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Special Issue Reprint

# Advances in the Astonishing World of Phytochemicals

## State-of-the-Art for Antioxidants—2nd Edition

Edited by
Antonella D'Anneo and Marianna Lauricella

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# Advances in the Astonishing World of Phytochemicals: State-of-the-Art for Antioxidants—2nd Edition

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Guest Editors

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# About the Editors

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*Editorial*

# Advances in the Astonishing World of Phytochemicals: State-of-the-Art for Antioxidants—2nd Edition

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This Editorial refers to the Special Issue titled "Advances in the Astonishing World of Phytochemicals: State-of-the-Art for Antioxidants—2nd Edition", which highlights the multifaceted properties of natural compounds containing antioxidants and describes the need to understand how active compounds, solvents, and complex formations interact, in order to better establish their potential in applied sciences.

The plant world is a fascinating source of biodiversity and physiologically active substances with antioxidant properties, which help to neutralize free radicals and reduce oxidative stress. Polyphenols are one of the main phytoconstituents, with antioxidant potential found in different parts of plants, including flowers, leaves, fruits, roots, and seeds [1]. Moreover, polyphenols are a diverse group of naturally occurring compounds that play a significant role in protecting plants from environmental stressors and pathogens [2].

Antioxidants derived from plant sources have a wide range of applications across various fields, including health, nutrition, cosmetics, agriculture, and food preservation. These plant-based antioxidants offer natural, sustainable, and often safer alternatives to synthetic chemicals, making them valuable in the development of new products [3,4].

In cosmetics, antioxidants derived from plants are widely used in the preparation of skincare products [5]. Exposure to UV rays and free radicals significantly contributes to skin damage, with the formation of wrinkles and dark spots hastening aging by interfering with defense and restorative processes. The plant world is a rich and diverse source of compounds and nutricosmetics that can play a key role in promoting health and skin vitality, and these have received a great deal of attention from pharmaceutical companies for their anti-free radical potential.

Some common plant-based antioxidants include flavonoids, polyphenols, and vitamins such as Vitamin C and E. In the beauty industry, plant-based ingredients can be used as supplements, nutricosmetics, and cosmetics in the preparation of skincare products with radical scavenging activity in order to create a skin-protective shield from UV rays and free radicals (contribution 1).

Plant-derived compounds also have remarkable uses in agriculture, benefiting both crop production and plant protection due to their antioxidant and antimicrobial properties. Particular interest has been paid to the application of antioxidants in horticulture as plant biostimulants are able to ameliorate stress-associated injury, favor plant growth, increase fruit quality, and reduce toxicity risks. On this topic, research conducted by Vichi et al. (contribution 2) proved that a zinc–caffeine-combined application was able to promote

metabolic processes, enriching the nutritional value of high-quality fruits, as well as improving plant growth. Indeed, the authors evaluated the effect of zinc, caffeine, and a combination of these administered in the irrigation procedures of *Solanum lycopersicum* L. plants. The research provided evidence that the zinc-caffeine combination could activate the phenylpropanoid pathway of tomatoes, not only increasing the content of 4-coumaric acid, caffeic acid, and t-ferulic acid but also enhancing the antioxidant properties of the fruits. The analysis of mineral element content assayed in the tomatoes obtained from *Solanum lycopersicum* L. plants that were cultivated with this procedure also demonstrated the absence of health risks after consumption of these fruits. The study is in line with the strategies set by the Food and Agriculture Organization (FAO) of the United Nations that support sustainable agricultural approaches aiming to increase food production and also reduce environmental impacts.

As reported by del Carmen Villegas-Aguilar in her contribution, plant-derived extracts rich in phenolic compounds possess a different plethora of bioactive properties (contribution 3). Indeed, for some classes of phenolic compounds, exact structure–bioactivity relationship mechanisms still remain unknown. However, analyzing five plant matrices (*Theobroma cacao*, *Hibiscus sabdariffa*, *Silybum marianum*, *Lippia citriodora*, *and Olea europaea*) with different phenolic compositions the authors provided evidence that *T. cacao* and *S. marianum* exerted similar inhibitory effects in the enzymes involved in phlogosis processes, as well as in skin aging, highlighting the fact that shared bioactive properties among phenolic compounds can be identified in different matrices.

Different plant extracts have been studied for their potential to treat cancer, and many of them have shown promising effects. *Ganoderma lucidum*, a mushroom known under different names depending on the country, has been extensively used in traditional medicine, particularly in East Asia, for its health benefits. On this topic, Cadar's review highlights the bio-compounds in *Ganoderma lucidum* that have been shown to have cancer-fighting effects (contribution 4). These bioactive molecules include polysaccharides, triterpenoids, sterols, proteins, nucleotides, fatty acids, vitamins, and minerals. These compounds have demonstrated multiple anticancer effects, namely immunomodulatory, anti-proliferative, cytotoxic, and antioxidant actions. However, there is uncertainty about the effects of *Ganoderma lucidum* bio-compounds in the treatment of different cancers. Such an effect could be ascribed to variations in the types of *Ganoderma lucidum* used, differences in the people studied, or interactions between the mushroom and other treatments. Thus, more rigorous and standardized clinical research is necessary to confirm its therapeutic potential and to better understand the mechanisms underlying its anticancer effects.

Apart from studies on plant-derived extracts, research on the nutritional value and antioxidant potential of fruits has gained particular attention due to the important role that fruits play in human health. On this topic, the nutritional value and antioxidant potential of *Solanum lycopersicum* L. fruits were investigated by Woo Baek et al. (contribution 5), who, in their analysis of the properties of five cherry tomato cultivars (green-colored Jocheong, yellow-colored BN Satnolang, orange-colored Gold Chance, black-colored Black Q, and red-colored Snacktom cherry tomatoes), measured physicochemical parameters and compared the firmness, color values, titratable acidity, brix-to-acid ratio (BAR), and total soluble solids of the fruits. Their analysis showed relevant changes in the BAR, as well as in some metabolite profiles, of the content of some amino acids, thus providing evidence for the different taste among cherry tomato cultivars, with metabolite content related to fruit color. In addition, the cherry tomato cultivars analyzed were shown to possess different amounts of pigment content, such as anthocyanins, chlorophylls, lycopene, and β-carotene contents, which could favorably impact consumers' taste. On the other hand, the presence

of lycopene and β-carotene, as well as chlorophylls, sustained the remarkable antioxidant properties of the fruit. The research proposed by these authors emphasizes the relevance of a cultivar screening program to ameliorate the nutritional profile and promote the inclusion of different tomato varieties in order to obtain additive or synergistic effects in secondary metabolite combinations.

Another functional food is *Citrus*, which possesses healthy properties due to the presence of carotenoids, a large family of isoprenoid pigments with antioxidant functions [6]. The chemical profiles of carotenoids change in different cultivars. In rare cases, *Citrus* fruits accumulate lycopene, a potent antioxidant carotene, which confers major nutritional and nutraceutical value to the fruits. Lycopene is present in red-orange fruits, whereas it is absent in orange fruits. Characterizing new red-orange cultivars could constitute a strategy for the citrus industry to diversify the color range and enhance the nutritional value of orange juice. In their study, Carmona et al. (contribution 6) characterized the fruit quality and biochemical features of two new red-fleshed sweet orange varieties, Carrancas and Pinhal, both originating in Sao Paulo (Brazil). In both of these cultivars, the authors demonstrated a high accumulation of lycopene and β-carotene carotenoids, thus highlighting their possible addition to the juice of popular blond-orange fruits.

*Theobroma cacao* L., commonly known as cocoa, has played an important role in human culture and diet for thousands of years. Sitarek's paper highlights how it also possesses therapeutic potential, relying on its anti-inflammatory and anticancer properties, which come from its polyphenol, flavonoid, and alkaloid content (contribution 7). *Theobroma cacao* L. extracts have been shown to have significant effects in cancer treatment, such as slowing tumor growth, inducing cancer cell death, and possibly preventing metastasis in various cancer cell lines and animal models. Moreover, cacao extracts demonstrated potential synergistic effects when combined with chemotherapy drugs to improve their effectiveness and reduce the organ toxicity caused by chemotherapy. Research into combining cacao extracts with traditional treatments could lead to new possibilities in cancer therapy and the treatment of chronic inflammatory diseases. Furthermore, combining cacao extracts with nanotechnology could be a promising direction for research in the future, as nanoparticles can improve the bioavailability, target delivery, and controlled release of active compounds. Future studies should focus on optimizing nanoparticle-based systems, examining their safety, and testing their long-term effectiveness for different treatments.

The importance and possible impact of antioxidants have been highlighted, specifically for when an imbalance between the body's natural defense systems and the generation of reactive oxygen species occurs, which promotes the development of chronic illnesses associated with oxidative stress, such as cancer, cardiovascular diseases, and neurodegenerative diseases (contribution 8). For these reasons, the potential of antioxidants has attracted the interest of researchers in nanotechnology, thus offering cutting-edge strategies to enhance drug delivery and promising revolutionary advancements in healthcare (contribution 8). In such a direction, green nanotechnology not only allows for the development of nanoparticle-based strategies to protect flavonoids from degradation in the gastrointestinal tract, but it also makes the creation of controlled release systems possible, maintaining these molecules in the bloodstream for prolonged periods in order to exert their proper therapeutic potential.

Natural antioxidants may play a significant role in managing rheumatoid arthritis (RA), a systemic autoimmune disease primarily affecting the synovial joints and defined by concomitant systemic inflammation and autoantibody production [7]. Although the availability of biological immunosuppressive and immunomodulatory agents alone or in association with nonsteroidal anti-inflammatory drugs or glucocorticoids has significantly

improved the course of the disease in patients with RA, patients often do not adequately respond to current treatment regimens due to tolerance development or severe side effects. Thus, there is an urgent need for therapeutic management using "safe" molecules. Olive oil, especially extra virgin olive oil (EVOO), is renowned for its numerous health benefits, largely attributed to its rich composition of monounsaturated fats and bioactive compounds such as phenolic compounds [8]. A study by Tamburini et al. (contribution 9) highlights the anti-inflammatory and antioxidant effects of extra virgin olive oil polyphenol-enriched extracts (PE-EVOOs) on an RA model. In their study, the authors demonstrated how the treatment of peripheral mononuclear cells (PBMCs) obtained from RA patients with PE-EVOOs resulted in a reduction in intracellular reactive oxygen species (ROS) and pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 β (IL-1β). These effects seem to be related to the inhibition of the phosphorylated and active forms of the inflammatory transcription factor NF-κB, as well as to an upregulation of the transcription factor nuclear factor erythroid-2-related factor 2 (Nrf2) and its target antioxidant enzyme catalase and manganese superoxide dismutase (MnSOD). Collectively, these results suggest a possible use of PE-EVOOs as potential adjuvants for the treatment of RA.

Antioxidant potential has also been recognized in olive leaves, the main byproducts generated from olive tree cultivation and processing, due to the presence of phenolic compounds [9]. These are both present in free and bound forms. In their paper, Li et al. (contribution 10) characterizes both free phenolics (FBs) and bound phenolics (BPs) in olive leaves and compares their antioxidant activity in both in vitro and in vivo models. While FBs exhibited significantly higher antioxidant activity than BPs in chemical antioxidant assays (DPPH, ABTS, and FRAP assays), similar antioxidant effects were observed for FBs and BPs in biological systems (cellular and in vivo mouse models). The authors demonstrated that both FPs and BPs protected HepG2 cells from $H_2O_2$-induced oxidative injury and were effective in mitigating oxidative damage by restoring SOD, CAT, and GSH-Px activities in aging mice. FPs exert their antioxidant activity partly via the activation of the Nrf2 signaling pathway; regarding BP mechanisms, the overall scenario seems to be unclear, and further studies are needed.

Indeed, beyond fruits, the plant kingdom offers a vast array of plants with healthy properties. However, the limited solubility of most of their compounds limits their potential clinical application [10]. To improve the bioavailability of nutraceuticals, the pharmaceutical industry has attempted to encapsulate them in β-cyclodextrin complexes [11]. *Inula sarana* is a plant of the Asteraceae family, which is largely spread in Asia, Europe, and Africa [12]. It exhibits antioxidant, anti-inflammatory, and anticancer properties due to the presence of different phytocompounds such as polyphenols, diterpenoids, and flavonoids [13]. Zengin et al.'s study aimed to compare the different compositions and properties of *Inula sarana* extracts in different solvents, including n-hesane, ethyl acetate, dichloromethane, 70% ethanol, and water (contribution 11). The properties of the different extracts were also compared with those incorporated into β-cyclodextrin. The authors demonstrated that water and 70% ethanol extracts had the highest phenolic content and highest antioxidant properties among the extracts analyzed. Additionally, ethanol and hexane extracts displayed the highest inhibition levels out of the different enzymes analyzed, while ethyl acetate extracts contained high levels of flavonoids. Notably, the inclusion complex displayed relatively little or no antioxidant efficacy and enzyme inhibitory potential compared to pure extracts.

Following the rigorous *Antioxidants* review process, eleven papers (seven manuscripts and four reviews in all) were accepted for the publication in this Special Issue. All of these contributions are listed below.

**Author Contributions:** Conceptualization, M.L. and A.D.; writing—original draft preparation, A.D. and M.L.; review and editing A.D. and M.L. All authors have read and agreed to the published version of the manuscript.

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*Review*

# The Potential of Plant Extracts Used in Cosmetic Product Applications—Antioxidants Delivery and Mechanism of Actions

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**Abstract:** Natural ingredients have been used in skincare products for thousands of years. The current focus is on novel natural bioactivities that shield the skin from UV rays and free radicals, among other damaging elements, while enhancing skin health. Free radicals significantly contribute to skin damage and hasten ageing by interfering with defence and restorative processes. Plants contain natural chemicals that can scavenge free radicals and have antioxidant capabilities. Plant materials are becoming increasingly popular as natural antioxidants related to the expanding interest in plant chemistry. This review focuses on the significance of medicinal plants in skin health and ageing and their potential as a source of antioxidant substances such as vitamins, polyphenols, stilbenes, flavonoids, and methylxanthines.

**Keywords:** plant extracts; cosmetics; antioxidants; bioactive compounds

## 1. Introduction

For many years, skincare products have been made with natural components, whether they come from mineral, animal, or plant origins [1–3]. In this century, the use of naturally occurring chemicals continues to increase, probably due to the social media influence. Between 2015 and 2019, the global market for naturally made cosmetics was growing by 10–11% per year. This market also offers a substantial opportunity for the cosmetics industry because many consumers are willing to pay more for these products [4,5]. Plant-based products may be used topically to treat a variety of skin conditions and for skin care. In addition to being more ecologically friendly than traditional cosmetics, cosmetics enhanced with bioactive ingredients are well adapted to the demands of the skin. Plant extracts, which are a rich source of physiologically active chemicals that have a substantial impact on human skin, are a class of natural compounds that are often utilized in cosmetics. We have to emphasize that there are a limited number of studies concerning substances added to food that had an effect on the skin. In some cases, scientific evidence is lacking and the effect is only an assumption.

Many factors, including environmental exposure, gut microbes, stability, activity, and variability in endogenous chemical levels that modulate biotransformation pathways, can influence an organism's metabolism. Antioxidant phytochemicals including vitamins, such as vitamin E, vitamin A, and vitamin C, and polyphenols, tocopherols, and carotenoids have been shown to enhance our aesthetic well-being. These phytochemicals have anti-inflammatory, antioxidant, photoprotective, anti-ageing, antiviral, and antibacterial characteristics. Synergistic stabilizing effects have been demonstrated when combining synthetic and natural antioxidants [6]. Antioxidants include both enzymatic and non-enzymatic compounds [7]. Their distribution is frequently influenced by the various skin cell types.

For example, melanocytes lack antioxidant enzymes [8]. Antioxidants are classified as biopharmaceuticals according to their permeability and solubility [9].

According to estimates, there are three different types of antioxidants: low solubility—low permeability, low solubility–high permeability, and high solubility—low permeability (vitamin C is present in cellular fluids and vitamin E is found in cell membranes). When used in topical treatments, water solubility, restricted permeability, and instability are the primary concerns. The instability caused by external stresses (such as air, light, moisture, heat, oxygen, etc.) affects the product's shelf life [6]. Due to their restricted permeability and water solubility [10], they have limited possibilities of entering deeper epidermal layers and reaching the target location. Antioxidant delivery systems must be capable of being absorbed into the food or beverage matrix without affecting the end product's appearance, texture, flavour, or shelf life [11]. Throughout manufacturing, storage, transit, and usage, it must tolerate environmental stresses like thermal processing, exposure to light, dynamic agitation, cooling, freezing, and dehydration [11]. This study aims to underline the physicochemical importance of antioxidant compounds used in cosmetics, considering their delivery and mechanism of action, summarizing the novelty of the results studied in vitro and in vivo.

## 2. Materials and Methods

### *2.1. Search Strategy*

Since most connected papers and themes were published recently, our research included all studies published in PubMed, Scopus, and a manual Google Scholar search. The keywords "antioxidants" AND ("dermatology" OR "inflammation" OR "cosmetics" OR "proliferation") formed the basis of the scientific literature search approach. Significant publications were chosen based on different plants? biological, chemical, and functional characteristics.

### *2.2. Inclusion and Exclusion Criteria*

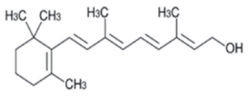
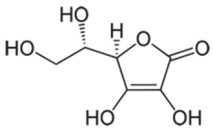
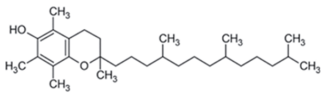
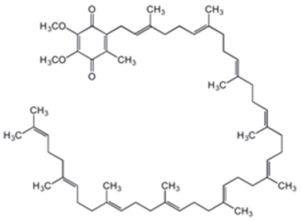
Following criteria, like experimental and review studies, full articles for each selected abstract were retrieved for review. All English-language research articles were included. Investigations were based on in vivo and in vitro research publications. Research publications dealing with specific plant extracts were included. Still, those that considered a combination of plant extracts or a formulation of some other chemical ingredients were excluded. The CAS numbers of the compounds were mentioned in the article, providing valuable information and facilitating the future search for the classification and labelling in the database.

## 3. Results

### *3.1. Vitamins*

Plants produce compounds (phytochemicals) through their secondary metabolism that can protect them from pests, bacteria, and atmospheric pollutants. In both people and animals, some of these compounds (such as polyphenols, cysteine sulphoxides, and carotenoids) can be combined with free radicals to create stable chemical species [12]. Numerous biological effects of phytochemicals which are beneficial to human health include photoprotective, anti-ageing, anti-inflammatory, antibacterial, antiviral, and anticancer activities [12]. Vitamins E, C, and A, for example, have the potential to be antioxidants and have skincare benefits (Table 1). Collagen synthesis is controlled by vitamin C. Free radicals are actively neutralized by vitamin E, which also helps to soften the skin [13]. Stretch marks, burn scars, and new skin cell growth are reduced by vitamin A, which also boosts collagen formation [14,15].

**Table 1.** Molecular structures, IUPAC names and CAS numbers for Vitamin A, Vitamin C, Vitamin E, and Coenzyme Q10.

| Bioactive Compound | Vitamin A | Vitamin C | Vitamin E | Coenzyme Q10 |
|---|---|---|---|---|
| Molecular structure |  |  |  |  |
| IUPAC name | 3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-yl) nona-2,4,6,8-tetraen-1-ol | (5R)-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one | (2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydrochromen-6-ol | 2-[(2E,6E,10E,14E,18E,22E,26E,30E,34E)-3,7,11,15,19,23,27,31,35,39-decamethyltetraconta-2,6,10,14,18,22,26,30,34,38-decaenyl]-5,6-dimethoxy-3-methylcyclohexa-2,5-diene-1,4-dione |
| CAS number | 68-26-8 | 50-81-7 | 59-02-9 | 303-98-0 |

#### 3.1.1. Vitamin A

Beta-carotene (pro-vitamin A), vitamin A and its derivatives, and other ingredients have been used as cosmetic additives. Beta-carotene can be found in foods like tomatoes, carrots, and yellow vegetables, whereas the main animal sources of vitamin A are liver and egg yolk. Beta-carotene and vitamin A were also found to be photoprotective by decreasing the quantity of peroxyl lipid radicals in the skin of mice exposed to UV radiation [16]. However, because beta-carotene is so fragile, other types of vitamin A are frequently included in cosmetic compositions. The capacity of vitamin A (CAS number: 68-26-8) and its derivatives to correct keratinization is the main advantage of these ingredients in the cosmetics industry. Tretinoin (CAS number: 302-79-4), vitamin A alcohol (retinol), vitamin A esters (retinyl palmitate (CAS number: 79-81-2), retinyl acetate (CAS number: 127-47-9)), and vitamin A aldehyde (retinal) (CAS number: 116-31-4) are some of the common vitamin A compounds that can be found in cosmetics. These are present in cosmetic compositions in various concentrations due to their involvement in controlling epithelial cell proliferation and differentiation [17].

#### 3.1.2. Vitamin C

Vitamin C (CAS number: 50-81-7), or ascorbate, is a hydrosoluble vitamin found in vegetables and citrus fruits. Its antioxidant properties and role as a cofactor in collagen hydroxylation events make it an essential nutrient. Since humans cannot produce ascorbate, nutritional intake is crucial. The capacity of vitamin C to immediately quench UV-induced free radicals and replenish vitamin E, another effective antioxidant, contributes to its popularity as a cosmetic element [18]. To maximize UV protection, combining sunscreen with a topical antioxidant is essential. Vitamin C does not absorb UV radiations but protects them by radical scavenging, in contrast to sunscreens, which do not [19]. Under laboratory circumstances, 10% topical vitamin C treatment reduced UVB-induced erythema by 52% and sunburn cell development by 40 to 60% [20]. Due to its capacity to promote collagen formation, vitamin C is also used as a component of anti-ageing products. Ascorbyl palmitate (CAS number: 137-66-6) [21], magnesium ascorbyl phosphate (CAS number: 114040-31-2) [22], and L-ascorbic acid (CAS number: 50-81-7) [23] are the three primary forms of ascorbic acid that are frequently found in cosmetics.

#### 3.1.3. Vitamin E

Vitamin E (CAS number: 59-02-9) is a liposoluble vitamin found in various foods, especially soybeans, nuts, wholemeal flour, and oils [24]. It is claimed that systemically reducing lipid peroxidation has several health advantages for the eyes and cardiovascular system. Numerous dermatological benefits of topically administered substances have been demonstrated. The powerful antioxidant properties of vitamin E serve as the main mechanism of action to support its significance. The "protective" term has been employed to characterise the protective effects of vitamin E and its derivatives due to its ability to scavenge free radicals, specifically lipid peroxyl radicals. Numerous studies have demonstrated their capacity to lessen erythema and edema, sunburn cell development, and lipid peroxidation caused by UV radiation [25]. Reduced skin wrinkling and skin tumour growth have been linked to clinical improvement in the obvious indications of skin ageing [26].

#### 3.1.4. Coenzyme Q10

Coenzyme Q10 (CAS number: 303-98-0) is a botanical food ingredient, and its derivatives are used in functional foods and nutritional supplements. The antioxidant properties of coenzyme Q10 have been correlated with the speed-up in recovery of ATP levels following radiation in human fibroblasts and maintaining the stability of cellular energy levels in human keratinocytes. It prevents the harmful effects of photoaging, minimizes wrinkles, and improves skin smoothness on human skin [27]. It is an internal lipophilic molecule that is essential or useful for mitochondrial strength biotransformation and effective for antioxidants and human health [28]. Another study [29] described the effects of administrations of biological and adjuvant coenzyme Q10 therapy, which showed an association between the Psoriasis Area Severity Index (PASI) and the Dermatology Life Quality Index (DLQI) ($p$ = 0.000132), which means that the daily administration of 100 mg coenzyme Q10 supplements to psoriatic patients for 12 weeks improved the correlation between PASI and DLQI. Coenzyme Q10 inhibited the deterioration of skin viscoelasticity, decreased the depth of microrelief lines (wrinkles), and enhanced the skin's smoothness and fairness [30].

### *3.2. Polyphenols*

Polyphenols are structured by one or more aromatic rings containing one or several hydroxyl groups. Depending on the number of phenolic rings and the elements that make up the structures linking these rings, it is possible to distinguish between various classes, such as phenolic acids, flavonoids, stilbenes and lignans [31].

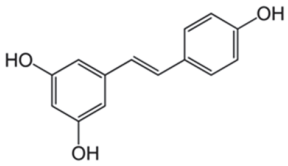
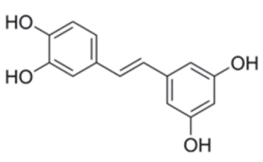
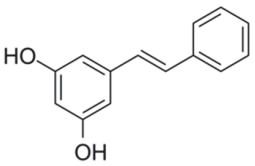
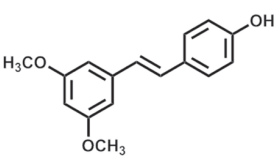
Oral consumption of polyphenols has been related to several health benefits. However, their bioavailability can be limited and is mostly influenced by their chemical structure. The bioavailability is mainly determined by the amount of nutrients ingested, absorbed, and used in metabolic processes [32,33]. The various biological activities of polyphenols reflect the diversity of their structure [34]. They are recognized for their antioxidant, anti-inflammatory, antibacterial, antifungal, antiviral, anti-allergenic, anticancer, and anti-coagulant effects. Plant polyphenols are considered significant for maintaining healthy skin because of their effects on hydration, smoothness, softness, calming, and astringency [35–37]. Collagenase, elastase, and hyaluronidase, which catalyse the degradation of collagen and elastin fibres and hyaluronic acid, respectively, are all skin-specific enzymes that are inhibited by polyphenols. Additionally, they calm inflammation and lessen skin redness while promoting quicker epidermal regeneration, stabilizing capillaries, enhancing microcirculation, increasing skin suppleness, and shielding against damaging environmental factors like UV radiation. Antioxidants have been demonstrated to be associated with a decreased incidence of ROS-induced photoaging [38]. According to Khlebnikov et al. [39], antioxidants are "any substance that directly scavenges reactive oxygen species (ROS) or indirectly acts to upregulate antioxidant defences or inhibit ROS production". The removal of radicals through direct interactions, scavenging, or the reduction of free radicals (such as hydroxyl, superoxide, peroxide, and alcoxyl radicals) to less

reactive molecules is the basis for polyphenols' antioxidant and antiradical effects. Additionally, polyphenols can chelate heavy metal cations (such as $Cu^{2+}$ and $Fe^{2+}$), blocking the Fenton reactions (which result in the production of the highly reactive hydroxyl radical $^{\bullet}OH$) and limiting the activity of numerous free radical-producing enzymes (xanthine oxidase, protein kinase, and lipoxygenase). Other antioxidants, such as ascorbate in the cytosol or tocopherol in biological membranes, are also stimulated and protected due to their activity [40]. Pure polyphenolic substances interact well with other antioxidants to delay skin ageing. An oral antioxidant combination of pycnogenol, evening primrose oil, vitamin C, and vitamin E was studied by Cho at al. [41] for its impact on UVB-induced wrinkle formation. According to the investigation, administering antioxidants to hairless mice exposed to UVB radiation three times per week for 10 weeks dramatically reduced the UVB-induced production of matrix metalloproteinases, mitogen-activated protein kinase, and transcription factor AP-1. In addition, TGF-2 and type I procollagen expression was increased. According to scientific studies, oral treatment with the antioxidant mixture can reduce the appearance of wrinkles by reducing matrix metalloproteinase expression and boosting collagen synthesis [40]. Pomegranates (*Punica granatum*) are a very good source of polyphenols (anthocyanins and hydrolysed tannins) that have beneficial effects on skin conditions [38]. Pomegranate extract has been shown to have photochemoprotective, antioxidant, anti-inflammatory, and anti-proliferative effects. Pomegranate fruit extract has been proven to promote skin colour and restore brightness to skin exposed to UV radiation [42] and minimize UVB-induced oxidative stress and the oxidation of skin proteins [43].

### *3.3. Stilbenes*

The most important stilbenes found in grapes are *cis*- and *trans*-resveratrol (3,5,4′-trihydroxystilbene) (CAS number: 501-36-0), resveratrol-3-O-β-D-glucopyranoside (piceid) (CAS number: 27208-80-6), piceatannol (3,4,3′,5′-tetrahydroxy-trans-stilbene) (CAS number: 10083-24-6) [44] and viniferins which are resveratrol dimers [45]. Research on the anti-carcinogenic, antioxidant, and anti-melanogenesis properties of natural stilbenes against ultraviolet light radiation were performed [46] (Table 2).

**Table 2.** Molecular structures, IUPAC names and CAS numbers for resveratrol, piceatannol, pinosylvin, and pterostilbene.

| Bioactive Compound | Resveratrol | Piceatanol | Pinosylvin | Pterostilbene |
|---|---|---|---|---|
| Molecular structure |  |  |  |  |
| IUPAC name | 5-[(E)-2-(4-hydroxyphenyl)ethenyl] benzene-1,3-diol | 4-[(E)-2-(3,5-dihydroxyphenyl)ethen-1-yl]benzene-1,2-diol | 5-[(1E)-2-phenylethen-1-yl]benzene-1,3-diol | 4-[(E)-2-(3,5-dimethoxyphenyl)ethen-1-yl]phenol |
| CAS number | 501-36-0 | 10083-24-6 | 22139-77-1 | 537-42-8 |

#### 3.3.1. Resveratrol

Resveratrol (CAS number: 501-36-0) is the main stilbene found naturally in grapes [47]; it is noted for its anticancer, antioxidant, anti-inflammatory and cardioprotective properties [48]. According to De Filippis et al. [49], resveratrol has a strong antioxidant activity on molecular targets related to tumour initiation, promotion, and progression [50]. In turn, it is proposed that it can initiate apoptosis (by regulating and modulating the p53 protein responsible for tumour destruction, by depleting levels of Bcl-2 and Bcl-xL anti-apoptotic molecules and by interfering with the process of nuclear transcription moderated by NF-κB

and AP-1 cascades) [51] and reduction of angiogenesis through inhibition of FGF-2 and VEGF, neovascularisation, as well as modulation of several signalling pathways linked to malignant progression or cell survival [52].

Its demonstrated capacity to permeate the skin barrier and anti-ageing properties are the main reasons for its prominence in dermatology and cosmetology. Resveratrol-containing formulations have been shown to promote fibroblast proliferation and raise the content of collagen III. Because of its affinity for the ERα and ERβ estrogen protein receptors, resveratrol helps to stimulate the formation of collagen types I and II. Furthermore, resveratrol also has antioxidant qualities, which means that by lowering the expression of AP-1 and NF-kB proteins and delaying the process of skin photoaging, it may shield cells from oxidative damage brought on by free radicals and UV radiation [53].

#### 3.3.2. Piceatannol

Astringenin, as piceatannol is also known, which is part of *trans*-resveratrol (trans-3,4,3′,5′-tetrahydroxystilbene), can be found naturally in red wine, sugar cane, grapes, berries, peanuts, and white tea [54]. Both resveratrol and piceatannol can induce direct antioxidant effects by scavenging free radicals and protecting proteins from cysteine groups under the effect of oxidative stress. A study described that the miR-181a was significantly downregulated in melanoma cancer tissues compared to their neighbouring ones, and strongly overexpressed in both WM266-4 and A2058 cells treated with piceatannol. Therefore, we propose that the apoptotic impact of piceatannol in melanoma cells may be associated with a high level of miR-181a expression [55].

#### 3.3.3. Pinosylvin

The natural polyphenol known as pinosylvin (3,5-dihydroxy-trans-stilbene) (CAS number: 22139-77-1) is a *trans*-stilbenoid and is found in pine trees, specifically in *Pinus sylvestris* [56]. A study in male mice confirmed the action of pinosylvin in reversing the agonist effect of the transient receptor potential ankyrin 1. High concentrations of pinosylvin (100 μM) showed less effect on the activation of the transient receptor potential ankyrin 1 (TRPA1), thus confirming its anti-inflammatory potency. Also, in the same mice, doses of pinosylvin decreased interleukin-6 levels [57].

#### 3.3.4. Pterostilbene

A natural analogue of resveratrol is pterostilbene (3,5-dimethoxy-4′-hydroxystilbene) (CAS number: 537-42-8), which has greater antioxidant activity than resveratrol and, therefore, has great potential for use in the clinical treatment of various diseases [58]. According to scientific studies [59], it has shown strong chemopreventive properties and beneficial effects of pterostilbene, similar to resveratrol in several in vitro and in vivo studies with different types of cancer.

Pterostilbene is an active apoptotic constituent and can inhibit growth, adhesion, and metastatic growth [60]. These qualities have been reported in various cancer research [61], including breast cancer, pancreatic cancer, stomach cancer, and colon carcinoma [62].

### *3.4. Phenolic Acids*

More than one-third of dietary phenols are phenolic acids. They are naturally occurring in plants as free polyphenols or bound; the latter are linked by ester, ether or acetal bonds [63]. Phenolic acids are made up of a diverse group of chemical substances, where more than 8000 different components can be found that influence human and animal diets. One of their main properties is that they can donate hydrogen molecules or chelate iron and copper ions, preventing low-density lipoproteins from oxidising [64]. They are closely linked to reducing the risk of neurodegenerative diseases, cardiovascular diseases, gastrointestinal [65], colon, breast or ovarian cancer, leukaemia [66], increasing bile secretion, decreasing cholesterol levels, decreasing blood lipid levels, and antimicrobial activities [67].

Phenolic acids can be found in edible vegetables, fruits and nuts suitable for the human diet, with strong anti-diabetic properties, consumption of which reduces the risk of diabetes by regulating the key pathway of carbohydrate metabolism and hepatic glucose homeostasis, including glycolysis, glycogenesis, and gluconeogenesis [68]. Structurally, phenolic acids are derived from the hydroxylation of cinnamic acid [69] or benzoic acid. The phenolic acids most recognized in human foods are caffeic and ferulic acids [70]. Although they are considered direct antioxidants, they also exhibit indirect antioxidant properties by producing endogenous protective enzymes and positive regulatory effects on signalling pathways [71].

According to Drawbridge et al. [72], cereals possess among their phytochemical components phenolic acids that have antioxidant and anti-inflammatory effects. The phenolic acids commonly found in cereals are p-hydroxybenzoic (CAS number: 99-96-7), protocatechuic (CAS number: 99-50-3), vanillic (CAS number: 121-34-6), gallic (CAS number: 149-91-7), syringic (CAS number: 530-57-4), caffeic (CAS number: 331-39-5), p-coumaric (CAS number: 231-000-0), ferulic (CAS number: 1135-24-6), and sinapic acids (CAS number: 530-59-6) (Table 3). Lodovici et al. [73] suggest that daily intakes of hydroxybenzoic and hydroxycinnamic acid range from 11 mg/day to 211 mg/day. In contrast, caffeic acid intake is about 206 mg/day in subjects who consume coffee. In another study, the presence of the gentisic and ferulic acids were reported in the roots of *Brassica rapa* ssp. Pekinensis [74]. The concentrations of these compounds were 0.68 mg/g and 0.56 mg/g after elicitation with copper nanoparticles.

**Table 3.** Molecular structures, IUPAC names and CAS numbers for p-hydroxybenzoic, protocatechuic, vanillic, gallic, syringic, caffeic, p-coumaric, ferulic and sinapic acids.

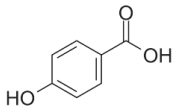
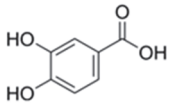
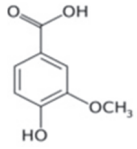
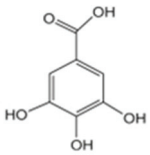
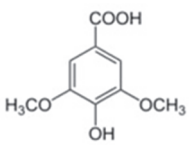
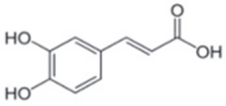
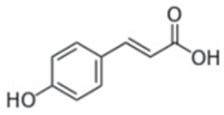
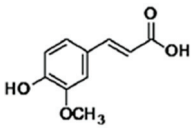
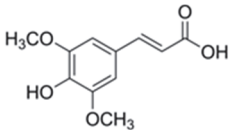
| Bioactive Compound | Molecular Structure | IUPAC Name | CAS Number |
|---|---|---|---|
| P-hydroxybenzoic acid |  | 4-hydroxybenzoic acid | 99-96-7 |
| Protocatechuic acid |  | 3,4-dihydroxybenzoic acid | 99-50-3 |
| Vanillic acid |  | 4-hydroxy-3-methoxybenzoic acid | 121-34-6 |
| Gallic acid |  | 3,4,5-trihydroxybenzoic acid | 149-91-7 |
| Syringic acid |  | 4-hydroxy-3,5-dimethoxybenzoic acid | 530-57-4 |
| Caffeic acid |  | (2E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid | 331-39-5 |

**Table 3.** *Cont.*

| Bioactive Compound | Molecular Structure | IUPAC Name | CAS Number |
|---|---|---|---|
| P-coumaric acid |  | (2E)-3-(4-hydroxyphenyl)prop-2-enoic acid | 501-98-4 |
| Ferulic acid |  | (2E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoic acid | 537-98-4 |
| Sinapic acid |  | 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid | 530-59-6 |

Okafor et al. [75] reported a range of hydroxybenzoic acids in different Bambara groundnut (*Vigna subterranean*) varieties, where 4-hydroxybenzoic acid (p-hydroxybenzoic acid), 2,6-dimethoxybenzoic acid, protocatechuic acid, caffeic acid, and ferulic acid were found in the highest quantity [76].

### *3.5. Flavonoids*

The chemical composition of flavonoids is 2-phenyl-benzo-a-pyrones. In their natural mode, it is possible to find various patterns in the composition of the two benzene rings that form the basic structure of this compound [77]. Depending on the connection between the rings and the ring structures, in addition to the various hydroxylation and glycosylation patterns, flavonoids can be classified into different subclasses as the following: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins (Table 4) [78,79].

