beneficial species), while limiting *S. aureus* and *C. acnes* (representing pathogens) in vitro. Clinical results demonstrated its beneficial effects on skin microbiota diversity, especially in older volunteers (Faith's index up to +20% vs. placebo). Improvements were shown in skin sensitivity and resilience (by ca. 30% vs. placebo), skin redness (reflecting inflammation status), pigmentation and radiance (+11% ITA), barrier function (−13% TEWL), and wrinkling. This demonstrates this extract's positive effects on the beauty, health, and microbiota balance of the skin.

Keywords: *Hylocereus*; dragon fruit; pitaya; microbiota; microbiome; in vitro; in vivo; prebiotic; anti-inflammation; brightening

1. Introduction

The skin microbiota includes trillions of bacterial, viral, and fungal species, interacting with each other and the host organism to form a complex multifaceted ecosystem [1]. A balanced, diverse skin microbiota supports healthy skin homeostasis and strengthens the skin barrier through microbial, physical, and immune effects [2–4].

Beneficial bacteria form a protective microbial barrier limiting the growth of potentially pathogenic strains, such as *Staphylococcus aureus*, through competition, pH regulation, and antibiotics production [3,5]. For example, coagulase-negative *Staphylococcus* such as *S. lugdunensis* produce lugdunin, a thiazolidine-containing cyclic antibiotic peptide directly inhibiting *S. aureus* growth [6]. *S. epidermidis* deploys glutamyl endopeptidase (Esp), a serine protease which dismantles proteins key to *S. aureus* biofilm formation [7,8]. *S. epidermidis* can also trigger keratinocytes to produce antimicrobial peptides, including β-defensin [5]. *Staphylococcus* species, including *S. epidermidis* and *S. hominis*, were shown to produce lantibiotics synergistic with human LL-37 cathelicidin antimicrobial peptide, together significantly reducing *S. aureus* colonization [9]. Conversely, some skin microbes can promote *S. aureus* biofilm formation, such as *Propionibacterium acnes* (through coproporphyrin III production [10]).

The skin microbiota influences the physical skin barrier through the regulation of epidermis renewal and barrier structural component production [4]. Activation of the aryl hydrocarbon receptor (AHR) in keratinocytes by microbial metabolites (in a wound healing

context) can promote epithelial differentiation and barrier integrity reinforcement [11]. *S. epidermidis*, for its part, secretes a sphingomyelinase enzyme, both serving the bacterium's nutrient acquisition and facilitating host tissue production of ceramides (critical components of the epithelial barrier, contributing to skin hydration) [12]. The skin microbiota also plays a complex role in wound development and healing, the full comprehension of which is an ongoing endeavor [13,14].

The skin microbiota can influence innate immune responses and even form symbioses. Influencing immune cell recruitment, differentiation, and more, commensals can contribute to defense against harmful species, especially where the physical skin barrier is compromised [4]. *S. epidermidis* stimulates innate immunity by activating γδ T cells and upregulating antimicrobial peptide perforin-2 [15].

The microbiota profoundly influences the skin's susceptibility to external conditions and its overall appearance. However, a definitive understanding of "balanced" skin microbiota remains elusive. Diverse microbial lineages exist in complex, dynamic equilibria, involving specific skin niche microenvironments and interwoven host immune–microbe and microbe–microbe interactions. These equilibria are highly individualized, and subject to continuous short- and long-term fluctuations, responding to changing environmental factors, aging processes, perturbations like changing hygiene practices, and even psychological stresses [16–21].

Available evidence suggests a strong positive correlation between skin health and microbial diversity, where healthy skin harbors more diverse and complex microbial communities compared to pathological states. This matches accumulating research positively correlating bacterial diversity with host protection, involving increased immune system adaptability and flexibility [2,22]. A "balanced", diverse skin microbiota is thus associated with optimal skin barrier function and well-moisturized, calm, even, and radiant skin. Conversely, imbalances (dysbiosis) are associated with skin conditions like eczema, acne, allergies, and sensitive/irritated and dry skin [23,24]. Recent work shows that microbiota disruption can affect communication between the skin and immune system, leading to the emergence of uneven pigmentation [25].

"Biotic" strategies are used in cosmetic products to support a healthy and youthful skin appearance. "Probiotics" refers to the use of live beneficial bacteria—however, current regulations proscribing the presence of such in most cosmetic formulations make this impractical [26]. Microbial metabolites ("postbiotics"), such as *Lactobacillus* fermentation products and yeast ferment extracts, are sometimes used instead.

Cosmetic formulations also use "prebiotics", nutrients including various oligosaccharides [27], to (selectively) encourage the growth of desirable commensal species [27,28]. Prebiotics can contribute to improved skin barrier function, reduced inflammation, and enhanced resilience against environmental stressors, and downregulate pro-inflammatory pathways, reducing redness and irritation associated with conditions like eczema and rosacea [29]. In our opinion, the cosmetic benefits of topical prebiotics remain under-studied.

Pitaya (*Hylocereus* spp.) is an epiphytic cactus, originating from Central and South America. At present, it is very widely cultivated, mainly in sub-tropical/tropical climates, for food, ornamental, medicinal, and other applications [30]. White-fleshed pitaya (*Hylocerus undatus*) is a naturally rich source of prebiotic oligosaccharides. *H. undatus* fruit flesh ethanol-extracted oligosaccharides trigger the growth of gut *Bifidobacteria* and *Lactobacilli* [31]. *H. undatus* oligosaccharides include raffinose, stachyose, maltopentaose, and maltotriose [32]. *H. undatus* also has anti-inflammatory and anti-oxidative potential, possibly due to bioactive compounds including betacyanin, p-coumaric acid, vanillic acid, and gallic acid [33]. Topical application of an *H. undatus* extract stimulated collagen synthesis (evidenced by elevated hydroxyproline and DNA content) and enhanced tissue integrity (reflected in increased tensile strength and epithelialization) in streptozotocin-induced diabetic rats, promoting wound healing [34]. Pitaya has also been used in traditional medicine to promote wound healing [30]. In light of the fruit's composition and its demonstrated effects on microbial growth, and the relationships between wound healing and

the skin microbiota, it is conceivable that some of the fruit's efficacy rests on its effects on that microbiota.

On this background, we present here our investigations into the potential of an *H. undatus* extract to promote skin microbiota diversity and support skin health and appearance.

2. Materials and Methods

2.1. H. undatus Fruit Extract

H. undatus fruit was grown in Israel for Lucas Meyer Cosmetics Ltd. (Kfar Buli Agriculture Ltd., Kfar Bilu, Israel), based on original material identified by Pr. Yosef Mizrahi (Ben-Gurion University of the Negev) and using a sustainable disconnected substrate technique in which the vines grow disconnected from the ground, submerged in inert medium and fed through a micro-irrigation system using recycled wastewater. This allows for a highly sustainable crop with minimal effects on the local land, and optimal use of fertilizers and pest control. Pollination and harvest were performed by hand.

The fruits, harvested in early Fall 2017 (coculture study), 2015 (clinical trial 1), and 2013 (clinical trial 2), were peeled and blended to allow extraction of active components in their own water, thereby reducing additional use of water resources. Solids were separated by centrifugation, after which the supernatant was heated above 100 °C for a minimum of 30 min, to remove protein residues. The resulting mixture was centrifuged again and filtered down to 0.22 µm, followed by the addition of preservatives to prevent microbial contamination. The resulting finished aqueous extract (IBR-Dragon™, Lucas Meyer Cosmetics, Yavne, Israel) was used in the studies described herein.

2.2. In Vitro Competitive Coculture Assay

This study aimed at evaluating the effect of the *H. undatus* fruit extract on the population balance of a model coculture of four bacterial species: *Staphylococcus epidermidis* (ATCC #12228, Manassas, VA, USA); *Staphylococcus hominis* (ATCC #27844); *Staphylococcus aureus* (ATCC #6538); and *Propionibacterium acnes* (ATCC #11827). For the purposes of this study, the former two species represent commensal/beneficial bacterial populations, while the latter two represent detrimental/pathogenic populations. Each strain was initially grown alone in specific media: Tryptone soya broth (Oxoïd CM0129, Thermo Fisher Scientific, Waltham, MA, USA) for *Staphylococcus* species and Brucella broth (Condalab 1223, Madrid, Spain) for *Cutibacterium acnes*.

H. undatus fruit extract was placed in microplates in minimal culture medium at 0.1, 0.5, and 1.0%. Each sample was then contaminated with the four strains, in initial proportions intended to produce an equally balanced population distribution, at about 106 CFU/mL for each species after 3 to 6 h. The plates were incubated at 32 °C for 48 h.

Culture medium samples were collected at 24 and 48 h. Each sample was placed on specific agar for each selected strains (*C. acnes*: Columbia with 5% sheep blood, Oxoid PB5039A; for *Staphylococcus* spp., trypsone soya, Oxoid PO5012A), and incubated for 48 h at 37 °C. After incubation, a CFU count was carried out for each strain. Populations at T24h and T48h and in the absence of product were compared. The analysis was performed in triplicate for each sample. Two controls were included in the study: a minimal medium control (untreated condition, expected to yield slower growth), and an optimal medium control (expected to lead to optimal growth).

2.3. Clinical Trial 1

2.3.1. General Design

The *H. undatus* fruit extract described above was tested in a clinical trial, with the aim of evaluating its effect on the skin microbiota in vivo, together with is benefits on skin resilience and skin pigmentation spots, as well as the skin's inflammation status.

An appropriate gel-cream model formulation containing 1% of the extract and a corresponding placebo product were designed for this study (Table 1).

Table 1. Clinical trial formulations.

Component	% in Active Formula	% in Placebo
Water	84.35	85.35
Butylene glycol	4.00	4.00
Dipropylene glycol	1.00	1.00
Hexylene glycol	1.00	1.00
Polysorbate 20	1.00	1.00
Hydrogenated polydecene	1.50	1.50
Cyclomethicone	4.00	4.00
H. undatus fruit extract	1.00	0.00
Carbomer	0.80	0.80
Triethanolamine	0.70	0.70
Phenoxyethanol	0.40	0.40
Methyl paraben	0.15	0.15
EDTA	0.10	0.10
FD&C red 4	0.000000	0.000003
FD&C yellow 5	0.000000	0.000015
Total	100.00	100.00

The design and execution of this study followed the principles of the Declaration of Helsinki and its amendments, as well as the spirit of Good Clinical Practice Guidelines, and complied with Portugal Law 46/2004 of 19 August 2004. The protocol was approved by an Internal Review Board (opinions 6882/2021 and 6883/2021), as well as by the Ethical Commission of PhD Trials (4 January 2021). Written informed consent was obtained from study participants, who also consented to the consequent use of study photographs. RNEC registration number 178,566 was attributed to this study, following local regulations (EMA/INFARMED, Portugal) for cosmetic clinical trials.

The protocol was built on the basis of a double-blind, placebo-controlled, randomized split-face/body design, where participants applied each product to a particular half-face (left/right) and the forearm on the same side. Application took place twice a day for 28 days, with measurements (including microbiota sampling) at Days 0 (D0), 7 (D7), and 28 (D28).

In total, 33 subjects were included in the final analysis (36 subjects were recruited in total, and 3 dropped out for reasons unrelated to the products). Participants were 30–55 years old and female. From the standpoint of ethnicity, 23 participants who finished the study were described as Caucasian, and 10 as Brazilian (i.e., mixed Caucasian–African ancestry). Fitzpatrick phototypes were, respectively, II–III and IV. Exclusion criteria included the presence of cutaneous marks in the areas of interest, such as extreme pigmentation irregularities, scars, and excessive pilosity; allergy or reactivity to skin care products; history of malignant melanomas, bilateral mastectomy with lymph node removal, or bilateral axillary lymph node removal; immune deficiency or auto-immune disease; treatment for malignancy within 6 months prior to enrollment; current treatment for asthma or diabetes; treatments susceptible of interfering with the study measurements, including anti-aging, anti-wrinkle, or hormonal treatments; plastic surgery; intensive sunlight or UV exposure; systemic disorders (cardiovascular, pulmonary, digestive, neurologic, psychiatric, genital, urinary, hematological, or endocrine); anticipated vaccination during the study, or vaccination within 3 weeks prior; and pregnancy or breast-feeding.

2.3.2. Microbiota Analysis

The skin microbiota was sampled by swabbing. While awaiting analysis, swab samples were kept at −80 °C.

Extraction and purification of DNA were carried out using a DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany, 47016). Cells were lysed using a combination of mechanical (beads) and chemical (detergent) means as provided by the kit. Extracted DNA was then purified on a silica membrane.

16S sequencing: The 16S Ribosomal DNA (rDNA) variable region (V1 to V3) was amplified using primers AGAGTTTGATCCTGGCTCAG (27F) and ATTACCGCGGCTGCTGG (534R) as described in Human Microbiome Project sequencing protocols [35]. An Illumina MiSeq (illuina, San Diego, CA, USA) with paired-end technology was used for amplicon sequencing.

Sequence processing and taxonomic assignment: Fastq files from sequencing were processed to filter, denoise, and merge sequences, remove chimeras, and assign taxonomy by using the Qiime2 software [36] (sequence quality was evaluated with the FASTQC software [37]); sequences were then trimmed and filtered according to sequence quality, before denoising and chimera removal by the DADA2 algorithm [38]; each sequence was then assigned to its Operational Taxonomic Unit (OTU) classification using the Qiime2 classify-sklearn classifier, trained on the SILVA release 138 database [39].

The abundance of each OTU corresponds to the number of associated reads. Taxa abundance was estimated by summing OTU abundances associated with each taxon (from Phylum to Species). The differential abundance of each taxon was analyzed using the Deseq2 R package [40]. A taxon was considered as differentially abundant if a. it was detected in at least three samples in one out of the two compared groups; b. the log2 Fold Change was <-0.5 or >0.5; and c. the non-adjusted p-value was <0.05.

Diversity analysis was performed on denoised and classified Operational Taxonomic Units (OTUs) with Qiime2 diversity core analysis to estimate alpha-diversity indexes for each sample and beta-diversity distances between the different samples.

Faith's phylogenetic diversity index [41,42] (which takes into account OTU phylogenetic proximity) was selected as our index for alpha diversity. Values were compared between groups using the Wilcoxon paired test; the diversity index ratio between two groups was calculated as the median of pair (subject) ratios.

2.3.3. Other Measurements

Red spots and visible spots were evaluated through image analysis (VISIA-CR, Canfield, Parsippany, NJ, USA), respectively, serving as indicators of the extract's effect on the skin's irritation status and pigmentation. Standardized photographic images were obtained under cross-polarized lighting for both hemifaces at D0, D7, and D28. A mask was created for the defined measurement area and applied in subsequent images in order to calculate differences vs. D0. Visia images were also used as the source for general macrophotography records.

Skin redness/irritation states were further evaluated through clinical evaluation by a dermatologist. Grading was carried out on the whole hemiface, along a 5-point scale from Score 0: very red or irritated skin to Score 4: No skin redness/irritation.

Skin sensitivity (or conversely, resilience) was evaluated by Laser Doppler Flowmetry (via skin microcirculation) with histamine stimulation. Histamine is known to induce itching and to increase capillary permeability and vasodilation. This is a useful model to assess cutaneous susceptibility to inflammation and the resilience of the cutaneous barrier, through simultaneous measurement of skin microcirculation. Histamine was dispensed by iontophoresis (30 s at 200 μA, Perilont iontophoresis system, Perimed, Jakobsberg, Sweden). Laser Doppler Flowmetry (LDF) was used to assess skin microcirculation, using a monochromatic low-energy (780 nm) laser (Perilont LDPM PF750/PF5000 + PF 5010 Periflux laser channel, Perimed, Jakobsberg, Sweden), to detect the movement of red blood cells in skin microcirculation (average depth of 1.5 mm, corresponding to the superficial dermal plexus). The results are expressed in arbitrary units (AUs) or BPUs (blood perfusion units). Time elapsed between histamine application and blood flow increase was defined as the reaction onset time. We also evaluated the maximal amplitude of the increase in microcirculation.

2.4. Clinical Trial 2

2.4.1. Clinical Design

A second clinical trial aimed at evaluating the effects of the *H. undatus* fruit extract on indicators of skin health and beauty, including skin barrier function, skin redness, skin radiance, and wrinkling. This trial used the same formulations as described above (Table 1) for clinical trial 1.

The design and execution of the study followed the principles of the Declaration of Helsinki and its amendments, as well as the spirit of Good Clinical Practice Guidelines and Portugal Law 46/2004 (9 August 2004). The protocol received Internal Review Board approval (opinions 1015/15 and 1016/15, 5 January 2015) and that of an Internal Ethical Commission (PhD Trials, opinion nº 028/2014, 29 December 2014). Written informed consent was obtained from study participants, including for subsequent study photograph use.

The study was designed based on a double-blind, placebo-controlled, randomized split-face design, where participants applied each product to a defined half-face (left/right). Application took place twice a day for 28 days, with measurements taken at D0 and D28.

Twenty-six subjects were recruited (distinct from the group recruited for Trial 1 above) and included in the final analysis. Participants were 35–55 years old, female, of Caucasian ethnic background, with Fitzpatrick phototypes II–III. All skin types were represented. Participants were selected for the presence of ageing signs (wrinkling) around the periocular area. Exclusion criteria included the presence of cutaneous marks in the areas of interest, such as extreme pigmentation irregularities, scars, and excessive pilosity; allergy or reactivity to skin care products; any history of malignant melanomas; treatments susceptible of interfering with the study measurements, including anti-aging, anti-wrinkle, or hormonal treatments; plastic surgery; intensive sunlight or UV exposure; and pregnancy or breast-feeding.

2.4.2. Measurements

Trans-Epidermal Water Loss (TEWL), measured using a Tewameter® TM 300 (Courage & Khazaka, Koln, Germany) with a cylindrical probe 10 mm in diameter and 20 mm in height, was used to evaluate skin barrier function.

The effect on skin complexion, radiance, and redness was evaluated through the expression of skin tone as Hunter L,a,b color space coordinates, as measured using a CR-400 Chromameter (Minolta, Tokyo, Japan). Skin redness was evaluated through the a* (red-green axis) coordinate, while skin radiance was evaluated through the L* (luminescence/light–dark axis), and the Individual Typological Angle (ITA) index [43,44], a one-number indicator of skin tone calculated from the L* and b* (blue–yellow axis) values as ITA = $\tan^{-1}[(L8 - 50)/b^*]$. A higher ITA index indicates lighter skin.

Wrinkling was evaluated in the crow's feet area using a PRIMOS 3D (Phase Shifting Rapid In-vivo Measurement of Skin) instrument (GFMesstechnik, Berlin, Germany) with a 40×30-mm evaluation area. Using this instrument, 3D images of the skin topography were obtained through a digital fringe projection using micro mirror displays, and quantified based on observed fringe deflection.

Illustrative photographs were produced using standardized photographic images obtained under normal and cross-polarized lighting at D0 and D28 using a VISIA-CA system (Canfield, Parsippany, NJ, USA) with software v6.

2.5. Statistics

Prism 9 (GraphPad, Boston, MA, USA) was used to analyze the numerical data obtained in our in-vivo studies, using $p = 0.05$ (per generally accepted practice in this field) as the statistical significance threshold. Normality was used to select specific statistical significance tests: when the data showed positive normality, we used the paired Student *t*-test, a parametric test; and when the data showed negative normality, we applied the Wilcoxon test, a non-parametric test.

3. Results

3.1. In-Vitro Competitive Coculture Assay

The *H. undatus* fruit extract was shown to increase bacterial growth overall, to levels comparable to optimal conditions (full medium). *S. hominis* and *S. epidermidis* (beneficial strains) exhibited optimal growth under treatment with the extract. On the other hand, the harmful strains *P. acnes* and *S. aureus* showed strongly reduced preponderance under the same treatment (Figure 1). This indicates that the extract could possess a prebiotic effect, leading to a beneficial rebalancing of the skin microbiota.

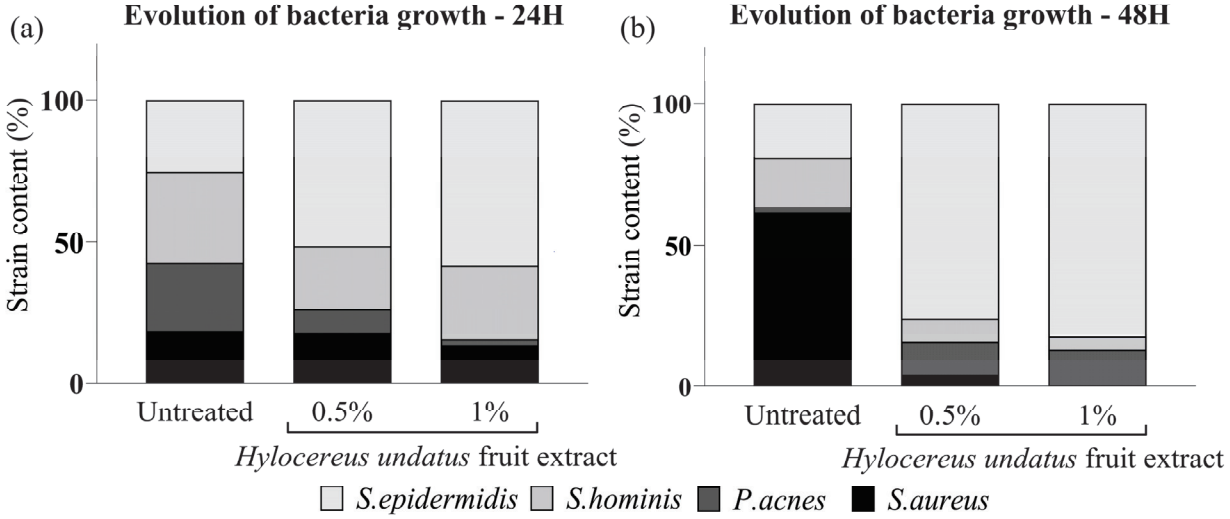


Figure 1. In-vitro competitive coculture assay showing the effect of the *H. undatus* fruit extract on representative commensal and pathogenic bacteria, as mean relative proportion of total population for each strain, at 24 h (**a**) and 48 h (**b**).

3.2. Clinical Trial 1

3.2.1. Microbiota Analysis Results

A total of 11,658 OTUs were identified in the dataset; 6236 of them (53%) were assigned down to the genus level and 4620 (40%) to the species level. Those OTUs were, respectively, assigned to 602 different genera and 998 different species. In all sample groups, the three dominant Phyla were *Actinobacteriota*, with *Cutibacterium* and *Corynebacterium* as the main genera, and *Firmicutes*, with *Staphylococcus* as the main genus. This is considered typical for sebaceous areas of the skin, such as the face [1].

After 28 days, compared to placebo, 1% *H. undatus* fruit extract in cream showed, in the global trial participant population, a 13% improvement in Faith's phylogenetic diversity index ($p < 0.05$) (Figure 2a; this improvement was observed in 67% of volunteers). In the subgroup of volunteers aged 45+ (n = 13), Faith's phylogenetic diversity was improved by 22% ($p < 0.01$) with the active vs. the placebo (Figure 2b; an improvement was observed in 85% of volunteers over 45).

Of particular interest among the sequencing data, *Corynebacterium tuberculostearicum*, a major skin bacterium and pathogen identified in almost all samples (97%), showed significantly reduced abundance (−37%) after 28 days (Figure 3).

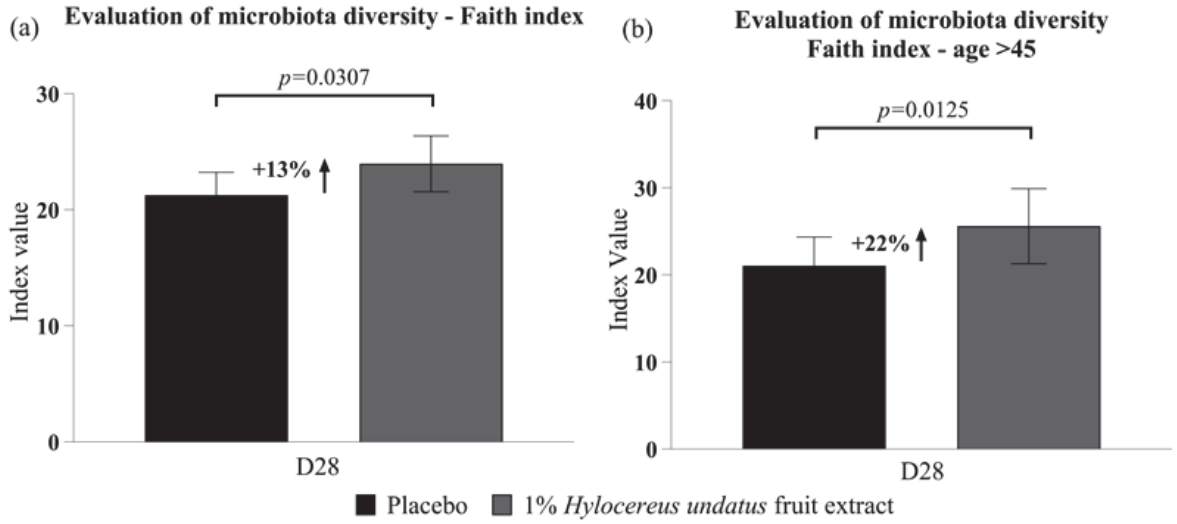


Figure 2. Evaluation of microbiota diversity (mean +/− SEM) according to Faith's index (**a**) in the whole participant group; (**b**) in participants aged over 45 years—showing improvements in phylodiversity with the product containing 1% *H. undatus* fruit extract.