**Table 4.** Molecular structures for flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins.

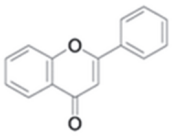
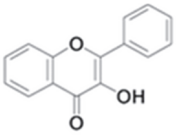
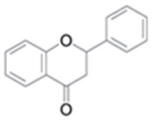
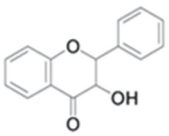
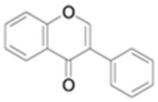
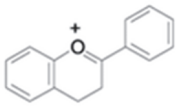
| Bioactive Compound | Molecular Structure |
|---|---|
| Flavones |  |
| Flavonols |  |
| Flavanols |  |
| Flavanones |  |

**Table 4.** *Cont.*

| Bioactive Compound | Molecular Structure |
| --- | --- |
| Isoflavones |  |
| Anthocyanins |  |

3.5.1. Flavones

This is a subclass that features a double bond between the C2 and C3 of the rings, and a ketone at C4 [80], but they are capable of containing other substituents depending on the taxonomical characteristics of the plant. They can be hydroxylated, methylated, glucosylated, or alkylated [81]. Flavones can be both of natural and synthetical origin. In their natural form, they can be found in various foods and plant tissues, such as flowers, fruits, grapes, apples, celery, mint, and tea, among others [82].

An article written by Maher [83] describes the ability to increase performance and working memory in 12–15-month-old mice by intraperitoneal injections of 7,8-dihydroxy-flavone flavone (5 mg/kg) over 10 days.

3.5.2. Flavonols

This subclass of polyphenols is the most diverse in the plant kingdom and possesses strong physiological activity. Flavonols are secondary metabolites present in a wide variety of fruits, vegetables, and plants [84].

According to Nagula and Wairkar [85], human skin is commonly subjected to oxidative stress due to the influence of UV radiations, ozone radiation and other harmful substances. The main characteristics of flavonols include their ability to act as oxidising agents and protection against the formation of reactive oxygen species [85].

On the other hand, Farhadi et al. [86] state that the flavonols with high antimicrobial activity include quercetin (CAS number: 117-39-5), myricetrin (CAS number: 529-44-2), morin (CAS number: 654055-01-3), galangin (CAS number: 548-83-4), entadanin, rutin (CAS number: 153-18-4), and piliostigmol. The authors reported strong antimicrobial activity against *Porphyromonas gingivalis* in an in vitro investigation of some of these flavonols, with quercetin at a concentration of 0.0125 μg/mL showing the best results.

3.5.3. Flavanols

Flavanols are found in significant amounts in various fruits and fruit products, such as juices, red wine, cocoa, and tea, among others. The absorption of flavanols in the human diet is limited, because parts of the fruits such as the hulls or seeds are discarded during processing or ingestion [87].

Gómez-Juaristi et al. [88] investigated the absorption and flavanol metabolism in two different soluble cocoa products, one with high flavanol content and one traditional, where for both a 35% absorption capacity was obtained, demonstrating that they are moderately bioavailable and considerably metabolised by the colonic microbiota.

In contrast, another article written by Geng et al. [89] reported on the antidepressant capacity of the flavanols catechin and epicatechin, originating from *Uncaria rhynchophylla*, which influenced melatonin receptors, by evaluating catechin metabolic pathways in mouse plasma.

3.5.4. Flavanones

Flavanones are formed by a chain saturated by three carbon atoms and one oxygen atom and are constituted especially by naringin and hesperidin glycosides, which are the

main compounds of citrus fruits and citrus peels, with a strong antioxidant and free radical inhibition capacity [90]; they are also found in tomatoes and a few aromatic plants such as mint [91].

Anacleto [92] evaluated the protective capacity of flavanones (naringenin) in pancreatic β-cells under oxidative stress, due to its anti-inflammatory and antioxidant capacities.

#### 3.5.5. Isoflavones

Isoflavones are found entirely in legumes and although they are not steroids, they have structural similarities to estrogens and pseudohormonal properties, which is why they are considered phytoestrogens [93]. Isoflavones can be hydrolysed through the gastrointestinal tract but mainly in the jejunum mediated by the collaboration of the brush border membrane and bacterial β-glucosidases [94], releasing aglycones which are absorbed into the intestinal epithelium [95].

Yonekura-Sakakibara et al. [96] proposed that the initial step in the biosynthesis of isoflavones is through the catalysis of 2-hydroxyisoflavanone synthase; isoflavone synthase transforms liquiritigenin and naringenin into 2-hydroxyisoflavanones, and then through dehydration of these are transformed into isoflavones by the influence of 2-hydroxyisoflavanone dehydratase.

#### 3.5.6. Anthocyanins

The pigments from which plants, flowers and fruits obtain their colours are anthocyanins, carotenoids, and others. The colour depends on the pH and the methylation or acylation of their hydroxyl group rings. Anthocyanins are located in the outer layers of the cells of different fruits such as blueberries, red grapes, raspberries, blackberries, strawberries, and many more. The main anthocyanins investigated by the scientific community are delphinidin, pelargonidin, cyanidin, peonidin, and malvidin [97].

Anthocyanins are widely used in the food industry as colour additives [98]; their positive effects on human health include tumour-growth inhibitors, circulatory system support, anti-inflammatory and antioxidant properties, and immune system support [99].

In general, flavonoids are currently being intensively investigated from a medical point of view for their beneficial properties for human health, such as enzyme inhibition, antimicrobial, anti-allergic, antioxidant, vascular, anti-tumour activity, etc. [100]. Flavonoids through direct inhibition of free radicals can prevent cell damage by forming more stable flavonoid radicals and less reactive free radicals [101].

Chen et al. [102] reported on the effects of lotus plumule flavonoids in alleviating inflammatory symptoms by inhibiting the biosynthesis and production of NO, PGE2 and TNF-α (inflammatory mediators) and proinflammatory cytokines such as IL-1β and IL-6.

AL-Ishaq et al. [103] reported the beneficial effects of flavonoids in the fight against diabetes by influencing carbohydrate digestion, insulin secretion and signalling, fat deposition, and glucose uptake.

#### 3.5.7. Tannins

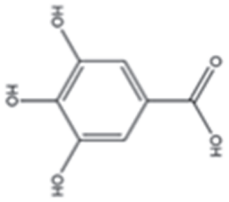
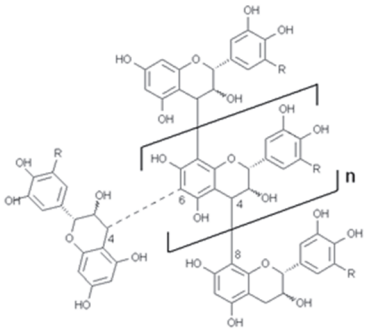
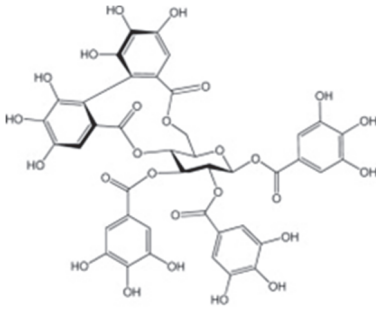
Several reports have shown that natural tannins and compounds distributed by various types of plants have beneficial effects on health by presenting antioxidant, hypoglycaemic, anti-tumoural, antibacterial, and hypoglycaemic properties [104]. Tannins are classified into the following three groups depending on their structure: hydrolysable tannins, condensed tannins, and compound tannins (Table 5) [105].

#### 3.5.8. Condensed Tannins

These types of tannins are considered oligomers or polymers which, depending on the hydroxylation pattern of the A and B rings of their flavan-3-ol units [106,107], are classified into prodelphinidins, procyanidins, and propelargonidins. These types of tannins possess high antioxidant potency by acting through hydrogen atom or single-electron

transfer mechanisms, and are also noted for their anti-inflammatory, antimicrobial and anticarcinogenic properties [108–110].

**Table 5.** Molecular structures for tannins, condensed tannins, and hydrolysable tannins.

| Bioactive Compound | Molecular Structure |
| --- | --- |
| Tannins |  |
| Condensed tannins |  |
| Hydrolysable tannins |  |

An excess of these tannins causes changes in taste and an astringent feel to the food; hence, different methods have been developed to remove excess tannins, which can be done by physical, chemical, and biological means [111].

According to Laddha and Kulkarni [112], one of the most important types of condensed tannins is proanthocyanidin, which can be found in various foods. The same authors report that dark chocolate has a strong composition of catechin and epicatechin, and therefore, has potent antioxidant activity.

### 3.5.9. Hydrolysable Tannins

Hydrolysable tannins contain various monosaccharides (oak, hazelnut, and quebracho) which have a high content of arabinose, glucose, fructose and glucose, but only those from vine bunches and nut galls contain fructose and glucose [113]. Through hydrogen bonds, hydrolysable tannins are able to interact with different cereals, but excess tannins in these products slow down or reduce the digestibility of protein and starch [114,115].

Gallotannin extracts, whose trade name is tannic acid (CAS number: 1401-55-4), depending on the plant source used for extraction are made with mixtures of polygalloylglucose esters or polygalloylquinic acid with a range in the number of galloyl molecules from 2 to 12 [116]. The galloyl units are linked by various polyols, catechins or tri-terpenoid units [117].

#### 3.5.10. Complex Tannins

This subclass of tannins has flavone as its basic unit and is found mainly in legumes, nuts, maize, rice, and tea. It is formed from the combination of an ellagitannin or gall tannin unit and a catechin. Its main positive effects on health include neuroprotective effects [118].

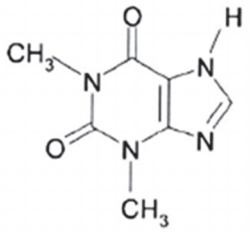
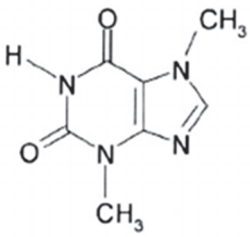
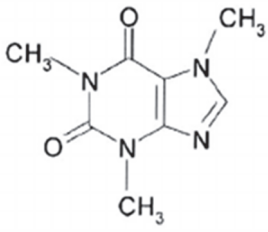
According to Molino et al. [119], several tannins extracted from wood showed positive biological effects in humans and animals, including anti-tumour, antidiabetic, antibacterial, antifungal, and anti-mutagenic properties.

Another article [120] reported the ability of tannins to form tannates, which are stable compounds formed from the binding of tannins to metal ions present in the body; this can be beneficial or harmful to human health, as they can be used to deal with overexposure to heavy metals, but their daily overconsumption can lead to nutrient deficiencies such as calcium and iron, causing osteoporosis and anemia.

### *3.6. Methylxanthines (Theophylline, Caffeine, and Theobromine)*

Methylxanthines are compounds of organic heterocyclic origin that are derived from purine; they are structured by coupled pyrimidinedione and imidazole rings (Table 6) [121] and originate naturally in different products such as coffee, chocolate, tea, soft drinks, mate, and energy drinks, among others [122–124]. The main components of methylxanthines are caffeine (CAS number: 58-08-2), theophylline (CAS number: 58-55-9), and theobromine (CAS number: 83-67-0). Among the main biological functions of methylxanthines are their anti-asthmatic, analgesic, energetic, chronoprotective, anti-inflammatory, antioxidant, and neuroprotective properties [125–128].

**Table 6.** Molecular structures, IUPAC names and CAS numbers for theophylline, theobromine, and caffeine.

| Bioactive Compound | Molecular Structure | IUPAC Name | CAS Number |
|---|---|---|---|
| Theophylline |  | 1,3-dimethyl-7H-purine-2,6-dione | 58-55-9 |
| Theobromine |  | 3,7-dimethyl-3,7-dihydro-1H-purine-2,6-dione | 83-67-0 |
| Caffeine |  | 1,3,7-trimethyl-3,7-dihydro-1H-purine-2,6-dione | 58-08-2 |

#### 3.6.1. Theophylline

Theophylline (1,3-dimethylxanthine) is derived from a methylated xanthine [129,130], the extraction of which is mainly from *Camellia sinensis* L. and *Ilex Paraguariensis*. Its main biological properties are the decrease of metastasis and inflammation and resistance to therapy in cancer cells. According to Pérez-Pérez et al. [131] theophylline inhibits the PI3K pathway which is a cancer activator that promotes metastasis and resistance to treatment; it is also able to inhibit the expression of inflammatory genes by activating the histone deacetylase 2 protein.

#### 3.6.2. Theobromine

Theobromine and theophylline are present in the tea plant, while the former is a precursor of caffeine biosynthesis, the latter is a caffeine biodegrader [132]. Theobromine originates from xanthine methylation, with a strong adenosine receptor antagonist and non-selective phosphodiesterase inhibitory activity, it increases adenosine monophosphate in the nervous system [133,134]; in turn, theobromine exhibits bronchodilator, diuretic, and antitussive effects and influences angiogenesis in tumour growth [135]. According to Ejuh et al. [136], this compound inhibits the crystallization of uric acid with a great capacity for the treatment and clinical prevention of uric acid-influenced nephrolithiasis.

#### 3.6.3. Caffeine

Currently, the most studied and consumed methylxanthine is caffeine, which can be found in various plants such as tea, coffee, cola, and guarana, and various products such as soft drinks, energy drinks, and chocolate, among others [137,138]. Caffeine is capable of forming the natural metabolites theophylline and theobromine [139]. Among its beneficial effects for humans are stimulation of the nervous system, analgesic effects, diuresis, psychomotor enhancement [140], and gastric acid secretion, as well as negative effects such as nausea, anxiety, increased blood pressure, tremors, and nervousness [141,142]. Caffeine increases the capacity for the occurrence of chromosomal mutations and potentiates cytotoxic, mutagenic, and carcinogenic activities in different animal cells [143].

Due to the wide range of studies carried out that are focused on the biological effects of caffeine on different molecular targets, the following stand out: its antagonist activity on adenosine receptors, the inhibitory effects on phosphodiesterases, the sensitization of cannulae sensitive to ryanodine for the release of calcium in the sarcoplasmic and endoplasmic reticulum, as well as its antagonist activity on GABAA receptors [144].

According to an experimental study, caffeine consumption increased blood pressure and heartbeat [145]. These effects of caffeine on blood pressure were more visible from the consumption of 205 mg per day, where the greatest effects can be seen in the elderly, the hypertensive population, and those who have never consumed caffeine [146].

Some examples of the plants and their bioactive compounds are reported in Table 7, with a focus on which biocompounds had the ability to benefit the skin cell culture.

**Table 7.** Examples of in vitro studies of plant extracts and their biocompound effects on skin.

| No | Botanical | Biocompound | Effects on Skin | Cells Type | Ref. |
|---|---|---|---|---|---|
| 1 | *Butyrospermum parkii* | Flavan-3-ols (catechin) | antioxidant, UV-induced skin damage prevention, collagen synthesis activation, matrix metalloproteinases inhibition | HaCaT | [1] |
| 2 | *Glycyrrhiza glabra* (licorice) leaf extract | Isoflavones (wighteone) | antioxidant, UV-induced skin damage prevention, anti-inflammatory, and estrogenic effects | HaCaT | [1] |
| 3 | *Simmondsia chinensis* | Tannins | antioxidant, astringent, wound-healing promotion | HaCaT | [1] |

**Table 7.** *Cont.*

| No | Botanical | Biocompound | Effects on Skin | Cells Type | Ref. |
|---|---|---|---|---|---|
| 4 | *Helianthus annuus* | Hydroxycinnamic acid derivatives (chlorogenic, acid, caffeic acid, ferulic acid) | antioxidant, UV-induced skin damage prevention, MMP inhibition, anti-inflammatory, anti-tyrosinase | HaCaT | [1] |
| 5 | *Theobroma cacao* | Flavan-3-ols | antioxidant, UV-induced skin damage prevention, collagen synthesis activation, MMP inhibition | HaCaT | [1] |
| 6 | *Calendula officinalis* | Flavonols (quercetin, rutin, narcissin, isorhamnetin, kaempferol) | antioxidant, cell longevity increase | HaCaT | [1] |
| 7 | *Glycyrrhiza glabra* | Dihydroxyflavanones (glabranin, licoflavanone) | antioxidant, anticancer | HaCaT | [1] |
| 8 | *Citrus limon* | Flavonoids | anti-inflammatory, antimicrobial, anticancer | HaCaT | [147] |
| 9 | *Verbena officinalis* | Flavonoids | antiproliferative and anticancer | HCT-116 | [148] |
| 10 | *Symphytum officiale* | n.d | boost the regenerative power of epidermal stem cells and their ability to build new tissue; | Callus culture | [149] |
| 11 | *Camellia sinensis* | Flavonoids glycosides | antioxidant, anti-ageing, photoprotective properties | keratinocyte | [150] |
| 12 | *Papaver roheas* | Flavonoids (anthocyanins), quercetin | antioxidant and anti-inflammatory | fibroblasts and keratinocytes | [151] |
| 13 | *Punica granatum* | Flavonoids (anthocyanins), quercetin | antioxidant and anti-inflammatory | fibroblasts and keratinocytes | [151] |
| 14 | *Clitoria ternatea* | Flavonoids (anthocyanins), quercetin | antioxidant and anti-inflammatory | fibroblasts and keratinocytes | [151] |
| 15 | *Carthamus tinctorius* | Carthamin, Quercetin | antioxidant and anti-inflammatory | fibroblasts and keratinocytes | [151] |
| 16 | *Gomphrena globosa* | Betacyanins, quercetin | antioxidant and anti-inflammatory | fibroblasts and keratinocytes | [151] |
| 17 | *Rubus idaeus* | n.d | anti-ageing, antioxidant | Keratinocyte | [152] |
| 18 | *Rosmarinus officinalis* | Flavonoids, polyphenols | antioxidant, anticancer, anti-ageing, anti-inflammatory | Keratinocyte | [153] |
| 19 | *Cannabis* | Flavanols and flavones | anti-ageing | Keratinocytes | [154] |
| 20 | *Epilobium angustifolium* | Flavonoids | anti-ageing and anti-inflammatory properties | HaCaT | [155] |
| 21 | *Schisandra chinensis* | Flavonoids (quercetin, rutinoside) | radiation-protective, anti-ageing, antioxidant, anti-allergic and anti-inflammatory | HaCaT | [156] |
| 22 | *Curcuma longa* | Flavonoids (rutin and quercetin-O-hexose) | anti-inflammatory and antioxidant | HaCaT | [157] |
| 23 | *Dendrobium officinale* | Stilbenoid | antioxidant, anticancer | HEK-293 | [158] |
| 24 | *Cocos nucifera* | caprylic acid, capric acid, lauric acid, stearic acid, linoleic acid | anti-inflammatory and skin protective | Keratinocytes | [159] |

n.d—not determined.

## 4. Antioxidants as Reactive Oxygen Species Antagonists in Skin Conditions—Possible Mechanism

There are a lot of harmful oxygen byproducts in the aerobic environment. The organism has developed antioxidant defence mechanisms to protect it from adverse effects. Antioxidants are "any substance that directly scavenges reactive oxygen species (ROS) or indirectly acts to upregulate antioxidant defences or inhibit ROS production" [39]. Still, antioxidants may also undergo further oxidation and intramolecular hydrogen bonding to generate a new, more stable radical [160]. Furthermore, antioxidants can control gene expression,

which causes the nuclear factor erythroid 2-related factor 2 (Nrf-2) to move from the cytosol to the nucleus after splitting apart from its inhibitor, the Kelch-like erythroid cell-derived protein 1. After entering the nucleus, Nrf-2 may bind antioxidant response elements and trigger the transcription of genes related to stress response, including NAD(P)H: quinone acceptor oxidoreductase 1, glutathione S-transferase, and heme-oxygenase-1 [161–163].

The cell's defence mechanism against oxidative stress is made up of an interconnected network of several antioxidants (e.g., superoxide dismutase, catalase, glutathione peroxidase, transferrin, and caeruloplasmin) that function in various ways and at various degrees (first, second, and third lines of defence) [163] (Figure 1).

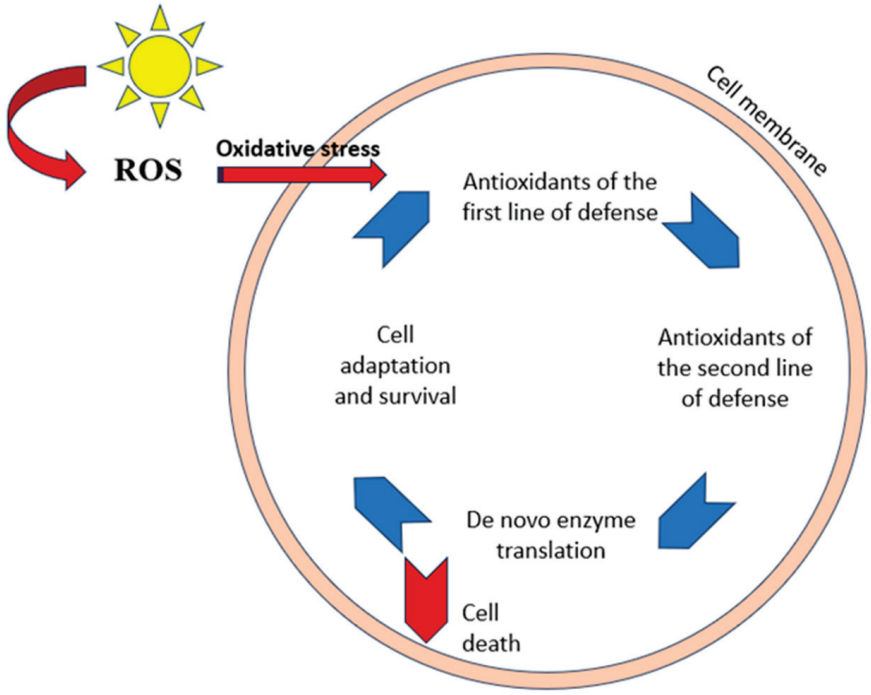



**Figure 1.** Antioxidant response of the cell after damage from oxidative stress. ROS levels rise, and oxidative stress is brought on by UV exposure.

ROS production is inhibited by endogenous antioxidants, and propagation reactions are suppressed by the combined action of exogenous and endogenous antioxidants. Enzymes generated from scratch restore damage caused in the cells. Ultimately, the cell will survive due to an adaptation process if the cooperation of various networks associated with antioxidants is able to resist oxidative stress damage. If the stress is continuous, the cell will eventually die [164].

## 5. Conclusions

For many years, skincare treatments derived from plants have been used. Natural components are still widely used in various novel formulations for skin care, cleansing, and protection (natural products often enhance their action when combined with each other and not simply isolated and concentrated). For medicinal and cosmetic uses, individual active chemicals and compounds found in plants are used; they are often taken in the form of extracts made from different plant tissues. The reason why plant extracts are employed is their ability to shield the skin from damaging external or internal causes. The primary advantages of using natural substances are their antioxidant qualities and their capacity to shield against oxidative stress-related skin problems. Plant extracts' ability to defend against UV radiation is particularly significant since UV-induced photo-oxidative damage to cellular lipids, proteins, and DNA is linked to early skin ageing and the emergence of skin cancer. Plants have much to offer regarding skin care, but further studies and clinical proof are required since many of these extracts' efficacy is still up for debate. Moreover, there are

still a lot of active molecules to be found, and natural compounds made from plant extracts make for a fascinating area of study. A great challenge of new aromatic and therapeutic plants that enhance the quality of plant-based goods may be discovered in the future.

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# Effects of Caffeine, Zinc, and Their Combined Treatments on the Growth, Yield, Mineral Elements, and Polyphenols of *Solanum lycopersicum* L.

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**Abstract:** (1) Background: The effects of Zn and caffeine as promoters of fruit quality in the *Solanum lycopersicum* L. cultivar 'Panarea' were tested. (2) Methods: During the 56 days of the experiment, plants were treated weekly with 100 mL of 1 mM Zn (Zn), 1 mg $L^{-1}$ caffeine trimethyl-$^{13}C$ (caffeine), and 1 mM Zn + 1 mg $L^{-1}$ caffeine trimethyl-$^{13}C$ (Zn + caffeine) and compared to plants that were given tap water (control). (3) Results: Caffeine was taken up by the roots and translocated to the leaves, which positively influenced the number of fruits per plant. After 56 days of treatment, Zn induced a positive increase in tomato dry weight, reducing shoot length (−16.7%) compared to the other treatments. Zn + caffeine had a positive effect on the phenylpropanoid pathway of fruits, and 4-coumaric acid, caffeic acid, and t-ferulic acid were significantly increased, as well as the total antioxidant capacity of the tomatoes. In the flavonoid pathway, only apigenin and luteolin contents were reduced by treatments. The tomatoes showed similar concentrations of the mineral elements Cu, Mn, Fe, Na, Ca, Mg, and K. The Zn and caffeine target hazard quotients were <1, indicating that health risks via the consumption of these tomatoes did not occur. (4) Conclusions: Tomato plants could be irrigated with water containing lower values of Zn, caffeine, and a combination of the two. The treated fruits are rich in antioxidant compounds, such as coumaric acid, caffeic acid, and t-ferulic acid, which are beneficial for human health. No considerable health risks associated with human consumption have been detected.

**Keywords:** LC-MS/MS; biostimulant; flavonoids; target hazard quotients; translocation factor

## 1. Introduction

The Food and Agriculture Organization (FAO) of the United Nations has emphasized the need for sustainable agricultural practices that can produce more food with fewer environmental impacts. Several key strategies can be implemented to achieve this goal, and the use of mineral elements and organic compounds represents an additional and increasingly important tool.

Among mineral elements, Zn is an essential micronutrient that plays a crucial role in various plant physiological processes. Zn is a co-factor in many important antioxidant enzymes in plants, such as copper/zinc-superoxide dismutase [1], and participates in several processes, such as germination and reproductive growth, with general effects on plant physiology and morphology [2]. The optimal level of Zn in crops ranges from 30 to 200 mg Zn $kg^{-1}$ dry weight [3]. Zn deficiency is a well-known nutritional disorder that modifies human health, mainly around the world, where principal food crops are the key source of daily calorie intake [3]. For these reasons, the Zn fertilization of crops is an objective of research [4].

Caffeine (1,3,7-trimethylxanthine) is a natural alkaloid found in various plants, including coffee (*Coffea* spp.), tea (*Camellia sinensis*), cocoa (*Theobroma cacao*), and other species. Caffeine is also an ingredient in foods and pharmaceuticals recognized for its stimulant effects on humans and the most frequently detected compound in water, and for this, it is considered a trace of anthropogenic presence [5,6]. Water data indicate that caffeine is relatively stable, with a half-life ranging from 100–240 days to 10 years [7,8].

In plants, it has been observed that caffeine effects are related to dose and species, showing clear biostimulant effects at low doses. Pierattini et al. [9] demonstrated that the treatment of *Populus alba* cv 'Villafranca' with 2 mg $L^{-1}$ caffeine (trimethyl-$^{13}$C) leads to a significant increase in stem and leaf growth. Caffeine can be used as a biostimulant in cucumbers, spinach, and lentils [10–12]. In *Phaseolus vulgaris*, caffeine increased the mitosis of the root meristematic cells [13], whereas, in *Capsicum annum*, low doses of caffeine (between 2 and 38 μM) increased the mean height of these plants [14]. In spinach (*Spinacia oleracea* L.), caffeine significantly reduced Cd stress, increasing the accumulation of osmolytes and antioxidant molecules, such as proline and ascorbic acid, and reducing lipid peroxidation and $H_2O_2$ content [11].

The application of low concentrations of caffeine and Zn has been a subject of interest for enhancing plant growth and development [12,15–17]. Combining these two substances can offer synergistic effects, potentially leading to improved plant health and productivity. This application appears interesting in tomato (*Solanum lycopersicum* L.), which is one of the most popular and widespread horticultural crops worldwide. Moreover, tomato plants represent an ideal research model because of their roots, stems, leaves, and edible fruit organs.

According to the Food and Agriculture Organization (FAO), it is cultivated in an area of approximately 5 million hectares, with an annual production of 186 million tons [18]. Considering the increased daily consumption and intrinsic qualities of tomatoes, they play a role in human health. Tomato is a well-characterized source of carotenoids and polyphenols, which are considered targets for cancer prevention [19]. According to their core structures, these antioxidant molecules can be divided into different groups, such as anthocyanins, hydroxycinnamic acids, flavonols, and flavanones. In addition, two flavonol glycosides, rutin and kaempferol-3-rutinoside, are present in tomatoes [19].

It is important to consider that high levels of both Zn and caffeine can be toxic to plants. When Zn reaches toxic concentrations, its effects range from a decrease in biomass to chlorosis, followed by necrosis, to hypoxic-like responses [20–24]. Similarly, a concentration of 2.5 M caffeine inhibits root mitosis in rice [25], and retardation in the seedling growth of *Arabidopsis* and tobacco has been observed when grown under 1 mM caffeine [26]. High levels of Zn and caffeine uptake by plants can also pose a risk for the tomato consumer, exceeding the safety limit for humans [27,28], and the non-carcinogenic target hazard quotient (THQ) [29] can be used.

In this study, we aimed to explore the potential roles of Zn and caffeine in improving tomato plant yield, mineral nutrition, antioxidant capacity, and polyphenol profile. We hypothesized that Zn and caffeine would exert a positive effect on tomato plants. We tested the following: (1) the positive effects of Zn and caffeine on tomato yield, phytochemical parameters such as polyphenols, antioxidant capacity, ethylene production, and solid soluble content (Brix); (2) the mineral nutrient profile of whole plants and fruits; and (3) the risk for humans consuming tomato fruits that could accumulate Zn and caffeine.

## 2. Materials and Methods

### *2.1. Plant Materials and Treatments*

Red tomatoes (*Solanum lycopersicum* L.) cv 'Panarea' were transplanted (one plant for pot) in 7 L pots filled with a peat-based commercial substrate (Potgrond H 90 Klasmann-Deilmann Belgium N.V., Bolzano, Italy). Each plant was fertilized at the start of the experiment with 2.5 g of NPK-MgO (16/10/18/2–Cifo, Orto, Bologna–Italy) and grown in a greenhouse (N.43.76926550560972, W.10.407108271387008, San Giuliano Terme, Italy)

from April to July. After three weeks, the plants were divided into groups ($n$ = 7) and treated weekly until the end of the trial (56 days) with 100 mL of tap water (control, containing 65 ± 13 µg $L^{-1}$ of Zn), 1 mM Zn (0.136 mg $L^{-1}$) (Zn), 1 mg $L^{-1}$ caffeine trimethyl-$^{13}$C (caffeine), and 1 mM Zn + 1 mg $L^{-1}$ caffeine trimethyl-$^{13}$C (Zn + caffeine). Regular daily irrigation with tap water was performed according to the plant growth dynamics. Zn was distributed in the form of $ZnCl_2$ (anhydrous powder, ≥98%, Sigma-Aldrich, Milan, Italy). Caffeine trimethyl-$^{13}$C (powder, ≥99, Sigma-Aldrich, Milan, Italy) was used to discriminate between exogenous and endogenous caffeine (Figure S1).

### *2.2. Yield Measurements and Sampling*

Stem length of each plant was measured weekly ($n$ = 7). During the experiment, the first, second, and third trusses were collected at 32, 48, and 56 days from the beginning of the experiment. The total yield and fruit number for each plant were determined by weighing and counting all fruits. For each truss, the first and second fruits of the truss were collected and analyzed. For the first fruit, the fresh weight (FW), dry weight (DW), caliber (cm), total soluble solids (°Brix, using a portable refractometer Shodex, West Berlin, NJ, USA), mineral element, caffeine, and polyphenol concentrations were determined. For the second fruit, ethylene analyses were performed. After 56 days (last sampling), roots, stems, and leaves were collected, and FW was measured and stored for specific analysis. Part of the plant material was oven-dried at 60 °C until its weight remained constant.

### *2.3. Ethylene Measurements*

Ethylene production in the first, second, and third trusses was assessed by enclosing the second fruits of each truss in airtight glass bottles sealed with plastic screwcaps and fitted with rubber septa. Incubation lasted for 1 h, and the headspace (1 mL) was removed from the jar and injected into a gas chromatograph (HP5890, Hewlett-Packard, Menlo Park, CA, USA) equipped with a flame ionization detector and metal column (internal dimension 150 × 0.4 cm) packed with Hysep T. Column, and detector temperatures were set at 70 and 350 °C, respectively. The nitrogen carrier gas was used at a flow rate of 30 mL $min^{-1}$. The ethylene data ($n$ = 5) are expressed as nl $g^{-1}$ $h^{-1}$.

### *2.4. Zinc and Mineral Element Analyses*

A microwave-assisted digestion method was used to digest 0.3 g of the dried first fruit of the first, second, and third trusses and leaf, stem, and root samples with 8 mL of 65% nitric acid (Sigma-Aldrich, Milan, Italy). The mixture was digested using a COOLPEX Smart Microwave Reaction System (Yiyao Instrument Technology Development Co. Ltd., Shanghai, China). The digested solution was diluted to 30 mL with deionized water. Mineral element quantification was performed using a Microwave Plasma-Atomic Emission Spectrometer (4210 MP-AES, Agilent Technologies, Santa Clara, CA, USA). The wavelengths used were 213.8 nm for Zn, 393.3 nm for Ca, 766.4 nm for K, 588.9 nm for Na, 324.8 nm for Cu, 371.9 nm for Fe, 403.0 nm for Mn, and 285.2 nm for Mg. *Daucus carota* (L.) leaf tissue was used as the analytical standard reference (WEPAL IPE, Wageningen University, Wageningen, Netherlands). A multi-element standard solution was prepared in 5% $HNO_3$ ($v/v$) medium and diluted with Milli-Q $H_2O$ for mineral quantification. The limits of detection (LOD) were calculated as three times the standard deviation of the blank samples: Zn 7.8, Ca 39, K 41.2, Na 35.5, Cu 0.9, Fe 1.6, Mg 0.8, and Mn 0.6 mg $kg^{-1}$. The results ($n$ = 7) are expressed on a dry mass basis (mg $kg^{-1}$ DW).

### *2.5. Caffeine-$^{13}$C Extraction and Polyphenol Extraction*

Fresh roots (0.5 g), stems, leaves, and first fruits of the first, second, and third trusses were extracted after grinding in mortars with 3 mL of 0.1% formic acid (Sigma-Aldrich, Milan, Italy) and 3 µL of 15% hydrochloric acid (Sigma-Aldrich, Milan, Italy). The extract was centrifuged at 17,000× $g$ for 5 min (Allegra 64R, Beckman Coulter Inc., Brea, CA, USA), and the supernatant was filtered through 0.20-µm syringe filters (Sartorius Stedim Biotech

GmbH, Gottingen, Germany) before storing at −20 °C until liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. The fresh fruit in first position of first, second, and third truss clusters was used to determine the selected polyphenols: protocatechuic acid (PCTA), 4-coumaric acid (PCA), caffeic acid (CFA), t-ferulic acid (TFRA), naringenin (NRG), apigenin (APG), luteolin (LTO), quercetin (QCT), chlorogenic acid (CGA), piceid (PCD), phloridzin (PDZ), kaempferol 7-G (QCT7G), kaempferol 3-G (QCT3G), kaempferol 3-O-rutinoside (KPF3R), rutin (RTN), and quercetin 3,4 DG (QCTDG) (Sigma-Aldrich, Milan, Italy). Tomato samples (7.5 g FW) were extracted with 25 mL of 80% methanol (Sigma Aldrich, Milan, Italy) for 30 min at room temperature (25 °C) using a laboratory shaker (VDRL mod. 711/CS ASAL, Milan, Italy). Extracts were filtered through a 0.45 μm pore size membrane (Sigma-Aldrich, Milan, Italy) before injection into LC-MS/MS mass spectrometer (AB Sciex LLC, Framingham, MA, USA). Analytical standards for caffeine (trimethyl-$^{13}$C) and specific polyphenols were used as the calibration curves. The matrix effects and recovery efficiencies were also evaluated.

### *2.6. Caffeine and Selected Polyphenols Analyses*

Caffeine (trimethyl$^{13}$-C) and selected polyphenol concentrations in the fruits were determined by LC-MS/MS mass spectrometry (Sciex 5500 QTrap+) using an information-dependent acquisition (IDA) method with selected reaction-monitoring (SRM) transitions per component as a survey scan and MS-MS enhanced product ion (EPI) spectrum acquisition. An AB Sciex 5500 QTrap+ mass spectrometer (AB Sciex LLC, Framingham, MA, USA), equipped with a Turbo V ion spray source coupled to an ExionLC AC System custom-made by Shimadzu (Shimadzu Corporation, Kyoto, Japan), was used to determine the specific molecules.

### *2.7. HPLC-MS/MS Method for Polyphenols*

A Phenomenex Kinetex ® Biphenyl 100 × 2.1 mm, 2.6 μm particle size column (Phenomenex, Torrance, CA, USA) was employed for the chromatographic separation. An elution gradient was performed using acetonitrile containing 0.1% $v/v$ formic acid and Milli-Q water with 0.1% $v/v$ formic acid (Sigma-Aldrich, Milan, Italy). MS-MS detection was performed in negative ion mode. The common source parameters were as follows: nebulization gas (GS1) 50, turbo gas (GS2) 50, curtain gas (CUR) 10, temperature (TEM) 500 °C, ion spray voltage (IS) −4500 V, and input potential (EP) 10 V. The declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) were adjusted for the specific SRM for each component. The SRM transitions and corresponding compound parameters are listed in Table S1.