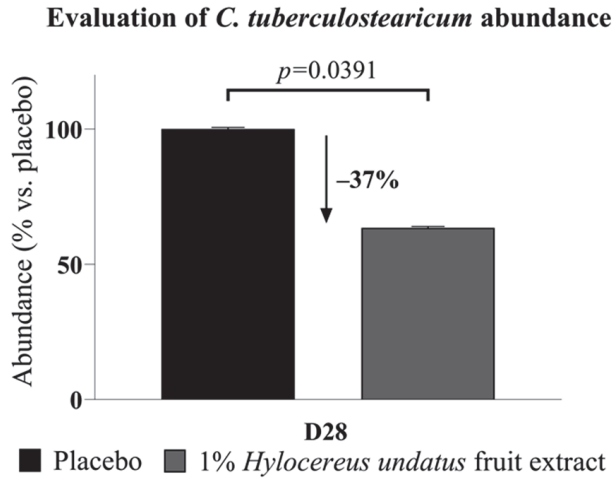


Figure 3. *Corynebacterium tuberculostearicum* abundance, reduced after 28 days' treatment with the product containing 1% *H. undatus* fruit extract (mean +/− SEM).

3.2.2. Effects on Skin Health and Beauty

1% *H. undatus* fruit extract in formula showed strong efficacy on red spot counts when compared to placebo: −35% at D28, and −36% at D7, with statistical significance vs. placebo in both cases (Figure 4a). Compared to D0, the reduction in red spot counts was statistically significant at D28 only (variations with placebo were not statistically significant vs. D0). These data indicate reduced skin inflammation with the active product. This is borne out by the results of clinician grading, which show a small (4%) but statistically significant improvement in redness/irritation grades at D28 vs. placebo and D0 with 1% *H. undatus* fruit extract in formulation (Figure 4b). Figure 4c,d show illustrative Visia photographs, demonstrating the effect on red spots and skin redness.

Upon histamine challenge, microcirculation data (Figure 5) showed a strong advantage for 1% *H. undatus* fruit extract in formula over placebo at D28, both in terms of onset time (time required to reach maximum irritation) and in terms of peak irritation—exhibiting, respectively, a 32% delay in onset time and a 29% drop in maximum amplitude. This effect is statistically significant vs. placebo and D0. At D7, an advantage for the active formulation's effect on onset time is already observable and significant vs. placebo and

D0. The active formulation's advantage in terms of maximum irritation is observable as a trend at D7, but is not yet statistically significant. In this test, irritation was observed as an increase in microcirculation; therefore, a reduction in the peak value and an increase in onset time indicate anti-inflammatory effects.

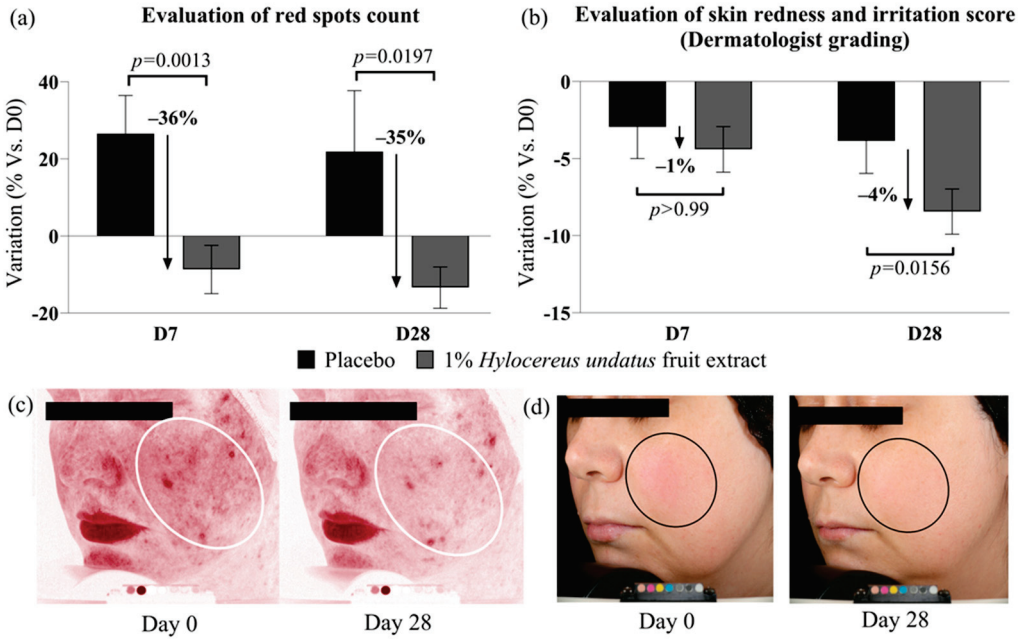


Figure 4. Evaluation of skin inflammation status indicators: red spot count (**a**) and clinical scoring of skin redness and irritation (**b**), both indicating reduced skin inflammation status with the product containing 1% *H. undatus* fruit extract (shown as mean +/− SEM). Illustrative Visia images: (**c**) red spots, volunteer #14 (age 35); (**d**) visible redness, volunteer #36 (age 42). Ovals indicate regions of interest.

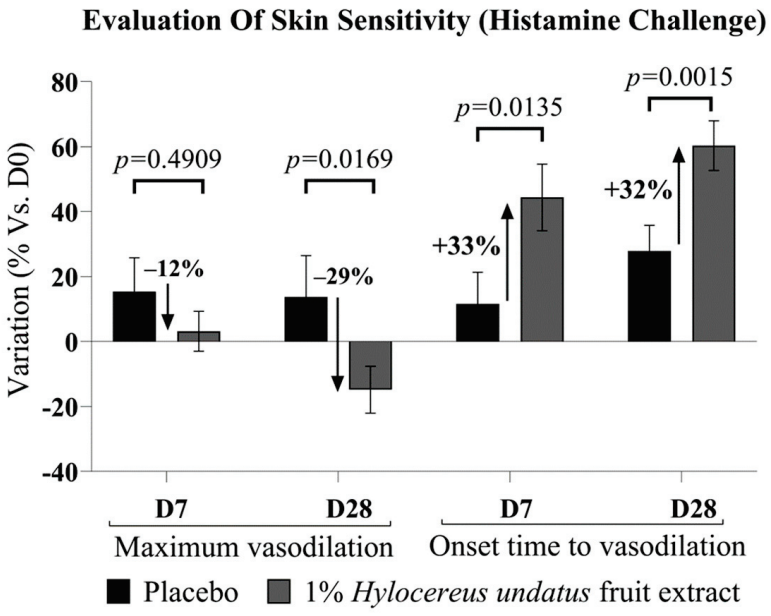


Figure 5. Evaluation of skin sensitivity, as resilience vs. histamine challenge, showing maximum vasodilation amplitude (**left**) and onset time (**right**) (mean +/− SEM). In all cases, improvements are observed, indicating greater skin resilience, with the product containing 1% *H. undatus* fruit extract.

Finally, 1% *H. undatus* fruit extract in formula shows an advantage vs. placebo in visible spot areas at D28 (−19% vs. placebo, with statistical significance vs. placebo) (Figure 6). At D7, a somewhat smaller advantage is already observable, but not yet statistically significant. This result indicates that treatment with the extract reduces uneven skin pigmentation.

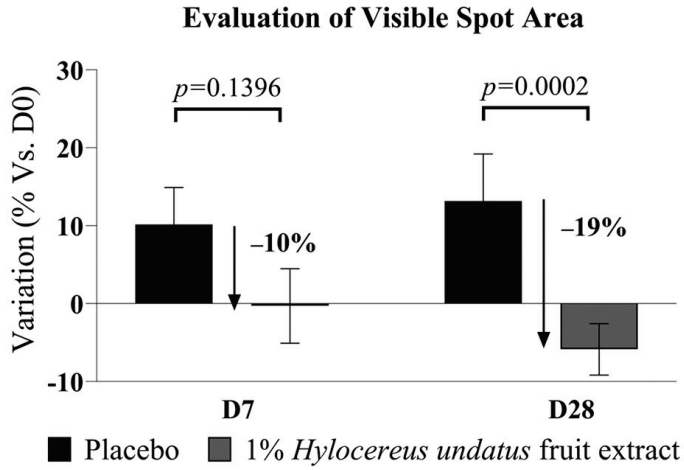


Figure 6. Evaluation of visible spot areas (Visia image analysis, mean +/− SEM), showing reduced visible spotting with the product containing 1% *H. undatus* fruit extract.

3.3. Clinical Trial 2

The Tewameter data (Figure 7) show a significant improvement in skin barrier with 1% *H. undatus* fruit extract in formula, as a reduction in TEWL at D28 (−13% vs. placebo), with statistical significance vs. D0 and vs. placebo—indicating that treatment with the extract reinforces the skin barrier.

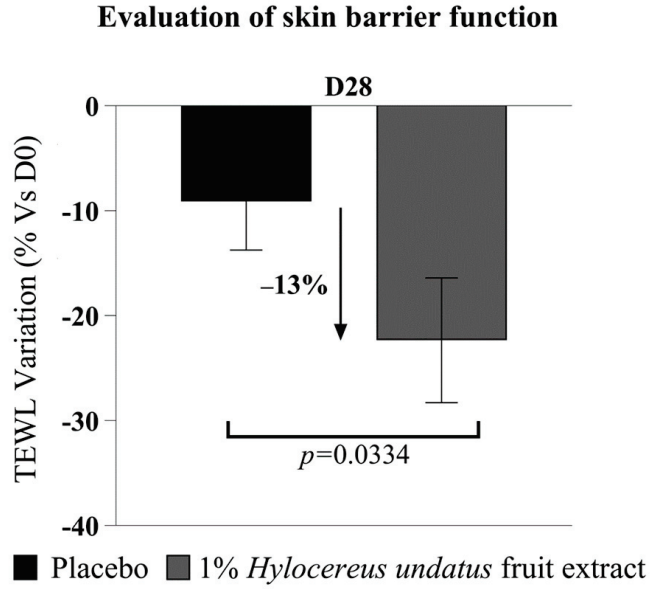


Figure 7. Evaluation of transepidermal water loss (mean +/− SEM), showing enhanced skin barrier function with the product containing 1% *H. undatus* fruit extract.

The chromameter data (Figure 8) show a statistically significant advantage in redness for 1% *H. undatus* fruit extract in formula vs. placebo at 28 days. Both products seem to increase redness slightly, but the increase is smaller with the active product. This indicates that treatment with the extract leads to reduced skin inflammation, reinforcing the indications derived from the results of the first clinical trial described above.

Additionally, the chromameter data at D28 also show modest but significant improvements in L* (luminance) with 1% *H. undatus* fruit extract in formula (+3%, with statistical significance vs. placebo and D0 baseline) (Figure 9, left). The ITA (skin tone index calculated based on L* and b* values) similarly shows a statically significant improvement (+11%, where higher ITA values indicate lighter skin) vs. placebo (statistically significant vs. placebo and D0) (Figure 9, right). This indicates that treatment with the extract has a skin-brightening effect.