### *2.8. HPLC-MS/MS for Caffeine*

An Agilent PhenylHexyl 2 × 100 mm 2.7 μm particle size column (Agilent, Santa Clara, CA, USA) was employed for chromatographic separation. An elution gradient was performed using acetonitrile containing 0.1% $v/v$ formic acid and Milli-Q water (Merck KGaA, Darmstadt, Germany) with 0.1% $v/v$ formic acid (Sigma-Aldrich, Milan, Italy). Tandem mass spectrometry (MS-MS) was performed in the positive ion mode. The common source parameters were as follows: nebulization gas (GS1) 50, turbo gas (GS2) 45, curtain gas 25, temperature 500 °C, ion spray voltage 5500 V, and input potential 10 V. The compound parameters were adjusted for the selected reaction-monitoring transitions for each component and are shown in Table S1.

### *2.9. DPPH Assay*

The scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical tomatoes was analyzed with 200 μL of the methanol extract previously used for polyphenol assay, mixed with 800 μL of a Tris-HCl 100 mM solution (Sigma-Aldrich, Milan, Italy), pH 7.0, and finally kept in the dark for 30 min after adding 250 μM DPPH (Sigma-Aldrich, Milan, Italy). Methanol: water (80:20, $v$:$v$) was used as a control reference, and the absorbance

was measured at 517 nm using a spectrophotometer (Infinite 200 PRO, Tecan Italia Srl Milan, Italy). The radical scavenging activity of the extracts was calculated using the following equation:

$$\text{DPPH inhibition}(\%) = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

*2.10. Translocation Factor*

The response of tomato plants ($n = 7$) to Zn and Zn + caffeine application was evaluated in terms of the translocation factor (Tf), a unit-less index indicating the ability of the plant to transfer caffeine or Zn from roots to aerial part of tomato plants. Tf was calculated to evaluate the capability of plants to accumulate Zn or caffeine, absorbed by roots, in the aerial parts (stems, leaves, and fruits harvested after 56 days of treatment). Tf was calculated using the following equation:

$$\text{Tf} = \frac{\text{Zn or Caffeine concentration in the aerial parts } \left(\text{mg kg}^{-1}\right)}{\text{Zn or Caffeine concentration in the root parts } \left(\text{mg kg}^{-1}\right)}$$

*2.11. Risk Assessment*

The health risks caused by the intake of tomato fruits grown under Zn and caffeine treatments were assessed using the THQ index [29]. THQ was calculated as the ratio of exposure to Zn or caffeine to the reference dose (RfD), which is the highest level at which no adverse health effects are expected. THQ describes the non-carcinogenic health risks posed by exposure to Zn or caffeine. If THQ is < 1, non-carcinogenic health effects are expected. In contrast, a THQ >1 indicates that there is a possibility that adverse health effects could occur. THQ was calculated as follows:

$$\text{THQ} = \frac{\text{Efr} \times \text{Ed} \times \text{Fir} \times \text{C}}{\text{RfD} \times \text{Bw} \times \text{ATn}} \times 10^{-3}$$

where Efr = exposure frequency (365 days/year); Ed = exposure duration (50 years); RfD = reference dose (being 0.3 mg $kg^{-1}$ $day^{-1}$ for Zn and 37 mg $kg^{-1}$ $day^{-1}$ for caffeine according to EFSA and Antoine et al. [28,29]. Bw = the estimated average body weight (70 kg) considering exposed consumers aged 15–17 years old; ATn = average time of exposure to non-carcinogenic HMs (Ed × 365 days/year), C = concentration of Zn or caffeine in tomato fruit; Fir = food ingestion rate in grams per day (for 50 g of tomato per day).

*2.12. Statistical Analysis*

Before performing the statistical test, the normality of the data was assessed and analyzed using two-way ANOVA. Tukey's post-hoc test for post-hoc mean comparison at $p = 0.05$ was used. *t*-test analyses were performed to determine the differences between the control and treated plants. A heat map and principal component analysis (PCA) were performed for the mineral elements and polyphenols. Graphs and statistical analyses were performed using Prism-GraphPad 10.1 Mac.

## 3. Results

One of the main objectives of this study was to assess whether Zn and caffeine have a positive effect on tomato plant performance, with a focus on fruit quality and safety. The macroscopic observation of tomato plants demonstrated that caffeine application did not change the growth performance compared with the control (Figure 1), either added to Zn or alone (Figure 1). In contrast, plant height was significantly lower in Zn-treated plants

than in the control plants, and stem length was significantly reduced by 16.7% (Figure 1c and Table S2).

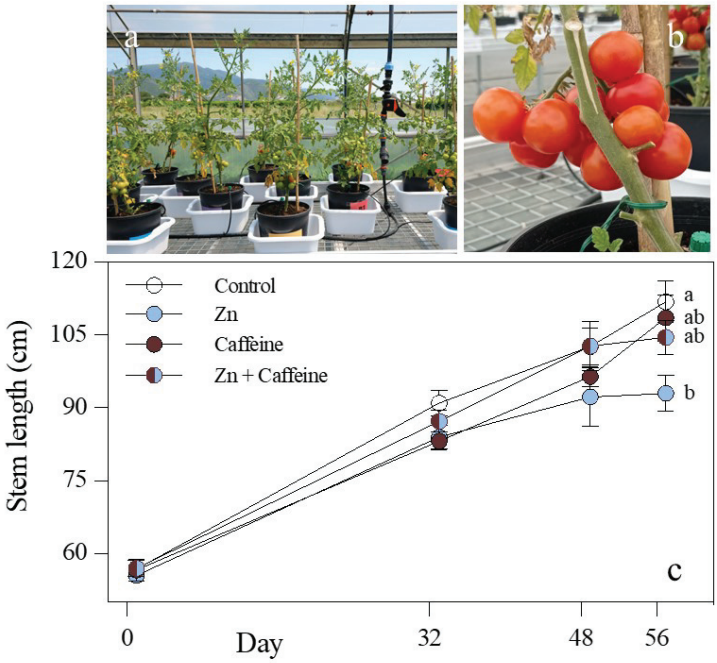


**Figure 1.** (**a**) Overview of plants (*Solanum lycopersicum* cv 'Panarea') grown under greenhouse conditions; (**b**) tomato truss; and (**c**) stem length at 0, 32, 48, and 56 days of the experiment. Plants were treated with tap water (control), 0.136 mg $L^{-1}$ Zn (Zn), 1 mg $L^{-1}$ caffeine-(trimethyl-$^{13}$C) (caffeine), and 1 mg $L^{-1}$ caffeine (trimethyl-$^{13}$C) + 0.136 mg $L^{-1}$ Zn (Zn + caffeine). Data represent the mean ± SD ($n$ = 7). The data followed a normal distribution and were subjected to two-way ANOVA, and the values indicated with different letters were significantly different from each other following Tukey's post-hoc test, $p \leq 0.05$ (Supplementary Table S2).

Tomato fruits were assessed at harvest for (i) fruit yield, (ii) fruit number per plant, (iii) caliber, (iv) total antioxidant capacity (DPPH%), and (v) fruit dry weight of the first tomato per truss. For the total fruit yield per plant, there was a not significant increase of 6% or 37 g/plant due to caffeine treatment (Figure 1a) compared to the control; these data are promising and deserve future experimental work. The number of fruits per plant was the highest in caffeine-treated plants (Figure 2b), with a significant interaction between Zn and caffeine factors ($p$ = 0.009, Table S3). The fruit number per plant increased under caffeine treatments by 20% compared to the control, whereas a slight reduction was observed under Zn + caffeine treatments (−17%). There were no significant differences in caliber between the treatment and control groups (Figure 2c). Regarding the total antioxidant activity of tomato fruits after 56 days of treatment (Figure 2d, Table S3), an increase was detected considering the caffeine factor ($p$ = 0.019). The results indicated that tomato plants treated with caffeine increased their average antioxidant capacity by 69% and 85% compared to the average control in the caffeine and Zn + caffeine treatments, respectively (Figure 2d). Finally, Zn application significantly increased the fruit DW when compared with the other treatments (+21%, +23%, and +34% relative to the control, caffeine, and Zn + caffeine, respectively) (Figure 2e, Table S3).

The total soluble solid content during the three harvesting periods ranged from 8.4 to 10.4 °Brix (Table 1), while concerning ethylene, the average values of the three harvesting periods ranged from 0.14 to 0.52 nl $g^{-1}$ $h^{-1}$ (Table 1 and Table S4). The total soluble solid content and ethylene concentration were not significantly affected by Zn or caffeine treatments (Table 1).

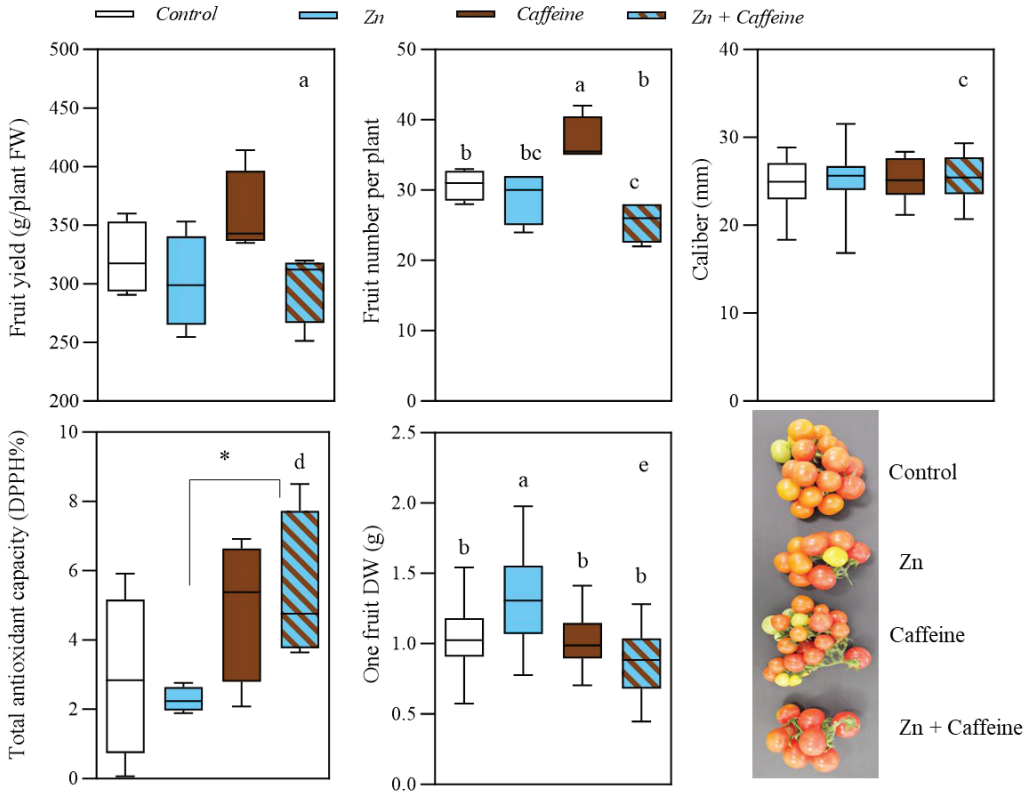


**Figure 2.** Box plot representation of the effects of treatments with tap water (control), 0.136 mg $L^{-1}$ Zn (Zn), 1 mg $L^{-1}$ caffeine-(trimethyl-$^{13}$C) (caffeine), and 1 mg $L^{-1}$ caffeine (trimethyl-$^{13}$C) + 0.136 mg $L^{-1}$ Zn (Zn + caffeine) on the fruit of *Solanum lycopersicum* cv 'Panarea' after 56 days of the experiment. (**a**) Fruit yield ($n$ = 7); (**b**) fruit number per plant ($n$ = 7); (**c**) caliber ($n$ = 7); (**d**) total antioxidant capacity (DPPH%) ($n$ = 4); (**e**) one fruit dry weight of the first tomato per truss ($n$ = 7). The data followed a normal distribution and were subjected to two-way ANOVA (Supplementary Table S3). Values indicated with different letters differ significantly from each other (Tukey's post-hoc test, $p \leq 0.05$). $t$-test analyses * = $p$ <0.05.

**Table 1.** Total soluble solids content (°Brix) and ethylene production (nl $g^{-1}$ $h^{-1}$) in *Solanum lycopersicum* 'Panarea' plants. One fruit per truss of the first, second, and third truss clusters was used for the analyses after 32, 48, and 56 days of treatment, respectively. Tap water (control), 0.136 mg $L^{-1}$ Zn (Zn), 1 mg $L^{-1}$ caffeine (trimethyl-$^{13}$C) (caffeine), 1 mg $L^{-1}$ caffeine (trimethyl-$^{13}$C), and 0.136 mg $L^{-1}$ Zn (Zn + caffeine). Data ($n$ = 5) were analyzed using two-way ANOVA. Tukey's post-hoc test at $p \leq 0.05$ probability level was applied, and statistical data are reported in Supplementary Table S4.

| | | Treatments | | | |
|---|---|---|---|---|---|
| | **Day of Treatment** | **Control** | **Zn** | **Caffeine** | **Zn + Caffeine** |
| Total soluble solids content (°Brix) | 32 | 8.9 ± 0.9 | 9.4 ± 1.4 | 8.8 ± 0.8 | 8.4 ± 1.2 |
| | 48 | 9.0 ± 1.0 | 10.0 ± 1.4 | 9.0 ± 0.8 | 9.0 ± 1.1 |
| | 56 | 10.4 ± 2.2 | 8.9 ± 0.8 | 9.0 ± 0.7 | 9.0 ± 1.4 |
| Ethylene (nl $g^{-1}$ $h^{-1}$) | 32 | 0.37 ± 0.3 | 0.40 ± 0.2 | 0.18 ± 0.1 | 0.52 ± 0.5 |
| | 48 | 0.16 ± 0.1 | 0.45 ± 0.5 | 0.13 ± 0.1 | 0.14 ± 0.1 |
| | 56 | 0.32 ± 0.3 | 0.48 ± 0.6 | 0.25 ± 0.2 | 0.44 ± 0.3 |

The uptake of Zn and caffeine in each organ of the tomato plants was evaluated (Figure 3). The stem is the organ in which Zn reached the highest value compared to roots and leaves (204 and 200 mg $kg^{-1}$ DW in plants treated with Zn + caffeine and Zn alone) (Figure 3a and Table S5). ANOVA did not show any significant interaction between Zn and

caffeine in the stems ($p$ = 0.838). In the roots, ANOVA revealed a significant interaction between Zn and caffeine ($p$ = 0.002) (Table S5). A significant increase of 53% and 98% in root Zn concentration was observed in plants treated with Zn + caffeine (89 ± 14.9 mg $kg^{-1}$ DW) and Zn alone (115 ± 17.3 mg $kg^{-1}$ DW) in comparison to the control plants (Figure 3a). The $t$-test analyses between Zn and Zn + caffeine treatments indicated that in roots ($p$ = 0.041) and leaves ($p$ = 0.035), the treatment with Zn + caffeine reduced the concentration of Zn allocated to these two organs (Figure 3a). When present in treatments, caffeine was taken up by the roots (2.6 ± 1.51 and 1.4 ± 0.53 ng $g^{-1}$ FW under caffeine and Zn + caffeine treatments respectively) and translocated to the leaves (1.3 ± 0.28 and 3.5 ± 1.28 ng $g^{-1}$ FW under caffeine and Zn + caffeine treatments, respectively) (Figure 3b). Under our experimental conditions, caffeine was not detected in the stems (Figure 3b). In particular, the $t$-test indicated that at the root level, the caffeine concentration was 4.5 times lower ($p$ = 0.031) under the Zn + caffeine treatments than under caffeine alone. In contrast, mixed Zn + caffeine exposure increased caffeine translocation to the leaves ($p$ = 0.037, Figure 3b).

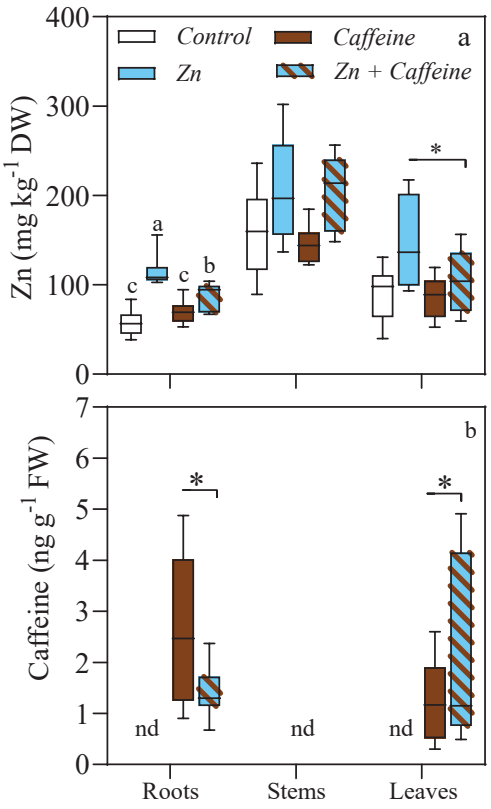


**Figure 3.** (**a**) Zn concentration (mg $kg^{-1}$ DW) and (**b**) caffeine concentration (ng $g^{-1}$ FW) in the roots, stems, and leaves of *Solanum lycopersicum* cv 'Panarea' after 56 days of treatment with tap water (control), 0.136 mg $L^{-1}$ Zn (Zn), 1 mg $L^{-1}$ caffeine-(trimethyl-$^{13}$C) (caffeine), and 1 mg $L^{-1}$ caffeine-(trimethyl-$^{13}$C) + 0.136 mg $L^{-1}$ Zn (Zn + caffeine). Statistical significances were determined with two-way ANOVA (data n = 7), and different letters indicate a statistical difference according to Tukey's multiple comparison test ($p \leq 0.05$) (Supplementary Table S5); nd = not detected; * = $p < 0.05$.

After verifying the uptake of Zn and caffeine in the non-edible parts of the plant, we focused on the fruits during the three different harvesting times after 32, 48, and 56 days of treatment (Figure 4a). The average Zn concentration in tomatoes ranges from a minimum of 19.7 to a maximum of 31.6 mg $kg^{-1}$ DW. A higher average value was detected in Zn + caffeine fruits after 56 days of treatment, whereas it was lower in fruits after 48 days of Zn treatment (Figure 4a). No significant changes ($p$ = 0.988) in the Zn concentration in fruits were observed between the treatments at the first (32 days) harvest time (Table S6). In contrast, ANOVA showed a significant difference ($p$ = 0.027) in the uptake of Zn between

treatments at the second harvest time (48 d), with a significantly higher uptake of Zn in plants exposed to 0.136 mg $L^{-1}$ of Zn. Finally, in plants treated with Zn + caffeine, after 56 days of exposure, we observed a significant reduction ($p = 0.003$) of 12% in the Zn content of the fruits (Figure 4a).

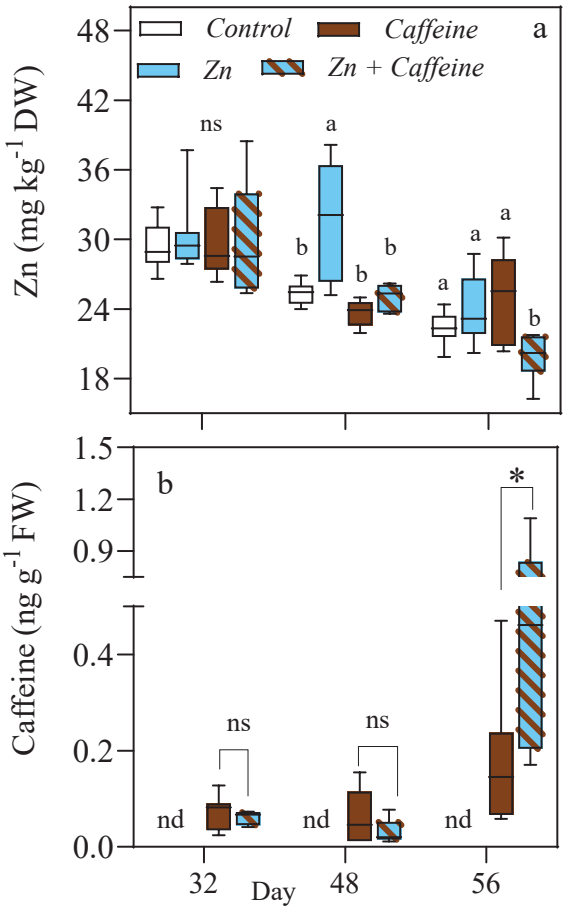


**Figure 4.** (**a**) Zn concentration (mg $kg^{-1}$ DW); (**b**) Caffeine concentration (ng g $FW^{-1}$) in tomato fruits *Solanum lycopersicum* cv 'Panarea' after 32, 48, and 56 days of treatments with tap water (control), 0.136 mg $L^{-1}$ Zn (Zn), 1 mg $L^{-1}$ caffeine-(trimethyl-$^{13}$C) (caffeine), and 1 mg $L^{-1}$ caffeine-(trimethyl-$^{13}$C) + 0.136 mg $L^{-1}$ Zn (Zn + caffeine). Statistical significances were determined with two-way ANOVA (data $n = 7$), and different letters indicated a statistical difference according to Tukey's multiple comparison test ($p \leq 0.05$) (Supplementary Table S6); ns = not significant; nd = not detected; * = $p < 0.05$. *t*-test between caffeine and mix results was also performed.

The uptake and accumulation of caffeine in the vegetables indicated that tomatoes could take up and accumulate this molecule in the soil. The amount of caffeine increases with exposure time; in fact, the average values for caffeine after 32 and 48 days range between 0.06 and 0.07 (ng $g^{-1}$ FW), while a maximum value of 0.52 ng $g^{-1}$ FW was measured after 56 days of Zn + caffeine exposure (Figure 4b).

Under our experimental conditions, the ratio of Zn that reached the aerial part with respect to that present in the roots (Tf) varied among different organs, with values higher than 1 in the stems and leaves (Figure 5a). The highest Zn Tf values were observed in the stems of the plants treated with Zn + caffeine (Tf = 2.4; $p = 0.041$). No significant Tf was found in the leaves or fruits (Figure 5a). The calculation of caffeine Tf plants showed Tf <1 in the leaves and fruits, with significant values in both the leaves and fruits under Zn + caffeine treatment (Figure 5b).

Data and statistical analyses of the concentrations of mineral elements in the first-, second-, and third-truss fruits are shown in Tables S7 and S8.

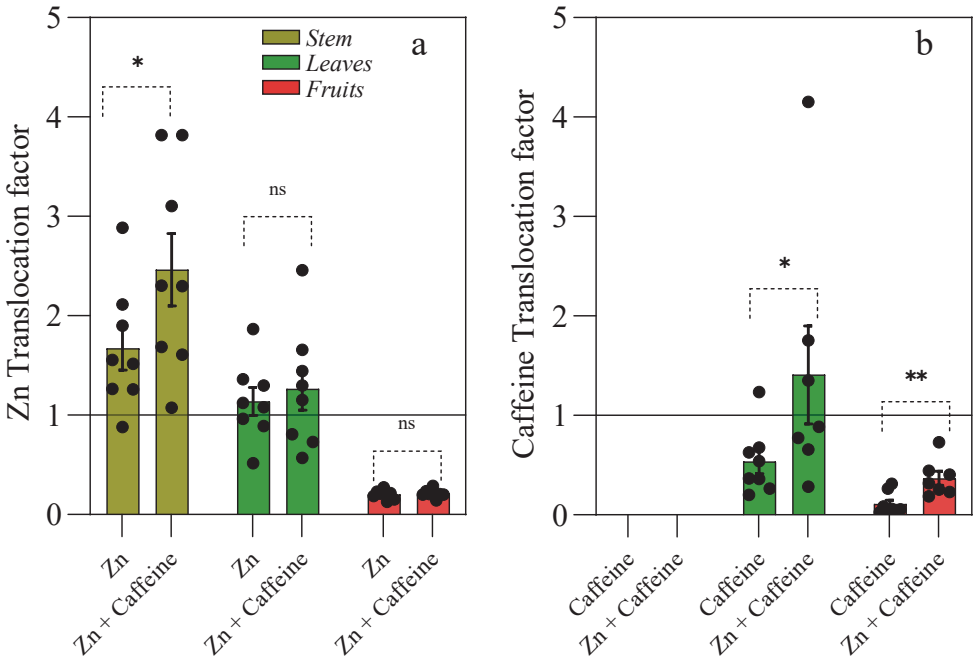


**Figure 5.** Translocation factors for Zn (**a**) and caffeine (**b**) in *Solanum lycopersicum* 'Panarea' stem, leaves, and tomatoes after 56 days of treatment with 0.136 mg $L^{-1}$ Zn (Zn), 1 mg $L^{-1}$ caffeine-(trimethyl-$^{13}$C) (caffeine), 1 mg $L^{-1}$ caffeine (trimethyl-$^{13}$C), and 0.136 mg $L^{-1}$ Zn (Zn + caffeine). Data were analyzed by *t*-test, and significant results are reported (* = $p < 0.05$; ** = $p < 0.01$; ns = not significant).

All tomatoes analyzed had similar concentrations of the mineral elements Cu, Mn, Fe, Na, Ca, Mg, and K after 32, 48, and 56 days of treatment, as indicated by the biplot data of the PCA analyses (Figure 6a). The scores of each tomato sample were examined in a two-dimensional plot of the first two principal components (52.8% of the total variability), and no separation of the samples into groups was found. K was the most abundant element in third-truss fruits, with concentrations ranging from 27,159 to 32,951 mg $kg^{-1}$ DW (Figure 6a). In the first and second truss, K ranged from 27,585 to 32,362 mg $kg^{-1}$ DW and 27,691 to 31,887 mg $kg^{-1}$ DW, respectively (Table S7). The Mg and Ca values did not differ significantly ($p > 0.05$) between the treatments.

The ANOVA analysis of mineral element data in fruits after 32, 48, and 56 days, in general, did not reveal significant differences in the interactions between the Zn and caffeine factors at the first and third truss harvests (Table S8). The only exceptions were Mn (Zn × caffeine, $p = 0.027$), Fe (Zn × caffeine, $p = 0.047$), and Na (Zn × caffeine $p = 0.049$) after 48 days of treatment. Some other interesting statistical differences were observed at the 56th day of treatment for Fe (Zn factor, $p = 0.006$) (Table S8).

In tomatoes, antioxidant molecules play a key role in determining fruit quality. The concentrations of the 16 polyphenols studied by UHPLC-ESI-MS/MS in all analyzed trusses are reported in Table 2 and Figures 6b and 7. The content of 11 antioxidant molecules did not change in relation to the treatments applied ( Tables 2 and S9). Rutin was the most abundant polyphenol in the fruit, with an average of 207,640 ± 131,711 ng $g^{-1}$ FW, followed by chlorogenic acid (91,221 ± 59,534 ng $g^{-1}$ FW), kaempferol 3-R (8388 ± 9428 ng $g^{-1}$ FW), and caffeic acid (6904 ± 4563 ng $g^{-1}$ FW) (Table 2).

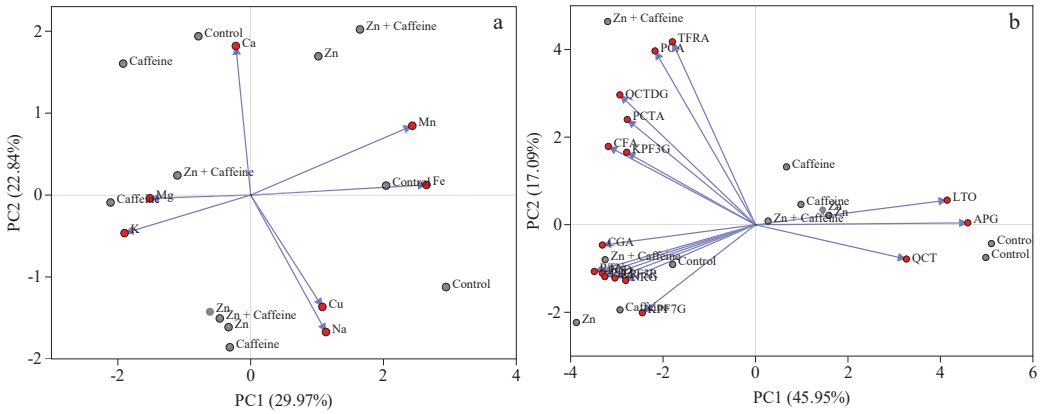


**Figure 6.** Principal component analysis (PCA) biplot of (**a**) mineral elements analyzed in the first fruit of the first, second, and third trusses (Supplementary Tables S7 and S8). Principal component analysis (**b**) of selected polyphenol–protocatechuic acid (PCTA), 4-coumaric acid (PCA), caffeic acid (CFA), trans-ferulic acid (TFRA), naringenin (NRG), apigenin (APG), luteolin (LTO), quercetin (QCT), chlorogenic acid (CGA), piceid (PCD), phloridzin (PDZ), kaempferol 7-G (QCT7G), kaempferol 3-G (QCT3G), kaempferol 3-O-rutinoside (KPF3R), rutin (RTN), quercetin 3,4 DG (QCTDG) of *Solanum lycopersicum* cv 'Panarea' treated with tap water (control), 0.136 mg $L^{-1}$ Zn (Zn), 1 mg $L^{-1}$ caffeine (trimethyl-$^{13}$C) (caffeine), 1 mg $L^{-1}$ caffeine (trimethyl-$^{13}$C), and 0.136 mg $L^{-1}$ Zn (Zn + caffeine). The loadings (red color) and score (grey color) of the PCA are reported.

**Table 2.** Concentration (ng $g^{-1}$ FW) of the selected polyphenols: protocatechuic acid (PCTA), naringenin (NRG), quercetin (QCT), chlorogenic acid (CGA), piceid (PCD), phloridzin (PDZ), kaempferol 7-G (QCT7G), kaempferol 3-G (QCT3G), kaempferol 3-O-rutinoside (KPF3R), rutin (RTN), quercetin 3,4 DG (QCTDG) analyzed in first fruit of first, second, and third truss of *Solanum lycopersicum* cv 'Panarea' treated with tap water (control), 0.136 mg $L^{-1}$ Zn (Zn), 1 mg $L^{-1}$ caffeine (trimethyl-$^{13}$C) (caffeine), 1 mg $L^{-1}$ caffeine (trimethyl-$^{13}$C), and 0.136 mg $L^{-1}$ Zn (Zn + caffeine). Data ($n$ = 5) are expressed as the mean ± standard deviation.

| | | Treatments | | | |
|---|---|---|---|---|---|
| | **Compound** | **Control** | **Zn** | **Caffeine** | **Zn + Caffeine** |
| third Truss | PCTA | 921 ± 220 | 722 ± 258 | 542 ± 294 | 863 ± 385 |
| | NRG | 747 ± 551 | 1935 ± 1312 | 1816 ± 2150 | 641 ± 212 |
| | QCT | 221 ± 139 | 259 ± 79 | 197 ± 61 | 204 ± 92 |
| | CGA | 125,798 ± 41,569 | 141,023 ± 42,128 | 131,831 ± 59,148 | 187,224 ± 62,235 |
| | PCD | 84 ± 25 | 111 ± 33 | 108 ± 26 | 98 ± 27 |
| | PDZ | 1584 ± 1146 | 2878 ± 1693 | 1939 ± 710 | 1878 ± 1201 |
| | KPF7G | 320 ± 29 | 374 ± 133 | 334 ± 209 | 303 ± 103 |
| | KPF3G | 65 ± 31 | 102 ± 44 | 94 ± 38 | 119 ± 64 |
| | KPF3R | 15,648 ± 5099 | 22,661 ± 4649 | 19,293 ± 10,572 | 20,822 ± 8325 |
| | RTN | 309,569 ± 97,652 | 375,194 ± 74,526 | 353,134 ± 72,683 | 365,085 ± 10,102 |
| | QCTDG | 916 ± 396 | 1263 ± 229 | 1304 ± 742 | 1283 ± 427 |
| second Truss | PCTA | 173 ± 117 | 833 ± 586 | 595 ± 153 | 709 ± 234 |
| | NRG | 76 ± 41 | 544 ± 818 | 299 ± 63 | 428 ± 421 |
| | QCT | 444 ± 245 | 204 ± 116 | 131 ± 27 | 94 ± 15 |
| | CGA | 8637 ± 5989 | 48,542 ± 23,516 | 47,921 ± 15,864 | 74,721 ± 45,677 |
| | PCD | 14 ± 10 | 67 ± 29 | 70 ± 26 | 80 ± 27 |
| | PDZ | 289 ± 238 | 719 ± 230 | 1304 ± 793 | 1466 ± 607 |
| | KPF7G | 81 ± 58 | 43 ± 23 | 41 ± 18 | 70 ± 56 |
| | KPF3G | 65 ± 14 | 85 ± 22 | 88 ± 15 | 88 ± 17 |
| | KPF3R | 120 ± 10 | 44 ± 5 | 142 ± 75 | 149 ± 93 |
| | RTN | 30,355 ± 20,331 | 153,394 ± 5505 | 161,355 ± 91,172 | 224,005 ± 94,321 |
| | QCTDG | 183 ± 143 | 799 ± 60 | 1309 ± 965 | 1127 ± 594 |

**Table 2.** *Cont.*

| | | Treatments | | | |
|---|---|---|---|---|---|
| | **Compound** | **Control** | **Zn** | **Caffeine** | **Zn + Caffeine** |
| first Truss | PCTA | 402 ± 170 | 522 ± 117 | 675 ± 152 | 1110 ± 422 |
| | NRG | 273 ± 117 | 405 ± 231 | 460 ± 112 | 757 ± 374 |
| | QCT | 863 ± 681 | 239 ± 81 | 166 ± 19 | 156 ± 50 |
| | CGA | 57,333 ± 29,331 | 92,037 ± 41,689 | 70,646 ± 22,055 | 123,978 ± 25,866 |
| | PCD | 43 ± 20 | 58 ± 18 | 58 ± 18 | 66 ± 25 |
| | PDZ | 457 ± 198 | 1049 ± 547 | 1123 ± 466 | 1206 ± 471 |
| | KPF7G | 124 ± 65 | 64 ± 13 | 74 ± 25 | 68 ± 41 |
| | KPF3G | 34 ± 14 | 36 ± 14 | 57 ± 19 | 140 ± 134 |
| | KPF3R | 3499 ± 1613 | 6151 ± 2594 | 6108 ± 2879 | 9657 ± 6951 |
| | RTN | 72,725 ± 32,123 | 151,346 ± 59,515 | 122,042 ± 32,231 | 222,966 ± 119,537 |
| | QCTDG | 367 ± 194 | 851 ± 756 | 1004 ± 513 | 2398 ± 3216 |

The five significantly different UHPLC-ESI-MS/MS data corresponding to 4-coumaric acid, caffeic acid, t-ferulic acid, apigenin, and luteolin analyzed in the first fruits of the first, second, and third trusses were incorporated in the plant metabolic pathway overview to provide evidence for changes in the antioxidant production of the polyphenols studied. The biosynthetic routes are indicated by lines (Figure 7). Globally, in the phenylpropanoid pathway, 4-coumaric acid, caffeic acid, and t-ferulic acid were significantly increased by Zn or caffeine (Figure 7a–i, Table S9), whereas the flavonoid pathway (apigenin and luteolin) was significantly reduced by treatment (Figure 7j–o, Table S9). Treatment with Zn + caffeine increased the concentrations of 4-coumaric acid, caffeic acid, and t-ferulic acid compared with the control tomatoes at the first and second times of tomato harvest (Figure 7 and Table S9). Apigenin and luteolin levels decreased after Zn and caffeine treatments (Figure 7 and Table S9).

Principal component analysis was applied to the tomato metabolite dataset under different treatments (Figure 6b). When the scores of each tomato sample were examined in a two-dimensional plot of the first two principal components (63.04% of the total variability), a clear separation of samples into groups was found, with a particular correlation among protocatechuic acid, 4-coumaric acid, caffeic acid, trans-ferulic acid, and quercetin 3,4 DG with Zn + caffeine treatment (Figure 6b). A positive correlation was detected between luteolin and apigenin and control tomatoes; these are, in fact, the two polyphenols that were significantly reduced under the treatments (Figure 6b).

In Table 3, the average Zn and caffeine concentrations and THQ values for the first, second, and third truss clusters are shown. Remarkably, no specific THQ related to Zn or caffeine was >1. For Zn, the THQ values ranged from 0.006 to 0.009, and 0.003 and 0.005 under Zn and Zn + caffeine treatments, respectively. Concerning caffeine, the THQ values ranged from $1.44 \times 10^{-5}$ to $4.33 \times 10^{-5}$ for Zn and from $8.16 \times 10^{-6}$ to $1.10 \times 10^{-4}$ under Zn + caffeine treatment (Table 3).