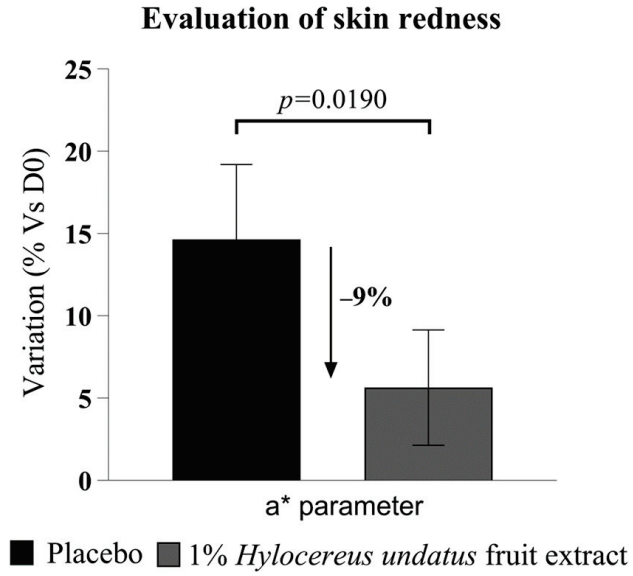


Figure 8. Evaluation of skin redness, as Hunter color space parameter a* (mean +/− SEM), showing reduced skin redness with the product containing 1% *H. undatus* fruit extract.

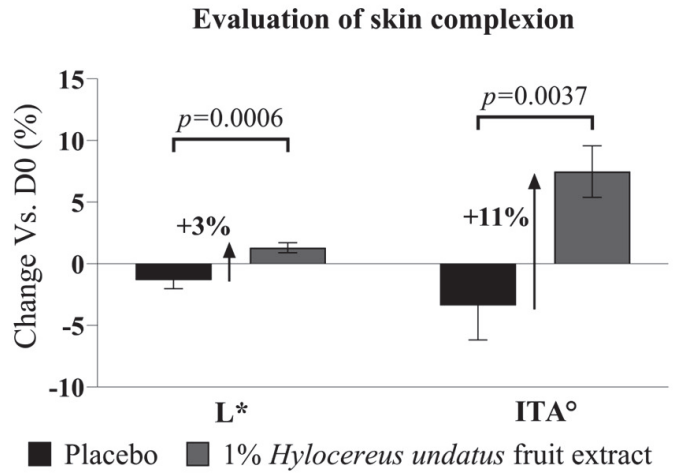


Figure 9. Evaluation of skin complexion, as Hunter color space parameter L* (**left**) and ITA skin tone index (**right**) (both as mean +/− SEM), showing a skin-brightening effect with the product containing 1% *H. undatus* fruit extract.

Finally, PRIMOS data show that after 28 days of treatment with 1% *H. undatus* fruit extract in formula, an anti-aging/anti-wrinkle effect can be observed as a significant reduction in wrinkle count (−11% vs. placebo), as well as lower wrinkle volume and length values (−7% and −5%, respectively vs. placebo), all with statistical significance vs. D0 and placebo (except for wrinkle volume, achieving borderline significance with $p = 0.055$) (Figure 10).

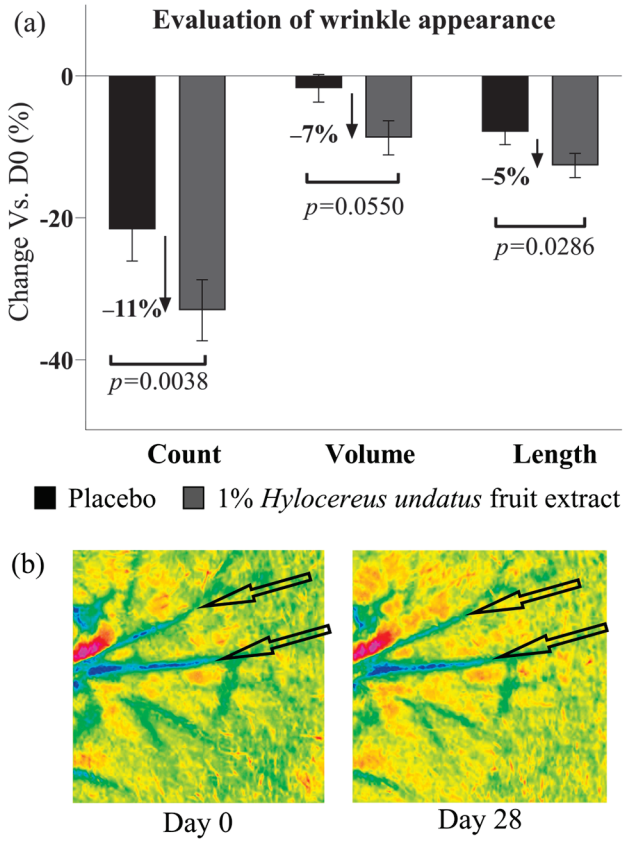


Figure 10. (**a**) Evaluation of skin wrinkles in the crow's feet area (next to the corner of the eyelids) by PRIMOS: wrinkle count (**left**), volume (**center**), and length (**right**) (all shown as mean +/− SEM), showing an ani-wrinkle effect with the product containing 1% *H. undatus* fruit extract. (**b**) Illustrative Primos false-color depth map images, volunteer #16 (age 42). Arrows indicate features of interest.

4. Discussion

As shown above, introduction of the *H. undatus* fruit extract described here into a model bacterial coculture resulted in enhanced growth for the two strains representing common beneficial commensal species (*S. hominis*, *S. Epidermidis*) while severely restricting the relative proportion of the two strains representing detrimental species (*S. aureus*, *C. acnes*). This indicated potential prebiotic activity on the extract's part. In a double-blind, placebo-controlled clinical study, this potential appeared borne out by the enhancement of microbiota diversity observed after 28 days of treatment with a formulation containing 1% of the extract, as evaluated through the Faith biodiversity index. This index is considered of special interest due to the fact that, where other commonly used indices may consider more limited factors, for example only the number of taxons recorded, the Faith index takes into account the phylogenetic distance between the various taxons. In other words, instead of simply recording how many different bacterial species were observed, Faith's index reflects how different the species recorded are from one another.

Of special interest was the observation that this enhancement was greater in older volunteers. While the correlations between aging and microbiota diversity and balance are not fully understood, recent research shows, perhaps counterintuitively, higher diversity in older age [18,45,46]. This could be due to accumulated exposure (seeding) to a consequentially accumulating variety of different microbial species over a lifetime. Age-related changes in host immune function could also play a part. Regardless, it is clear we do not yet understand everything there is to understand about the relationship between age/aging and the skin microbiota. It is even conceivable that the effects of the microbiota on health and beauty could be more strongly related to specific elements in the microbiota balance

than to diversity itself. In light of this state of affairs, it may be particularly valuable to focus more future research on correlations between skin health or beauty elements and particular microbial populations or balance components, in order to shed more specific light on the relationships between skin condition and changes in microbiota composition driven, inter alia, by aging—as well as on the ultimate value of diversity assessments themselves, or conversely perhaps to enable the definition of a more discriminating, weighted diversity index geared towards specifically evaluating the microbiota for health- and beauty-enabling microbiota diversity and balance elements. Still, the correlation with the end-product skin beauty benefits demonstrated here remains interesting. If nothing else, this suggests that the extract described here could at the least represent a valuable tool to answer specific needs relating to skin health and beauty as well as microbiota health in aging or older populations.

We also showed a direct effect in vivo against at least one skin pathogen, *C. tuberculostearicum*, further reinforcing the indication that the extract exerted an overall positive effect on the skin microbiota balance. *Corynebacterium* is a major genus of skin microbiota that is reported as predominant on moist skin [1]. Some Corynebacterium species, including *C. tuberculostearicum*, are reported as causal agents of axillary odor [47]. It is also reported in the literature as involved in human skin diseases, including tinea pedis [48]. This species is also a major bacterium of the nasal microbiota, and is involved in pathologies there [49,50]. In keratinocytes and cutaneous squamous carcinoma cell cultures, *C. tuberculostearicum* is also reported as triggering inflammation through canonical NF-κB pathway activation, and thus inducing pro-inflammatory cytokine production. Mechanistically, pathogen-associated molecular patterns of *C. tuberculostearicum* could interact with host cells' Toll-Like-Receptors (TLRs), leading to NF-κB translocation in the nucleus and consequent inflammatory gene expression modulation. Furthermore, *C. tuberculostearicum* could upregulate the expression of Toll-Like-Receptor 2, hence amplifying inflammation [51]. This modulation of the abundance of *Corynebacterium tuberculostearicum* with the active vs. placebo could thus correlate with the active's anti-inflammatory effects.

In our clinical studies, 1% of *H. undatus* fruit extract also delivered significant skin health and beauty benefits, compared with placebo, including in terms of skin inflammation status (as shown by a reduction in red spots; improved grading by a trained clinician; and reduced redness as measured by chromametry), as well as resilience to chemical insult (as shown by reduced reactivity to histamine); an amelioration in uneven pigmentation (spots); strengthened skin barrier function (as evaluated via transepidermal water loss); improved skin radiance (also as evaluated by chromametry, expressed as ITA); and reduced signs of aging (wrinkling).

One main limitation of the research described here remains, of course, that it is difficult to establish a direct link between the two types of effects observed: the effects on the skin microbiota, on the one hand, and the effects on skin health and beauty measures on the other. The data indicate that treatment with the extract positively affects both the microbiota balance and the overall condition of the skin. It is possible that these effects are linked, or that they are parallel and unrelated to one another. The data presented here do not provide a definite answer, but they appear to at least suggest a correlation. This is especially so in light of the association of a disturbed microbiota balance with increased skin inflammation, compromised skin barrier, and dry skin inter alia. Our data show that the extract alleviates precisely such phenomena while improving microbiota diversity, which appears suggestive in this context. This is especially so considering the extract's composition, which could provide favorable nutrition for some bacterial species, and the extract's demonstrated prebiotic properties in other contexts. As pointed out by Boxberger et al. [52] in their review of the known effects of pre- and probiotics on the human skin microbiota, scientific data enabling clear conclusions remain "elusive", but the results presented above may represent one more correlative element backing the current models for these effects.

In future studies, it could be valuable to extend the clinical investigations described here to cover more different types of skin and of volunteers, including wider ranges of

age and ethnicities, and possibly attempting to address some specific conditions with a known microbiota-related component, such as acne or atopic dermatitis. It may be valuable to examine the extract's effects on especially dry or oily skin. The effect of the extract on pruritus (itching) may also be interesting in light of recent research showing that *S. aureus* can drive skin itch, leading to skin damage from scratching [53]. It might also be interesting to examine the effect of the extract on wound healing and the formation of visible scar tissue, including parameters such as thickness and pigmentation, seeing as microbial populations can also strongly influence these processes and outcomes. In the context of the findings by Morvan and Vallee, who identified differences in the microbiota composition of highly stressed volunteers compared to a reference population, it may be interesting to see if this extract can correct the effects of high psychological stress in ways similar, or different, from what these authors reported with a different topical treatment [21]. There may also be valuable lessons to be learned from the consideration of other forms of microbial life present in the skin microbiota, such as fungi or viruses, especially phages, in any such future in vitro or in vivo studies with this extract. Finally, it may be interesting to examine synergies between this extract and other substances capable of influencing the skin microbiota, such as the Lysine dendrimer recently described by Leignadier et al. in the context of acne [54].

5. Conclusions

The natural extract of *H. undatus* fruit described here showed effects in preclinical and clinical studies, consistent with a prebiotic activity leading to a beneficial rebalancing of the skin microbiota. In vitro, the extract positively affected the balance of a model bacterial coculture. In vivo, the extract was shown to enhance microbiota diversity, especially in older volunteers, and reduce the prevalence of at least one pathogenic species, *Corynebacterium tuberculostearicum*. Further, beneficial effects of treatment with this extract were observed in vivo, including reduced skin inflammation; increased skin resilience to chemical insult; strengthened skin barrier; increased skin radiance; and reduced signs of aging (wrinkles). The available literature suggests that at least some of these effects are consistent with improved skin microbiota balance and diversity. These results indicate that this extract has prebiotic potential, potentially valuable in cosmetic applications aimed at delivering skin health and beauty benefits through the preservation and/or rebalancing of the skin microbiota.

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Data Availability Statement: Upon reasonable request, the corresponding author may make available the data presented in this study.

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Efficacy and Skin Microbiome Modulation Effects of a Fixed-Concentration Combination of Benzoyl Peroxide 4% Plus Niacinamide 4% in a Film-Forming Cream in Subjects with Mild-to-Moderate Acne: A Non-Sponsored, Prospective, Assessor-Blinded, Pilot Trial

Mario Puviani [1,*] and Klaus Eisendle [2]

[1] Unit of Dermatology and Surgical Dermatology, Sassuolo Hospital, 41049 Sassuolo, Italy
[2] Clinic of Dermatology, Central Hospital of Bolzano, 39100 Bolzano, Italy; klaus.eisendle@sabes.it
* Correspondence: mariopuf@libero.it

Abstract: Benzoyl peroxide (BPO) and niacinamide (Niac) are commonly used alone or in combination with other molecules in the treatment of acne. BPO and Niac in the same product could exert complementary and synergistic effects improving efficacy in acne. Furthermore, Niac could reduce some side effects of BPO, such as skin irritation and erythema. A film-forming cream product containing BPO 4% and Niac 4%, vehiculated in a mixture of polyvinyl alcohol (PVA) and perfluoropolyether (PFPE) has been recently developed (B-N cream). A 28-day, assessor-blinded pilot trial was conducted to evaluate the clinical efficacy, skin tolerability, sebum production, skin redness, and skin microbiome modulation of B-N cream in subjects with mild to moderate acne of the face. Twenty-two adult men and women with mild-to-moderate acne of the face participated in this trial, after their written informed consent. B-N cream was applied once daily in the evening. The use of B-N cream was associated with a statistically significant decrease in acne lesions after treatment in comparison with baseline (non-inflammatory lesions: −40%; inflammatory lesions: −43% and total lesions: −41%). The use of the cream was not associated with a skin barrier function alteration. The skin redness score (−11%) and the sebum production (−42%) were significantly reduced after treatment. The use of B-N cream determined a reduction in the relative abundance of the *Actinobacteria phylum* (*Corynebacterium* and *Propionibacterium*, from 56% to 47%) and a significant increase in the abundance of *Bacteroidetes phylum* (from 3% to 5.2%). In addition, the product was well tolerated. In conclusion, this film-forming, medical device cream was effective in subjects with mild-to-moderate acne, reducing acne lesions and sebum production without altering skin barrier function, offering good skin tolerability. Furthermore, this product exerts positive skin microbiome modulation effects.

Keywords: acne; benzoyl-peroxide; niacinamide; film-forming cream

1. Introduction

Acne vulgaris (AV) is a very common skin inflammatory disease affecting the pilosebaceous unit [1]. It is well known that AV pathogenesis involves an increase in sebum production, follicular hyperkeratinization with sebaceous follicules obstruction, *Propionibacterium acnes* (*Cutibacterium acnes*) proliferation and inflammation [2]. AV is characterized by an alteration in the skin barrier function even in the absence of anti-acne aggressive treatments such as oral or topical retinoids [3]. Commonly, AV is treated using topical products, alone or in combination, such as benzoyl peroxide, retinoids and antibiotics [4]. The clinical success of acne treatments is deeply influenced by adherence or compliance with the therapy. Low skin tolerability and/or low efficacy are the main reasons for the lack of compliance [5,6]. Benzoyl peroxide (BPO) and niacinamide (Niac) are commonly used alone or in combination with other molecules in the treatment of acne. BPO is a very

effective topical product able to express bactericidal activity against *C. acnes* [7]. BPO has also keratolytic effects. However, BPO can have, especially during the initial treatment phases, a low skin tolerability with skin erythema and a negative effect on skin barrier function [8]. During treatment with BPO, the transepidermal water loss can increase up to 80%, demonstrating that BPO can alter skin barrier function. In subjects with acne, the transepidermal water loss is higher in comparison with normal subjects [9] and the application of irritative molecules, like BPO and retinoids, could further increase this parameter [10]. Niacinamide provides potent anti-inflammatory properties without the risk of bacterial resistance and systemic side effects and this molecule could represent an interesting treatment modality for acne vulgaris [11]. Several reviews support that the use of Niac is effective in acne [11,12]. Combination products of BPO (2.5%) and Niac (5%) have been developed [13]. Combining BPO and Niac in the same product could have the advantage of expressing complementary and synergistic effects improving efficacy in acne [13]. Furthermore, Niac could reduce some side effects of BPO, such as skin irritation and erythema. A film-forming cream product using BPO 4% and Niac 4% (B-N cream) has been recently developed. In this medical device, BPO 4% and Niac 4% are vehiculated in a mixture of polyvinyl alcohol (PVA) and perfluoropolyether (PFPE). These two components of the cream after application form a transparent and non-occlusive film on the skin allowing BPO and Niac to act locally. Topical film-forming systems exert emollient or protective effects and facilitate the local action or transdermal penetration of actives [14].

PVA and PFPE are color and odorless film-forming polymers [15]. Due to their inertness and stability, they are generally considered safe and biocompatible. PVA is able to absorb water and therefore when applied to the skin it could act as a moisturizer. Both polymers are also effective transdermal drug delivery systems when used in topical products. PFPE is another kind of special liquid material [16]. It is colorless, tasteless, non-poisonous, hypo-allergenic, and has excellent handling properties, chemical stability, lower viscosity, and excellent film-forming ability, so it is suitable for application in the field of cosmetics. PFPE has been demonstrated to reduce the quantity of sebum on the skin [17]. So far, no clinical and instrumental data on this medical device are available. To evaluate the clinical efficacy, skin tolerability, sebum production, and skin microbiome modulation of B-N cream in subjects with mild to moderate acne of the face, we conducted a 28-day, prospective, assessor-blinded trial.

2. Materials and Methods

2.1. Population and Study Design

Twenty-two adult men (N = 3) and women (N = 19), with a mean age 23 years (range 18–32) participated, after their written informed consent in a 28-day, assessor-blinded trial. B-N cream (a film-forming cream containing BPO 4% and Niac 4% vehiculated in a mixture of polyvinyl alcohol and perfluoropolyether) was applied once daily in the evening. The main inclusion criteria were age between 18 and 40 years old; both females and males with a clinical diagnosis of mild-to-moderate acne affecting the face. The main exclusion criteria were (1) personal history of adverse reactions to the same type of products as the investigational product, (2) under treatment, before the study, able to interfere with the interpretation of the study results, particularly: systemic retinoids (isotretinoin per os) within the 6 months, other systemic anti-acne medication within the 3 months, topical retinoids within the 2 months, other topical anti-acne medication within the month, anti-acne cosmetic products within the 2 weeks, topical or systemic medication with anti-inflammatory or antihistamine products within the 2 weeks, antibiotics within the 2 weeks, medication for malignancy (of any kind) within the 5 years, desensitization treatment within the 6 months. The trial was coordinated by Eurofins Cosmetics & Personal Care Italy S.r.l. (20055 Vimodrone (MI), Italy,). The tested product should be applied at home by the test subjects under the normal conditions of use: (1) application site: face; (2) duration and frequency of use: once a day, for 28 consecutive days; and (3) the Investigational product directions for use were to apply the product in the evening on the

surfaces to be treated distributing uniformly, after thoroughly cleansing the affected areas. The cream should be distributed with clean hands. Regarding the cleansing procedures, the subject was instructed to apply it morning and evening to wet skin, massaging gently letting it act for 1–2 min, then rinse thoroughly.

2.2. Study Outcomes

Clinical efficacy was evaluated by acne lesion count (non-inflammatory lesions, NI-L, Inflammatory lesions, I-L, and total lesions, T-L) by comparing baseline values and values at the end of the treatment period. Skin tolerability was assessed by performing a transepidermal water loss measurement (TEWL expressed in $g/m^2/h$; Tewameter® TM Hex, Courage & Khazaka, Cologne, Germany); skin redness was determined using a dedicated software imaging analysis of the face calculating a skin redness score (Antera® 3D PRO, Miravex, Irland, using a dedicate software for the analysis of the parameters "redness"); and sebum production was evaluated using Sebumeter SM 815 (Courage & Khazaka, Germany). TEWL, sebum production, and skin redness were determined at baseline and after treatment in the same check area. Finally, the determination of the skin microbiota through skin swabs by skin DNA sequencing was performed to analyze the bacterial genes present on the skin and their relative proportions (microbiome). The genomic DNA from the swab heads is extracted using Ion 16S Metagenomics Kit (ThermoFisher Scientific, Waltham, MA, USA). The Ion 16S™ Metagenomics Kit uses two primer pools to amplify seven hypervariable regions of bacterial 16S rRNA. The amplified fragments can then be sequenced on either the Ion S5™ or the Ion PGM™ Systems and analyzed using the Ion16S metagenomics workflow in Ion Reporter™ Software (ThermoFisher Scientific, Waltham, MA, USA). Considering the potential variation of skin microbiota according to the skin area, skin swabs were performed on the right cheek area of each subject. All the efficacy parameters were performed by an investigator unaware of the type of treatment. All the tests were performed by competent investigators and trained and qualified technical staff.

2.3. Statistical Analysis

Statistical analysis was performed using Graph-Pad Statistical Software (Version 10, GraphPad Software, Inc., La Jolla, CA, USA). Continuous variables were expressed as mean ± standard deviation (SD). The primary (evolution of AV lesions count) and secondary outcomes of the study were evaluated using both paired parametric and non-parametric tests (Student's *t*-test and Wilcoxon test, respectively) comparing baseline with end-of-treatment values according to the type of the distribution (normal or not). In relation to the skin microbiome analysis, the bacterial community was investigated by assessing for each experimental time (D0/T0, D28) the average and standard deviation, the variations versus D0/T0 and the distribution of difference (D28-D0/T0) of each bacterial population (Shapiro–Wilk normality test, threshold 1%; Student's *t*-test set at 5%—Wilcoxon test set at 5%). In view of the proof-of-concept nature of the present trial, a formal sample size calculation was not performed. We decided to enroll at least 20 evaluable subjects.

2.4. Ethical Issues

The study was performed in the spirit of (1) the general principles of medical ethics in clinical research coming from the Declaration of Helsinki (June 1964) and its successive amendments, (2) the international recommendations relating to Good Clinical Practices for conducting clinical trials for drugs ICH TOPIC E6 (R2) of November 2016; CPMP/ICH/135/95), and (3) the Directive of the European Parliament and Council 2001/20/EC concerning the harmonization of legislative, statutory and administrative provisions of the member States relating to the application of good clinical practices when conducting clinical trials for drugs for human use—OJ/EC of 01/05/2001. The study did not begin without the approval of the Internal Revision Committee. The Internal Revision Committee gave its opinion on 18 November 2022. The information about the study was

given to each test subject before the start of the study. This information was accessible, understandable, and suitable for each test subject. It was orally given and then in a written specific document. This information was completed, if necessary, by the dermatologist (or the competent person designated) who answered all the questions asked by the test subject.

3. Results

All the subjects concluded the 28-day treatment period. The use of B-N cream was associated with a statistically significant decrease in acne lesions after treatment in comparison with the baseline (NI-L: −40%; I-L: −43% and TL: −41%). Table 1 reports numerical variations of NI-L, I-L and TL from baseline to day 28.

Table 1. Acne lesions evaluated at baseline (T0) and after 28 days (T28) in all the subjects included in the study (n = 22).

Parameter	Time	Mean Score ± SD	% Variation vs. T0	*p*-Value *
Non-inflammatory lesions (comedones + microcysts)	T0	23.18 ± 17.73	−40.39%	<0.05
	T28	13.82 ± 12.71		
Inflammatory lesions (papules + pustules)	T0	10.68 ± 5.62	−43.40%	<0.05
	T28	6.05 ± 3.12		
Total acne lesions	T0	33.86 ± 20.34	−41.34%	<0.05
	T28	19.86 ± 14.24		

* Student's *t*-test, statistically significant variation: $p < 0.05$/non-significant variation: $p > 0.05$.

The use of the cream was not associated with a skin barrier function alteration. At baseline, the TEWL value was 17.3 ± 5 and 18.4 ± 4 at day 28 (difference not significant) (Table 2). The skin redness score was significantly reduced after treatment in comparison with the baseline (54 ± 14 vs. 61 ± 15; representing an 11% reduction; $p < 0.05$). Sebum production was significantly reduced by 42% after treatment (Table 2).

Table 2. Evolution of Transepidermal water loss measurements, sebum production (skin lipids), and skin redness.

Parameter	Time	Mean Score ± SD	% Variation vs. T0	*p*-Value *
Transepidermal water loss ($g/m^2/h$)	T0	17.33 ± 4.95	6.48%	>0.05
	T28	18.45 ± 4.00		
Skin lipids (mg/cm^2)	T0	137.05 ± 66.89	−41.63%	<0.05
	T28	80.00 ± 31.34		
Skin redness (score)	T0	61.23 ± 15.28	−11.14%	<0.05
	T28	54.41 ± 14.23		

* Wilcoxon paired test, statistically significant variation: $p < 0.05$/non-significant variation: $p > 0.05$.