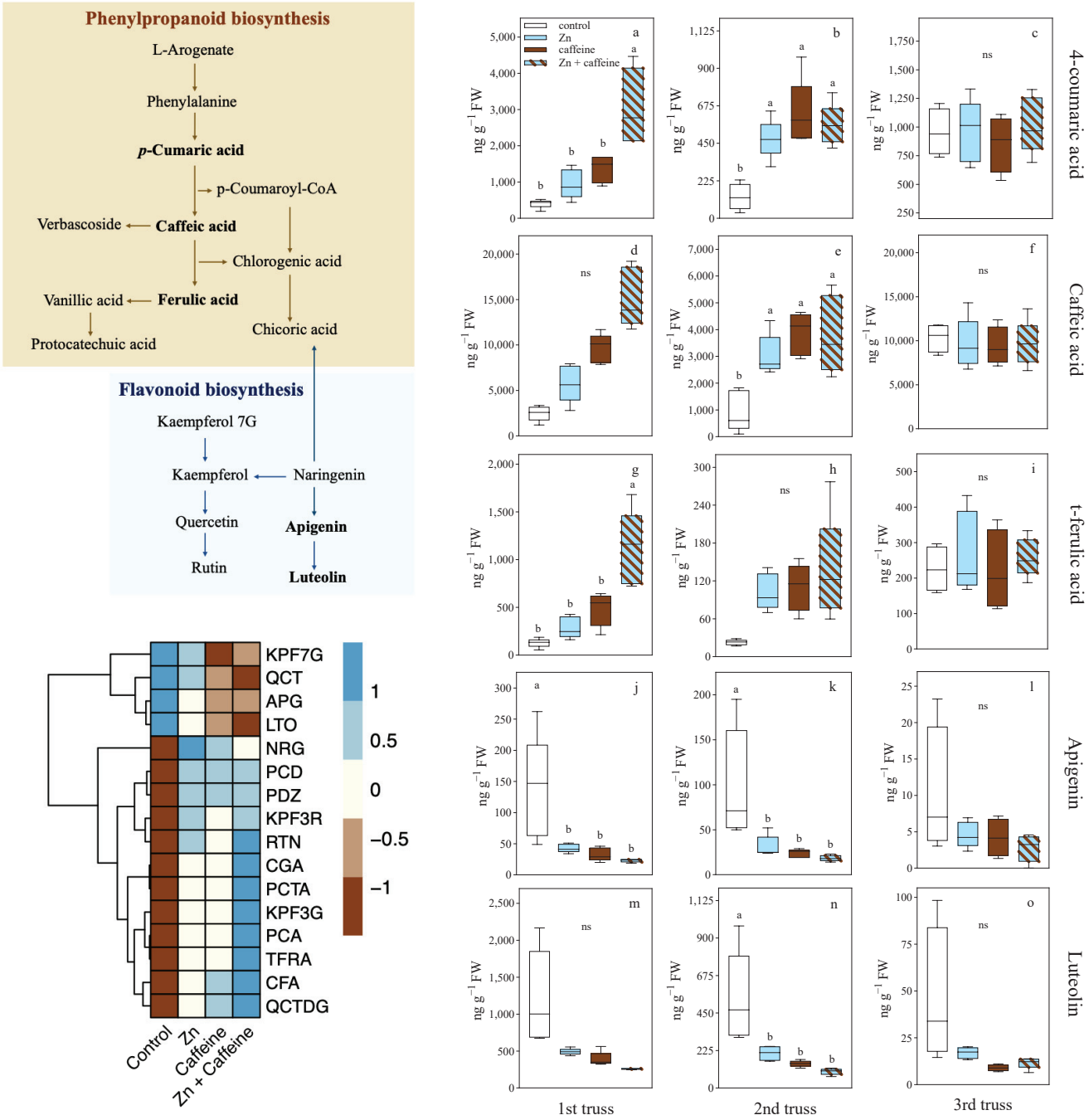


**Figure 7.** Schematic representation of the putative biosynthetic pathways of the main secondary compounds and hierarchical clustering analysis (HCA) plot of polyphenols in *Solanum lycopersicum* cv 'Panarea'; box plot representation of 4-coumaric acid (PCA) (**a**–**c**), caffeic acid (CFA) (**d**–**f**), t-ferulic acid (TFRA) (**g**–**i**), apigenin (APG) (**j**–**l**), and luteolin (LTO) (**m**–**o**) in *Solanum lycopersicum* cv 'Panarea' after 32, 48, and 56 days of treatment with tap water (control), 0.136 mg $L^{-1}$ Zn (Zn), 1 mg $L^{-1}$ caffeine-(trimethyl-$^{13}$C) (caffeine), and 1 mg $L^{-1}$ caffeine-(trimethyl-$^{13}$C) + 0.136 mg $L^{-1}$ Zn (Zn + caffeine). Statistical significances were determined with two-way ANOVA ($n$ = 5) and different letters indicated a statistical difference according to Tukey's multiple comparison test ($p \leq 0.05$) (Supplementary Table S9); ns = not significant.

**Table 3.** Zn and caffeine average concentration expressed as fresh weight and target hazard quotient (THQ) values in first, second, and third truss clusters of *Solanum lycopersicum* 'Panarea' plants exposed to 0.136 mg $L^{-1}$ Zn (Zn), 1 mg $L^{-1}$ caffeine (trimethyl-$^{13}$C) (caffeine) or 0.136 mg $L^{-1}$ Zn and 1 mg $L^{-1}$ caffeine (trimethyl-$^{13}$C) (Zn + caffeine).

| | **Truss Clusters** | **Zn** | **Zn + Caffeine** |
|---|---|---|---|
| $Zn_{average}$ (mg $kg^{-1}$ FW) | first | 3.79 | 2.29 |
| | second | 3.71 | 1.99 |
| | third | 2.85 | 1.52 |
| THQ of Zn | first | 0.009 | 0.005 |
| | second | 0.008 | 0.004 |
| | third | 0.006 | 0.003 |
| | | **Caffeine** | **Zn + Caffeine** |
| $Caffeine_{average}$ (ng $g^{-1}$ FW) | first | 0.072 | 0.059 |
| | second | 0.060 | 0.034 |
| | third | 0.181 | 0.460 |
| THQ of caffeine | first | $1.73 \times 10^{-5}$ | $1.42 \times 10^{-5}$ |
| | second | $1.44 \times 10^{-5}$ | $8.16 \times 10^{-6}$ |
| | third | $4.33 \times 10^{-5}$ | $1.10 \times 10^{-4}$ |

## 4. Discussion

In horticulture, to use irrigation water supplemented with ions and molecules that could have a role as biostimulants, we need proof of the effective changes it can have on plant growth, fruit quality, and toxicity risks. The combined application of caffeine and zinc could stimulate overall metabolic processes, with the final goal of inducing plant growth and producing higher-quality fruits with improved size, mineral elements, and nutritional value.

It has been demonstrated that treatment with caffeine has a positive effect (biostimulator) on the number of fruits per plant, as observed by Jené et al. [30] on lentil yields, which showed an almost 50% increase when plants were treated with $10^{-3}$ M caffeine. These authors also observed that the effects of caffeine disappeared when an additional treatment was added to the plant, which could explain why the plants treated with the combination of Zn + caffeine did not show the same results as those treated with caffeine alone. Caffeine has a positive effect on the growth of *Vigna radiata* plants, as they grow faster in soil with caffeine [31]. Moreover, caffeine can inhibit seed germination but does not impair plant development when sprayed on plants or used to wet the soil [32]. Once caffeine treatment is applied, this compound in tomato is mainly absorbed in the roots; however, its translocation to the aerial part seems to be facilitated by a caffeine–Zn complex, as observed in our previous study on poplar plants [33]. When caffeine was present with Zn in the treatments, it also improved the total antioxidant capacity of fruits. Tomato plant organs have different capabilities to uptake Zn, as indicated by our results, but fruits have less ability to accumulate Zn because of the high presence of phloem tissue [34]. In this study, we showed that Zn was translocated to all organs in *S. lycopersicum* cv 'Panarea'. The concurrent presence of Zn and caffeine reduces the translocation of this element at the fruit level. Zn can easily form complexes with several molecules, including organic acids such as citrate and malate, or amino acids such as histidine. Moreover, it has been reported that when a formulation containing caffeine and Zn is prepared, a Zn–caffeine complex is formed in the solution [35]. Under our experimental conditions, this complex could reduce Zn uptake in plants, as also reported in our previous observations in *Populus alba* leaves exposed to Zn + caffeine treatments [33]. The Zn concentration found in the stems under our experimental conditions did not explain the shoot reduction observed after 56 days. Zn is necessary for the synthesis of tryptophan, a precursor of indole acetic acid; therefore, it plays an active role in the production of auxin, an essential growth hormone [36] that may indirectly interfere with internodal elongation.

Because tomato quality is also related to the content of different mineral elements that could contribute to taste, texture, and nutritional value [37] and play a role in antioxidant defense enzymes, the contents of Fe, Mn, Cu, and Ca were measured in this study. We hypothesized a positive effect of Zn and caffeine on tomatoes in terms of mineral elements; however, we found that treatments did not significantly interfere with mineral elements in tomatoes, and in general concentrations, the data are in line with the literature [38]. In the presence of Fe, the Zn–caffeine complex is converted into an Fe–caffeine complex [35], which could explain why Fe in fruit is reduced after 56 days of Zn + caffeine treatment. Regarding the total soluble content and ethylene production, our data were similar to those obtained by Roohanitaziani et al. [39], who measured the °Brix of 107 tomato accessions, and the Brix values range from 3.5–9.8. As ethylene promotes the ripening of tomato fruit, our data demonstrate that Zn + caffeine treatments did not interfere with the maturation process. The qualitative analysis of the phenolic compounds obtained in our study was consistent with those reported in the literature. For example, rutin and naringin have been reported as the main flavonoids in different varieties of red tomatoes [40–42].

It is interesting to note that the presence of Zn + caffeine in tomato plants induces an increase in the abundance of polyphenols like 4-coumaric, caffeic, and t-ferulic acid, which in the literature were found to be bioactive phenolic compounds that could help humans to ameliorate many diseases [37]. In tomatoes exposed to Zn and caffeine, the activation of a plant's antioxidant system could be the result of the stimulation of the phenylpropanoid biosynthetic pathway, inducing the synthesis of the above-indicated phenolic acids [43]. It is important to note that some other molecules (the flavones apigenin and luteolin) were significantly reduced by Zn or caffeine. These two molecules are considered functional components in foods, and their relationship with health has been proven by numerous researchers [44]. In general, the increase in polyphenols under Zn + caffeine treatment could also be associated with tomatoes, with an increase in the total antioxidant capacity of the fruits after 56 days of treatment. For this reason, tomatoes treated with low levels of caffeine and Zn could be an excellent source of secondary metabolites that play beneficial roles in inhibiting reactive oxygen species by scavenging free radicals [37]. The ability of tomato plants to take up Zn or caffeine has important implications for human health risk assessment [28,45,46]. According to Kloke [47], Tf > 1 represents the capability to transfer the mineral element/organic compound in the aerial parts of the plants [48]. Under our experimental conditions, Zn and Zn + caffeine treatments showed Tf > 1 in the stem and leaves, indicating a clear translocation in the aerial part of the compost used. Therefore, it was necessary to evaluate the THQ-based risk assessment, as both compounds can be toxic to humans if taken in high concentrations. The THQ-based risk assessment method provides a more precise indication of risks [49,50]. THQ data for Zn ingestion indicated that there were no significant health risks associated with the intake of Zn- and caffeine-treated tomatoes. Moreover, our Zn THQ data are in line with a previous meta-analysis study on tomatoes irrigated with Zn-containing water [43]. Finally, the measured risk due to the ingestion of tomatoes irrigated with caffeine indicated that THQ values in all fruits were lower than 1, indicating no considerable health risks for human consumption [28].

## 5. Conclusions

In conclusion, under our experimental conditions, the quality of fruits was conserved, and for some antioxidant molecules, such as 4-coumaric, caffeic, and t-ferulic acid, the concentration was increased. The estimated THQ values demonstrated that the daily consumption of tomatoes irrigated with Zn- and caffeine-treated water used in this study did not pose a health risk. Further specific analyses must be performed, especially for the dose related to lycopene and beta-carotene, considering the impact of these compounds on the total antioxidant profile of tomatoes. We also acknowledge that the results of our short-term experiment are not comparable to field ones because plant responses may change over time due to complex plant–soil interactions but provide an indication of the positive

effect of caffeine and Zn on tomato plants and could be taken under consideration for the future formulation of biostimulant products.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox13091100/s1, Figure S1: Schematic representation of experimental design and sampling; Table S1: Mass spectrometer parameters; Table S2: Statistic of stem length; Table S3: Statistic on fruit physiology; Table S4: Statistic of total soluble solids content and ethylene production; Table S5: Statistic of Zn concentration in roots, stem and, leaves; Table S6: Statistic of Zn concentration in fruits; Table S7: Data of mineral elements in fruits; Table S8: Statistic of mineral elements concentration in fruits; Table S9: Statistic of polyphenols data in fruits.

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# Evaluation of Bioactive Effects of Five Plant Extracts with Different Phenolic Compositions against Different Therapeutic Targets

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**Abstract:** Plant extracts rich in phenolic compounds have been reported to exert different bioactive properties. Despite the fact that there are plant extracts with completely different phenolic compositions, many of them have been reported to have similar beneficial properties. Thus, the structure–bioactivity relationship mechanisms are not yet known in detail for specific classes of phenolic compounds. In this context, this work aims to demonstrate the relationship of extracts with different phenolic compositions versus different bioactive targets. For this purpose, five plant matrices (*Theobroma cacao*, *Hibiscus sabdariffa*, *Silybum marianum*, *Lippia citriodora*, and *Olea europaea*) were selected to cover different phenolic compositions, which were confirmed by the phytochemical characterization analysis performed by HPLC-ESI-qTOF-MS. The bioactive targets evaluated were the antioxidant potential, the free radical scavenging potential, and the inhibitory capacity of different enzymes involved in inflammatory processes, skin aging, and neuroprotection. The results showed that despite the different phenolic compositions of the five matrices, they all showed a bioactive positive effect in most of the evaluated assays. In particular, matrices with very different phenolic contents, such as *T. cacao* and *S. marianum*, exerted a similar inhibitory power in enzymes involved in inflammatory processes and skin aging. It should also be noted that *H. sabdariffa* and *T. cacao* extracts had a low phenolic content but nevertheless stood out for their bioactive antioxidant and anti-radical capacity. Hence, this research highlights the shared bioactive properties among phenolic compounds found in diverse matrices. The abundance of different phenolic compound families highlights their elevated bioactivity against diverse biological targets.

**Keywords:** phenolic compound; HPLC-MS; free radical scavenging capacity; enzyme inhibition; antioxidant; neuroprotection; structure–activity relationship

## 1. Introduction

Phenolic compounds are a class of compounds present in plants that have been reported to have enormous bioactive potential [1]. These compounds are secondary metabolites naturally synthesized in plants, and more than 8000 different chemical structures have been reported [2]. Based on their basic chemical structures, these compounds have been

classified into at least 10 different classes [3]. It should be noted that, even though they show so much structural variability, they are widely studied for their antioxidant [4,5], anti-inflammatory [6], anti-aging [7], neuroprotective [8], and anticarcinogenic [9] potentials, among others. In fact, plant sources with different phenolic contents have been demonstrated to be involved in multiple pathways in the pathogenesis of different diseases [10,11]. In this scenario, there is emerging evidence on the neuroprotective activity of different phenolic compounds present in various plant sources regarding inhibition of acetylcholinesterase (AchE), Aβ aggregation, proinflammatory markers, and 1-methyl-4-phenyl-1-1,2,3,6-tetrahydropyridine (MPTP)-induced apoptosis, among others [12]. Thus, phenolic compounds from different matrices have been shown to have neuroprotective capacity, such as *Eucommia ulmoides* Oliver (rich in phenolic acids, flavonoids, and iridoid glycosides), herbaceous peony (with paeoninflorin as the main active ingredient), and *Sanghuangprous vaninii* (an extract rich in caffeic acid); they may ameliorate the dopaminergic deficiency in the MPTP-induced model in the zebrafish brain [12–14]. However, common phenolics present in different sources, for example, quercetin, which is contained in different matrices such as apple, chocolate, or cherry [15], have been shown to have both antioxidant and tyrosinase inhibitory actions [16]. Another example is kaempferol, another flavonoid, which is also present in a wide variety of plants and has a high antioxidant capacity [17]. When compared to other groups—for example, verbascoside, which belongs to the phenylpropanoids and is present in different matrices such as *Lippia citriodora* and *Olea europaea*—they have also been shown to have a high antioxidant capacity [18]. In this context, the structure–bioactivity relationship mechanisms are not yet known in detail for specific classes of phenolic compounds present in different plant sources.

Hence, the aim of this study is to evaluate the bioactive potential of different plant extracts with different phenolic profiles and to analyse the structure–bioactivity relationship mechanisms of phenolic compounds present in different plant sources. For this purpose, five plant matrices (fruits of *Theobroma cacao* (TC), calyxes of *Hibiscus sabdariffa* (HS), fruits of *Silybum marianum* (SM), leaves of *L. citriodora* (LC), and leaves of *O. europaea* (OE)) were selected. On these selected plants, a hydroalcoholic mixture extraction was carried out, and in order to know the phenolic content, a characterization by HPLC-ESI-TOF-MS was performed.

## 2. Materials and Methods

### *2.1. Chemicals*

All chemicals used in this study were of analytical reagent grade and used as received. LC-MS grade acetonitrile and formic acid for mobile phases were purchased from Riedel-de-Haën (Honeywell, Charlotte, NC, USA). For solutions, ultrapure water was obtained with a Milli-Q system Millipore (Bedford, MA, USA), and absolute ethanol was purchased from VWR chemicals (Radnor, PA, USA). The chemical standards (verbascoside, myrecetin-3-glucoside, quercetin, epigallocatechin, gardoside, sylibin, oleuropein, kaempferol, catechin, quercetin glucoside, quinic acid, and procyanidin B1) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The following reagents were used for the different in vitro assays—sulfuric acid and absolute ethanol—they were obtained from Riedel-de-Haën (Honeywell, NC, USA). Acetic acid, sodium carbonate, sodium hydroxide, TPTZ (2,4,6-tris(2-pyridyl)-s-triazine and hydrochloridic acid were purchased from Fluka (Honeywell, NC, USA). Folin reagent, gallic acid (GA), sodium phosphate monobasic and dibasic, potassium persulfate, ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulphonate)), fluorescein, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Tris (tri(hydroxymethyl)aminomethane), sodium acetate, heptahydrate ferrous sulphate, ferric chloride, DMF (dimethylformamide), NADH (β-nicotinamide adenine dinucleotide), DAF-2 (diaminofluorescein diacetate), DHR (dihydrorhodamine), potassium dihydrogen phosphate anhydrous, NBT (nitrotetrazolium blue chloride), Cayman's xanthine oxidase fluorometric assay kit, tyrosinase inhibitor screening kit (colorimetric), acetylcholinesterase from Electrophorus, acetylthiocholine iodide, neu-

trophil elastase colorimetric kit, 1-10 phenantroline, sodium chloride, hyaluronidase from sheep testes, hyaluronic acid, FALGPA (*N*-[3-(2-furyl)acryloyl]-L-leucyl-glycyl-L-prolyl-L-alanine), and collagenase from *Clostridium histolyticum* were purchased from Sigma-Aldrich (St. Louis, MO, USA). NOC-5 was purchased from Chemcruz (Santa Cruz Biotechnology, Dallas, TX, USA).

Human keratinocytes (the spontaneously immortalized cell line HaCaT) were obtained from Cell Lines Service (CLS) GmbH (Eppelheim, Germany). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin solution (10,000 U/mL penicillin and 10 mg/mL streptomycin in citrate buffer) were purchased from Gibco™/Thermo Fisher Scientific (Waltham, MA, USA). Hoechst 33342 and 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) probes were acquired from Molecular Probes™ (Invitrogen™/Thermo Fisher Scientific, Waltham, MA, USA).

### *2.2. Extraction Procedure from Plant Matrices*

The pre-industrial extracts of the selected plant matrices were provided by NATAC Biotech S.L. (Cáceres, Spain). These extracts were obtained by ethanol/water mixture extraction, which is considered a favorable solvent for the extraction of polar substances such as phenolic compounds, in addition to being environmentally friendly and non-toxic to humans (GRAS (Generally Recognized As Safe) solvent).

The extraction parameters were optimized for each plant matrix individually and are described below. For all extracts, a solid–liquid extraction (maceration) was carried out using a hydroalcoholic mixture for two hours. A mixture of EtOH:$H_2O$ (80:20; *v*:*v*) was used for all samples except for SM, which was EtOH 96%. The extraction temperatures were set at 45 °C for the OE and HS extracts and 55 °C for the rest of the extracts. A solvent:plant ratio of 20:1 was used for all extracts except for SM, which was 25:1. The obtained extracts were dried using a vacuum drying, stored at room temperature, and protected from light until their analysis. For the different analytical and bioactive analyses, the extracts were reconstituted with the same solvents and mixture used for the extraction procedure.

### *2.3. HPLC-ESI-TOF-MS Analysis*

TC, HS, SM, LC, and OE extracts at 5000 mg/L were analysed by high-performance liquid chromatography (Agilent 1290 HPLC, Agilent Technologies, Palo Alto, CA, USA) coupled to mass spectrometry with a quadrupole time-of-flight analyser (Agilent 6545 QTOF Ultra High Definition, Agilent Technologies, Palo Alto, CA, USA). Chromatographic analysis was carried out in reversed phase with a C18 ACQUITY UPLC BEH column (1.7 µm, 2.1 mm, 150 mm, 130 Å, Waters Corporation, Milford, MA, USA). The working temperature of the column was 60 °C. The mobile phases were (A) acidified water with 0.1% of formic acid (*v*/*v*) and (B) acetonitrile. The following mobile phase gradient was used for optimal separation: 0.00 min [A:B 100/0], 5 min [A:B 90/10], 18 min [A:B 15/85], 24 min [A:B 0/100], 25.50 min [A:B 0/100], 26.50 min [A:B 95/5], and 32.50 min [A:B 95/5]. A mobile phase flow rate of 0.4 mL/min and an injection volume of 5 µL was used.

MS acquisition was performed in electrospray negative ionization (ESI) mode in a mass range between 50 and 1200 *m*/*z*. Other parameters were as follows: gas flow rate 10 L/min; gas temperature 200 °C; nebulizer 20 psig, enveloping gas temperature 350 °C, enveloping gas flow rate 12 L/min, VCap 4000 V, nozzle voltage 500 V.

Finally, the acquired data were processed through Qualitative Analysis of MassHunter workstation software version B.06.00 (Agilent Technologies), Mzmine 2.53, and Sirius 2.0. The compounds were annotated by comparison of the MS/MS spectra with those from analytical standards or published in the literature and databases, such as SciFinder®, CEU Mass Mediator, Human Metabolome Data Base (HMDB), and Kyoto Encyclopedia of Genes and Genomes (KEGG).

*2.4. In Vitro Assays for Bioactive Determination of Phenolic Compounds in Extracts*

The assays described below were adapted to a 96-well polystyrene microplate, and absorbance and fluorescence measurements were performed on a Synergy H1 Monochromator-Based Multimode Microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

2.4.1. Total Phenolic Content and Antioxidant Capacity Measurements

Total phenolic content (TPC) was measured by the Folin–Ciocalteu method using gallic acid as a reference compound for the standard curve [19]. Measurements were carried out in triplicate.

The FRAP assay was performed following the method described by Benzie and Strain (1996) [20]. The reduction of the radical cation of ABTS was performed by TEAC assay using a method previously described by Zulueta et al. (2022) [21]. To test the ability of the extracts to scavenge peroxyl radicals, an ORAC method was used with some modifications [21]. In all assays, measurements were performed in triplicate.

2.4.2. Evaluation of Free Radical and ROS/RNS Scavenging Potential

All free-radical scavenging assays were performed and adapted according to Rojas-García et al. (2022) [22]. To measure the scavenging capacity of the radical superoxide anion ($\cdot O_2^-$), a colorimetric method was used. A fluorometric-based method was used for the nitric oxide ($\cdot$NO) and HOCl assays. The results for the three assays were expressed as the concentration of the different extracts needed to inhibit ROS/RNS formation by half ($IC_{50}$).

2.4.3. Evaluation of Enzymatic Inhibition Potential

The activity of the extracts to inhibit the enzyme tyrosinase was performed using the Tyrosinase Inhibitor Screening Kit (Colorimetric). The Xanthine Oxidase (XO) inhibitory activity of the extracts was measured using the Cayman's XO Fluorometric Assay Kit. The elastase inhibition assay was measured using the Neutrophil Elastase Inhibitor Screening Kit. For the measurement of hyaluronidase inhibition, the test performed by Nema et al. (2013) [23] was used with some modifications. The inhibitory effect against collagenase of the extracts was performed following the methodology performed by Kumar et al. (2019) [24], but some parameters were modified. Finally, acetylcholinesterase (AchE) inhibitory activity was measured using a photometric assay described by Ellman et al. (1961) [25]. All assays were performed in triplicate, and the result was expressed by calculating the $IC_{50}$ using different concentrations of the extracts except for the tyrosinase and elastase assays, where the % inhibition of the enzymes was calculated at 500 and 1000 mg/L concentration of the extracts, respectively.

2.4.4. Cellular Assays to Measure Antioxidant Capacity
Cytotoxic Activity In Vitro

The cytotoxic activity of the extracts was assessed on human immortalized keratinocytes (HaCaT cell line). Cells were maintained following the manufacturer's indications. DMEM with 4.5 g/L glucose and 1 mM pyruvate was supplemented with 10% ($v/v$) of fetal bovine serum and 1% ($v/v$) penicillin–streptomycin solution. Cells were grown at 37 °C in a humidified 5% $CO_2$ incubator and were passed every 2–3 days. Assays were carried out in 96-well plates with 12,000 seeded cells in each well.

Cells were seeded and were treated after 24 h with each extract at different concentrations (5–800 μg/mL). Cells were incubated for 24 h, and nuclei were stained by adding Hoechst 33342 fluorescent probe during the last 30 min (4.5 μM final concentration). Extracts were freshly prepared at 100 mg/mL in DMSO, and corresponding DMSO controls were included in the assay to evaluate cytotoxic effects caused by this solvent.

Fluorescence measurements were carried out in PBS 1x using a Cytation 3 Cell Imaging Multimode reader (BioTek, Winooski, VT, USA) with 377 nm excitation and 447 nm emission filters. Data were expressed as a percentage of cellular viability compared to

nontreated cells. $IC_{50}$ values were calculated through nonlinear regression of the algorithm-transformed concentrations and the normalized responses.

Antioxidant Activity In Vitro

To evaluate the antioxidant effectiveness of the extracts, HaCaT cells were cultured for 24 h as described above and then treated with noncytotoxic concentrations (10, 20 y 40 μg/mL) of the extracts. After 24 h of treatment, cultures were washed twice with PBS 1x. Cells were maintained with a thin layer of PBS 1x while were exposed to solar ultraviolet radiation type A (UVA) radiation (8 J/cm$^2$) emitted by a Bio-Link Crosslinker BLX-E312 (Vilber Lourmat, Collégien, France). To prevent excessive heating due to the UVA exposure, plates were meanwhile put on ice as described previously by Cooper et al. (2009) [26]. In parallel, treated cells were manipulated in the same manner but were covered during UVA exposure (nonirradiated controls). Subsequently, cells were incubated with Hoechst 33342 (4.5 μM) and H2DCF-DA (30 μM) in fresh medium for 30 min. H2DCF-DA (nonfluorescent) to monitor ROS generation through its oxidation to fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) by those radical species and cell viability (Hoechst). Both fluorescent signals were measured by the Cytation 3 reader as described above for Hoechst 33342 and using 485 nm excitation and 535 nm emission filters for DCF. DCF signals were normalized with the nuclei number determined for each well by Hoechst staining, and data were expressed as percentage of ROS (%) compared to nontreated and nonirradiated cells.

### *2.5. In Vivo Neuroprotection Capacity*

#### 2.5.1. Drosophila Stock and Exposure to Hypoxia

*Drosophila melanogaster* has been commonly employed as a neurodegeneration model system due to its minimal resource demands and notable conservation, including the response to alterations in oxygen levels, which is similar to the human. Hypoxia-induced injury is a crucial mechanism in several medical conditions, including ischemic stroke, cardiac infarction, and renal disease, among others. In fact, drosophila has been proposed by several authors to study the impact of hypoxia-reperfusion [27,28] and represents a good screening model for neurovascular disease [29].

*Drosophila melanogaster* stock (Oregon R strain) was kindly provided by Dr. Luisma Escudero. Flies were bred in polystyrene tubes on a standard medium at constant temperature and humidity (25 °C; 50% humidity) and a 12 h light/dark cycle. Three days after emergence, male flies were sexed under $CO_2$ anesthesia, and vials containing 10–15 male flies were prepared. Treated flies received instant food formulation (Genesee Scientific, Morrisville, NC, USA) prepared in water containing the plant extracts at the indicated concentrations. After hatching, male flies were maintained in standard food for 5 days, and a set of flies were supplemented in the food with the plant extracts at 0.05, 0.2, and 0.3 mg/mL. The control group was kept on media prepared in vehicle (0.5% ethanol in water). Treatment media was refreshed once during the experimental procedure. After 5 days, flies were subjected to 2.5 h of hypoxia (1% $O_2$, 25 °C, 30–40% humidity) by introducing the vials in a hypoxic glove box (Coy, Grass Lake, MI, USA) where the environmental oxygen was displaced by $N_2$. Then, flies were monitored for locomotor activity for 4 h and mortality rate was assessed after reoxygenation and represented as relative mortality to the hypoxia group. Each experiment consisted of three tubes per condition (10–15 flies/vial) and was repeated at least three times.

#### 2.5.2. Drosophila Locomotor Activity Monitoring

To study the effect of hypoxia exposure on fly behavior, flies were transferred into a 25 mm empty polycarbonate tube and placed in the Drosophila Activity Monitoring (DAM) system v3.11.1.35 (LAM25H-3, Trikinetics Inc., Waltham, MA, USA). Locomotor activity was recorded for 4 h by registering the infrared light beam crosses in each tube at 3 different heights. The DAMSystem3 Data Collection Software was used for data acquisition, and

raw data were grouped into 30-minute intervals using FileScan Software v1.13. Live flies were counted at the beginning and the end of the assessment period. Relative mean beam crosses were calculated by normalizing mean movement counts per fly to the hypoxia group and represented as a heatmap using GraphPad Prism v7.

### *2.6. Statistical Analysis*

The results were presented as mean ± standard deviation (SD) of at least three replicates. IBM SPSS Statistics 24.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis of antioxidant, free radical, and enzyme data. The differences between samples were statistically analysed by one-way ANOVA, and post hoc comparisons of the means were performed with Tukey's HSD and T3 de Dunnett tests.

GraphPad Prism version 8.01 (GraphPad Software, San Diego, CA, USA) was used for the representation and analysis of cellular assays. Data were expressed as the mean ± SD of 5–10 replicates depending on the assay. Statistical differences were determined by one-way ANOVA and statistical comparisons with Tukey's test.

## 3. Results

### *3.1. Characterisation of the Extracts by HPLC-ESI-qTOF-MS*

The TC, HS, SM, LC, and OE extracts were tentatively characterized by HPLC-ESI-qTOF-MS. Base peak chromatograms (BPCs) of the five extracts are shown in Figure S1. The characterization was carried out based on retention times, fragments, mass spectra, predictions from different software, and other studies previously published in the literature. According to the identification guidelines proposed by Sumner et al. (2007) [30], compounds were annotated at level 1 with commercial standards, at level 2 by comparing the MS/MS spectra with those present in the databases, at level 3 based on the molecular formulation and MS1 spectra, and at level 4 where the molecules remain as unknowns. All this information is provided in Tables S1, S2, S3, S4, and S5 for TC, HS, SM, LC, and OE extracts, respectively.

In total, 292 compounds were characterized, specifically 52 compounds in TC, 40 compounds in HS, 67 compounds in SM, 85 compounds in LC, and 98 compounds in OE. It was worth noting the difference between the matrices in phenolic richness, with SM, LC, and OE having more than 65 compounds in each, while TC and HS have less than 60 compounds. This fact may be mainly due to the extraction conditions and the polarity of the majority of phenolic compounds in each matrix. Among the five extracts, LC and OE contain the highest number of annotated compounds. Briefly, the flavan-3-ols was the class with the highest richness in the TC extract since epigallocatechin, quinic acid, and gluconic acid were the main compounds present in this extract. The HS extract has a high presence of hibiscus acid, hibiscus acid lactone, and glycosylated flavonoids, such as quercetin 3-O-rutinoside and quercetin 7-glucoside. The SM extract stood out for its high flavonoid presence, especially silybin and its isomers such as silycystin, isosilybin b, or its modified forms such as dehydrosilybin, silybin hydrogenated or acetylsilybin A/B. The LC extract was characterized by a particularly high presence of phenylpropanoids. Among the phenylpropanoids, verbascoside presented the highest presence. In addition, a high presence of iridoids and secoiridoids, such as shanziside, and glycosylated compounds of this type, such as gardoside, was detected. In the OE extract, the presence of the oleuropein aglycone was particularly high. The parental form of this compound, oleuropein, and other modifications of oleuropein, such as oleuropein-glucoside, were also found in high concentrations.

Table 1 shows the common compounds among the five matrices under study. The high presence of fatty acids shared by the five matrices is noteworthy. The matrices with the highest number of compounds in common were LC and OE, with verbascoside, malic acid, gluconic acid, and fatty acids such as linolenic acid and palmitic acid standing out among the compounds in common.

**Table 1.** Common compounds that have been identified in more than one of the matrices.

| Compound | Rt (min) | [M-H]$^-$ | Mol. Formula | *T. cacao* | *H. sabdariffa* | *S. marianum* | *L. citriodora* | *O. europaea* |
|---|---|---|---|---|---|---|---|---|
| Gluconic acid | 1.01 | 195.0519 | $C_6H_{12}O_7$ | x | | | x | x |
| Malic acid | 1.07 | 133.0140 | $C_4H_6O_5$ | | | | x | x |
| Quinic acid | 1.13 | 191.0292 | $C_7H_{12}O_6$ | x | | | | x |
| Chlorogenic acid | 4.02 | 353.0867 | $C_{16}H_{18}O_9$ | | x | x | | |
| Epigallocatechin | 9.18 | 305.0690 | $C_{15}H_{14}O_7$ | x | | | | x |
| Verbascoside | 9.33 | 623.1978 | $C_{29}H_{36}O_{15}$ | | | | x | x |
| Quercetin 3-O-rutinoside | 9.60 | 609.1458 | $C_{27}H_{30}O_{16}$ | | x | | | x |
| Quercetin glucoside | 9.85 | 463.0878 | $C_{21}H_{20}O_{12}$ | x | x | x | | |
| Unknown | 10.23 | 539.1751 | $C_{25}H_{32}O_{13}$ | | x | | | x |
| Quercetin | 12.16 | 301.0339 | $C_{15}H_{10}O_7$ | x | x | | | |
| Unknown | 12.95 | 329.0654 | $C_{17}H_{14}O_7$ | | x | | x | |
| Dihydrocapsiate | 13.53 | 307.192 | $C_{18}H_{28}O_4$ | | | | x | x |
| Eupatorin | 13.56 | 343.0818 | $C_{18}H_{16}O_7$ | | x | | x | |
| Gingerol | 13.92 | 293.1748 | $C_{17}H_{26}O_4$ | x | x | | x | x |
| Hydroxylinolenic acid | 15.74 | 293.2113 | $C_{18}H_{30}O_3$ | | x | | x | x |
| Unknown | 16.30 | 540.3288 | $C_{29}H_{49}O_9$ | x | | x | x | x |
| Unknown | 16.60 | 566.3453 | $C_{31}H_{51}O_9$ | | | x | x | x |
| Ethyl vanillate | 17.77 | 195.0686 | $C_{10}H_{12}O_4$ | x | | | | x |
| Linolenic acid | 18.46 | 277.2159 | $C_{18}H_{30}O_2$ | x | x | | x | x |
| 10′-Apo-beta-carotenal | 18.73 | 375.2712 | $C_{27}H_{36}O$ | x | x | x | x | x |
| Linoleic acid | 19.16 | 279.2328 | $C_{18}H_{32}O_2$ | x | x | x | x | x |
| Palmitic acid | 19.82 | 255.2325 | $C_{16}H_{32}O_2$ | x | | | x | x |
| Unkwnon | 19.87 | 403.3052 | $C_{22}H_{44}O_6$ | x | x | x | | x |
| Oleic Acid | 19.95 | 281.2482 | $C_{18}H_{34}O_2$ | x | x | x | x | x |
| Unknown | 20.98 | 383.1934 | $C_{16}H_{32}O_{10}$ | x | x | | x | x |
| Ginsenoside Rh2 | 20.98 | 621.4417 | $C_{36}H_{62}O_8$ | | | x | x | |

x: presence of the compound in the extract.

### *3.2. Evaluation of the Antioxidant and Anti-Inflammatory Capacities of the Extracts*

#### 3.2.1. Evaluation of TPC, Antioxidant Capacity and ROS Scavenging Potential

In Table 2, we can find the TPC values obtained by the Folin–Ciocalteu method and the results of the FRAP, TEAC, and ORAC tests for the five matrices under study. As a result, all the matrices under study show antioxidant capacity, so they can all be considered bioactive against oxidative stress. It is worthwhile to highlight the case of the SM extract, which obtained the highest values for the four tests. In addition, a general trend showing that the higher the content of TPC, the greater the capacity for the transfer of electrons and H atoms can be observed, confirming previous studies [31]. This is also in agreement with the findings in the case of the HS extract, which presented the lowest values in the four assays. It is important to note that there is no universal method for measuring antioxidant capacity, as different methods can measure various mechanisms of action. For instance, assays like FRAP and TEAC are based on single electron transfer (SET), employing indirect and direct approaches, respectively. On the other hand, the ORAC method relies on hydrogen atom transfer (HAT), which is similar to both electron transfer and hydrogen atom transfer. Choosing different assays to evaluate antioxidant activity can offer a comprehensive prediction of this bioactive potential, providing complementary information [32].