The skin microbiome evaluation demonstrated that the use of B-N cream was associated with a significant reduction in the relative abundance of the *Actinobacteria phylum* (*Corynebacterium* and *Propionibacterium*) which was reduced from 56.6 ± 23.5% at baseline to 47.6 ± 14.5% after treatment. The treatment was also able to increase significantly the abundance of *Bacteroidetes phylum* (from 3.1 ± 3.2% to 5.2 ± 3.5%). No other modification in the skin microbiome was observed (Figure 1).

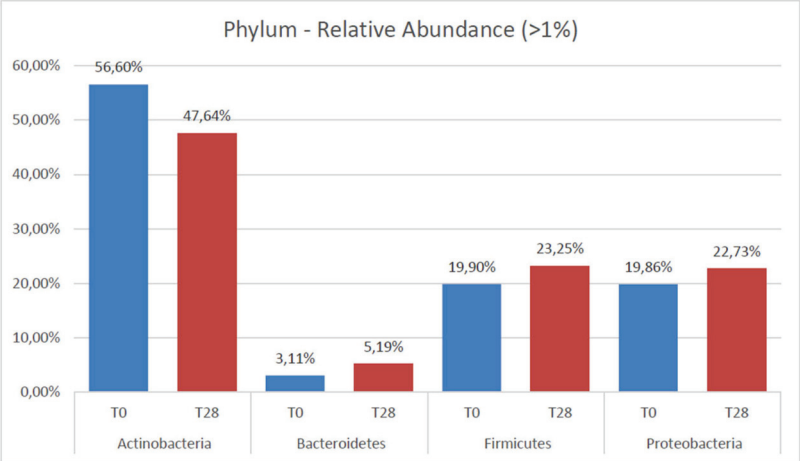


Figure 1. Relative abundance of each phyla detected at a level > 1%, at baseline (T0) and after 28 consecutive days of treatment (T28).

The product was well tolerated. No relevant side effects were recorded.

4. Discussion

In our trial, we demonstrated that a film-forming cream containing BPP 4% and Niacinamide 4% is effective and well tolerated in the treatment of AV. This product does not alter skin barrier function and is also able to reduce skin inflammation. This combination reduces sebum production and can positively modulate the skin microbiome. Benzoyl peroxide is available in various strengths (2.5–20%) and vehicles (gels, lotions, solutions, creams, soap bars, pads, masks, and washes), as well as in combination formulation with the topical antibiotic erythromycin or clindamycin phosphate [18]. It is also combined with the topical retinoid adapalene. BPO is an effective antibacterial compound reducing the risk of selected resistant bacterial strains. However, BPO can alter skin barrier function and cause skin irritation. Part of the mechanism of action of BPO could be ascribed to the formation of reactive oxygen species (ROS) which can exert bactericidal activity but also skin irritation [4,7,19]. Niacinamide is also a common topical product used in different skin inflammatory diseases, such as AV [12]. Topical Niac induced a significant reduction in the sebum excretion rate and decreased the casual sebum levels (sebum on the skin surface) after 4 weeks of treatment [20]. Niac helps protect the natural barrier of the skin and may have a bacteriostatic effect on *C. acnes*. Finally, in vitro data show that Niac decreases the secretion of interleukin-8, a cytokine produced by keratinocytes in response to pathogens (including *C. acnes*) [21]. Niac therefore can exert an anti-inflammatory effect through the inhibition of leukocyte chemotaxis. There is a strong rational to use BPO and Niacinamide in combination due to the fact that these two molecules have different anti-acne mechanisms of action. Furthermore, niacinamide can in part reduce the negative effects of BPO on skin barrier function with the advantage of increasing skin tolerability. It is relevant to point out that in our study the TEWL measured values are in line with published data supporting the fact that TEWL in acne subjects is higher, even before the use of specific products, in comparison with healthy subjects [9]. In addition, quite recently a relevant role of the WNT-βcatenin and AKT/mTORC systems have been demonstrated in the pathogenesis of acne [22,23]. Aberrant Wnt-βcatenin signaling is involved in the formation of comedons. Akt/mTORC is activated in acne subjects and can be involved in hypersebhorrea and comedogenesis [24]. Both BPO and niacinamide have been demonstrated to attenuate Wnt-βcatenin and Akt/mTORC signaling [25]; therefore, the composition of the tested product could be interesting also for this aspect and rationale. This B-N film-forming cream is at the moment the only medical device using an equal concentration (4%) of both products in a film-forming cream formulation. Our trial supports the data that this combination vehiculated with two specific film-forming substances such as polyvinyl alcohol (PVA)

and perfluoropolyether (PFPE) is very effective in reducing acne lesions with good skin tolerability. It is interesting to note that in our study we observed a reduction in acne total lesions of 41%.

The use of BPO as monotherapy in a recent network metanalysis of 221 trials assessing the efficacy of different topical anti-acne products, was associated with a 20% reduction (95% CI: 17–25%). When used in combination with adapalene, BPO reduces total acne lesions by 25%. When used in combination with retinoid and topical antibiotics, BPO can reduce total acne lesion number by 33% (95% CI: 25–41%). However, the fixed combination of BPO and retinoids is associated with a significant odd ratio of discontinuation due to adverse events compared to the placebo of 4.4 [26].

The tested product of this trial was very well tolerated with a reduction in skin irritation and a lack of skin barrier alteration function. Another interesting effect of this medical device was the relevant reduction in sebum production observed after 28 days of treatment (−42%). This effect was not observed so far for BPO. A sebum regulatory effect could be performed by the film-forming components of the tested cream (PFPE or PVA). This latter aspect can further underline the peculiarity of this medical device as an interesting therapeutic approach in subjects with mild to moderate acne. Finally, we observed that the use of this cream was associated with positive modulating effects on the face microbiome, with a significant reduction in the *Acinetobacter* group (to which *C. acnes* belongs to). Recent data have shown that even if a clinical improvement is registered, after oral retinoids treatment or topical BPO, a negative effect on the skin microbiome is observed [27]. In this view, our data can support the concept that B-N film-forming cream could be an interesting first-line treatment in mild to moderate acne but also an interesting coadjuvant treatment strategy in severe forms. Some study limitations should be taken into account by evaluating the study results. This was not a double-blind trial, and there was not a control group. However, the present study was a pilot proof-of-concept trial. The study duration was only 28 days. To assess the full efficacy of an anti-acne regimen, in general 12 weeks is considered the most appropriate treatment period. However, one of the main outcomes of our study was to evaluate the skin tolerability of this combination, and 4 weeks in this regard could be considered sufficient. Further larger prospective controlled studies are warranted to evaluate whether this therapeutic strategy could be helpful in the management of common acne.

5. Conclusions

This film-forming, medical device cream containing BPO 4% and Niacinamide 4% in subjects with mild-to-moderate acne has shown to be effective in reducing acne lesions, and sebum production without altering skin barrier function, offering good skin tolerability. Furthermore, the use of this product was also associated with positive skin microbiome modulation effects.

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Informed Consent Statement: All participants provided written informed consent before starting the study.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy.

Conflicts of Interest: The authors declare no conflicts of interest.

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Commentary

Exosomes in Dermatological Research: Unveiling Their Multifaceted Role in Cellular Communication, Healing, and Disease Modulation

Daniela Pinto *, Giammaria Giuliani, Giorgia Mondadori and Fabio Rinaldi

Human Microbiome Advanced Project, HMAP, 20129 Milan, Italy
* Correspondence: dpinto@giulianipharma.com

Abstract: Exosomes, a subtype of extracellular vehicles (EVs), play a pivotal role in cellular communication and have gained considerable attention in dermatological research. Formed through the inward budding of the endosomal membrane, exosomes facilitate the transfer of proteins, lipids, and nucleic acids, including microRNAs (miRNAs), thereby influencing the behavior and function of recipient cells. These vesicles are secreted by various cell types, including keratinocytes, and are crucial for maintaining skin homeostasis, regulating immune responses, and promoting wound healing. Exosomes have demonstrated therapeutic potential in addressing dermatological conditions such as hair disorders, skin cancers and photoaging through enhanced regeneration and reduced oxidative stress. However, they are also implicated in disease progression, with pathogens utilizing exosome release to evade host immune responses. Recent studies highlight the diverse origins and functions of exosomes, suggesting their promise as innovative therapeutic agents in dermatology. As research continues to elucidate their multifaceted roles, exosomes represent a frontier in understanding intercellular communication and developing novel treatments for skin-related diseases, underscoring their potential impact on both health and clinical applications. This review synthesizes the existing literature on exosome biology and isolation with a focus on their implications in dermatological contexts.

Keywords: exosomes; dermatology; cellular communication; hair growth; wound healing; therapeutic applications

1. Introduction

Exosomes are a specific subtype of EVs (EVs) measuring between 30 and 150 nm in diameter [1,2]. Among the various types of EVs, exosomes have garnered significant interest as a focal point in dermatological research due to their significant role in cellular communication and their potential therapeutic applications.

2. Role of Exosomes in Human Health

Exosomes are formed through the inward budding of the endosomal membrane, leading to the creation of multivesicular bodies (MVBs) that release exosomes into the extracellular space upon fusion with the plasma membrane [3,4].

Exosomes are secreted by a wide range of cell types and are involved in the transfer between cells of proteins, lipids, and nucleic acids, including microRNAs (miRNAs) [5].

miRNAs have the capacity to affect recipient cells by altering gene expression and influencing various cellular activities such as cell growth, programmed cell death, and

movement [6–8]. The resilience of miRNAs within exosomes protects them from degradation, boosting their utility as biomarkers for diagnosing and predicting outcomes in diseases, especially cancer [9,10].

The content of exosomes, especially in terms of miRNA, strongly influences the behavior and function of recipient cells, making exosomes important mediators in both health and disease [11].

First of all, it is widely recognized that exosomes play a role in communication within the immune system and facilitate immune modulation [12].

They are also essential for cell communication within brain tissue [13] and are released by the cardiovascular system for normal function [14].

Interestingly, skin cells, particularly keratinocytes, continuously release exosomes to regulate intercellular communication and maintain homeostasis. Exosome release from the skin system exerts a wide range of functions, such as wound healing [15], regulation of the immune response, modulation of pigmentation [16], control of microbiome balance [17] and responses to oxidative stress [18,19].

Besides their typical beneficial roles, exosomes are also implicated in the pathological development and progression of various diseases [11]. Current research is highlighting that pathogens can exploit exosome release to infect host cells, manipulating these host-derived exosomes to evade immune responses [20].

Exosomes have also been recognized for their pivotal roles in various diseases, particularly concerning abnormalities in production and signaling pathways like Notch and Sonic Hedgehog (SHH) [21].

In neurodegenerative disorders such as Alzheimer's disease (AD), exosomes's role in the production of abnormalities have been linked to disease pathology [22]. In liver diseases such as non-alcoholic fatty liver disease and hepatocellular carcinoma, EVs are significant for disease progression because they carry microRNAs and proteins that modulate cellular responses [22]. This underscores their potential as therapeutic targets and biomarkers, particularly in conditions involving aberrant SHH signaling.

Additionally, exosomes are associated with several other conditions, including heart failure [23] and cancer [24–26].

Indeed, the diverse origins of exosomes contribute to their unique characteristics and therapeutic potential in addressing various dermatological issues [27,28].

Treatment with exosomes has been reported to be effective in skin tumors, especially as a drug carrier [29,30], as well as in wound healing [31–34] and in reducing photo-oxidative stress [35–38], including disorders affecting the scalp and hair follicles [39–42].

Research has shown that exosomes can transport hydrophobic Wnt proteins on their surface, facilitating the activation of β-catenin [43]. This process plays a crucial role in the signaling pathway that regulates hair morphogenesis and regeneration [44].

Exosomes derived from platelet-rich plasma (PRP) have gained interest due to their potential to stimulate hair growth, particularly in conditions like androgenetic alopecia [41,45]. PRP is abundant in growth factors and cytokines essential for maintaining hair follicle health [46]. Recent research indicates that exosomes released from PRP can promote the proliferation and survival of dermal papilla cells (DPCs), which are crucial for hair follicle development and cycling [47–49]. Specifically, these exosomes are known to transport microRNAs and growth factors that can influence signaling pathways such as the Wnt/β-catenin pathway while also antagonizing the inhibitory effect of dihydrotestosterone on HF growth [50,51]. Moreover, the use of PRP-derived exosomes has been linked to increased angiogenesis and an improved vascularization of follicles, thereby enhancing nutrient delivery to support hair growth [52,53] (Figure 1).

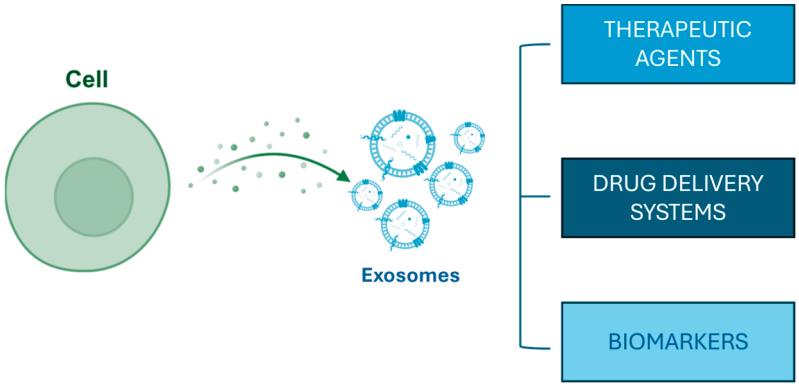


Figure 1. Exosome biogenesis and their potential applications.

3. Types of Exosomes

The origin of human exosomes is notably diverse; studies have demonstrated that exosomes can be released from various biological fluids, including blood, saliva, urine, and breast milk, and they can be produced by a wide range of cells, such as immune cells, epithelial cells, and cancer cells [54].

For example, exosomes sourced from human umbilical cord MSCs have been reported to enhance skin wound healing by promoting angiogenesis and fibroblast activity through miRNA-mediated mechanisms [55,56].

Furthermore, exosomes derived from induced pluripotent stem cells have been found to mitigate skin aging by stimulating collagen production and lowering matrix metalloproteinase activity, which is crucial for maintaining skin elasticity and integrity [57,58].

In consideration of their strong immunomodulatory effects, they are also considered valuable for treating inflammatory skin conditions like psoriasis and atopic dermatitis [59]. Exosomes derived from human umbilical cord mesenchymal stem cells alleviate psoriasis-like skin inflammation [60,61].

Another important source of human exosomes is breast milk. Milk exosomes (MI-Exos) were found to be particularly rich in bioactive molecules, including proteins, lipids, and various types of microRNAs (miRNAs), able to exert diverse biological functions and with potential health benefits [29]. The first study focused on exosomes derived from milk was published in 2007 [62]. MI-Exos possess an extraordinary ability to influence immune responses due to their richness in immune-related miRNAs that can enhance the development of regulatory T cells, which are essential for maintaining immune tolerance and preventing excessive inflammation [63,64]. This is mainly related to their capacity to deliver oligosaccharides to macrophages, thereby modulating the immune response and potentially offering protection against infections, including those caused by adherent–invasive *Escherichia coli* [65]. miR-148a-3p is the most prevalent miRNA found in human breast milk [66].

The immunomodulatory function of breast milk is especially advantageous for infants, aiding in the establishment of a balanced immune system during their early growth [67]. Additionally, studies suggest that miRNAs derived from milk can be absorbed by human intestinal cells, facilitating their bioavailability and their potential to have biological effects within the body [66,68]. Breast milk exosomes have shown protective effects against inflammatory disorders such as necrotizing enterocolitis (NEC), a severe gastrointestinal condition affecting premature infants [69]. Their protective properties also extend to skin health, where the anti-inflammatory effects of breast milk exosomes may alleviate conditions like atopic dermatitis and psoriasis by modulating local immune responses [70].

Significant attention has been given to investigating microRNA and mRNA within exosomes derived from colostrum [71].

Colostrum, the initial milk produced postpartum, is especially abundant in exosomes and has been found to contain a greater concentration of bioactive molecules than mature milk. Studies have shown that these exosomes can enhance the proportion of regulatory T cells, which are crucial for maintaining immune tolerance and preventing allergic reactions in infants [72].

Additionally, human colostrum-derived exosomes have also been found to survive digestion and be absorbed by intestinal cells, allowing them to exert systemic effects, including potential benefits for skin health [73].

The exosome system represents an archaic and highly conserved signaling system among all mammals. Exosomes originating from non-human cells, including those from rodents and other mammals, have been thoroughly investigated for their possible therapeutic uses [74]. MI-Exos have been successfully isolated from the milk of cows, pigs, mice, sheep, kangaroos, and yaks [75–78]. These animals can produce several liters of milk each day, resulting in substantial quantities of exosomes compared to those obtained from cell lines or other biological fluids. Notably, these exosomes exhibit a consistent particle size of around 100 nm, which is particularly advantageous for therapeutic applications.

One of the main features of MI-Exos is their capacity to affect immune responses mainly due to the presence of miRNAs [77,79].

Indeed, miRNAs found in bovine milk, for instance [80,81], are a type of single-stranded noncoding RNA that attach to the 3′ untranslated regions of target genes [82].

Through this mechanism, miRNAs regulate a variety of cellular processes, including cell differentiation, tissue development, and immune responses, which in turn influence various facets of health and disease [83–86].

Exosomal miRNAs exhibit a high degree of sequence homology across different species. The main advantage of MI-exos, as of those derived from breast milk, is their capability to pass through biological barriers, including the intestinal and blood–brain barriers, avoiding immune reactions. This low antigenic potential makes them especially attractive for therapeutic purposes, such as drug delivery systems for small interfering RNAs and other bioactive substances [74,87].

The minimal antigenicity of milk exosomes is linked to their composition, which is similar to that of cell membranes, leading to high biocompatibility and lowered immunogenicity [88].

This feature is essential because it enables the safe delivery of miRNAs to recipient cells without provoking negative immune responses. Therefore, research has shown that miRNAs from Mi-EXOs can easily preserve their integrity and functionality in the gastrointestinal environment, since they are not influenced by low pH and enzymatic breakdown [88,89].

The strong resilience of miRNAs during the digestive process and their capacity to enter systemic circulation highlight the therapeutic promise of milk exosomes as carriers for delivering genetic material and other therapeutic substances [90]. The stability of miRNAs found in Mi-EXOs is another significant factor that boosts their therapeutic promise, enabling them to exert biological effects beyond the gastrointestinal system [91]. These features imply that Mi-EXOs could be utilized for topical treatments, potentially delivering therapeutic miRNAs directly to skin cells to encourage healing and decrease inflammation [75,92].

Research also indicates that exosomes from bovine colostrum can stimulate cell proliferation and boost immune responses, proving beneficial for conditions like intestinal colitis [93,94]. Notably, certain miRNAs present in bovine colostrum exosomes, such as miR-30a-5p, are associated with anti-inflammatory properties, which highlights their potential in the treatment of inflammatory skin disorders [95].

Exosomes can also originate from a variety of non-human sources. In plants, they facilitate the communication between plant cells and the surrounding environment and mediate the stress responses by releasing signaling molecules that help in the adaptation to adverse conditions, such as drought or pathogen attack [96]. Due to their functions, plant exosomes have proven to be useful in both the field of agriculture and human health.

Non-plant exosomes closely mimic human cell structures and pathways, allowing them to encapsulate and transport molecules like proteins, lipids, and RNAs more efficiently within animal and human systems. This results in more precise targeting and a reduced likelihood of immune response. Consequently, animal exosomes are particularly advantageous in areas like drug delivery, disease diagnostics, and regenerative medicine. Therefore, animal exosomes are frequently regarded as superior to plant exosomes for specific medical and therapeutic uses, mainly because of their high biocompatibility [77].

When it comes to isolating plant-derived EVs, the outcomes recorded in different studies often differ based on factors such as the plant source, the isolation method used, the plant's physiological condition, and the composition of the extracted nanoparticles. This variability is largely due to the absence of a standardized isolation protocol [97].

Furthermore, there is a significant gap in knowledge regarding the biogenesis of plant-derived EVs [97,98], and the scarcity of specific protein markers for EVs complicates the biological characterization of these exosomes. More research is needed to identify the surface markers and other defining features of plant exosomes, which remain largely unknown.

Another non-human sources of exosomes are bacteria. In bacteria, exosomes are often referred to as outer membrane vesicles (OMVs) [99,100]. These vesicles are released by Gram-negative bacteria and contain various virulence factors, including toxins and enzymes that can modulate host immune responses [101]. One of the most fascinating discoveries concerning the release of EVs from Gram-positive bacteria is the identification of EVs released by mycobacteria [102,103]. Interestingly, EVs containing mycobacterial materials can be produced by infected host cells, such as macrophages, which serve as the primary host cells for Mycobacterium tuberculosis and other pathogenic mycobacteria [104,105].

4. Exosome Isolation and Quality Control

Eosomes isolation is a critical step in harnessing their therapeutic potential. Given their small size and the complexity of biological fluids, effective methodologies for isolating exosomes must be robust and reproducible to ensure high yield and purity.

The choice of isolation method significantly influences the characterization of exosomal content, with various techniques leading to differences in concentration, purity, and size.

There is currently no established gold standard for isolating exosome, and it seems that new methods are constantly emerging. Ultracentrifugation, particularly in the form of differential centrifugation, continues to be the predominant method for the primary isolation of EVs and is the methodology utilized by over 80% of researchers in the field globally [106]. Unfortunately, this technique presents several limitations, including the simultaneous isolation of non-exosomal contaminants, issues with reproducibility, low yields of RNA, potential harm to the exosomes themselves, and limited throughput, making it less suitable for clinical applications [107].

Also, multiple isolation methods are used (e.g., density gradient followed by ultrafiltration and size-exclusion chromatography) [106].

Zhang and collaborators [108] recently developed a new technique for isolating exosomes using asymmetric-flow field-flow fractionation (AF4), which separates nanoparticles according to their density and hydrodynamic properties.

Research indicates that different isolation methods produce distinct mRNA profiles with unique miRNAs and altered levels of several proteins widely used as "markers" of exosomes, such as tetraspanins [109–112].

Once isolated, electron microscopy is the most widely used technique for verifying the quality of exosome isolation and confirming that the vesicles remain intact [113–115]. Even though effective, this technique is labor-intensive and not well suited for high-throughput analysis of exosome counts. Therefore, the actual number of exosomes can be underestimated due to the potential loss of vesicles during the sample preparation process.

5. Future Perspectives and Conclusions

The safety and tolerability of exosome-based therapies are critical factors in their clinical application. Although many studies have highlighted positive results with minimal side effects, continued research is necessary to develop standardized procedures for isolating, characterizing, and utilizing exosomes [116–118].

Additionally, the mechanisms of exosome uptake and action in target cells remain poorly understood, complicating the development of targeted therapies. Issues related to the large-scale isolation, purification, and characterization of exosomes also hinder their clinical translation. Developing stringent quality control measures is crucial for the consistent production of therapeutic exosomes. Innovations in characterization techniques to assess exosome size, concentration, and biological activity are key to establishing product standards. This will also facilitate the creation of a biorepository with well-defined exosome characteristics that can serve as a valuable resource for ongoing research.

Future research should focus on engineering exosomes for enhanced specificity and therapeutic effect, elucidating their interactions with host cells, and developing robust methods for their production and quality control, thereby addressing these critical gaps in knowledge and application.

Additionally, the regulatory landscape for exosome therapies is intricate; currently, no exosome products have received FDA approval for clinical applications, underscoring the need for further investigation into their long-term safety and effectiveness [107].

The FDA categorizes exosomes as biologics, necessitating thorough regulatory oversight, which includes extensive preclinical data on safety and efficacy prior to clinical trial approval. Although the FDA has issued guidance on cell-based therapies, specific protocols for exosome therapies continue to evolve, highlighting the need for researchers to remain updated on regulatory changes.

In an international context, disparate standards across countries pose challenges for the development and market access of exosome products. To address this, it is crucial to establish harmonized guidelines for the characterization, safety assessment, and clinical evaluation of exosomes. Collaboration with global regulatory agencies, such as the European Medicines Agency (EMA) and the World Health Organization (WHO), is vital to create cohesive evaluation processes that can expedite the transition from research to clinical application and promote broader acceptance of exosome therapies worldwide.