**Table 2.** Evaluation of total phenolic content and antioxidant capacity of extracts.

| Sample | TPC (mg GAE/g DE) | FRAP (mmol $Fe^{2+}$/g DE) | TEAC (mmol TE/g DE) | ORAC (mmol TE/g DE) |
|---|---|---|---|---|
| *T. cacao* | $255 \pm 12^{a}$ | $1.38 \pm 0.09^{b,c}$ | $1.25 \pm 0.08^{a}$ | $2.35 \pm 0.09^{c}$ |
| *H. sabdariffa* | $51 \pm 1^{d}$ | $0.47 \pm 0.05^{d}$ | $0.24 \pm 0.02^{c}$ | $1.16 \pm 0.03^{d}$ |
| *S. marianum* | $617 \pm 8^{b}$ | $1.4 \pm 0.3^{a,b,c,d}$ | $1.3 \pm 0.1^{a}$ | $11.7 \pm 0.3^{b}$ |
| *L. citriodora* | $344 \pm 15^{c}$ | $2.4 \pm 0.2^{a}$ | $1.15 \pm 0.08^{a}$ | $5.9 \pm 0.2^{a}$ |
| *O. europaea* | $216 \pm 28^{a}$ | $1.65 \pm 0.10^{a,b}$ | $0.84 \pm 0.05^{b}$ | $6.0 \pm 0.2^{a}$ |

TPC: Total Polar Compounds; FRAP: Ferric Reducing Antioxidant Power Assay; TEAC: Trolox Equivalent Antioxidant Capacity; ORAC: Oxygen Radical Absorbance Capacity; GAE: Gallic Acid Equivalent; DE: Dry Extract; TE: Trolox Equivalent. Data are means ± standard deviation ($n$ = 3). Different letters represent statistically significant differences at $p < 0.05$ level.

Table 3 reveals the amount of extract required to inhibit half the concentration of reactive species ($IC_{50}$).

**Table 3.** Evaluation of radical scavenging of controls and extracts.

| Sample | $\cdot O_2^-$ (mg/L) [1] | ·NO (mg/L) [1] | HOCl (mg/L) [1] |
|---|---|---|---|
| *T. cacao* | $29.7 \pm 0.4^{c}$ | $0.42 \pm 0.02^{b}$ | $0.71 \pm 0.02^{b}$ |
| *H. sabdariffa* | $50 \pm 2^{a}$ | $10.3 \pm 1.0^{c}$ | $1.32 \pm 0.04^{d}$ |
| *S. marianum* | $57 \pm 6^{a}$ | $5.0 \pm 0.8^{a}$ | $0.70 \pm 0.01^{b}$ |
| *L. citriodora* | n.d. | $3.76 \pm 0.08^{a}$ | $3.5 \pm 0.4^{a}$ |
| *O. europaea* | $269 \pm 17^{d}$ | $3.0 \pm 0.2^{a}$ | $16 \pm 2^{c}$ |
| Gallic acid | $50 \pm 3^{a}$ | $1.4 \pm 0.3^{b}$ | $3.8 \pm 0.3^{a}$ |
| Epicatechin | $70 \pm 5^{b}$ | $0.87 \pm 0.02^{b}$ | $0.18 \pm 0.01^{e}$ |

Data are means ± standard deviation ($n$ = 3). [1] Inhibitory Concentration at 50%. Different letters represent statistically significant differences at $p < 0.05$ level. n.d.: no data.

The intracellular accumulation of ROS, which occurs in cells under oxidative stress, is responsible for several chronic pathologies, including cancer, neurodegenerative or cardiovascular pathologies [33]. Thus, Reuter et al. (2010) revealed that oxidative stress

can activate several transcription factors, which can lead to the expression of more than 500 different inflammation-related genes [34]. The activation of this entire cascade can lead to chronic inflammation, which in turn may mediate most chronic diseases, including cancer, diabetes, cardiovascular, neurological, and pulmonary diseases [34]. In this regard, phenolic compounds have been shown to have a potent antioxidant effect because their chemical structure means they can eliminate ROS, and their antioxidant capacity is therefore related to the other properties of this type of compound, such as anti-inflammatory and neuroprotective properties.

In relation to radical scavenging assays, with the exception of LC and OE for the $\cdot O_2^-$ assay, all showed bioactivity. TC presented the best $IC_{50}$ value for all the radical scavenging tests, being significantly better than the other extracts. This indicates that the types of phenolic compounds present in TC have a higher anti-radical power than those present in OE for the $\cdot O_2^-$ and HOCl test and that the HS for the ·NO test presented the highest $IC_{50}$ values. For these tests, epicatechin (EPI) and gallic acid (GA) were used as standard controls, listed in Table 3. It is observed that, especially in the HOCl assay, EPI has significantly better values compared to the five plant matrices, which is related to the TC extract being one of the lowest as this extract has a high EPI content.

### 3.2.2. Evaluation of Enzymatic Inhibition Capacity

Table 4 presents the inhibitory effect of the five extracts under study on the hyaluronidase, XOD, tyrosinase, elastase, and collagenase enzymes and the positive control used for each enzyme. As mentioned above, phenolic compounds are involved in the regulation of the level of reactive species. In this sense, the excess of these reactive species can lead to excessive activation and dysregulation of different enzymes studied in this work. For instance, the enzyme XOD is a dehydrogenase responsible for catalyzing hypoxanthine to xanthine and subsequently to the oxidation of uric acid. However, when oxidative stress is present, XOD is transformed into an oxidase, which leads to the production of superoxide radicals and causes many inflammatory diseases [35].

**Table 4.** Evaluation of enzymatic inhibition capacity of controls and extracts.

| Sample | Hyaluronidase (mg/L) [1] | XOD (mg/L) [1] | Tyrosinase (% inh.) [2] | Elastase (% inh.) [3] | Collagenase (mg/L) [1] | AChE (mg/L) [1] |
|---|---|---|---|---|---|---|
| *T. cacao* | $12 \pm 2^{a}$ | n.d. | $28 \pm 8^{a,b,c}$ | $23 \pm 4^{b,c,d}$ | $156 \pm 1^{b}$ | $244 \pm 9^{a}$ |
| *H. sabdariffa* | $66 \pm 6^{d}$ | n.d. | $5 \pm 2^{c}$ | $44.1 \pm 0.5^{a,b,c,d}$ | $1190 \pm 35^{b}$ | n.d. |
| *S. marianum* | $4.9 \pm 0.4^{a}$ | $4.4 \pm 0.4^{a}$ | $39 \pm 4^{a}$ | n.d. | $56 \pm 3^{b}$ | $1259 \pm 53^{b}$ |
| *L. citriodora* | $87 \pm 5^{c}$ | $3.2 \pm 0.4^{a,b}$ | $17 \pm 2^{b,c}$ | $48 \pm 3^{a}$ | $633 \pm 27^{a}$ | $316 \pm 4^{c}$ |
| *O. europaea* | $187 \pm 5^{b}$ | $2.3 \pm 0.3^{b}$ | $28 \pm 3^{a,b}$ | $25 \pm 3^{b,c,d}$ | $618 \pm 9^{a}$ | $373 \pm 6^{d}$ |
| Gallic acid | $102 \pm 4$ [4] | n.d. | n.d. | n.d. | n.d. | n.d. |
| Epicatechin | $167 \pm 6$ [4] | $9 \pm 1^{c}$ | n.d. | n.d. | n.d. | n.d. |
| Physostigmine | n.d. | n.d. | n.d. | n.d. | n.d. | $0.043 \pm 0.004$ [6] |
| 1,10-phenanthroline | n.d. | n.d. | n.d. | n.d. | $83 \pm 2$ [5] | n.d. |
| Elastatinal (51.26 ppm) | n.d. | n.d. | n.d. | $53 \pm 5$ | n.d. | n.d. |
| Kojic acid (21.3 ppm) | n.d. | n.d. | $49 \pm 6$ | n.d. | n.d. | n.d. |

Data are means ± standard deviation ($n = 3$). [1] Inhibitory Concentration at 50%. [2] At 500 mg/L. [3] At 1000 mg/L. [4] Inhibitory Concentration at 20%. [5] % Inhibition. [6] Inhibitory Concentration at 90%. Different letters represent statistically significant differences at $p < 0.05$ level. n.d.: no data.

In the case of the hyaluronidase assay, all matrices under study show bioactivity. Nevertheless, SM has been shown to be the most bioactive, while OE is significantly less so. For the enzyme XOD, the lowest $IC_{50}$ value corresponds to OE, while TC and HS did not even reach this 50% inhibition. Furthermore, when compared to the positive control used, EPI, both SM and LC, and OE have a more significant ability to inhibit the enzyme. The tyrosinase inhibition assay shows the % inhibition of the enzyme at 500 mg/L, with SM showing the highest activity and HS the lowest and with the other three extracts being similar in terms of inhibition. In the elastase assay, the results were expressed as % inhibition of the enzyme at 1000 mg/L, with the LC extract having the highest inhibitory power, while SM is not able to inhibit. Thus, in this case, with the exception of SM, all the extracts showed bioactivity. Finally, in the collagenase assay, the five matrices under study were able to inhibit collagenase, highlighting, in this case, SM since, when compared to HS, which is the least bioactive, there is a big difference in the dose needed to inhibit the enzyme at 50%.

### 3.2.3. Cellular Assays to Measure Antioxidant Capacity

The cytotoxic effects of the extracts in HaCaT cells were plotted in Figure 1 (Statistical significance for Figure 1 was included in Table S6). DMSO was used as a vehicle to solubilize the extracts and not alter cell viability, even at the highest used concentration (Table S7).

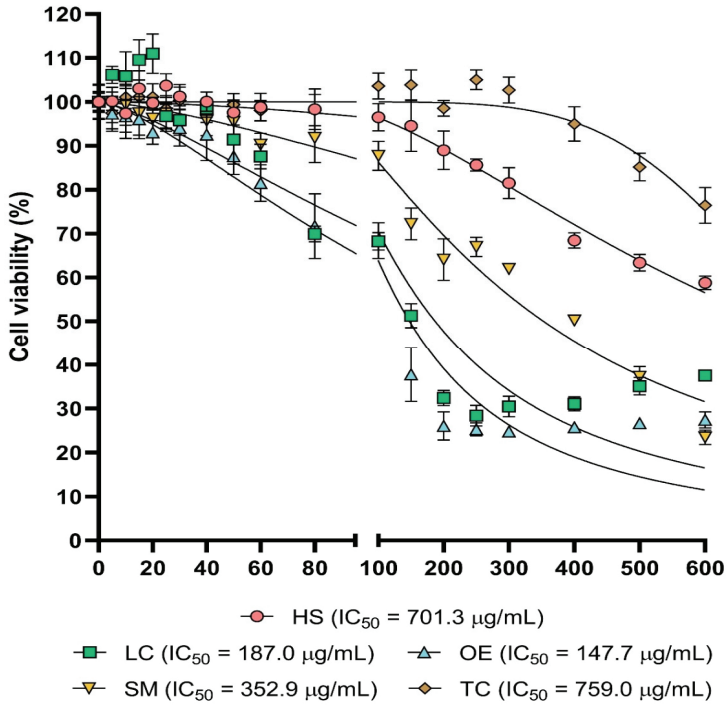


**Figure 1.** Cytotoxic activity. Dose–response cell viability plots for the five extracts tested. Cell viability values were obtained as described in methods section. Statistical significance was included in Table S6.

LC and OE extracts exhibited the highest cytotoxicity, and both treatments resulted in a statistically significant reduction in cell viability from 40 μg/mL. $IC_{50}$ was estimated as 187 μg/mL for LC and 147.4 μg/mL for OE. In the case of the SM extract, cytotoxic effects were statistically significant from 60 μg/mL, and $IC_{50}$ was 352.9 μg/mL. With the HS treatment, cell viability was decreased from 200 μg/mL with statistical significance, and calculated $IC_{50}$ was 701.3 μg/mL for this extract, which did not reduce cell viability in a significant manner up to 400 μg/mL and whose $IC_{50}$ was 759 μg/mL.

The effectiveness of the extracts as antioxidant ingredients was explored in the HaCaT model due to the significant role of oxidative stress on skin health and aging. UVA was chosen as a well-known inductor of ROS generation and oxidative stress in the skin [36]. On the one hand, as shown in Figure 2, none of the extracts were able to reduce the basal oxidative stress in the absence of UVA. On the other hand, UVA radiation increased the presence of ROS in a significant way in all the conditions, and only the SM extract was able to decrease these ROS levels in a dose-dependent manner. Pretreatment with 20 μg/mL of SM reduced the increment in ROS levels from 146% (untreated but irradiated condition) to 129%. A statistically significant reduction to 105% ROS was evidenced for 40 μg/mL (Figure 2D, ####, $p < 0.0001$). Apparently, the rest of the extracts (HS, OE, LC, and TC) did not prevent the oxidative action of UVA. Furthermore, the LC extract exhibited a significant prooxidant effect at 20 and 40 μg/mL, probably related to a phototoxic effect of some of its components.

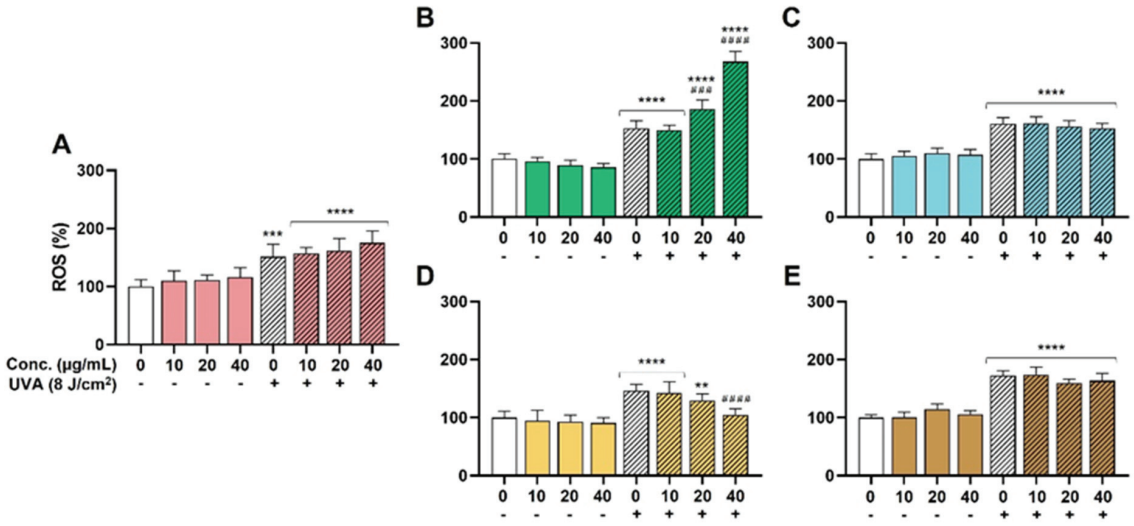


**Figure 2.** Antioxidant activity. ROS levels were measured as described in methods section. Significance was established at $p < 0.05$. ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$) are in the figures and indicate statistically significant differences compared to the nontreated and nonirradiated control. ### ($p < 0.001$), and #### ($p < 0.0001$) indicate statistically significant differences compared to the nontreated irradiated control. (**A**): *Hibiscus sabdariffa*; (**B**): *Lippia citriodora*; (**C**): *Olea europaea*; (**D**): *Silybum marianum*; (**E**): *Theobroma cacao*.

### *3.3. Evaluation of the Neuroprotective Effect of the Extracts*

There is a close link between the ability of phenolic compounds to exert their neuroprotective effect through their antioxidant and free radical scavenging action and their ability to inhibit enzymes involved in neurodegenerative diseases, such as AChE [8].

#### 3.3.1. Evaluation of Acetylcholinesterase (AChE) Inhibition Capacity

Table 4 shows the inhibitory effect of the extracts on AChE, showing that the lowest extract concentration to inhibit 50% of the enzymatic activity was for the TC extract, followed by LC, OE, and SM extracts. The HS extract did not even reach 50% of the enzymatic activity at very high doses of concentration. Thus, with the exception of HS, all showed bioactivity against enzyme inhibition.

#### 3.3.2. Effect of Supplementation with Different Plant Extracts on Hypoxia–Reoxygenation Injury in *D. melanogaster*

Hypoxic stress is known to produce injury in flies. The drosophila model does not reproduce some aspects of human brain ischemia because of its primitive blood system (blood vessels are lacking, and there is no lymphoid blood cell lineage). However, the model recapitulates important pathogenic features when subjected to hypoxia, such as increased

activation of brain caspases, locomotor deficiencies, and mortality [37]. Moreover, increases in several oxidative stress markers and changes in metabolic activity have been observed in flies subjected to hypoxia [27]. Therefore, the use of this screening model provides an easy and convenient way of testing antioxidant compounds, such as polyphenols. To investigate the influence of hypoxia–reoxygenation injury in Drosophila, treated flies were subjected to hypoxic stress for 2.5 h under controlled conditions (1% $O_2$; 25 °C; 30–40% relative humidity) (Figure 3a). As expected, flies in the hypoxia group showed a significant increase in mortality compared to the control group 4 h after reoxygenation. Treatment with the extract TC at the higher concentration resulted in a significant increase in fly survival after the hypoxia challenge (Figure 3b).

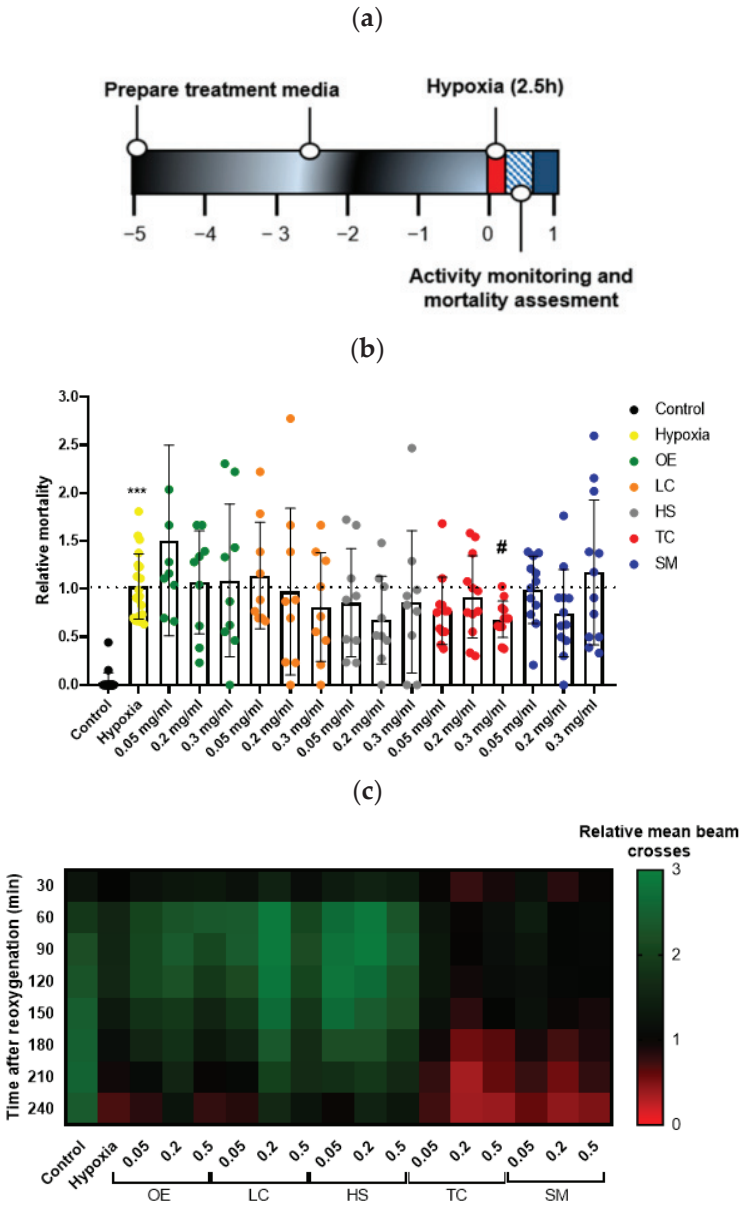


**Figure 3.** Effect of plant extract supplementation on Drosophila melanogaster exposed to severe hypoxia. (**a**) Schematic illustration of the hypoxia protocol in flies. (**b**) Effect of the treatment on fly survival after exposure to 1% $O_2$ for 2.5 h. Data were analyzed using one-way ANOVA test followed by Dunnett´s multiple comparisons test *** $p < 0.001$ vs. control; # $p < 0.05$ vs. hypoxia. (**c**) Heatmap displaying the locomotor activity of treated flies. Flies were transferred into the monitor immediately after hypoxia and the activity was recorded for 240 min after reperfusion. Each cell shows the mean beam crosses per fly in the group.

We also studied fly behavior by quantifying animal movement for 4 h after hypoxia. While control flies moved uniformly over time, flies in the hypoxia group showed a reduction in locomotor activity, indicated by fewer beam crosses, which were more evident after 120–150 min of reoxygenation, revealing that the reperfusion injury worsened fly behavior. However, treatment with TC could not recover the loss of locomotor activity induced by hypoxia (Figure 3c).

The results obtained show that there is no single matrix that stands out for its bioactivity in all the tests, but rather, depending on the bioactive target and the assay in question, there are matrices that stand out. Still, the rest, with a few exceptions, also show bioactivity. This shows that despite the structural diversity present in the different families of phenolic compounds, they all show high bioactivity; it is the combination of these compounds that gives them their pleiotropic character.

## 4. Discussion

The extracts under study have been characterized, giving a wide range of phenolic compounds, some of which are characteristic of each of the matrices and a minority of which are common among the different matrices (Table 1). Although most of the compounds are not common, all matrices have bioactive potential against the targets under study, so the differences in potential between them may be mainly due to these specific compounds being in each of the matrices and also due to the differences in the ratio between them.

When we pay attention to the results of the antioxidant tests for TPC and FRAP, TEAC, and ORAC (Table 2), the higher the TPC, the greater the antioxidant power shown in the tests. Thus, HS has the lowest TPC and the lowest value in the other antioxidant test values, while SM has the highest TPC content and high values in the rest of the tests measuring antioxidant power. This relationship is consistent with the tests carried out by Aroso et al. (2017) [38], in which there is also a positive relationship between the TPC content and the tests measuring antioxidant power.

In the case of the other three remaining plant matrices, they have intermediate values for the results of the TPC and FRAP, TEAC, and ORAC assays. For the FRAP assay, the matrices TC, SM, and OE showed very similar values. This may be due to the fact that there are similar compounds in their composition. For example, both TC and OE have epigallocatechin [39], a compound that has been shown to have a potent action in the FRAP assay. In contrast, SM, with its high content of flavolignans such as silybin A and B, has also been shown to have a potent action in the FRAP assay [40]. In the case of the ORAC assay, the matrices LC and OE obtained a similar and high value, which may be due to the fact that both matrices share the most compounds in their composition, like verbascoside, which has been shown to be potent in proton transfer, the mechanism of action on which this antioxidant capacity test is based [41].

In the case of cellular assays in HaCaT cells, TC, OE, and SM extracts were able to decrease ROS levels; however, the only one that achieved this in a dose-dependent manner was SM. This is in agreement with Svobodová et al. (2007) [42], who found that flavonolignans present in the SM extract suppress UVA-induced oxidative stress in HaCaT cells, making this extract potentially useful in the treatment of UVA-induced skin damage.

In relation to the tests to measure the capacity to eliminate specific ROS and RNS, for the $\cdot O_2^-$ radical test, the HS and SM matrices obtained a similar $IC_{50}$ value, which is also similar to the value obtained for the gallic acid standard. Table 1 shows that the compounds that these two matrices have in common include chlorogenic acid and quercetin glucoside. In this sense, there are studies that demonstrate the high power of chlorogenic acid [43], quercetin glucoside [44], and gallic acid [45] for the uptake of the superoxide radical, which is in agreement with the results obtained.

In the test to eliminate HOCl, TC and SM were the ones that obtained the lowest IC50. Despite the difference in phenolic composition, they achieved similar results. TC extract has a high epigallocatechin and epicatechin content. As shown in Table 3, epicatechin has a high free radical scavenging power, with its power in the HOCl scavenging test standing

out, where it has more power than even the whole TC extract. This is supported by different studies showing the high power of TC extract to scavenge free radicals [46]. Furthermore, He et al. (2018) evaluated the power of different catechins against radicals, and they found that epigallocatechin gallate possessed the highest radical scavenging power, followed by epigallocatechin, epicatechin, and catechin in descending order of power. This suggests that the effect of these compounds is strongly related to the structure of catechins, mainly due to the hydroxyl and galloyl groups [47]. For SM, silybin has shown potent action for HOCl removal [48].

In the case of the XOD enzyme, the OE and LC extracts have been shown to have potent inhibitory power. Both extracts share compounds such as verbascoside, the main bioactive compound present in LC. This compound was shown to be a potent inhibitor of the XOD enzyme, as verbascoside is able to enter the active site of XOD and form hydrogen bonds with amino acid residues (such as Lys-1045, Arg-880, Arg-912, Glu-1261, and Gln-1194) [49].

Within the enzymes involved in maintaining skin firmness, hyaluronidase, elastase, and collagenase, there is no single matrix that stands out for all of them. For both hyaluronidase and collagenase, the TC and SM matrices have the lowest $IC_{50}$ values. Thus, in the case of TC, the content of compounds from the flavan-3-ols group was shown to be potent inhibitors of these three enzymes involved in the loss of elasticity and firmness of the skin [50]. For SM, the main group of compounds are flavolignans, which have also been shown to have a potent inhibitory action on the aforementioned enzymes. This shows that phenolic compounds from different groups and, therefore, with different chemical properties can exert the same bioactive effect [51].

The mechanism of action by which the polyphenols present in our matrices have the ability to inhibit enzymes would be the next step in our research. There are in silico studies—using different phenolic compounds that have been tested on the enzymes—for example, the inhibition of rosmarinic acid against human hyaluronidase. Molecular docking studies revealed that rosmarinic acid is bounded to the hyaluronidase binding pocket with four binding interactions [52]. In our study, the hyaluronidase used was not human but bovine, but these are highly phylogenetically conserved proteins [53]. Another compound that has been tested in silico against collagenase, elastase, and tyrosinase enzymes is caffeine, which was shown to form a stable protein–ligand complex validated by molecular dynamics simulation. Thus, the potential of phenolic compounds in the inhibitory action of these enzymes is shown [54].

Finally, the neuroprotective effect of the different extracts was evaluated in both in vitro and in vivo assays. In the case of the in vitro AChE enzyme inhibition assay, the extract with the highest enzyme inhibitory capacity was TC, followed by LC and OE extracts with a similar $IC_{50}$ value. In the case of the in vivo assay in which the survival of flies after hypoxia challenge was measured, it was TC that significantly improved survival. The TC extract is high in epigallocatechin and epicatechin, compounds that have been shown to be potent inhibitors of AChE [55,56]. The similar inhibition of LC and OE at doses slightly higher than TC may be due to their high presence of flavonoid glycosides, which are considered essential for AChE inhibition [57].

The results highlight the importance of reporting the bioactive properties of plant extracts according to their phenolic composition and not simply their total phenolic content, as many of the bioactive properties can be related to a particular phenolic type or phenolic family or to the synergistic action of them.

In this context, when exploring the potential use of bioactive compounds, such as phenolic compounds, for health improvement, utilizing combined plant extracts becomes intriguing. A rich array of phenolic compounds from diverse sources could offer broader benefits to the organism, leveraging the potential synergistic effects arising from different types of phenolic compounds [58]. Therefore, plant extracts with this bioactive potential could be used for the development of nutraceuticals for the prevention of diseases related to metabolic stress and inflammation [59]. Additionally, these extracts could be em-

ployed in nutricosmetics, as the main enzymes evaluated—such as tyrosinase, collagenase, and hyaluronidase—are associated with maintaining skin color, elasticity, and hydration, respectively [60].

## 5. Conclusions

In this study, the antioxidant, anti-inflammatory, and neuroprotective effects of five plant extracts showing different phenolic compositions have been evaluated by means of different assays in order to assess the relationship between the presence of different types of phenolic compounds and their bioactivity. This study shows that not all extracts that are rich in phenolic compounds show the same bioactivities in the assays used, but, depending on their phenolic composition, there are extracts with greater or lesser bioactive potential against different targets. In general, it is observed that when there are common phenolic compounds in the different extracts, some of the bioactive capacities are similar, as has been observed in the case of LC and OE. Nevertheless, the greatest bioactive difference has been observed when comparing the richness of the families of phenolic compounds, so the SM extract, rich in flavolignans such as silybin, are shown to possess a high antioxidant capacity in both spectrophotometric and cellular assays. It also proved to be a potent inhibitor of tyrosinase, hyaluronidase, and collagenase. In contrast, in other bioactivity tests measuring free radical scavenging capacity and neuroprotective effect through inhibition of the enzyme AChE and survival in flies subjected to hypoxia stress, it was the TC extract that stood out, possibly due to its high presence in compounds of the flavan-3-ol family, such as epigallocatechin and epicatechin. In future studies, it would be interesting to study the relationship between the phenolic composition of the matrices and the macronutrients present in the same matrices, with the aim of evaluating whether these interactions affect the hypoactivity of the phenolic compounds. Thus, this work demonstrates that phenolic compounds present in different matrices have common bioactive properties and that the abundance of different families of phenolic compounds makes them stand out with higher bioactivity against different biological targets.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antiox13020217/s1, Figure S1. Base peak chromatogram from the extracts. A. *T. cacao*. B. *H. sabdariffa*. C. *S. marianum*. D. *L. citriodora*. E. *O. europaea*; Table S1. Identification of phytochemical compounds in *T. cacao* extract by HPLC-ESI-qTOF-MS; Table S2. Identification of phytochemical compounds in *H. sabdariffa* extract by HPLC-ESI-qTOF-MS; Table S3. Identification of phytochemical compounds in *S. marianum* extract by HPLC-ESI-qTOF-MS; Table S4. Identification of phytochemical compounds in *L. citriodora* extract by HPLC-ESI-qTOF-MS; Table S5. Identification of phytochemical compounds in *O. europaea* extract by HPLC-ESI-qTOF-MS; Table S6. Numerical results (mean and SD) and statistical significance for Figure 1 results; Table S7. Equivalent DMSO concentrations for each extract concentration.

**Author Contributions:** Conceptualization, M.d.C.V.-A. and M.d.l.L.C.-G.; methodology, M.d.C.V.-A., N.S.-M. and C.D.R.; software, M.d.C.V.-A., N.S.-M., Á.F.-O., C.D.R. and M.H.-L.; investigation, M.d.C.V.-A., N.S.-M. and C.D.R.; resources, V.M., E.B.-C., D.A.-R. and A.S.-C.; data curation, M.d.C.V.-A., N.S.-M., M.d.l.L.C.-G., Á.F.-O., C.D.R., J.M. and M.H.-L.; writing—original draft preparation, M.d.C.V.-A.; writing—review and editing, M.d.l.L.C.-G., Á.F.-O. and E.B.-C.; visualization, M.d.l.L.C.-G., Á.F.-O. and J.M.; supervision, M.d.l.L.C.-G., Á.F.-O., J.M. and M.H.-L.; project administration, V.M., E.B.-C., D.A.-R. and A.S.-C.; funding acquisition, V.M., E.B.-C., D.A.-R. and A.S.-C. All authors have read and agreed to the published version of the manuscript.

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*Review*

# Natural Bio-Compounds from *Ganoderma lucidum* and Their Beneficial Biological Actions for Anticancer Application: A Review

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**Abstract:** *Ganoderma lucidum* (*G. lucidum*) has been known for many centuries in Asian countries under different names, varying depending on the country. The objective of this review is to investigate the scientific research on the natural active bio-compounds in extracts obtained from *G. lucidum* with significant biological actions in the treatment of cancer. This review presents the classes of bio-compounds existing in *G. lucidum* that have been reported over time in the main databases and have shown important biological actions in the treatment of cancer. The results highlight the fact that *G. lucidum* possesses important bioactive compounds such as polysaccharides, triterpenoids, sterols, proteins, nucleotides, fatty acids, vitamins, and minerals, which have been demonstrated to exhibit multiple anticancer effects, namely immunomodulatory, anti-proliferative, cytotoxic, and antioxidant action. The potential health benefits of *G. lucidum* are systematized based on biological actions. The findings present evidence regarding the lack of certainty about the effects of *G. lucidum* bio-compounds in treating different forms of cancer, which may be due to the use of different types of *Ganoderma* formulations, differences in the study populations, or due to drug–disease interactions. In the future, larger clinical trials are needed to clarify the potential benefits of pharmaceutical preparations of *G. lucidum*, standardized by the known active components in the prevention and treatment of cancer.

**Keywords:** natural bio-compounds; *Ganoderma lucidum*; polysaccharides; triterpenoids; antitumor activity; immunomodulatory; antioxidant; cytotoxic

## 1. Introduction

*Ganoderma lucidum* (*G. lucidum*), (Fr.) Karst is a medicinal mushroom known in traditional Asian medicine under different names depending on the country: Lingzhi (China), Reishi, and Mannentake (Japan), Linh chi (Vietnam), and Yeong Ji or Yung Gee (Republic of Korea) [1]. It is considered a source of longevity and health promotion [2–4]. The traditional medicinal uses of these mushrooms in Chinese and Japanese folk medicine as

health remedies and herbal supplements are widely recognized [5–7]. The knowledge and use of medicinal mushrooms have preoccupied researchers, who have developed valuable studies on their bioactive components and their importance [8–12]. According to the World Health Organization, it has been estimated that around three-quarters of the world's population relies on traditional medicines to maintain their health [13]. In traditional oriental medicine, *G. lucidum* has been used to treat several diseases, such as asthma, bronchitis, arthritis, hypertension, insomnia, diabetes, liver disease, nephritis, and cancer [14–16]. In ancient Chinese medicine, *G. lucidum* has been used for longevity and as an anticancer and antioxidant agent [17–20]. In the last 30 years, data have been reported on the chemical composition of *G. lucidum* extracts, justifying their biological activity and numerous health benefits. Among the most important bioactive compounds are polysaccharides and triterpenes [21–26]. Numerous other bioactive metabolites, such as proteins, peptides, sterols, lectins, adenosine, vitamins, and metals, have also been identified [27–31]. However, it should be noted that there are differences in the composition of *G. lucidum* products due to cultivation conditions, the extraction process of the bio-compounds, and the origin or part of the fungus used (fruiting body, mycelium, or spores) [14,28,30]. Nevertheless, many bioactive compounds in *G. lucidum* exhibit antioxidant, antitumor, and anti-inflammatory properties [32–37]. Such benefits have been investigated in multiple studies on their effect against prostate cancer, ovarian cancer, hepatocellular carcinoma, the induction of apoptosis in colon cancer, and the inhibition of angiogenesis [38–43]. Different immunomodulatory activities have been reported [44–47]. Other researchers have also reported studies on antioxidant and antioxidative stress actions [48–50]. With the development of modern research techniques to identify the chemical compositions of *G. lucidum* compounds, data may be accessible for use in medical research. A detailed understanding of these biological mechanisms could greatly influence and extend their benefits to human health.

The present study aims to review the data on active bio-compounds with important biological actions against cancer. Factors influencing the composition of the *G. lucidum* fungus, such as growing conditions (in the wild, in deciduous forests, or in special crops) and territorial areas, are analyzed. The characteristics of the *G. lucidum* fungus, as well as the chemo-bioactive compounds identified in *G. lucidum* with anticancer activities, its biological activities against cancer, and its toxicity and safety, are described in separate sections. The mechanisms of anticancer effects, including other biological activities that contribute to the fight against cancer, such as immunomodulatory, antioxidant, and cytotoxic actions, are also presented. Some negative cases in which *G. lucidum* bio-compounds were administered to patients undergoing treatment for several conditions are included as well. In these patients, the effects of *G. lucidum* treatments were not beneficial due to the occurrence of adverse effects.

## 2. Characteristics of *Ganoderma lucidum* Fungus

### *2.1. Description and Spread of the Fungus*

The first description of *Ganoderma lucidum* (Curtis) P. Karst. was made by Curtis in England, and this description was officially recorded by Fries [51]. Initially, Cao et al. claimed that this fungus was already known as "Lingzhi", a medicinal fungus identified and used in China for more than 2000 years [52]. Later, based on molecular studies, it was established that the East Asian medicinal mushroom is a different species from the *G. lucidum* mushroom [52]. After morphological and molecular examinations, Wang et al. confirmed that the Lingzhi species from China are related to *G. lucidum* from the UK and to other *Ganoderma* species [53]. Kwon et al. conducted phylogenetic analyses of *Ganoderma* species and showed that there are 62 strains of *Ganoderma* [54]. According to the taxonomic classification established by Nahata A., the species *Ganoderma lucidum* (Curt: Fr.) Karst belongs to the kingdom *Fungi*, phylum *Basidiomycota*, class *Agaricomycetes*, order *Polyporales*, family *Ganodermataceae*, genus *Ganoderma*, and species *lucidum* [55]. *G. lucidum* from the UK and other related *Ganoderma* species have also been morphologically and molecularly examined by different researchers who have conducted important studies,

such as Kim et al., Park et al., and Liao et al. [56–58]. Several phylogenetically related *Ganoderma* species have been found in North America, Europe, and Asian countries [56,58]. Gottlieb et al. also performed molecular and morphological studies on the *Ganoderma* species collected from South America, followed by Eyssartier et al. from France [59,60]. In 2017, Copot et al. identified the fungus *G. lucidum* in hilly and mountainous areas in Romania, specifically in oak forests [61]. The description of *G. lucidum* collected from Romania highlighted the mushroom cap, which is kidney-shaped and can be up to 20 cm in diameter, ranging in color from red to pinkish-brown when mature [61]. Towards the edges, the coloration of the cap ranged from bright yellow to white. The spore print is brown (see Figure 1a,b) [61]. Figure 1c shows the fungus *G. lucidum* (Reishi or Lingzhi) adapted from studies by Parepalli et al. [62].

(**a**) (**b**) (**c**)

**Figure 1.** Appearance of *Ganoderma lucidum* in Europe (Romania) and Asia [61,62]. (**a**) *G. lucidum* from Neamt County, Romania, adapted from [61]. (**b**) *G. lucidum* from Bacau County, Romania, adapted from [61]. (**c**) Reishi or Lingzhi from India, adapted from [62].

### *2.2. Data on G. lucidum Cultivation*

*G. lucidum* can also grow in greenhouses under controlled conditions. Data on cultivation methods have been reported in the literature, such as the study of Boh et al., where different biotechnological cultivation methods are presented [14]. As *G. lucidum* is rare in nature, cultivation in greenhouses is practiced using two methods: cultivation of fruiting bodies on wooden logs or on sawdust bags (or large plastic bottles) [14]. The main cultivation methods to produce *G. lucidum* (fruiting body and mycelia) are presented in Figure 2 [14].

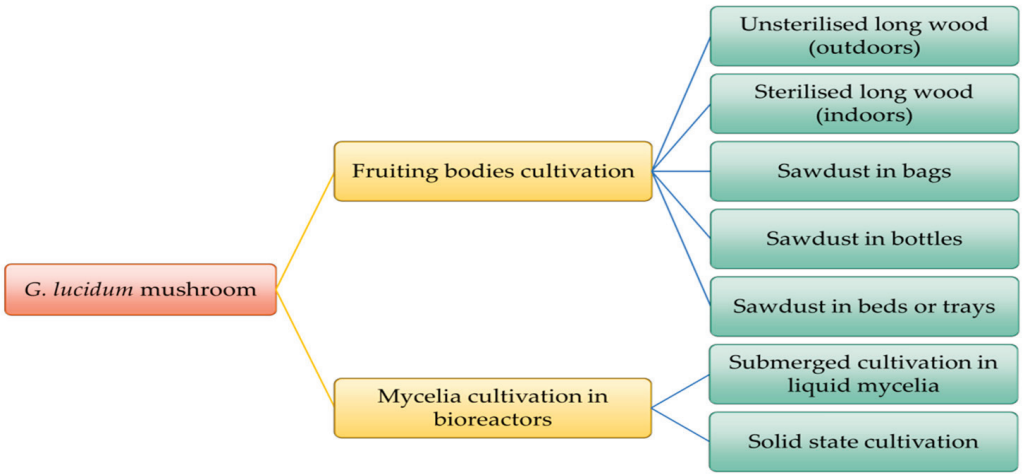


**Figure 2.** Different cultivation methods for fruit body and mycelium of *Ganoderma lucidum*, adapted from [14].

Various other cultivation methods have also been practiced. For example, Nithya et al. conducted research on the selection of an ideal material to grow *G. lucidum* fungus by testing wood retting, wheat bran, sorghum, and sorghum grains combined with chalk dust

and gypsum [63]. Adongbede et al. (2021) used indigenous hardwoods supplemented with rice and wheat bran as substrates for growing *G. lucidum* in Nigeria, where the fungus is not naturally available [64]. Matute et al. (2002) grew *G. lucidum* in bags using sunflower hulls as a nutrient source [65]. Yang et al. (2003) used carbohydrate and nitrogen-rich residues from a rice bran distillery to grow *G. lucidum* in polypropylene bags [66]. Hsieh et al. (2004) used soybean residues as a nutrient for growing *G. lucidum* in polypropylene bags [67]. Chang et al. (2006) reported studies on the optimization of growth methods for *G. lucidum* [68]. It is worth noting that cultivation conditions and substrate composition influence the biochemical composition of *G. lucidum* fungi qualitatively and quantitatively, as confirmed by Baskar et al. in 2011 through their studies [69].

## 3. Chemical Bioactive Compounds Identified in *G lucidum* with Anticancer Actions

In the last 30 years, numerous studies have been conducted highlighting the nutritional potential of *G. lucidum* mushrooms [30]. Research on the biochemical composition of this mushroom has led to the identification of several categories of compounds with health-promoting biological activity, as reported by Ahmad et al. [7,13].

### *3.1. Proximate Composition for G. lucidum*

The values of the bioactive compound classes differ quantitatively and qualitatively within certain limits, depending on the country and area of origin of the fungus and whether it is cultivated or naturally occurring in the forest. In the case of cultivated mushrooms, bioactive compounds depend on the nutrient richness of the substrate and environmental factors, such as humidity and temperature [14]. Most phytochemical reports show that, with regard to the chemical composition, the fruiting body of *G. lucidum* consists of 90% water and 10% different compounds, as presented in Table 1, according to Mau et al. [70].

**Table 1.** Proximate composition of the fruiting body of *G. lucidum* from different country origins, reported as a percentage.

| Mushroom Origin/ Constitutes | From Bangladesh | From Bangladesh | From Taiwan | From China | From Spain | From India | From Nigeria |
|---|---|---|---|---|---|---|---|
| Moisture % | 12.19 | 47 | - | - | - | 7.5 | 2.78 ± 0.05 |
| Ash % | 3.93 | 6.3 | 1.8 | 1.21 ± 0.06 | 2.31 ± 0.12 | 18.7 | 8.42 ± 0.13 |
| Water-soluble proteins % | 28.6 | 19.50 | 7–8 | 7.47 ± 0.22 | 11.70 ± 0.35 | 23.6 | 16.79 ± 0.13 |
| Total lipids % | 2.4 | 3.00 | 3–5 | - | - | 5.8 | 1.52 ± 0.09 |
| Fatty acid | - | - | - | 1.44 ± 0.10 | 1.27 ± 0.09 | - | 1.22 ± 0.07 |
| Total carbohydrates % | 44.91 | 5.41 | 26–28 | 9.88 ± 1.04 | 11.02 ± 1.16 | 42.8 | 63.27 ± 0.20 |
| Dietary fibers % | 14.67 | 2.4 | 59 | 76.81 ± 3.46 | 69.35 ± 3.12 | - | 7.77 ± 0.34 |
| References | [21] | [30,71] | [70] | [72] | [72] | [73] | [74] |

Table 1 summarizes the information on the biochemical composition of the fungus, as noted by multiple researchers. The content analysis of the bioactive compound classes includes data on moisture, ash, water-soluble protein content, total lipid content, total carbohydrate content, and dietary fiber content. From Bangladesh, data were reported by Rahman et al. (2020), El Sheikha (2022), and Roy et al. (2018) [21,30,71]. The interest in the nutritional potential of *G. lucidum* in Taiwan was proven by studies conducted by Mau et al. (2001) [70]. The total fatty acid content was reported only by Fraile-Fabero et al. for G. lingzhi from China and *G. lucidum* from mushroom crops from Madrid, Spain, in 2021 [72]. Comparable data were also reported by Parapelli et al. (2021) for *G. lucidum* from India and by Ogbe et al. (2013) from Nigeria [73,74]. Additional studies were published by Wachtel-Galor et al. (2011), by Paterson et al. (2006), and by Garuba et al. (2000) [75–77]. The pharmacognostic review of the active compounds isolated from basidiocarp and mycelium of *G. lucidum* revealed that it contains polysaccharides, triterpenes, vitamins, minerals,

sterols, proteins, proteo-polysaccharides, lectins, nucleotides, and fatty acids, as reported by Ahmad (2018) [13]. Figure 3 systematizes the categories of biochemical compounds that support the biological activities occurring in the treatment of various cancer tumors [13].

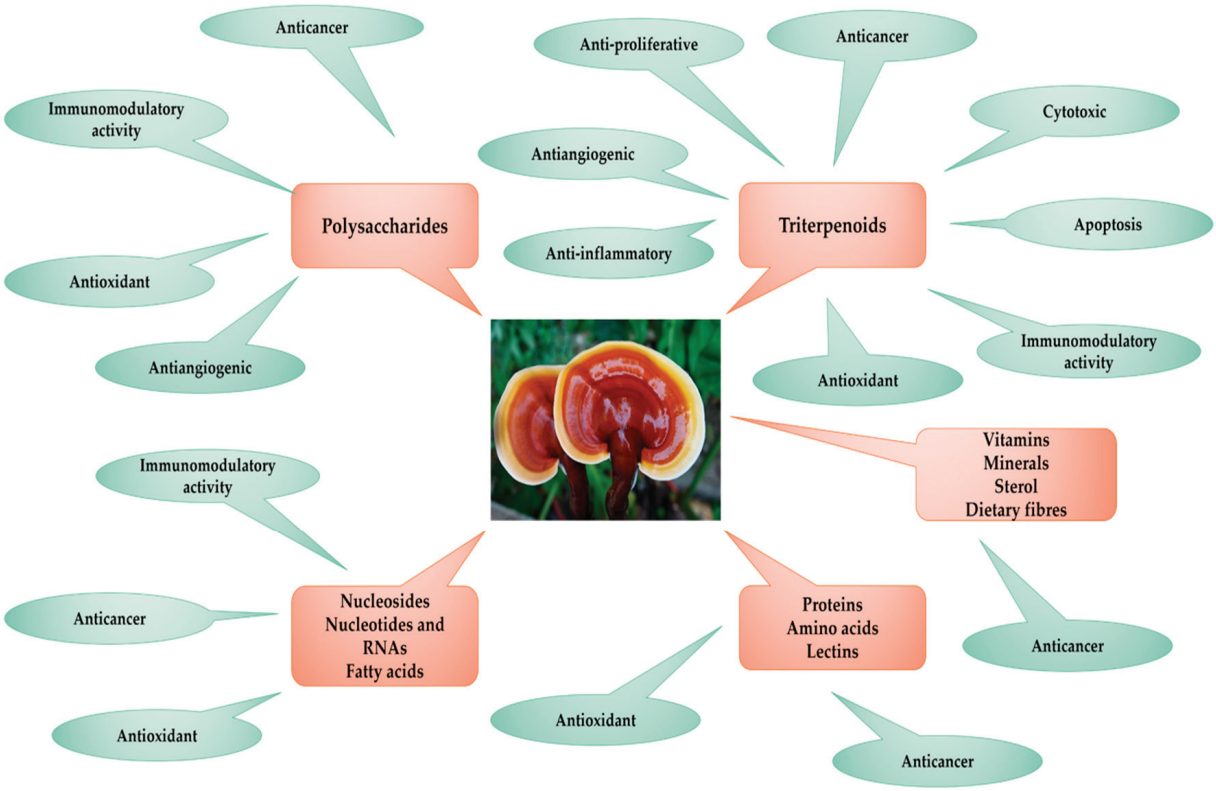


**Figure 3.** Bioactive compounds in *G. lucidum* and biological actions involved in anticancer activity, adapted from Ahmad [13].

### *3.2. Polysaccharide Content of G. lucidum*

Numerous studies have identified the existence of several types of polysaccharides in the fruiting body, spores, or mycelium of *G. lucidum*, as reported by Liu et al. and Lin et al. [78,79]. Polysaccharides represent a class of macromolecules with diverse structures and a wide range of physicochemical properties and biological actions, according to studies by Ferreira et al., Yu et al., and Giavasis [80–82]. According to Ahmad, Bhat et al., and Liu et al., these polysaccharide compounds are considered some of the most potent bioactive metabolites with antitumoral effects due to their biochemical structure [13,18,78]. Polysaccharide compounds (Gl-Ps) have a rich history and have been extensively studied in recent years by Sanodiya et al., Parepalli et al., and Chen et al. [27,62,83].

#### 3.2.1. Extraction and Purification of Polysaccharides

The most widely used method for polysaccharide extraction from spores, fruiting bodies, and mycelium is hot-water extraction, as documented by Nie et al. [84]. Figure 4 illustrates the extraction of five polysaccharide fractions from *G. lucidum* [85]. The fruiting body of *G. lucidum* was initially peeled, shredded, and sieved in order to obtain a fine powder. The extraction of Gl-Ps from the mushroom powder was performed with distilled water at 80 °C [85]. There are researchers who have used diluted saline solution or alkaline acid solutions for polysaccharide extraction, as reported by Wang et al. (2011). Other extraction methods that were applied included microwaves, ultrasound, or enzymatic methods, as in the studies of Lin et al. (2005), Huang et al. (2010), Zhao et al. (2010), and Leong et al. (2021) [86–90]. After filtering, the solution is subjected to precipitation with alcohol or acetone to obtain crude polysaccharides [84]. Polysaccharide purification

can be achieved by several techniques, such as ethanol fractionation or different column chromatographic techniques, as illustrated by Chen et al. (2008), Huang et al. (2011), and Jiang et al. (2012) [91–93]. Ion-exchange chromatography (DEAE-Sepharose Fast Flow), gel filtration, and affinity chromatography were the most useful methods, as highlighted by Choong et al. [85]. Figure 4 illustrates the extraction and fractionation steps of polysaccharides from *G. lucidum*. Five polysaccharide fractions were obtained [85].

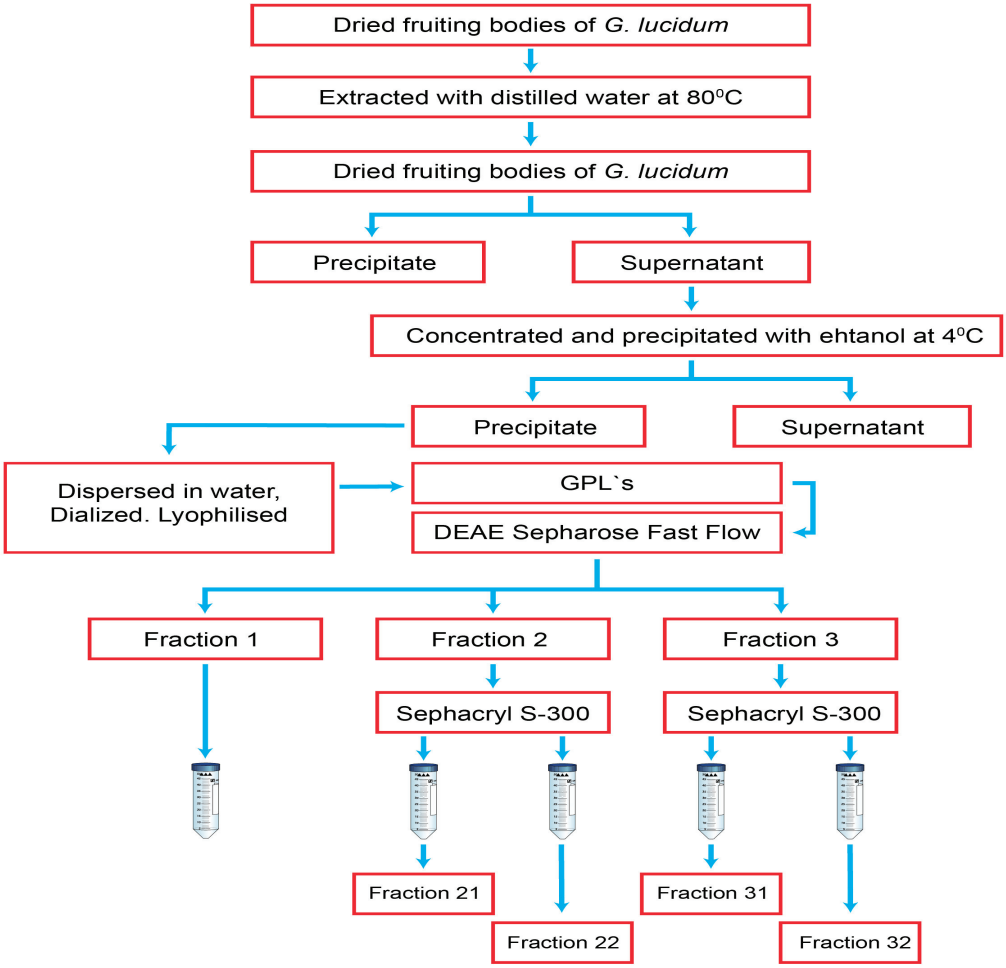


**Figure 4.** Diagram of the extraction of polysaccharides from *G. lucidum*, adapted from [85].

### 3.2.2. Structure of Polysaccharides from *G. lucidum*

The investigation of the structures and chemical properties of polysaccharides from *G. lucidum* involves knowledge of the composition of existing monosaccharides, branching structures, types of glycosidic linkages, chain conformations, and molecular weights [18]. As investigation techniques have developed, it was found that *G. lucidum* can have polysaccharides in its composition, either as pure or linked with other proteins or peptides [94–98]. Bhat et al. (2021) showed that homo-glucans from *G. lucidum* are linear or branched biopolymers, possessing a backbone formed by α- or β-linked (1→3), (1→6)-β-glucan and (1→3)-α-glucan glucose units, and may possess side chains attached in different positions [18]. Further investigations by different authors explore bioactivities as well. Bao et al. (2002) determined that the structures of the backbone chains are linear or branched bipolymers [94]. These possess a backbone consisting of α- or β-linked glucose units [94]. In 2006, new structures with heteroglucans were outlined by Cao et al., and new structures with heteropolysaccharides were documented by Sullivan et al. [95,96]. Further types of ligands were presented by Li et al. (2007), Ye et al. (2008), Ye et al. (2009), Wang et al. (2009), Ye et al. (2010), and Liu et al. (2010) [97–102]. Pan D. et al. (2012), Ma et al. (2013), and Pan K. et al.

(2013) also described the types of polysaccharide structures with different types of linkages, shown in detail in Table 2 [103–105]. Ooi et al. (2000) and Zhang et al. (2007) pointed out in their studies that the degree of base-chain substitution and branching chain length play important roles in determining the bioactivity of β-(1→3)-linked glucans [106,107]. Table 2 illustrates various techniques and methods utilized in the extraction, fractionation, and purification of polysaccharides and provides information about their structure (the backbone and monosaccharide compositions).

Side-chain branching occurs at C-6 of the main-chain glucosyl residues, as shown in Figure 5 and indicated by Choong et al. (2019) [85]. Jia et al. (2009) argued that among all homo-glucans, β-glucans are glucose polymers that exist as unbranched (1→3)-β-linked backbones in the form of (1→3)-β-linked backbones [108]. Figure 5 shows a Gl-Ps chain [85].

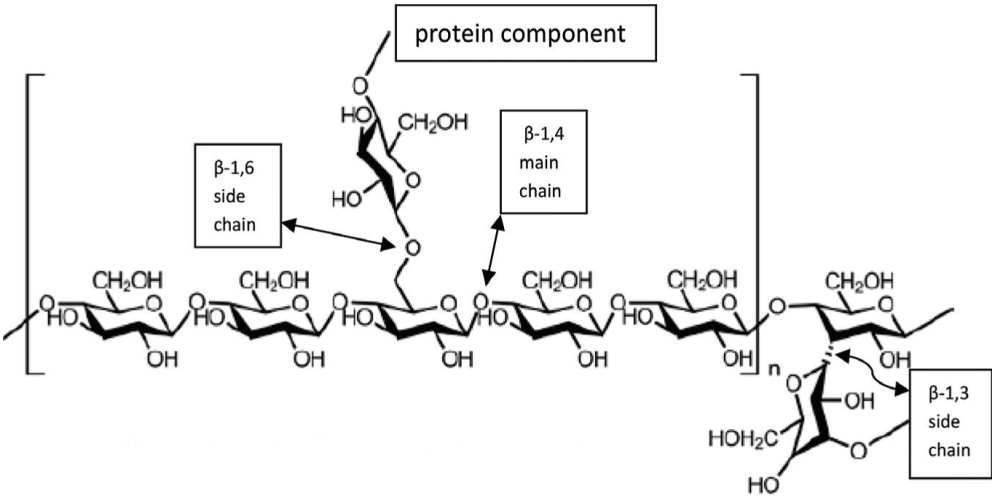


**Figure 5.** The structure of a Gl-Ps chain consisting of β-(1→3) bonds with some β-(1→6) branches, adapted from [85].

The molecular weight of Gl-Ps can range from $4 \times 10^5$ to $1 \times 10^6$ Da. Sanodiya et al. (2009) demonstrated that this has a significant impact on reducing cancer progression [27]. Studies by Moradali et al., Doco et al. (2001), and Hung et al. (2005) described polysaccharide-protein or peptide complexes using modern GC-MS methods [109–111]. Wang et al. (2002) and Sanodiya et al. (2009) investigated monosaccharides from the raw extract of *G. lucidum* [24,27]. Furthermore, Wang et al. (2009) and Ye et al. (2010) also provided data on monosaccharides identified in *G. lucidum* [100,101]. Similarly, Dai et al. (2010) and Yang et al. (2010) investigated *G. lucidum* and described the monosaccharide composition as predominantly consisting of xylose, fructose, glucose, and maltose [112,113]. Several other studies have been published reporting data on the polysaccharide structure and their medical applications, including Dong et al. (2012), Liu et al. (2012), Skalicka-Wozniak et al. (2012), and Pascale et al. (2022) [114–117].

**Table 2.** Structural characteristics, extraction, and fractionation techniques of polysaccharides from *G. lucidum*.

| | Mushroom Part of *G. lucidum* | Monosaccharide Composition | Backbone | Extraction and Fractionation | Bioactivity | References |
|---|---|---|---|---|---|---|
| 1 | Fruiting body | Glucose, rhamnose glucose, galactose, rhamnose | β(1→3)-Glucan; -(1→3)-(1→4)-, (1→6)Heteroglycan α-(1→4), β-(1→6)-heteroglycan | Hot-water extraction; DEAE-cellulose and gel filtration; chromatography | Antioxidant activity | [94] |
| 2 | Fruiting body (cultivated) | Mannose, rhamnose, glucose, galactose | Heteroglucans (GLP, GLP1, GLP2, GLP3, GLP4) Main glycosidic bond | Ultrasonic extraction; Sevag method; ethanol precipitation; ultrafiltration membranes | Antioxidant activity in vitro by DPPH scavenging activity; reducing power; $Fe^{2+}$ chelating activity; ORAC | [95] |
| 3 | Fruiting body (cultivated) | Glucose, galactose, mannose, arabinose | Heteropolysaccharide (GL-1; GL-V) (1→4)-galactan, Heteropolysaccharide | Soluble in water and in ethyl-acetate; Sevag method; dialysis | Bioactive compounds are an important source of anticancer agents | [96] |
| 4 | Extracellular | Galactose, mannose, glucose, arabinose, rhamnose | α-(1→4)-Galactose | DEAE-Sephcel and Sephadex G200. | Enhance T- and B-lymphocyte proliferation and antibody production | [97] |
| 5 | Fruiting body | Galactose, glucose, fucose | α-(1→6)-galactose α-(1→3)-Glucose | Hot-water extraction; DEAE-Sepharose Fast-Flow and Sephacryl S-300 | Immunostimulatory activity of spleen lymphocyte proliferation | [98] |
| 6 | Fruiting body | Glucose, galactose, arhamnose | Heteroglycan α-(1→4), β-(1→6) | Hot-water extraction | Immunologically active; proliferation of B-lymphocytes with important immunologic activity | [99] |
| 7 | Fruiting body (cultivated) | Glucose, galactose, mannose, arabinose, xylose, fucose | Heteropolysaccharides glucans (1→3)-β-D-glucan with a few short (1→4)-linked glucosyl units | Extraction and separation of fractions with hot water, cold and hot 1 M NaOH | Antitumor activity against sarcoma solid tumor | [100] |
| 8 | Fruiting body | Galactose, glucose, fucose | α-(1→6)-, (1→2,6)-Galactose β-(1→3)-, (1→4,6)-Glucose | Hot-water extraction; DEAE-Sepharose Fast-Flow and Sepharose CL-6B | An immunostimulating potential | [101] |
| 9 | Fruiting body | Glucose, galactose, mannose | β-(1→3)(1→4)(1→6)-Glucan Heteropolysaccharides | Hot-water extraction; DEAE-cellulose-32 and Sephacryl S-200 h | Pronounced antioxidant activity in free radicals scavenging and $Fe^{2+}$ chelating | [102] |
| 10 | Fruiting body (wild) | Galactose, rhamnose, and glucose in mole ratio of 1.00:1.15:3.22 | Water-soluble polysaccharide α-(1→6)-, (1→2,6) Galactose β-(1→3)-, (1→4,6) Glucose | Hot water and ethanol precipitation; DEAE-Sepharose Fast Flow and Sephacryl S-300 | Neutral heteropolysaccharide, which reported antihyperglycemia effects | [103] |

**Table 2.** *Cont.*

| | Mushroom Part of *G. lucidum* | Monosaccharide Composition | Backbone | Extraction and Fractionation | Bioactivity | References |
|---|---|---|---|---|---|---|
| 11 | Mycelium (cultivated) | Rhamnose, arabinose, mannose, glucose, galactose | Heteropolysaccharide α-D-Glc (1→6), α-D-Glc,α-D-Man (rhamnose and arabinose residues in the side chain) | Hot water; ethanol precipitation; Sevag method; dialysis | Antitumor activity against Human hepatocarcinoma cell line (HepG2) and tumor xenografts in ICR mice | [104] |
| 12 | Fruiting body | Glucose | Branched homo-glucan (GLP0; GLP1) (1→3)-β-D-glucan with (1→6)-β-D branches | Hot water followed by ethanol precipitation | Induced a cascade of immunomodulatory cytokines against sarcoma 180 solid tumor | [105] |

### *3.3. Triterpene and Triterpenoid Content of G. lucidum*

Triterpenes belong to the class of terpenes that have a molecule consisting of six isoprene units and are widespread in the plant kingdom [35]. Triterpenoids are part of the triterpene class, having heteroatoms (usually oxygen atoms) in the molecule. They are important bioactive compounds in the composition of *G. lucidum*, as shown by Wu et al. (2023) [118]. In 2018, Gu et al. revealed that the present triterpenes synthesize structures derived from lanosterol, which has a skeleton with a tetracyclic structure and the molecular formula $C_{30}H_{48}$ [119]. Terpenoid compounds from *G. lucidum* that have a C30 backbone and molecular masses between 400 and 600 kDa were studied by Baby et al. and Galappaththi et al., who generically named them Ganoderma triterpenoids (Gl-Ts) [120,121]. Further research on the structure and characterization of triterpenoids from *G. lucidum* that elucidates their biological activities was published in 2022 by Cör et al. and Lin et al. [122,123].

#### 3.3.1. Physicochemical Determination and Analysis of Triterpene Compounds

Studies conducted by Ghorai et al. (2012), Taofiq et al. (2017), and Chang et al. (2012) utilizing UV spectrophotometric measurements have made significant contributions to the methods for determining total terpenoids [124–126]. Huie et al. (2004) employed chromatographic and electrophoretic methods for the analysis of triterpenoids, and Yang et al. (2007) utilized a combined approach involving HPLC-ESI-MS [127,128]. Chen et al. (2012) highlighted the existence of multiple methods for investigating terpenoids [83]. Triterpenoid analysis was also outlined in the studies of Zhang et al. (2008), Shi et al. (2010), and Hadda et al. (2015) [129–131]. Che et al., Hui et al., and Zhang et al. reported novel insights into *triterpenoids* obtained from *G. lucidum* [132–134]. In 2023, Wu et al. emphasized the necessity of a new research strategy intertwining the concepts of chemical component analysis and pharmacological activity [118].

#### 3.3.2. Structure of *G. lucidum* Triterpenoids (Gl-Ts)

Wu et al. (2023) extensively reported on the diverse types of Gl-Ts identified from the mycelia, fruit body, and spores of *G. lucidum*, showcasing distinct structural variations, including alcohols, aldehydes, ketones, acids, esters, and various other substituents positioned differently [118]. Xia et al. (2014) conducted an in-depth analysis of the skeletal structure of *G. lucidum*, revealing that a majority of these terpenoids consist of 30 carbon atoms [135]. Using information derived from Wu et al., in Figure 6, the structures of a typical terpenoid skeleton are depicted, illustrating the numbered positions corresponding to the matched carbon atoms alongside 10 additional terpenoid structures exhibiting diverse substituents [118].

In Figure 6, illustrating the typical terpenoid skeleton structure, distinct substituents are evident at positions C-3, C-7, C-11, C-12, C-15, C-20, and C-27. Fatmawati et al. (2010) elucidated the structure of a new terpenoid, Ganoderic acid Df, featuring a β-hydroxy substituent at the C-11 position, distinguishing it from all other compounds characterized by a carbonyl group at the same position [136]. At the C-3 position, potential substituents include the β-hydroxy, carbonyl, and β-acetoxy groups. At C-20, a single carbon atom can host two substituents, which may be the methyl or hydroxyl groups or even hydrogen. Wu et al. demonstrated that at the C-25 position, various carboxyl groups, such as formyl, acetyl, or butyryl, can be found [118]. Additionally, Sharma et al. (2019) presented data on triterpenes from *G. lucidum*, characterized by isoprene units in their composition, featuring a C30 skeleton structure of ganoderic acids, aldehydes, esters, alcohols, lactones, glycosides, ketones, and molecular masses ranging from 400 to 600 g/mol [137]. Koo et al. (2019) identified a new compound with a lanostane triterpenoid structure named Ganosidone A, which, along with eight other derivatives, was investigated for its cancer chemopreventive potential [138]. Concerning the molecular configuration of ganoderic acids, Cör et al. (2022) documented the structures illustrated in Figure 7 [122].

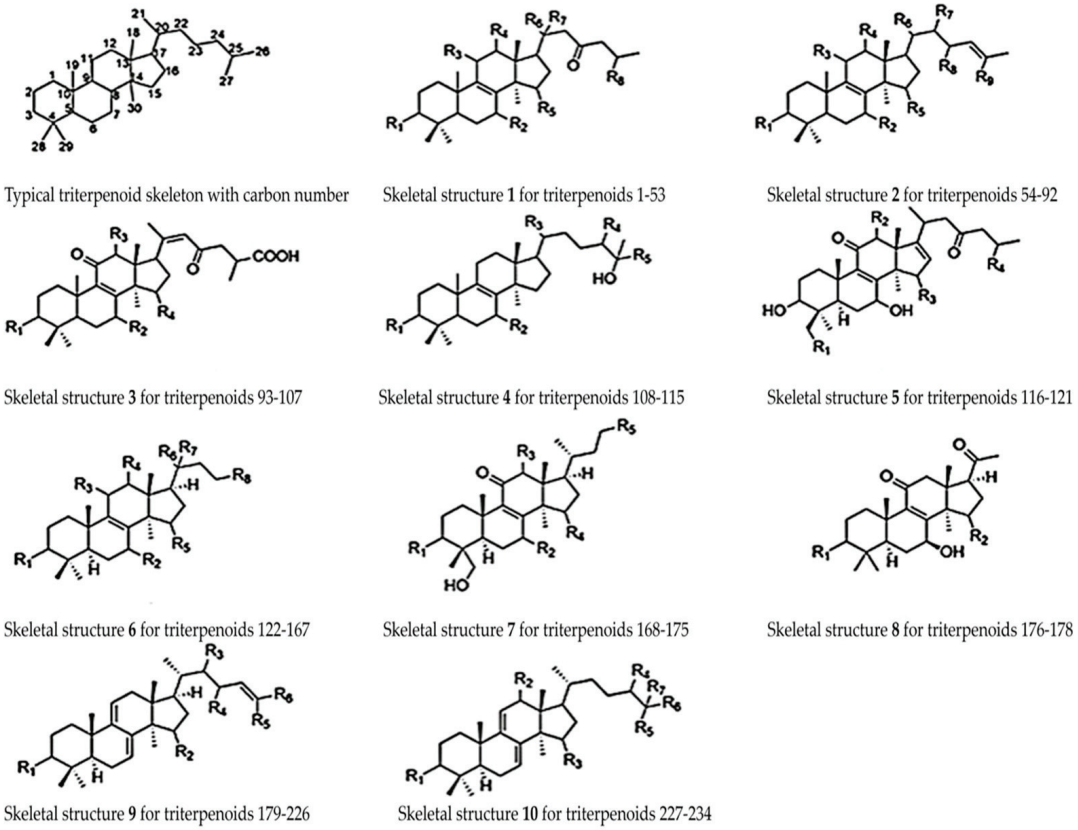


**Figure 6.** Skeletal structure of a typical terpenoid and other terpenoids 1–10 [118].

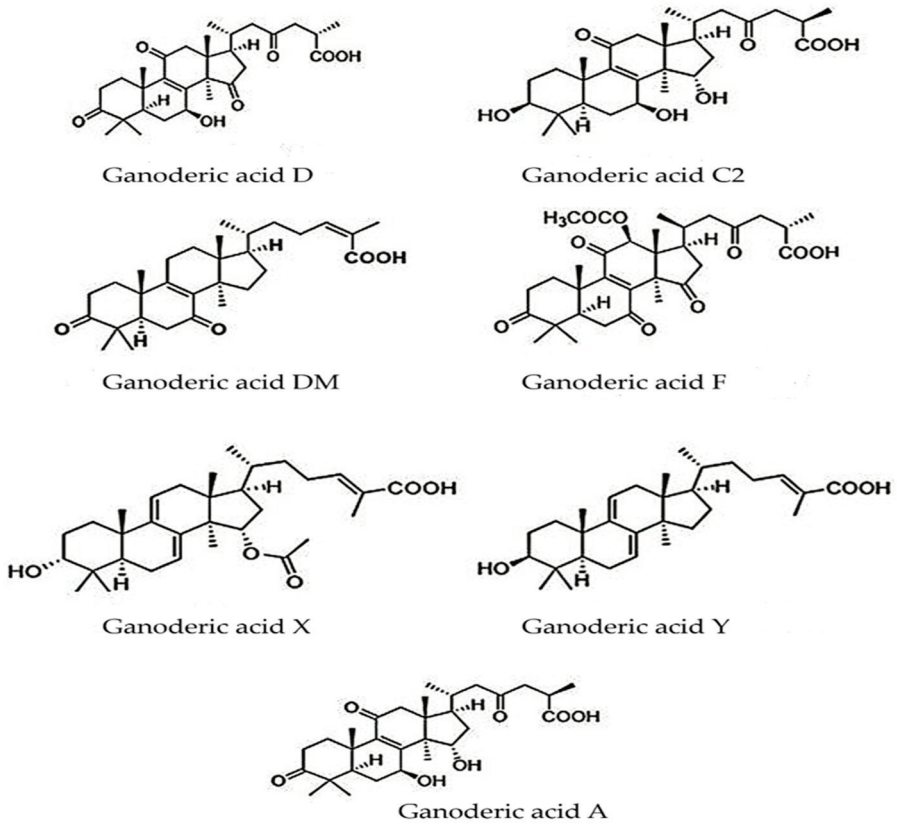


**Figure 7.** Structure of ganoderic acids from *G. lucidum*, adapted from [122].

Murata et al. (2019) identified and extracted a novel compound with a lanostane triterpene structure from *G. lucidum* using NMR and MS physicochemical analysis [139]. Studies presented by Yue et al. in 2008 and 2010, as well as those by Zhang et al. and Cheng et al., revealed the cytotoxic effects exerted by ganoderic acids from *G. lucidum*, which alter proteins involved in cell proliferation and cell death in carcinogenesis or oxidative stress [140–143]. In 2012, Liu et al. and Rios et al. studied the lanostanoid compounds from *G. lucidum* for their anticancer activities [144,145]. In 2013, Li et al. isolated a novel ganoderic acid from *G. lucidum* mycelia and studied its characteristics. Fatmawati et al. studied the structure–activity relationship of lanostane-type triterpenoids, and Li et al. reported cytotoxic effects in a wide range of triterpene compounds [146–148]. In 2017, Chen et al. presented data on compounds with triterpene structures existing in *G. lucidum* that exhibited activity as inhibitors of biological processes [149]. In 2019, Liang et al. illustrated their findings on the structure and mechanisms of action of ganoderic acids from *G. lucidum* [150]. In 2021, Chinthanom et al. documented their findings on lanostane triterpenoids isolated from mycelial cultures of Ganoderma spp. that can be modified by semisynthesis, thus obtaining synthetic compounds [151]. In 2023, Pascale et al. reported data on the specific structures and pharmacological mechanisms of triterpenoids with biological activities from *G. lucidum* [152]. Figure 8 illustrates other structures specific to the terpenoid compounds described by Pascale et al. [152].

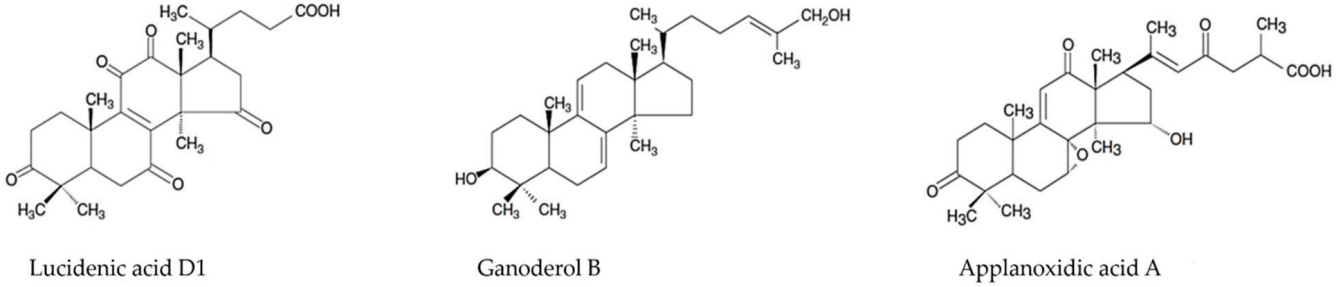


**Figure 8.** Specific structures for other ganoderic compounds [152].

### *3.4. Vitamins, Minerals, and Sterols Content*

Vitamins, minerals, and sterols were among the 400 biologically active constituents recognized by various researchers after 2018, including Cör et al., Yang et al., and Ahmad, F. In terms of quantity, the vitamins were in the following order: B1, B2, B6, β-carotene, C, D, and E [153–155]. Hussein et al. (2022) also studied the existence of vitamins in *G. lucidum* [156]. El Sheikha et al. (2022) highlighted the highest vitamin content as consisting of niacin (B3) and ascorbic acid (C) [30]. Mineral contents have been reported by several studies, such as El Sheikha et al., Roy et al., Ogbe et al., Cör et al., and Hussein et al. [30,71,74,153,156]. Table 3 displays the values for mushroom compositions reported by various authors, expressed in milligrams per 100 g (mg/100 g), parts per million (ppm), and percentages (%) [30,71,74,153,156].