To facilitate clinical translation, it would also be important to improve communication between researchers, product developers, and regulatory bodies to help clarify regulatory pathways and identify challenges early in the development process. Lastly, providing educational resources and training for stakeholders on regulatory standards and best practices will enhance the likelihood of successful market entry for exosome-based products.

In summary, exosomes are emerging as an innovative and promising area for human health, including the dermatological field. Their ability to facilitate intercellular communication and modulate cellular responses makes them highly valuable in regenerative medicine.

Human and milk-derived exosomes show great potential due to their high biocompatibility and resemblance to natural cellular processes within the human body. These exosomes can be tailored to deliver specific therapeutic agents, thereby providing targeted and personalized treatments. Milk-Exos are plentiful, easy to extract, and have demonstrated effectiveness in enhancing gut health and systemic immunity, making them suitable for a broad range of therapeutic applications.

The production process for both human and Milk-Exos is crucial, as it ensures the preparations are pure, functional, and consistent. Implementing standardized protocols and strict quality control measures is essential to creating exosomes that are both safe and effective for clinical use. In vitro studies are crucial for understanding the underlying biological mechanisms and optimizing formulations, while in vivo experiments are necessary to assess therapeutic performance and safety in living organisms.

This comprehensive approach to research and development is vital for transforming the potential of exosomes into practical medical solutions.

Additionally, the characterization of miRNA content within exosomes is of significant importance. miRNAs play a key role in regulating gene expression and influencing cellular behavior, so a detailed profiling of exosomal miRNAs can provide insights into their therapeutic potential. This enables the development of more precise and effective treatments.

As research continues to uncover the mechanisms behind exosome functionality and their clinical applications, exosome-based therapies have the potential to revolutionize the treatment of dermatological conditions, leading to improved patient outcomes and an enhanced quality of life.

To advance this field, focused research on EXs from diverse origins could unveil new, biocompatible options for therapeutic delivery. Additionally, optimizing delivery systems for targeted skin treatments can enhance the efficacy and specificity of exosome-based therapies, ultimately improving clinical outcomes.

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Commentary

Perspectives of Regenerative Medicine in Dermatology and Cosmetology

Enzo Berardesca [1,*] and Norma Cameli [2]

[1] Phillip Frost Department of Dermatology, University of Miami, Miami, FL 33136, USA
[2] San Gallicano Dermatological Institute, 00144 Rome, Italy; norma.cameli@ifo.it
* Correspondence: exb743@med.miami.edu

Abstract: Regenerative medicine has revolutionized the field of biological sciences, pharmacology and healthcare, bringing a new approach to treatments of various health conditions. Dermatology and aesthetic medicine are interested in these new scientific approaches as well. From this point of view, many areas of skin health and cosmetology may be involved, but several questions should be answered before these procedures become routine in offices and hospitals. Indeed scientific, ethical, and financial issues still have to be addressed in detail. Nevertheless, ongoing research and technological advancements are expected to further improve therapies. The integration of artificial intelligence and machine learning could enhance the precision of regenerative treatments, making them more effective and accessible in forthcoming years.

Keywords: platelet-rich plasma (PRP); stem cell therapy; exosome therapy; biochemical cues (Bio-Cues); dermatology; aesthetic medicine

1. Introduction

Regenerative medicine is a rapidly evolving field that holds the promise of revolutionizing healthcare by repairing or replacing damaged tissues and organs. This innovative approach leverages the body's natural healing processes, often through the use of stem cells, tissue engineering, and biomaterials. As we stand on the brink of a new era in medicine, it is crucial to explore both the potential and the challenges that lie ahead.

One of the most exciting aspects of regenerative medicine is its potential to treat conditions that are currently incurable. For instance, stem cell therapy has shown promise in treating neurodegenerative diseases such as Parkinson's and Alzheimer's [1]. Additionally, advancements in tissue engineering could lead to the development of bioengineered organs, reducing the dependency on organ transplants and the associated risks of rejection [2].

Moreover, regenerative medicine can significantly improve the quality of life for patients with chronic conditions. For example, regenerative therapies for cardiovascular diseases aim to repair damaged heart tissue, potentially reducing the need for invasive procedures like heart transplants [3]. Similarly, advancements in cartilage regeneration could offer new hope for patients suffering from osteoarthritis [4].

2. Challenges and Ethical Considerations

Despite its potential, regenerative medicine faces several challenges. One of the primary obstacles is the complexity of translating laboratory research into clinical applications. Ensuring the safety and efficacy of new treatments requires extensive clinical trials, which can be time-consuming and costly [5].

Ethical considerations also play a significant role in the development of regenerative therapies. The use of embryonic stem cells, for instance, has sparked considerable debate due to ethical concerns about the destruction of embryos [6]. Additionally, there are concerns about the long-term effects and potential risks associated with genetic modifications and stem cell therapies [7].

3. The Skin and Regenerative Medicine

Regenerative medicine is transforming the field of dermatology and cosmetology by offering novel treatments for a variety of skin conditions and aesthetic concerns. This approach focuses on harnessing the body's natural healing processes to repair or replace damaged skin tissues, providing new hope for patients with chronic skin conditions, scars, and signs of aging.

The skin shows its regenerative ability mostly during the complex process of wound healing. Following skin trauma, a network of events is launched that involves three steps: the early coagulation and hemostasis phase, the inflammatory phase, and the growth phase [8]. The key applications of regenerative medicine in Dermatology are as follows:

1. Wound healing and scar reduction. Regenerative medicine techniques, such as the use of platelet-rich plasma (PRP) and stem cell therapy, have shown significant promise in enhancing wound healing and reducing scars. PRP, which is rich in growth factors, can accelerate tissue repair and improve the quality of healing [8]. Stem cell therapies, particularly those using mesenchymal stem cells, have been effective in promoting skin regeneration and reducing scar formation [9].
2. Antiaging and skin rejuvenation. The aesthetic applications of regenerative medicine are particularly exciting. Techniques like microneedling combined with PRP, and the use of exosomes, have been shown to improve skin texture, elasticity, and overall appearance [10]. These treatments stimulate collagen production and enhance the skin's natural regenerative capabilities, offering a non-invasive alternative to traditional cosmetic procedures [9].
3. Treatment of chronic skin conditions. Regenerative therapies are also being explored for chronic skin conditions such as psoriasis and vitiligo. By modulating the immune response and promoting tissue regeneration, these treatments can potentially offer long-term relief and improve the quality of life for patients [11].

Regenerative medicine in dermatology encompasses a variety of innovative techniques aimed at repairing and rejuvenating skin tissues. Here are some of the key methods:

Platelet-Rich Plasma (PRP) Therapy: PRP involves extracting a patient's blood, processing it to concentrate the platelets, and then injecting it back into the skin. The growth factors in PRP stimulate cell proliferation, collagen production, and tissue regeneration, making it effective for wound healing, scar reduction, and skin rejuvenation [12].

Stem Cell Therapy: Stem cells, particularly mesenchymal stem cells (MSCs) and adipose-derived stem cells, are used for their ability to differentiate into various cell types and promote tissue repair. These cells can be injected directly into the skin or used in combination with other treatments to enhance skin regeneration and treat conditions like alopecia and chronic wounds [13].

Exosome Therapy: Exosomes are small vesicles secreted by cells that contain proteins, lipids, and genetic material. They play a crucial role in cell communication and can promote tissue repair and regeneration. Exosome therapy is used for skin rejuvenation, reducing inflammation, and enhancing the healing process [14].

Microneedling with PRP: This technique combines microneedling, which creates tiny punctures in the skin to stimulate collagen production, with the application of PRP. The combination enhances the skin's natural healing process, improving texture, tone, and overall appearance [15].

Fat Grafting: Also known as autologous fat transfer, this technique involves harvesting fat from one part of the body, processing it, and injecting it into areas requiring volume restoration or rejuvenation. The stem cells within the fat contribute to tissue regeneration and improved skin quality [16].

Biochemical Cues (Bio-Cues): These are signals that guide cell behavior and tissue regeneration. Bio-Cues can be delivered through scaffolds or hydrogels that provide a supportive environment for cell growth and tissue repair. This approach is used in wound healing and skin regeneration [17].

Skin Organoids: Skin organoids are 3D structures grown from stem cells that mimic the architecture and function of human skin. They are used in research to study skin diseases and test new treatments, potentially leading to personalized regenerative therapies [18].

These techniques represent the forefront of regenerative dermatology, offering new hope for patients with various skin conditions and aesthetic concerns.

While the potential of regenerative medicine in dermatology is vast, several challenges remain. Ensuring the safety and efficacy of these treatments through rigorous clinical trials is essential. Guidelines and standardization of procedures are essential to improve the quality of the output [19]. Additionally, the high cost of regenerative therapies can be a barrier to widespread adoption. Addressing cost barriers for patients seeking regenerative medicine treatments in dermatology involves a multi-faceted approach:

Insurance Coverage: Advocating for the inclusion of regenerative treatments in health insurance plans can significantly reduce out-of-pocket expenses for patients. This requires demonstrating the long-term cost-effectiveness and benefits of these therapies to insurance providers.

Government Funding and Subsidies: Increased government funding for research and development can help lower the costs associated with regenerative medicine. Subsidies or grants for patients undergoing these treatments can also make them more accessible.

Public–Private Partnerships: Collaborations between public institutions and private companies can drive down costs through shared resources and expertise. These partnerships can also facilitate large-scale clinical trials, accelerating the approval and availability of new treatments.

Technological Advancements: Continued innovation in regenerative medicine can lead to more efficient and cost-effective treatment methods. For example, advancements in bioprinting and stem cell technologies can reduce production costs.

Education and Awareness: Educating healthcare providers and patients about the benefits and availability of regenerative treatments can increase demand and drive competition, potentially lowering prices. Awareness campaigns can also highlight financial assistance programs and alternative funding options.

Non-Profit Organizations: Non-profits can play a crucial role by providing financial assistance, raising funds for research, and advocating for policy changes that support affordable access to regenerative treatments.

By addressing these areas, we can work towards making regenerative medicine more affordable and accessible to a broader range of patients.

Regenerative therapies offer several long-term benefits that can significantly improve patient outcomes and quality of life; indeed, regenerative therapies, such as stem cell treatments and platelet-rich plasma (PRP), can accelerate the healing process by promoting tissue regeneration and reducing inflammation. This can lead to faster recovery times and improved outcomes for conditions like chronic wounds, injuries, and skin damage [20]. As a consequence, these therapies can reduce the need for invasive surgeries and procedures. For example, patients with joint injuries or degenerative conditions may benefit from regenerative treatments that restore cartilage and joint function, potentially avoiding joint replacement surgeries [21]. In this light, such therapies, even if they can be expensive initially, may lead to long-term cost savings by reducing the need for ongoing treatments, surgeries, and hospitalizations. This can be particularly beneficial for chronic conditions that require extensive medical care.

Looking forward, ongoing research and technological advancements are expected to further refine these therapies. The integration of artificial intelligence and machine learning could enhance the precision of regenerative treatments, making them more effective and accessible. Artificial intelligence has shown significant potential in optimizing treatment protocols, improving precision in regenerative therapies, and personalizing patient care [22–24]. Regenerative medicine is poised to revolutionize dermatology by offering innovative solutions for wound healing, anti-aging, and the treatment of chronic skin conditions. As research continues to advance, these therapies hold the promise of

significantly improving patient outcomes and transforming the landscape of dermatology and cosmetology.

4. Conclusions

Looking ahead, the future of regenerative medicine appears promising. Ongoing research and technological advancements are continually pushing the boundaries of what is possible. For instance, the development of induced pluripotent stem cells (iPSCs) has opened new avenues for creating patient-specific therapies without the ethical concerns associated with embryonic stem cells [25].

Furthermore, the integration of artificial intelligence and machine learning in regenerative medicine is expected to accelerate the discovery of new treatments and improve the precision of existing therapies [26]. These technologies can help identify optimal conditions for cell growth and differentiation, ultimately enhancing the effectiveness of regenerative treatments.

Regenerative medicine represents a beacon of hope for patients with debilitating conditions, offering the potential to restore function and improve quality of life. However, realizing this potential requires addressing the scientific, ethical, and logistical challenges that currently hinder progress. As we continue to explore and refine these innovative therapies, the future of regenerative medicine looks bright, promising a new era of healing and recovery.

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MDPI AG
Grosspeteranlage 5
4052 Basel
Switzerland
Tel.: +41 61 683 77 34

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