Similar data were also presented by Sharif et al. (2016) and Treviño et al. [157,158]. The bioaccumulation of copper and zinc in *G. lucidum* was also documented by Matute et al. in 2011 [159]. In 2008, Falandysz J. reported the existence of selenium in the composition of some antioxidant enzymes with protective actions that are involved in antitumor effects [160]. In their study, conducted in 2000, Chiu et al. identified the presence of germanium in the Ganoderma extract. Their research encompassed an assessment of genotoxicity and antigenotoxicity related to this finding [161]. Du et al. (2008) studied the positive effect of selenium on the immune regulation activity of *G. lucidum*, and the involvement of this element in anticancer activities was documented [162].

**Table 3.** Minerals of *G. lucidum* fruiting body.

| Elements | mg/100 g | mg/100 g | % or ppm | ppm |
|---|---|---|---|---|
| Potassium | 432 | 3.590 | 1.11 ± 0.04 (%) | - |
| Phosphorus | 225 | 4.150 | 30.17 ± 1.29 (ppm) | - |
| Sulfur | 129 | - | - | - |
| Magnesium | 7.95 | 1.030 | 0.34 ± 0.01 (%) | 50.76 ± 1.19 |
| Sodium | 2.82 | 375 | 229.88 ± 0.34 (ppm) | - |
| Calcium | 1.88 | 832 | 1.99 ± 0.04% | - |
| Copper | 27 | - | 7.43 ± 0.13 (ppm) | 5.49 ± 0.35 |
| Manganese | 22 | - | 71.06 ± 1.56 (ppm) | 20.19 ± 0.54 |
| Iron | 2.22 | 82.6 | 121.37 ± 1.82(ppm) | 130.60 ± 1.63 |
| Zinc | 0.7 | - | 51.49 ± 2.16 (ppm) | 8.45 ± 0.38 |
| References | [30,71] | [153] | [74] | [156] |

Sterols with the chemical formula $C_{17}H_{28}O$ are a group of steroids that are cyclic secondary monohydric alcohols. Since 2002, Ma et al. have reported three new lanostanoids and two ergostane sterols, which were isolated by spectroscopic methods [163]. The role of sterols is important in the body's metabolism in regulating some hormonal and immune system functions. Akihisa et al. (2007) studied the effects of sterols and triterpene acids from *G. lucidum* on anti-inflammatory and antitumor actions [164]. In 2011, the sterol content of *G. lucidum* was researched by Liu et al., who analyzed it qualitatively and quantitatively using HPLC methods [165]. In 2015, Baby et al. showed that sterols in *G. lucidum* can be classified based on their skeletons [120].

### *3.5. Protein, Lectin, and Amino Acid Content*

#### 3.5.1. Proteins and Peptides in *G. lucidum*

Proteins constitute a distinct category of biochemically active molecules in G. *lucidum*, as demonstrated by Cör et al. in 2018 [153]. Numerous studies have reported various biological effects of the primary fungal protein, Ling-zhi-8 (LZ-8). Structural reports were published by Huang et al. in 2009, and the immunomodulatory actions of LZ-8 were documented by Hsu et al. in 2013, Lin et al. in 2014, and Yang et al. [166–169]. In 2015, Saard et al. studied crude proteins from *G. lucidum* (both mycelia and fruiting bodies) for their antioxidant actions [170]. In 2021, Fraile-Fabero et al. conducted studies on proteins from *G. lucidum* [72]. Additional research by Sun et al. in 2004 revealed the existence of compounds with a polysaccharide-peptide complex structure along with phenolic components with antioxidant potential in *G. lucidum* [171].

Ji et al. (2007) further documented the existence of proteo-polysaccharides in *Ganoderma lucidum*. They studied the immunomodulatory effects, emphasizing the anticancer properties of this fungus [172]. The immunomodulatory properties of LZ-8 protein and polysaccharides were studied by Yeh et al. in 2010 and Girjal et al. in 2012 [173,174]. Zhong et al. (2015) conducted research on the involvement of the peptide-polysaccharide complex from *G. lucidum* in oxidative stress [175]. Subsequent data on the presence of proteins in *G. lucidum* and their biological actions were published by Kumakura et al. in 2019, and further insights were provided by Yu et al. in 2021 and 2023, where they specifically documented the effects of proteoglycans derived from *G. lucidum* [176–178]. Huang et al. (2022) reported a novel pentapeptide in the mycelium of Ganoderma spp. that demonstrates antioxidant properties [179].

#### 3.5.2. Lectins from *G. lucidum*

Lectins are glycoproteins found in the fruiting body of *G. lucidum*. Lectins are carbohydrate-binding proteins that have been classified according to their origin and

structure. In 2007, Thakur et al. isolated and purified a group of lectin-structured proteins with a weight of 114 kDa from the fruiting bodies of *G. lucidum* [180]. In 2011, Girjal et al. isolated a new bioactive lectin from *G. lucidum*, which also exhibited hemagglutinating activity against both human and animal erythrocytes [181]. Nikitina et al. (2017) investigated lectins in mycelia and detected hemagglutinating activity in culture medium and in a crude mycelial extract of *G. lucidum* [182]. Recent research conducted by Yousra et al. has demonstrated varying binding patterns of lectins from *G. lucidum* with glycan moieties. These patterns play a crucial role in influencing biological activities, including antitumor, antiviral, and immunomodulatory effects [183].

#### 3.5.3. Amino Acid Content of *G. lucidum*

The amino acid content has been documented by several authors. According to the research of Sanodiya et al., Yousra et al., and Deepalakshmi et al., the most abundant amino acid was glutamic acid [27,183,184]. This was followed by aspartic acid, glycine, and alanine. The amino acids in *G. lucidum* have also been analyzed by Zhang et al. in 2018, who reported 18 different types of amino acids [185]. It should be noted that factors such as the origin of the tested samples and the species of fungus, as well as different analysis techniques, may result in different amino acid values. Compounds with antioxidant properties from *G. lucidum* have been analyzed in numerous studies. Thus, Kim et al. (2008), Sheikh et al. (2014), Lin et al. (2015), and Veljović et al. (2017) presented data on the chemical compounds of *G. lucidum* [186–189]. The amino acid values for *G. lucidum*, as reported by Sanodiya et al. in 2009 and Deepalakshmi et al. in 2011, are presented in Table 4 [27,184].

**Table 4.** Amino acids content in *Ganoderma lucidum* mushroom.

| **Amino Acid** | **Aspartic Acid** | **Threonine** | **Serine** | **Glutamic Acid** | **Proline** | **Glycine** | **Alanine** | **Valine** |
|---|---|---|---|---|---|---|---|---|
| | Asp | Thr | Ser | Glu | Pro | Gly | Als | Val |
| mg AA/g protein | 117 | 66 | 54 | 120 | 60 | 108 | 100 | 61 |
| References | [27,184] | [27,184] | [27,184] | [27,184] | [27,184] | [27,184] | [27,184] | [27,184] |
| Amino acid | Methionine | Isoleucine | Leucine | Phenylalanine | Tyrosine | Histidine | Lysine | Arginine |
| | Met | Ile | Leu | Phe | Tyr | His | Lys | Arg |
| mg AA/g protein | 6 | 36 | 55 | 28 | 16 | 12 | 21 | 22 |
| References | [27,184] | [27,184] | [27,184] | [27,184] | [27,184] | [27,184] | [27,184] | [27,184] |

#### 3.5.4. Content of Compounds with Antioxidant Properties in *G. lucidum*

Dong et al. (2019), Zheng et al. (2020), Rahman et al. (2020), and Kolniak-Ostek et al. (2022) reported data regarding the total triterpenoid, polysaccharide, polyphenol (TPC), and flavonoid (TFC) content of *G. lucidum* [21,190–192]. Furthermore, Kim et al. (2008) analyzed the phenolic compounds from *G. lucidum* using HPLC and identified 28 phenolic compounds. All these compounds are responsible for antioxidant activity [186]. Triterpenoid compounds were documented by Lin et al. in 2015 and Kolniak-Ostek et al. in 2022 [188,192]. Polysaccharide compounds were described by Lin et al. in 2015 [188]. Polyphenolic compounds were investigated by Rahman et al. in 2020 and by Kolniak-Ostek et al. in 2022 [21,192]. Flavonoid compounds and ascorbic acid were detailed in a study by Rahman et al. in 2020 [21]. Depending on the area of origin of the mushroom (cultivated or forest) and the extraction techniques used, the concentrations of the compounds listed in Table 5 vary considerably. The terpenoid content is lower in cultivated *G. lucidum*, depending on the cultivation method, as Kolniak-Ostek et al. showed [192]. The results on compounds responsible for antioxidant activity are presented in Table 5.

**Table 5.** Compounds with antioxidant activity from *G. lucidum*.

| Total Triterpenoids | Total Polysaccharides | | Total Polyphenol Content (TPC) | | Total Flavonoid Content (TFC) | Ascorbic Acid |
|---|---|---|---|---|---|---|
| /g d.w. | mg glucose equiv./g d.w. | | mg/100 g d.w. | | mg/100 g d.w. | mg/100 g d.w. |
| 196.03–643.06 | 769.1 | 112.53 | 33.3–43.49 | 912.38 | 34.09–38.08 | 30.51–32.2 |
| [188] | [192] | [188] | [21] | [192] | [21] | [21] |

Kolniak-Ostek et al. presented more data on several phenolic acids present in *G. lucidum* responsible for antioxidant activity, which are presented in Table 6 [192].

**Table 6.** The phenolic acids from *G. lucidum* [192].

| Nr. | Phenolic Acids | Quantity (mg/100 g DW of Extract) |
|---|---|---|
| 1 | Tricaffeoyl-glucosyl-glucoside | 13.54 ± 0.23 |
| 2 | Tricaffeoyl-glucosyl | 23.79 ± 0.24 |
| 3 | Caffeoyltrihexoside | 38.02 ± 0.30 |
| 4 | Protocatechuic acid hexoside | 19.09 ± 0.15 |
| 5 | 1-Caffeoylquinic acid | 505.89 ± 3.21 |
| 6 | trans-5-P-coumaroylquinic acid | 0.46 ± 0.01 |
| 7 | 5-Caffeoylquinic acid | 95.01 ± 0.92 |
| 8 | Caffeoyl-2-hydroxyethane-1.1.2-tricarboxylic acid | 213.89 ± 1.52 |
| 9 | Yunnaneic acid F | 1.29 ± 0.01 |
| 10 | Salvianolic acid B | 1.39 ± 0.01 |
| | Sum | 912.38 ± 20.14 |

Means ± SD ($p \leq 0.05$; $n = 3$).

The antioxidant properties of G. *lucidum* were analyzed using several methods, including the reporting of DPPH and ABTS radical scavenging activities, as well as the FRAP assay. The results were expressed in units of measurement. These units vary according to the different experimental methods used. However, the importance of the results lies in the unequivocal demonstration of the antioxidant activity possessed by the constituents found in *G. lucidum* (see Table 7).

**Table 7.** Antioxidant capacity in the extract of G. lucidum.

| DPPH | | FRAP | | ABTS |
|---|---|---|---|---|
| (%) | (μMol TE/g) | (μg/100 g) | (μMol TE/g) | (μMol TE/g) |
| 24.04 ± 0.33 | 51.3 ±1.04 | 614.83 ± 0.05 | 49.87 ± 1.58 | 81.26 ± 1.10 |
| [21] | [192] | [21] | [192] | [192] |

Means ± SD ($p \leq 0.05$; $n = 3$).

Furthermore, Dong et al. (2019) demonstrated a strong correlation between the antioxidant capacities measured using DPPH, ABTS, and FRAP assays and the content of polyphenolics and triterpenoids in *G. lucidum* [190]. Similar studies have also been published by Saltarelli et al. in 2015 [193]. The properties of compounds in *G. lucidum* and their effects on antioxidant activity were also documented by Tang et al. (2016), Sanchez C. (2017), Mohammadifar et al. (2020), and Mustafin et al. (2022) [194–197].

### *3.6. Content in Nucleosides and Fatty Acids*

#### 3.6.1. Nucleosides and Nucleobases in *G. lucidum*

Nucleotides are monomeric units that consist of a base (purine or pyrimidine) and one or more phosphate groups. Nucleotides are formed from nucleosides by phosphorylation under the action of kinases. Nucleosides are glycosyl amines derived from a nitrogenous base and a ribose or deoxyribose. These compounds have been shown to contain uridine

and uracil, both of which are capable of reducing elevated serum aldolase levels. The water-soluble fraction of *Ganoderma* suppresses platelet aggregation. Cheung et al. have carried out studies on the identification and role of bases and nucleoside markers [198]. Gao et al. (2007) identified six nucleobases qualitatively in fruiting body samples of *G. lucidum* and *G. sinense* [199]. Additional studies of the distribution of nucleotides and nucleobases were also carried out by Yuan et al. in 2008 and Chen et al. in 2012, who were able to document 16 nucleotides and nucleobases in the *Ganoderma* species [200,201]. Phan et al. (2018) demonstrated in their work that nucleobases, nucleosides, and nucleotides found in fungi play crucial roles in regulating various physiological processes in the human body through purinergic and/or pyrimidine receptors. [202]. In 2022, Sheng et al. analyzed 10 target compounds from 23 batches of *Ganoderma* samples from different regions of China and demonstrated that the geographical origin of the fungi might be the exclusive factor affecting the accumulation of nucleosides and nucleobases in the *Ganoderma* spp. mycelium [203].

#### 3.6.2. Fatty Acid Content

The total lipid content of *G. lucidum* has been reported in several studies by Rahman et al. (2020), El Sheikha (2022), Roy (2018), and Ogbe et al. (2013) [21,30,71,74]. Comparative data for the fatty acid content of carpophores from G. lingzhi (from industrial cultures in China) and *G. lucidum* (from cultures in Madrid, Spain) were described by Fraile-Fabero et al. in 2021 [72]. Fraile-Fabero et al. found that G. lingzhi contains the highest percentage of arachidic acid, followed by the margaric, behenic, margaroleic, lignoceric, and cis-vaccenic acids, while *G. lucidum* contains the highest percentage of α-linolenic acid, followed by the myristic, stearic, capric, erucic, nervonic, elaidic, octadecatrienoic, octadecadienoic, and eicosatrienoic acids [72]. Data on the presence of essential fatty acids in *G. lucidum* were also presented by Hossain et al. in 2007 [204]. Stojković et al. (2014) conducted comparative studies between the *G. lucidum* species from Serbia and China [205]. Lin et al. (2017) reported hydroxy fatty acids (HFA) present in the molecular species of acylglycerols of *G. lucidum* [206]. Salvatore et al. (2020) identified fatty acid methyl esters (FAMEs) in the triglycerides of *G. lucidum* spores [207]. Table 8 displays the composition similarities between the two species. [72].

**Table 8.** Fatty acids content of two *Ganoderma* species [72].

| Fatty Acids | *G. lingzhi* ± 15% | *G. lucidum* ± 15% |
|---|---|---|
| Total monounsaturated fatty acids | 37.5 | 28.68 |
| Total polyunsaturated fatty acids | 43.84 | 49.93 |
| Total saturated fatty acids | 18.64 | 20.77 |
| Total | 99.98 | 99.38 |

Phytochemical reports over the past four decades indicate the presence of 279 bioactive secondary metabolites (Wasser et al.), along with over 200 polysaccharides and polysaccharide-protein complexes (Baby et al.), and more than 30 steroidal compounds (Wasser et al.) within the chemical composition of *G. lucidum*. These compounds play a pivotal role in the development of various biological actions. [120,208,209].

## 4. Biological Activities against Cancer

The biocomponents present in *G. lucidum* contribute to the numerous biological actions of this medicinal mushroom, making it applicable in medical contexts for combating cancer. Both polysaccharide compounds (Gl-Ps) and triterpene compounds (Gl-Ts) have been shown to exhibit anticancer activities. Over a 25-year period, numerous studies have investigated the anticancer effects of *G. lucidum* compounds, including those reported by Wang et al. (2002), Zhang et al. (2007), and Akihisa et al. (2007) [24,107,164]. Between 2009 and 2015, other researchers published studies on the anticancer effects of *G. lucidum*

bio-compounds, such as Cho et al., Ferreira et al., Trajkovic et al., Kao et al., Zhao et al., and Joseph et al. [15,28,34,35,37,80,89]. Between 2018 and 2023, new studies disclosed data on anticancer actions, including those by Ahmad F. and Cör et al. in 2018, Kolniac-Ostek et al. in 2022, and Ekiz et al. in 2023 [13,153,192,210]. Our aim is to review the most important studies and systematize these data on the possible mechanisms.

### *4.1. Anticancer Action*

The anticancer activity attributed to Gl-Ps was studied by Cao et al. in 2002 and 2003 [211,212]. Many other studies have demonstrated the effect of Gl-Ps on cytokines, including those by Chen et al. and Zhu et al. [213,214]. In 2007, Zhu et al. demonstrated the beneficial immunological effects in mice attributed to Gl-Ps extracts administered at low doses [215]. You et al. studied the effects of Gl-Ps on antioxidant enzymes in ovarian cancer in rats, while Xu et al. conducted research on mediating the immunomodulatory, cytotoxic, and anti-angiogenic effects of Gl-Ps [216,217]. Polysaccharides have been identified as a distinct class of compounds present in *G. lucidum* compositions, demonstrating anticancer activity by stimulating host immune function, as reported by Ahmad F., Sun et al., and Wiater et al. [213,218,219]. In 2012, Zhang et al. reported the effects of Gl-Ps on suppressing hepatocyte proliferation in rats [220]. Pan et al. reported the effects of Gl-Ps on rats with gastric cancer, inducing enhanced immunity and antioxidant activity [103]. The study by Suarez-Arroyo et al. in 2013 explored inflammatory breast cancer (IBC) and the effects of Gl-Ps in breast cancer [221]. In 2015, Habijanic et al. reported on the effects of Gl-Ps in modulating cytokine responses and lymphocyte activity [222]. Several studies have delved into the mechanisms of action of *G. lucidum* compounds in cancer treatment, such as those published by Ahmad F., Sohretoglu et al., and Wang et al. in 2018, as well as Fu et al. in 2019 [13,223–225]. The triterpene compounds (Gl-Ts) identified in *G. lucidum* exhibit significant anticancer effects. Both Min et al. in 2000 and Gao et al. in 2002 reported that triterpene compounds displayed cytotoxic effects against tumor cells [226,227]. In 2004, Lin et al. investigated the actions of Gl-Ps from aqueous extracts and Gl-Ts from alcoholic extracts of *G. lucidum*, evaluating their angiogenic effects [228]. Li et al. conducted research on ganoderic acid X, which acts by inhibiting topoisomerases and inducing apoptosis [229]. In Table 9, we have compiled the effects of essential compounds (Gl-Ps and Gl-Ts) in *G. lucidum* that significantly contribute to its anticarcinogenic activity.

**Table 9.** Anticancer activity of polysaccharides and triterpenes from *G. lucidum*.

| | *G. lucidum* Compounds | Actions and Effects of Gl-Ps on Antitumor Activity | Mechanism of Action | References |
|---|---|---|---|---|
| 1 | Gl-Ps | Effects on dendritic cells | Gl-Ps acts on the maturation and function of cultured murine bone marrow-derived dendritic cells (DCs). | [211] |
| 2 | Gl-Ps | Effect on cytotoxicity | Gl-Ps acts with a specific T-lymphocyte cytotoxic (CTL) mechanism, which has been pulsed with the tumor antigen P815. | [212] |
| 3 | Gl-Ps | Evaluation of immunomodulatory effect on cytokines | Explain the mechanism of action on macrophages in which Gl-Ps (fractions) activate kinase to induce, in turn, activation of IL-1, IL-2, and TNF-α. | [213] |
| 4 | Gl-Ps | Effect of cytokine-induced killer cells (CIK) | Gl-Ps decreases the number of lymphokine-activated cytokines (LAK) and CIK-induced cytokine-killing cells. | [214] |
| 5 | Gl-Ps | Actions in immunopotentiation therapy against induced immunosuppression | Gl-Ps extract at low doses leads to increased immunological effector cell activity in immunosuppressed mice. | [215] |
| 6 | Gl-Ps | Effect on antioxidant enzyme activity | Gl-Ps from *G. lucidum* significantly reduced malondialdehyde (MDA) production and increased the activity of serum antioxidant enzymes in ovarian cancer therapy in rats. | [216] |
| 7 | Gl-Ps | Gl-Ps suppresses tumorigenesis, inhibits tumor growth | Gl-Ps affects immune cells, including B-lymphocytes, T-lymphocytes, dendritic cells, and natural killer cells. They are mediated by immunomodulatory, anti-angiogenic, and cytotoxic effects. | [217] |
| 8 | Gl-Ps | Antitumor effects by stimulating host immune function | Gl-Ps acts directly in activating lymphocytes that have been tested by incubating Gl-Ps with an antigen-deficient tumor cell line. Also, Gl-Ps acts on B16F10 melanoma cells. | [218] |
| 9 | Gl-Ps | Antitumor effects by stimulating host immune function | Gl-Ps can induce lymphocyte proliferation through action on B16F10 melanoma cells and IFN-γ production. | [219] |
| 10 | Gl-Ps | Antitumor activity manifested by a mixture of Gl-Ps and sulfates. | Gl-Ps sulfate showed remarkable inhibition of rat Heps proliferation. | [220] |
| 11 | Gl-Ps | Therapeutic potential in inflammatory breast cancer (IBC). | Study results provide evidence that Gl-Ps treatment suppresses protein synthesis and tumor growth by affecting survival signaling pathways in mice injected with IBC cells, suggesting a natural therapeutic potential for breast cancer. | [221] |
| 12 | Gl-Ps | The ability of isolated Gl-Ps fractions (F3) to induce innate inflammatory cytokines | Enhanced Th1 response with high levels of IFN-γ and IL-2. Cell wall Gl-Ps were inducers of innate inflammatory cytokines, and extracellular Gl-Ps demonstrated a high capacity to modulate cytokine responses to IL-17 production. | [222] |

**Table 9.** *Cont.*

| | *G. lucidum* Compounds | Actions and Effects of Gl-Ps on Antitumor Activity | Mechanism of Action | References |
|---|---|---|---|---|
| 13 | Gl-Ps | Potential anticancer activity | They discussed the mechanisms of anticancer activity attributed to Gl-Ps by highlighting immunomodulatory, anti-proliferative, pro-apoptotic, antimetastatic and anti-angiogenic effects. | [223] |
| 14 | Gl-Ps | Antitumor action and immunomodulatory effects of Gl-Ps in rats | Gl-Ps increased the serum concentration of Il-2, INF-γ and tumor necrosis factor-α. It increased the cytotoxic activity of natural killer cells and T cells and led to prolonged lifespan of brain glioma-bearing rats. | [224] |
| 15 | Gl-Ps, spores | Antitumor action of a novel polysaccharide with an estimated average molecular weight of 1.5 × 104 Da | In vivo antitumor activity tests showed that Gl-Ps could significantly inhibit S180 tumor growth in mice. No drug-related toxic reactions were observed. | [225] |
| 16 | Gl-Ts | Ganoderic acids from spores and their cytotoxicity | The cytotoxicity of the compounds isolated from the *Ganoderma* spores was carried out in vitro against Meth-A and LLC tumor cell lines. | [227] |
| 17 | Gl-Ts | Anticancer study of lucialdehydes B, C (2,3), ganodermanonol, and ganodermanondiol | Cytotoxic mechanism. Lucialdehyde C exhibited the most potent cytotoxicity against CLL, T-47D, sarcoma 180, and Meth-A tumor cells. | [227] |
| 18 | Gl-Ps Gl-Ts | Antitumor effect of aqueous extract; cytotoxic activity of alcoholic extract | Manifestation of a significant antitumor effect in several tumor-bearing animals; manifestation of an anti-angiogenic effect that may be involved in the antitumor activity. | [228] |
| 19 | Gl-Ts | Ability of ganoderic acid X (GAX) to inhibit topoisomerases and interfere with apoptosis | Mechanisms of chromosomal DNA degradation, cancer cell apoptosis, mitochondrial membrane disruption, and caspase-3 activation have been elucidated upon GAX treatment of HuH-7 human hepatoma cells. | [229] |
| 20 | Gl-Ts | Cytotoxicity of GA-T on different human carcinoma | It was shown in vivo to significantly inhibit proliferation of lung cancer cells by inducing apoptosis by GA-T | [230] |
| 21 | Gl-Ts | The effect of ganoderic acids A, F, and H on breast cancer cells was evaluated | GA-A, GA-F, and GA-H suppressed cell proliferation, colony formation, and invasive behavior of MDA-MB-231 cells. They have biological effects by inhibiting transcription factors AP-1 and NF-κB. | [231] |
| 22 | GA-T | Studies of anti-invasive and antimetastatic mechanisms of GA-T in vitro in lung cancer | GA-T dose-dependently inhibited 95-D cell migration by wound healing assay, promoting cell aggregation and inhibiting cell adhesion to the extracellular matrix (ECM). GA-T prevents tumor metastasis in highly metastatic lung carcinoma. | [232] |
| 23 | Gl-Ts. | Anticancer, anti-inflammatory, and antimetastatic activities of *G. lucidum* extracts | Gl-Ts from *G. lucidum* reduces the production of IL-8, IL-6, MMP-2, and MMP-9 in breast cancer and melanoma cells. They decrease cancer cell viability in a time and dose-dependent manner. | [233] |
| 24 | GL-Ts | Investigation of Gl-Ts with activity in inhibiting growth of pulmonary carcinoma metastates and suppressing colonic inflammation | The triterpene extracts exhibit inhibitory activity against foodborne carcinogen-induced mouse colon carcinogenesis. All suppressive functions were enhanced by high doses of triterpene extract. | [234] |

**Table 9.** *Cont.*

| | *G. lucidum* Compounds | Actions and Effects of Gl-Ps on Antitumor Activity | Mechanism of Action | References |
|---|---|---|---|---|
| 25 | Gl-Ts NTF, ATF | Evaluation of anticancer effects of NTF (neutral triterpene fraction) and ATF (acidic triterpene fraction) on human colorectal cancer | The cytotoxic effects of Gl-Ts on human colon cancer cells SW480, SW620, SW116, and mouse embryonic fibroblast cells NIH3T3 were studied. Compounds isolated from NTF acted as antitumorals by inducing apoptosis. | [235] |
| 27 | GlSO | Mechanistic investigation of the anticancer-gene effect of GlSO (*G. lucidum* spore oil) on mammary cancer cells | Growth of MDA—MB-231 cells, in vitro, were inhibited by treatment with GlSO (0.2, 0.4, and 0.6 μL/mL). In vitro, GlSO increased Bax and caspase-3 expression but did not affect caspase-8 expression. | [236] |
| 27 | Gl-Ts | Anticancer potential of *G. lucidum* against prostate cancer (PC-3) | *G. lucidum* has been shown to prevent prostate cancer cell growth and stimulate apoptosis in PC-3 cells by preventing STAT-3 translocation (signal transduction and activation of transcription). | [237] |
| 28 | Gl-Ts | Effects on colorectal cancer. Involves suppression of NF-κB-regulated inflammation and carcinogenesis | In vitro administration of GLSF extract at non-toxic concentrations to mice inoculated with CT27 tumor cells significantly potentiated paclitaxel-induced growth inhibition and apoptosis in CT27 and HCT-15 cells. | [238] |
| 29 | Gl-Ps, Gl-Ts | Evaluation of the effects on skin carcinogenesis analyzed on JB6 cells in SKH-1 mice | Reduced incidence and multiplicity of skin tumors. In tumor-free skin tissue of mice, Gl-Ps and Gl-Ts attenuated UV-induced epidermal thickening. Gl-SF increased CD8 and Granzyme B expression. | [239] |

Tang et al. (2006) reported the effects of ganoderic acid T in inhibiting lung cancer tumors (95-D), inducing apoptosis, and arresting the cell cycle at the G(1) phase [230]. Gl-Ts-type triterpenes and ganoderic acids A, F, and G were investigated in 2008 by Jiang et al. for their effects on breast cancer [231]. In 2009, Trajkovic et al. documented the action of triterpenes in B-16 melanoma, while Xu et al. studied ganoderic acid T and its effects on lung cancer [34,232]. In 2017, Barbieri et al. published data on the inhibition of IL-8, IL-6, MMP-2, and MMP-9 release in cancer cells under pro-inflammatory conditions in breast cancer and melanoma cells, and Ye et al. disclosed data on the effects of ganoderic and lucidenic acids in inhibiting the growth of lung carcinoma metastases and suppressing colon inflammation [233,234]. In 2020, the anticancer effects of triterpene fractions extracted from *G. lucidum* on SW620 human colorectal cancer cells were reported in vitro by Li et al. [235]. The action of Gl-Ts against various cancers has been studied by Jiao et al. (breast cancer cells), Wang et al. (prostate cancer), and Liu et al. (colorectal cancer) [236–238]. In 2022, Shahid et al. conducted research on the anticancer activities of Gl-Ts in skin cancer [239].

### *4.2. Possible Mechanisms in Anticancer Actions*

The medical world has shown significant interest in establishing possible mechanisms of anticancer activity, especially as the literature provides ample evidence regarding the actions of *G. lucidum* biocomponents against various forms of cancer. In 2018, Ahmad F. systematized the possible mechanisms by which *G. lucidum* bio-compounds participate in cancer treatment [13]. The steps considered by Ahmad include the activation of the host cell immune response, induction of cell differentiation, inhibition of angiogenesis, direct cytotoxicity to tumor cells, inhibition of urokinase-type plasminogen activator and receptor expression in cancer cells, and inhibition of phase II metabolizing enzymes [13].

Kao et al. elucidated the mechanisms of the anticancer action of *G. lucidum* compounds by discussing the distinct effects of polysaccharide and triterpene compounds separately, as well as their combined effects (see Figure 9) [35].

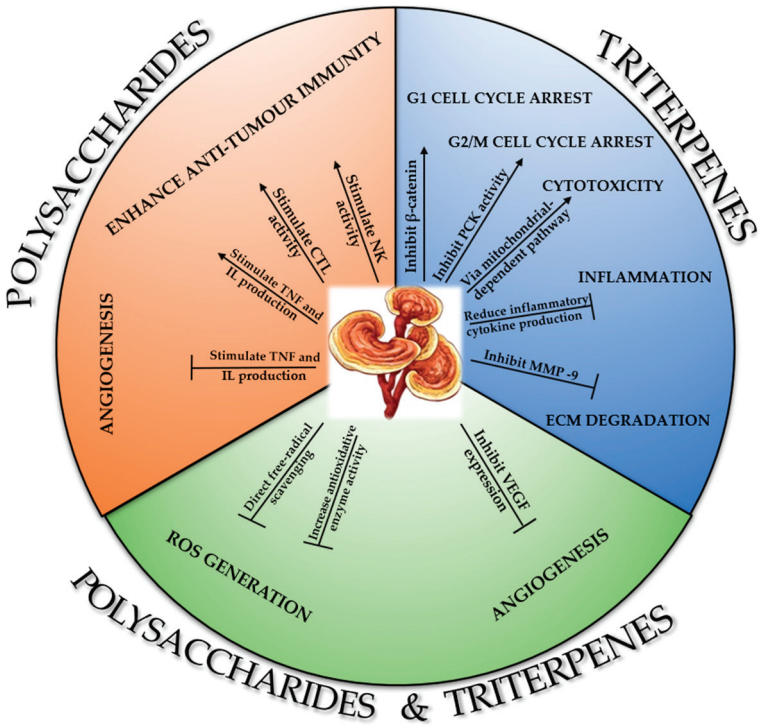


**Figure 9.** Mechanisms of action of polysaccharide and triterpene extracts isolated from *G. lucidum* with anticancer effect, adapted from [35].

They showed that polysaccharide compounds (Gl-Ps) act through three mechanisms [35]: by enhancing the host immune response, by stimulating macrophage activity, and by stimulating T-lymphocytes and natural killer (NK) cells. Triterpenes (Gl-Ts) act through five mechanisms:

- G1-phase cell cycle arrest by inhibition of β-catenin;
- Inhibition of protein kinase C (PCK), which generates G2-phase cell cycle inhibition;
- Induction of apoptosis in cancer cells via the mitochondrial pathway, followed by activation of caspase cascades;
- Preventing tumor metastasis by inhibiting MMP-9 and interleukin IL-8 and by degrading the extracellular matrix (ECM);
- Suppressing the secretion of anti-inflammatory cytokines [240].

Together, the two major classes of bio-compounds, Gl-Ps and Gl-Ts, share two common mechanisms, namely:

I. Antioxidant actions by reducing oxidative stress generated by free radicals and reactive oxygen species (ROS) through the actions of antioxidant enzymes [35].
II. Suppressing angiogenesis and inhibiting nitric oxide production.

### *4.3. Other Biological Actions Involved in Anticancer Activities*

The antitumor activity of *G. lucidum* bio-compounds is based on the differential effects of other biological activities generated by *G. lucidum* bio-compounds, as reported by Cör et al. [122,153]. Other researchers, such as Sharma et al., Ahmad, and You et al., have also conducted studies on the various factors that can lead to cancer [137,155,216]. Additionally, researchers like Xu et al., Fu et al., and Hapuarachchi et al. have analyzed various biological actions associated with cancer [217,225,241].

#### 4.3.1. Immunomodulatory Activities

Anticancer activity based on immunomodulation activity has been reported by several researchers, such as Bao et al., Ooi et al., Moradali et al., and Lin et al. [94,106,109,168].

##### Effects of Gl-Ps on T- and B-Lymphocytes

Numerous studies have been reported that highlight the activating role of Gl-Ps (F3 fraction) on T-lymphocytes by increasing interleukin production: IL-1, IL-2, IL-6, and IL-12, and increasing the expression of IFN-γ and INF-α. Additionally, they enhanced DNA synthesis in mouse spleen cells, as reported by Chen et al. [213] and Wang et al. [24]. Gl-Ps from *G. lucidum* can activate PKC and PKA protein kinases in murine T-lymphocytes, according to Sohretoglu et al. [223]. The effect of Gl-Ps on B-lymphocytes is also crucial for tumor immunity. For example, Gl-Ps can activate B-lymphocytes by enhancing their proliferation and differentiation, leading to the production of substantial amounts of immunoglobulins in mice. Furthermore, Gl-Ps can directly stimulate the expression of PKCα and PKCγ in B-lymphocytes, as reported by Zhang et al. [242]. Activated B-lymphocytes increase the production of immunomodulatory substances, such as IL-1β and TNF-α, and reactive nitrogen species, such as NO [242].

##### Effect of Gl-Ps on Dendritic Cells

Dendritic cells (DCs) are professional antigen-presenting cells involved in the initiation of the primary T-lymphocyte immune response [223]. Stimulation of dendritic cell maturation by Gl-Ps from *G. lucidum* was documented by Sanodiya et al. [27]. Lai et al. reported that treatment with Gl-Ps F3 fraction extract improved the mixed lymphocyte response and stimulated the production of ten cytokines and six chemokines [243]. Jan et al. outlined the immunomodulatory activity of Gl-Ps, including the activation and maturation of DCs, as evidenced by increased cytokine production (IL-12, IL-6, IL-23, and IL-10) [244]. Chan et al. reported that treatment of monocytic leukemic cell lines with Gl-Ps resulted in

increased leukocyte HLA-DR antigen expression and induced leukemic cell differentiation through increased superoxide production and cell cycle arrest [245].

Effect of Gl-Ps on Macrophages

Sohretoglu et al. demonstrated that Gl-Ps activate macrophages in vitro and elevate the levels of various cytokines, including IL-1β, tumor necrosis factor (TNF-α), IFN-γ, and IL-6 in the culture medium. This activation significantly increases macrophage phagocytosis and enhances macrophage-mediated tumor cytotoxicity [223]. Zhang et al. showed that the in vivo treatment of S180 sarcoma-bearing mice with Gl-Ps activated bone marrow-derived macrophages, inducing the production of immunomodulatory compounds such as IL-1β, TNF-α, and nitric oxide (NO) [242]. Hsu et al. elucidated the role of caspases in macrophage F3 fraction-induced Gl-Ps from *G. lucidum* spores [246]. Guo et al. discovered a novel water-soluble polysaccharide within *G. lucidum* spores that acts as an inducer of TNF-α and IL-6 secretion in murine peritoneal macrophages [247]. The in vivo administration of extracts from *G. lucidum* spores potentiated the proliferative response of splenocytes and induced antitumor activity against lung cancer in mice [247]. Hsu et al. reported that Gl-Ps induces increased secretion of the inflammatory cytokine IL-1 and stimulates the expression of pro-IL-1 and IL-1-converting enzymes in human and murine macrophages, an association linked with its anticancer activity [248].

Effect of Gl-Ps on Natural Killer (NK) Cells

Altfeld et al. defined the role of (NK) natural killer cells in innate immunity [249]. Chien et al. demonstrated that treatment with Gl-Ps resulted in increased monocyte, macrophage, and NK cell populations in human umbilical cord blood [250]. Wang et al. studied the effects of the bio-compounds from *G. lucidum* that, upon oral administration to mice, improved NK cell and phagocytosis activities and increased cytokine levels [251]. In additional research by Zhu et al., it was shown that Gl-Ps accelerated the recovery of bone marrow cells, red blood cells, and white blood cells, as well as splenic NK and NKT killer cells, and enhanced T- and B-lymphocyte proliferative responses [215]. The application of Gl-Ps treatments is recommended in cancer chemotherapy only at low doses [215].

4.3.2. Anti-Proliferative, Cytotoxic, and Apoptosis-Increasing Activities

Ganoderic bio-compounds have demonstrated various anti-proliferative and cytotoxic effects in studies regarding the treatment of different types of cancer [252–255]. The anti-proliferative effects of Gl-Ts compounds in *G. lucidum* manifest through cell cycle arrest [252,253]. Gl-Ts compounds can arrest the cell cycle in the G1 phase by inhibiting the β-catenin pathway, as reported by Wu et al., and in the G2/M phase by suppressing protein kinase C (PKC) activity, as reported by Lin et al. [252,253]. Jedinak et al. reported that ganodermanontriol inhibited the proliferation of HCT116 and HT-29 colon cancer cells by inhibiting β-catenin [254]. Li et al. (2005) identified ganoderic acid X as a compound that can arrest the cell cycle by inhibiting topoisomerase [229]. Chen et al., in 2010, reported that ganoderic acid T (GA-T) exhibits anti-proliferative effects against cancer cells in vitro and against metastasis in vivo [255]. In 2008, Chen et al. reported in the wound vacuolization assay that ganoderic acid Me (GA-Me), administered in a dose- and time-dependent manner, inhibited tumor invasion and cell adhesion to the extracellular matrix (ECM) [256]. GA-Me suppressed master metalloproteinases at the mRNA and protein levels in 95-D cells and is considered a potent antimetastatic carcinoma inhibitor [256]. Hsu et al. studied the anti-proliferative effects of lucidenic acids in human leukemic HL-60 cells [257].

Tang et al. conducted a study on the anti-proliferative effect of ganoderic acid T against cancer cells and observed enhanced cytotoxicity in lung cancer [230]. Triterpene compounds can induce apoptosis of cancer cells via the mitochondria-dependent pathway, followed by caspase activation, as reported by Kao et al. and Liu et al. in 2011 and 2012 [35,258,259]. Zhou et al. investigated the cytotoxic effects of GA-Me in human

colon carcinoma, observing a dose-dependent pattern, and determined that the anticancer bioactivity of GA-Me was mediated through induced apoptosis [260].

#### 4.3.3. Anti-Inflammatory Activities

Several inflammatory mediators, such as TNF-α, IL-6, TGF-β, and IL-10, have been shown to play roles in cancer initiation and progression [261,262]. *G. lucidum* biocomplexes have demonstrated dose-dependent anti-inflammatory effects [223]. Administration of the triterpene extract suppressed inflammatory cytokine secretion in macrophages with a significant reduction in inflammation in the affected tissue, as reported by Dudhgaonkar et al. in 2009 [263]. Joseph et al. found that Gl-Ps administration resulted in a 58% inhibition of inflammation, as assessed by carrageenan-induced (acute) and formalin-induced (chronic) inflammation assays [37].

#### 4.3.4. Anti-Angiogenic Activities

Angiogenesis, the process by which new vasculature is formed from pre-existing vasculature, plays a key role in tumor growth and metastasis as well [264]. Nitric oxide is known to be an angiogenesis-inducing agent in tumors, promoting capillary formation within the tumor and allowing it to expand. In 2004, Cao et al. reported the existence of a peptide in *G. lucidum* that significantly reduced microvessel formation, as detected by the chorioallantois membrane assay [265]. Stanley et al. (2005) found that the *G. lucidum* extract prevented capillary morphogenesis by inhibiting the secretion of angiogenic factors VEGF and (TGF)-β1 [38]. Cao et al. (2006) demonstrated that the *G. lucidum* extract contains a polysaccharide peptide that exhibits anti-angiogenic activity by inhibiting nitric oxide production, subsequently suppressing cell multiplication in a dose-dependent manner [95].

#### 4.3.5. Antioxidant Activities

Excess free radicals adversely affect bases in the nucleic acid structure, amino acids in the protein structure, and double bonds in unsaturated fatty acids, leading to oxidative stress, which is responsible for the alteration of DNA, RNA, proteins, and lipids. Hsieh et al. reported that bio-compounds from *G. lucidum* can exert chemopreventive effects through their antioxidant properties, such as free radical scavenging, as well as the ability to affect phase II detoxification enzymes [39]. Other authors, such as Smina et al., studied the antioxidant activity of Gl-Ts and demonstrated that they can reduce free radicals in cancer cells. [266]. In another study, Smina et al. reported the effect of total triterpenes from *G. lucidum* on the intracellular levels of reactive oxygen species (ROS) and the activities of endogenous antioxidant enzymes in spleen lymphocytes, highlighting their role in reducing radiation-induced oxidative DNA damage in spleen cells [267]. In 2001 and 2003, Lu et al. highlighted the beneficial contribution of the polysaccharides extracted from *G. lucidum* mycelium in reducing ROS-induced oxidative damage [268,269]. Lee et al. reported the existence of an amino polysaccharide compound in *G. lucidum* that can inactivate hydroxyl and superoxide anion radicals [270]. Other authors, such as XiaoPing et al. and Zhao et al., studied Gl-Ps and showed that these bio-compounds could be beneficial for glutathione peroxidase and reduce malonaldehyde levels in rats with cervical carcinoma and mice exposed to γ-irradiation [49,271].

## 5. Toxicity and Safety

Although there are numerous published studies on the beneficial effects of *G. lucidum*, there is also relatively little information reporting the toxic effects in humans. In this regard, Ahmad F. highlighted human sensitisation to *Ganoderma* antigen, reported in the USA, leading to allergic reactions [13,155]. When undergoing *G. lucidum* treatment, special attention should be paid to potential interactions with other drugs. Diabetic patients or those being treated with anticoagulants or antiplatelet drugs require special caution when being administered *G. lucidum*, as the anticipated effects may be altered [272].

In cancer therapy, although *G. lucidum* has been utilized as an anticancer agent, caution is still required when using it in conjunction with chemotherapy due to potential toxicity. Plasma concentrations of *G. lucidum* should be carefully monitored to detect elevated, toxic levels [273]. In an *in vitro* study, *G. lucidum* extracts were found to have toxic effects when exposed to cells at concentrations higher than those required for stimulatory results, resulting in a significant reduction in cell viability in a number of cell lines [274]. *G. lucidum* exhibits antihypertensive activity and may potentiate the effects of antihypertensive drugs [275]. Gl-Ps from *G. lucidum* have antibacterial activity and can enhance the activity of some antibiotics (e.g., tetracycline and cefazolin) [276].

## 6. Conclusions

*G. lucidum* bio-compounds are regarded as valuable in alternative cancer treatments based on non-natural products. The present work has compiled available data from various in vitro and in vivo studies on *G. lucidum* bio-compounds and their beneficial effects in anticancer treatment through their biological actions, such as anti-proliferative, antioxidant, immunomodulatory, anti-inflammatory, and anti-angiogenic effects. This paper presents information on the active bio-compounds in *G. lucidum* to obtain conclusive data and confirm their benefits regarding the mechanisms of anticancer action. Additionally, understanding the mechanisms of anticancer action, combined with other biological anticancer actions exerted by both the main bioactive compounds Gl-Ps and Gl-Ts and other compounds described in the paper (proteins, vitamins, metals, sterols, fatty acids, and nucleotide compounds), is necessary for targeted use in anticancer treatments. It is also crucial to comprehend that the biocomponents of *G. lucidum* are directly influenced by several factors, including the origin and culture medium, environmental conditions, temperature, humidity, and the quality of the environment from which the fungus originates. Therefore, further experimental, epidemiological, and clinical studies are needed to characterize the interactions of the administration of *G. lucidum* forms with different conventional anticancer drugs. More research is needed to combine *G. lucidum* bio-compound treatments with chemotherapy. Extensive pharmacological studies are also necessary to establish optimal dosages and assess the efficacy and safety of administration. Moreover, it is important to extend the research to identify metabolite subtypes that support the observed bioactivities, aiming to establish anticancer therapy procedures that promote general health and longevity.

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## Abbreviations

| | | | |
|---|---|---|---|
| Gl-Ps | *G. lucidum* polysaccharides | ROS | Reactive Oxygen Species |
| Gl-Ts | *G. lucidum* triterpenoids | GA-A | Ganoderic acids A |
| TPC | Total polyphenol content | GA-F | Ganoderic acids F |
| TFC | Total flavonoid content | GA-H | Ganoderic acids H |
| IL | Interleukin | GA-T | ganoderic acid T |
| TNF-α | Tumor Necrosis Factor Alpha | GA-Me | Ganoderic acid Me |
| INF-γ | Interferon Gamma | MMP | Matrix metalloproteinase; |
| TGF-α | Transforming Growth Factor-Alfa | TCL | T-lymphocyte cytotoxic |
| TGF-β | Transforming Growth Factor-Beta | NK | Natural killer cells; |
| VEGF | Vascular Endothelial Growth Factor | PKC | Protein kinase C |
| NO | Nitrogen species | ECM | Extracellular matrix; |
| MDA | Malondialdehyde | DCs | Dendritic cells |
| HPLC-ESI-MS | Liquid chromatography coupled with electrospray ionization mass spectrometry | TEAC | Trolox equivalent antioxidant capacity |

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antioxidants

*Article*

# Antioxidant Profile, Amino Acids Composition, and Physicochemical Characteristics of Cherry Tomatoes Are Associated with Their Color

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**Abstract:** This study was conducted to characterize different colored lines of cherry tomatoes and derive information regarding their metabolite accumulation. Different colored cherry tomato cultivars, namely 'Jocheong', 'BN Satnolang', 'Gold Chance', 'Black Q', and 'Snacktom', were assessed for their firmness, taste characteristics, and nutritional metabolites at the commercial ripening stage. The cultivars demonstrated firmness to withstand impacts during harvesting and postharvest operations. The significant variations in the Brix to acid ratio (BAR) and the contents of phenylalanine, glutamic acid, and aspartic acid highlight the distinct taste characteristics among the cultivars, and the nutritional metabolites are associated with the color of the cultivars. The cultivar choices would be the black-colored 'Black Q' for chlorophylls, β-carotene, total flavonoids, and anthocyanins; the red-colored 'Snacktom' for lycopene; the orange-colored 'Gold Chance' for total phenolics; and the green-colored 'Jocheong' for chlorophylls, vitamin C, GABA, glutamic acid, essential amino acids, and total free amino acids. The antioxidant capacity varied among the cultivars, with 'Gold Chance' consistently exhibiting the highest activity across the four assays, followed by 'Snacktom'. This study emphasizes the importance of screening cultivars to support breeding programs for improving the nutritional content and encourages the inclusion of a diverse mix of different colored cherry tomatoes in packaging to obtain the cumulative or synergistic effects of secondary metabolites.

**Keywords:** anthocyanins; cherry tomatoes; GABA; β-carotene; lycopene; phenolics

## 1. Introduction

The tomato (*Solanum lycopersicum* L.) originates from South America, where it underwent domestication and improvement primarily in the Andean regions of Ecuador and Peru, which was completed in Mesoamerica [1]. Currently, a diverse range of tomato cultivars exists, with different morphological, physicochemical, and sensory attributes contributing to a wide range of tomato-based foods, either in raw or processed form [2]. In 2021, the global production of tomatoes reached 189.13 million tons, cultivated on 5.17 million ha, with Asia contributing significantly by holding a substantial 63.02% share [3]. Specifically, the Republic of Korea produced 348,983 tons of tomatoes from an area of 5610 ha during the same year [3].

Regular tomato consumption is associated with various health benefits, including anticancer properties, decreased susceptibility to cardiovascular, neurodegenerative, and bowel diseases, and improved immune response, exercise recovery, and skin health [4,5]. Experimental evidence also suggests that tomatoes rich in carotenoids have protective effects against oxidative stress in the retinal pigment epithelium and delay the progression of age-related macular degeneration [6]. These protective effects of tomatoes are primarily linked to their valuable bioactive components, such as β-carotene, lycopene, flavonoids, phenolics, and vitamins C and E, which possess antioxidant properties [5].

The increased amino acid levels in tomatoes play a role in their health-promoting qualities, as amino acids are the building blocks of proteins essential for maintaining cellular structure, facilitating the transport and storage of nutrients, promoting wound healing, and aiding in the repair of damaged tissues [7]. Among the amino acids, γ-aminobutyric acid (GABA) and its precursor, glutamic acid, play signaling roles in a range of tissues [8,9]. GABA-rich tomatoes improve metabolic health as the bioavailability of GABA from pureed tomatoes was found to be similar to that of GABA supplement solution in water [8]. In tomatoes, a high level of GABA was found to accumulate in tomato fruit before the breaker stage and to be catalyzed rapidly thereafter [10]. So, screening cultivars rich in GABA and glutamic acid, which do not show rapid GABA catabolism after reaching the breaker stage, could be advantageous for consumers.

Apart from the above-mentioned antioxidant properties and GABA content, consumer acceptance of tomato fruit relies significantly on the taste and physicochemical attributes like the firmness, color, total soluble solids (TSS), titratable acidity (TA), and the Brix to acid ratio (BAR) [11,12]. The postharvest quality of tomatoes develops during the growth, and the phytochemical composition and antioxidant activity can be affected by agronomic practices, environmental factors such as light intensity, water availability, temperature, and growing media, along with the ripening stages and conditions [4,13,14]. Furthermore, research findings have revealed that the diversity among tomato cultivars affects their physicochemical and antioxidant properties and GABA levels [5,9,15].

This study was conducted in a climate-controlled greenhouse with a hydroponics system under the same agronomic practices based on the hypothesis that the color variation among different cherry tomatoes will reveal significant variations in their metabolite profiles. We used green-colored 'Jocheong', yellow-colored 'BN Satnolang', orange-colored 'Gold Chance', black-colored 'Black Q', and red-colored 'Snacktom' cherry tomatoes to characterize these different colored lines of cherry tomatoes and derive novel information regarding their metabolite accumulation. Specifically, we expected that a distinct color difference would influence the concentration and composition of metabolites, leading to measurable differences in the nutritional content and antioxidant activity among the cherry tomato cultivars, which could require consuming a mix of different colored cherry tomatoes to benefit from their cumulative effects. In addition, the synergistic effects of all the constituents in tomatoes are likely to surpass the advantages of individual components like lycopene and β-carotene. Thus, this study incorporated both individual parameters and antioxidant activities in four assays to study the combined effect of these individual parameters.

## 2. Materials and Methods

### *2.1. Chemicals*

All the chemicals utilized in this study were of analytical reagent grade. HPLC-grade methanol for the mobile phase was purchased from the J.T. Baker chemical company (Center Valley, PA, USA). Potassium dihydrogen phosphate was sourced from Yakuri Pure Chemicals (Kyoto, Japan). Metaphosphoric acid and dimethyl sulfoxide (DMSO) were purchased from Kanto Chemical (Tokyo, Japan). Potassium chloride was procured from Junsei Chemical (Tokyo, Japan). β-carotene, gallic acid, rutin, and ascorbic acid were purchased as standards from Sigma-Aldrich (St. Lous, MO, USA). Additionally, the amino acid standard was purchased from Agilent Technologies (Santa Clara, CA, USA).

The following reagents were also purchased from Sigma-Aldrich (St. Louis, MO, USA): sodium acetate, acetone, ethanol, hexane, Folin–Ciocalteu reagent, sodium carbonate, aluminum nitrate, potassium acetate, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)), potassium persulfate, acetate, TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine), hydrochloric acid, ferric chloride, sodium phosphate, potassium ferricyanide, trichloroacetic acid, and iron(III) chloride.

### *2.2. Plant Materials*

Five cherry tomato (*Solanum lycopersicum* L.) scion cultivars, namely green-colored 'Jocheong' (Taeyang Seed Co., Ltd., Seoul, Republic of Korea), yellow-colored 'BN Satnolang', orange-colored 'Gold Chance', black-colored 'Black Q' (Bunong Seed Co., Ltd., Suwon, Republic of Korea), and red-colored 'Snacktom' cherry tomatoes (Pan Pacific Seed Co., Ltd., Yongin, Republic of Korea), were used in this research. Additionally, 'Bikeio' rootstock (Bunong Seed Co., Ltd., Suwon, Republic of Korea) was used for this study. The trays for cultivation were arranged within the greenhouse facility of Hoban Agriculture Corporation, situated at coordinates 37°55′29″ N and 127°47′04″ E, at an elevation of 85 m above sea level in Chuncheon, Gangwon Province, Republic of Korea. The seeds were sown on 27 September 2021 on horticultural soil sourced from Pindstrup, Denmark. A 162-hole tray measuring W 280 × L 540 × H 45 mm and a 128-hole tray measuring W 280 × L 540 × H 48 mm, both sourced from Bumnong Co., Ltd. (Jeongup, Republic of Korea), were used for sowing the cherry tomato scion and rootstock seeds, respectively. After sowing, we adequately irrigated the seeded trays through overhead irrigation and then covered them with vermiculite to retain moisture. The germination process took place over 48 h in a germination room under dark conditions, with a controlled temperature ranging between 25 and 28 °C and a relative humidity maintained at 90%. Then, the seedlings were transferred to the greenhouse and grafting was attained within a month of seeding, and the plants had reached a stage of growth suitable for transplantation by 5 December 2021.

The transplanted tomatoes were grown using a hydroponic system within a climate-controlled greenhouse located at coordinates 37°92′ N and 127°75′ E in Gangwon Province, Republic of Korea, during the spring/summer of 2022. The electrical conductivity (EC) of the fertigation solution was regulated within a range of 2.5 to 2.8 deciSiemens per meter (dS $m^{-1}$) while maintaining the pH between 5.5 and 5.8, tailored according to the growth stages. The harvesting commenced on 10 March 2022, with the fruits used for the measurements being harvested on 10 May 2022. Harvesting involved selecting uniform-sized fruits from the third cluster of each plant without physical defects, with the exclusion of fruits located at the tip of the cluster.

After harvesting at the commercial harvesting stage, the fruits were transported to Kangwon National University, where ten representative fruits per cultivar were randomly selected. The physicochemical data were taken the same day and the samples prepared for metabolite analysis were freeze-dried, ground, filtered using a 40 μm filter, and stored in a deep freezer until analysis.

### *2.3. Physicochemical Parameters*

Fruits of the five cherry tomato cultivars were measured for the firmness, color values, total soluble solids (TSSs), titratable acidity (TA), and brix to acid ratio (BAR). The fruit firmness at the equator of each fruit was measured following the method described by Baek et al. [16]. This involved applying a maximum force of 10 kg using a rheometer (Sun Scientific Co. Ltd., Tokyo, Japan) fitted with a 3 mm diameter round stainless-steel probe with a flat end at a speed of 1.0 mm/s, and the results were quantified in Newtons (N). The fruit skin color values greenness to redness (a*), blueness to yellowness (b*), and darkness to brightness (L*) were measured according to Baek et al. [16] from ten fruit using a CR-400 Chroma meter (Minolta, Tokyo, Japan). The determination of the TSSs, TA, and BAR values followed the procedure described by Tilahun et al. [13] The TSSs was assessed using an Atago refractometer (Atago Co., Ltd., Tokyo, Japan) at 20 °C, with measurements taken

from ten sample fruits. The TA was measured through a Mettler Toledo analyzer (Mettler Toledo Ltd., Zurich, Switzerland). This involved dilution (1 mL juice: 19 mL distilled water) followed by titration until reaching a pH of 8.1, utilizing 0.1 N NaOH. The TA result was quantified as mg 100 $g^{-1}$ citric acid of fresh tomato. The BAR was computed by dividing the TSSs by the TA.

### *2.4. Amino Acids*

Three biological replicates of freeze-dried cherry tomato fruit samples (1 g) were extracted using 75% ethanol with ultrasonic extraction for 1 h followed by room temperature extraction for 24 h. After filtering the extract with a 0.2 μm filter, analysis of the amino acid content was conducted using Henderson et al.'s [17] method with a Dionex Ultimate 3000 HPLC. The method utilizes automated online derivatization with o-phthalaldehyde (OPA) for primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for secondary amino acids, and it involves the separation and detection of amino acids using specific reagents that react with amino groups to form derivatives, which are then quantified by the HPLC system.

### *2.5. Secondary Metabolites*

Chlorophylls (Chls) were extracted from the ethanol extract of the cherry tomato cultivars. The Chls extraction process utilized the dimethyl sulfoxide (DMSO) extraction procedure as described by Baek et al. [18]. Absorbance readings were taken at 645 and 663 nm utilizing a microplate reader (SpectraMax ABS Plus, Molecular Devices, Sunnyvale, CA, USA) with a DMSO blank serving as the reference. Then, Chl a, Chl b, and the total Chls were calculated using the equations of Arnon [19], as outlined Tilahun et al. [20].

The total anthocyanin content (expressed as cyanidin-3-glucoside equivalents) was assessed using the pH differential method [21] as described by Tilahun et al. [20]. Freeze-dried radish microgreen samples (0.25 g) were mixed with 5 mL of methanol containing 0.1% HCl. The samples were subjected to ultrasonic treatment three times for 10 min, followed by centrifugation to collect the supernatant. A 50 μL portion of the supernatant was filtered using a 0.45 μm membrane filter (PTFE, 13 mm, Whatman, Maidstone, UK). Subsequently, a mixture of 25 mM potassium chloride buffer (pH 1.0) and 400 mM sodium acetate buffer (pH 4.5) was prepared in 950 μL and developed for 15 min. Readings were taken at 520 nm and 700 nm using a microplate reader (SpectraMax ABS Plus, Molecular Devices, Sunnyvale, CA, USA). Then, the anthocyanin content was calculated using the formula:

$$\text{Anthocyanin content (mg g}^{-1}) = (V \times A \times MW \times DF)/(\varepsilon \times m)$$

where the variables V, A, MW, DF, ε, and m represent the total volume of the extract (mL), the difference in absorbance values at pH 1.0 and pH 4.5 at 520 nm and 700 nm, the molar mass of cyanidin-3-glucoside (449.2 g $mol^{-1}$), the dilution factor, the molar extinction coefficient in L $mol^{-1}$ $cm^{-1}$ (26,900), and the sample quantity (g), respectively.

The lycopene and β-carotene content of the cherry tomato fruit samples were determined according to the methods described by Tilahun et al. [14]. Freeze-dried tomato samples (1 g) were placed in vials, followed by the addition of 5 mL of ethanol, 5 mL of acetone, and 10.0 mL of hexane to each vial. Subsequently, the vials underwent centrifugation for 15 min at 6000× *g*. Following centrifugation, each vial was supplemented with 3 mL of deionized water, and the samples were subjected to agitation for an additional 5 min. Phase separation was achieved by allowing the vials to stand at room temperature without agitation for 5 min. The hexane layer's absorbance at 503 and 448 nm, relative to a blank hexane solvent, was measured using a microplate reader (SpectraMax i3, Molecular Devices, Sunnyvale, CA, USA) to determine the lycopene and β-carotene content, respectively. The lycopene content of the samples was subsequently quantified as mg 100 $g^{-1}$ of dry weight following the procedure outlined by Fish et al. [22]

$$\text{Lycopene content (mg kg}^{-1}) = (\text{Absorbance at } 503 \times 0.0312)/\text{kg} = (\text{Absorbance at } 503 \times 31.2)/\text{g}$$

Meanwhile the β-carotene content was determined by comparing the sample readings to the β-carotene standard curve and expressed as mg 100 $g^{-1}$ dry weight of the sample.

The levels of total phenolics and flavonoids in the freeze-dried cherry tomato fruit samples were determined with three replications, following the methodology established in our laboratory, as described by Baek et al. [23]. An ethanolic extract (1 mg $mL^{-1}$) or standard was mixed with 1 mL of 10% Folin–Ciocalteu phenol reagent and 1 mL of 2% sodium carbonate solution for the total phenolics measurement. After 90 min of dark incubation at room temperature, the absorbance was then measured at 750 nm using a microplate reader (Spectramax i3, Molecular Devices, Sunnyvale, CA, USA). The results were expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE $g^{-1}$) after the measurements were compared to the gallic acid calibration curve. For the total flavonoid analysis, 1.5 mL of ethanol, 0.1 mL of 10% aluminum nitrite solution, 0.1 mL of 1 M potassium acetate solution, and 2.8 mL distilled water were combined with an ethanolic extract (1 mg $mL^{-1}$). After stirring, the mixture was left to react for 30 min. The absorbance was then measured at 415 nm using a microplate reader (SpectraMax i3, Molecular Devices, Sunnyvale, CA, USA). The obtained results were compared to a rutin calibration curve and expressed as milligrams of rutin equivalents per gram of sample (mg RE $g^{-1}$).

The vitamin C content was analyzed from the freeze-dried cherry tomato samples in triplicate using reversed phase (RP) liquid chromatography with ultraviolet (UV) detection according to Tilahun et al. [20]. A 1 g sample was combined with 10 mL of 5% metaphosphoric acid (5 g/100 mL) and homogenized for 1 min. Following homogenization, the mixture underwent 10 min centrifugation at 7828× *g*. The liquid layer of the extract was then filtered with 0.22 μm membrane and subjected to analysis using a ZORBAX Eclipse XDB-C18 column (4.6 cm × 250 mm, 5 μm, Agilent Technologies, Santa Clara, CA, USA) coupled with a UV-2075 detector (Jasco, Tokyo, Japan) at 265 nm. The analysis involved a 20 μL injection of 100% MeOH:0.1 M $KH_2PO_4$ (1:9 ratio) at a flow rate of 1 mL $min^{-1}$ as the mobile phase.

### *2.6. Antioxidant Activities*

Freeze-dried and ground cherry tomato samples were subjected to extraction following the method previously implemented in our laboratory [23]. The assessment of the Trolox-equivalent antioxidant capacity (ABTS), DPPH radical-scavenging capacity, and ferric-reducing antioxidant power (FRAP) was carried out in triplicate following the procedures described by Baek et al. [23]. Additionally, the reducing power (RP) assay was conducted in triplicate according to the method detailed by Choi et al. [24].

A sample solution (0.2 mL) containing a 10 mg $mL^{-1}$ concentration of the extract was added to a 0.8 mL ethanolic DPPH (0.4 mM) solution. The mixture was allowed to react at room temperature in the dark for 10 min. The absorbance was then measured at 517 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). For the blank, distilled water was used instead of the sample. Then, the calculation for the radical scavenging activity was as follows:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (\text{Absorbance of the sample}/\text{Absorbance of the blank})] \times 100$$

A stock solution of ABTS was prepared by dissolving it in water to a concentration of 7.4 mM. The cation (ABTS+) was generated by reacting this stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand for 14 h at room temperature in the dark. The $ABTS^+$ solution was then diluted with ethanol to achieve an absorbance of 0.70 ± 0.02 at 750 nM. Then, 1.0 mL of diluted $ABTS^+$ solution was added to 0.01 mL of sample (10 mg $mL^{-1}$ concentration), and the mixture was left at room temperature for 30 min in the dark. The absorbance was measured at 750 nM using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). For the blank, distilled water was used instead of the sample. Then, the radical scavenging activity was calculated by the following equation:

$$\text{ABTS radical scavenging activity (\%)} = [1 - (\text{Absorbance of the sample}/\text{Absorbance of the blank})] \times 100$$

The FRAP reagent was prepared fresh daily using 300 mM acetate buffer (pH 3.6), a 10 mM 2, 4, 6-tri (2-pyridyl) -1, 3, 5-triazine (TPTZ) solution in 40 mM HCl, and a 20 mM $FeCl_3 \cdot 6H_2O$ solution in a 10:1:1 ($v/v$) ratio. The reagent was warmed to 37 °C in a water bath before use. Then, 0.05 mL of the sample (10 mg $mL^{-1}$ concentration) was mixed with distilled water (0.15 mL) and the FRAP reagent (1.5 mL). The reaction mixture was incubated at 37 °C for 4 min, and the absorbance was measured at 595 nM using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

For the reducing power (RP) assay, samples (0.1 mL) of 10 mg $mL^{-1}$ concentration were mixed with potassium ferricyanide (1 %, 0.5 mL) and sodium phosphate buffer (0.2 M, 0.5 mL) and incubated at 50 °C for 20 min. Trichloroacetic acid (0.5 mL) was then added to the mixed solution and centrifuged at 1790× $g$ for 10 min. In a new test tube, the supernatant (0.5 mL), iron (III) chloride solution (0.1%, 0.1 mL), and distilled water (0.5 mL) were mixed. Then, the absorbance of this solution was measured at 700 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

### *2.7. Experimental Design and Statistical Analysis*

A completely randomized design was used at the greenhouse. To assess the differences between the cherry tomato cultivars, the collected data were subjected to analysis of variance (ANOVA) at $p < 0.05$ using statistical software (SAS/STAT® 9.1; SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test, heat maps and principal component analysis (PCA) were used to further examine the variations between the cultivars. MetaboAnalyst v6.0 and XLSTAT version 2015.1 (Addinsoft Inc., 244 Fifth Avenue, Suite E100, New York, NY, USA) were used for the heat maps and PCA, respectively.

## 3. Results and Discussion

### *3.1. Physicochemical Parameters*

Assessing the firmness of fresh tomatoes is a crucial parameter to evaluate their quality, especially concerning their suitability for the intended culinary uses. Additionally, it serves as a valuable criterion for screening resilient cultivars, ensuring resistance against mechanical injury during harvesting and postharvest operations [16]. In the present study, significant difference in firmness was observed among the five cherry tomato cultivars (Table 1). The highest firmness value was recorded in 'Black Q' (17.09 N), followed by 'Jocheong' (13.38 N), and the lowest firmness value was recorded in the 'BN Satnolang' (10.44 N) cultivar, although the difference between 'BN Satnolang', 'Gold Chance', and 'Snacktom' was not significant. Previous studies have also reported significant differences in firmness among different tomato cultivars [5,13]. The genetic variation in cherry tomato cultivars can influence the cuticle of the tomato fruit, which has been found to have a significant influence on the fruit firmness and ripening physiology, both directly as the cuticle acts as a load-bearing matrix under tension and indirectly by regulating the fruit's water status [25]. In addition, differences among cultivars may impact the absorption of calcium [26]. An adequate calcium intake is essential for promoting robust growth in tomato plants, reinforcing and stabilizing cell walls, and ultimately, influencing the firmness of the fruit [27].

**Table 1.** Physicochemical parameters of fresh cherry tomato cultivars at harvest.

| Cultivars | Firmness (N) | TSSs (°Brix) | TA (mg 100 $g^{-1}$) | BAR (TSS/TA) |
|---|---|---|---|---|
| Jocheong | 13.38 ± 0.97 b | 7.84 ± 0.11 cd | 1.14 ± 0.03 a | 6.84 ± 0.69 c |
| BN Satnolang | 10.44 ± 0.39 c | 10.75 ± 0.20 a | 0.96 ± 0.03 b | 11.33 ± 1.26 a |
| Gold chance | 10.88 ± 0.52 c | 8.66 ± 0.27 b | 1.00 ± 0.04 b | 8.76 ± 1.34 b |
| Black Q | 17.09 ± 0.60 a | 7.43 ± 0.45 d | 0.92 ± 0.02 b | 8.15 ± 0.64 b |
| Snacktom | 10.82 ± 0.42 c | 8.26 ± 0.16 bc | 1.01 ± 0.03 b | 8.28 ± 0.99 b |

TSSs, TA, and BAR stand for total soluble solids, titratable acidity, and brix to acid ratio. The results are shown as the mean ± SD ($n$ = 10), with differing letters in the same column indicating significant statistical difference at $p < 0.05$.

A significant difference was also observed in the TSSs, TA and BAR of the cherry tomato cultivars (Table 1). The TSSs content of 'BN Satnolang' (10.75 °Brix) was the highest and 'Black Q' (7.43 °Brix) was the least. The TA values ranged from 0.92 mg 100 $g^{-1}$ in 'Black Q' to 1.14 mg 100 $g^{-1}$ in 'Jocheong'. On the other hand, although the difference between 'Gold Chance', 'Black Q', and 'Snacktom' was not significant, 'BN Satnolang' had the highest BAR (11.33), while the lowest BAR (6.84) was observed in 'Jocheong' (Table 1), indicating differences in the taste characteristics of the tested cherry tomato cultivars. In agreement with this study, previous studies reported variations in the TSSs, TA, and BAR among cultivars, highlighting that the balance of sugar and acid contents influences the taste characteristics of tomatoes [28,29].

The external color of cherry tomatoes is more closely tied to consumer preferences, given its impact on the human eye's perception of color. Oltman et al. [30] reported color to be the most important external attribute for tomato liking in their survey conducted on consumers' attitudes and preferences for fresh market tomatoes. Although they reported red-colored tomatoes as attractive, they also stressed that different groups of tomato consumers exist, with specific preferences for health benefits, taste, firmness, and juiciness. In the current study, the color of cherry tomatoes spans from green to red, encompassing a* values that vary from −8.86 in the 'Jocheong' to 14.42 in the 'Snacktom'. The degree of yellowness, represented by the b* values, also ranges from 16.48 in 'Black Q' to 38.32 in 'Gold Chance', as illustrated in Figure 1.

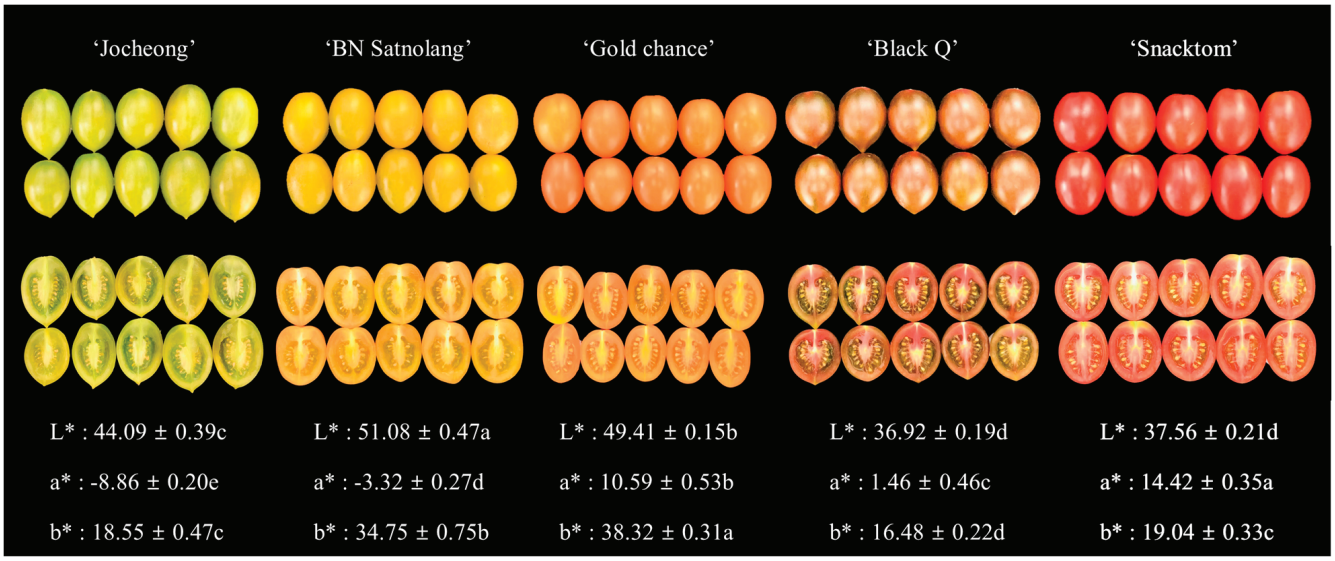


**Figure 1.** Harvesting stages and external colors of the tested cherry tomato cultivars. The color of the fruit was assessed using the L* (brightness), Hunter a* (redness), and b* (yellowness) values.

### *3.2. Amino Acids*

In this study, the amino acid content was evaluated among the different colored cherry tomato cultivars tested. A total of 22 free amino acids were identified and the content of amino acids in the cherry tomatoes was significantly dependent on the cultivar (Table 2). The total free amino acid content (TAA) of 'Jocheong' was the highest (62.36 g $kg^{-1}$ DW), followed by 'Black Q' (52.88 g $kg^{-1}$ DW), 'Gold Chance' (35.32 g $kg^{-1}$ DW), 'Snacktom' (34.56 g $kg^{-1}$ DW), and 'BN Satnolang' (32.05 g $kg^{-1}$ DW), respectively (Table 2). Consistent with the findings of this research, Tilahun et al. [5] tested the 'TY VIP', 'Mamirio', 'Tori', and 'Arya' tomato cultivars and also observed variation in the total amino acids, ranging from 38.81 to 57.31 g $kg^{-1}$ DW. The assessment of the protein quality in a food can be performed by examining the levels of nine essential amino acids (EAA), namely methionine, leucine, isoleucine, histidine, tryptophan, valine, phenylalanine, threonine, and lysin [31]. The findings of this study, as indicated in Table 2, revealed the highest essential amino acids content

in 'Jocheong' (5.96 g $kg^{-1}$ DW), followed by 'Black Q' (3.74 g $kg^{-1}$ DW), 'BN Satnolang' (2.95 g $kg^{-1}$ DW), 'Snacktom' (2.68 g $kg^{-1}$ DW), and 'Gold Chance' (2.52 g $kg^{-1}$ DW), respectively. Hence, the contents of EAA in cherry tomatoes is also cultivar-dependent, and having information about the EAA content could assist customers in selecting the cultivar that best suits their preferences.

**Table 2.** Individual, essential and total amino acid contents in fresh cherry tomato cultivars at harvest.

| Amino Acids | Jocheong | | BN Satnolang | | Gold Chance | | Black Q | | Snacktom | |
|---|---|---|---|---|---|---|---|---|---|---|
| | mg $kg^{-1}$ | % | mg $kg^{-1}$ | % | mg $kg^{-1}$ | % | mg $kg^{-1}$ | % | mg $kg^{-1}$ | % |
| Aspartic acid | 4971.14 b | 7.97 | 3403.79 c | 10.62 | 3254.97 c | 9.22 | 5274.66 a | 9.98 | 3052.74 d | 8.83 |
| Glutamic acid | 26,883.30 a | 43.11 | 12,054.79 c | 37.62 | 16,446.00 b | 46.56 | 27,544.36 a | 52.09 | 17,118.92 b | 49.54 |
| Asparagine | 4535.81 a | 7.27 | 2928.94 b | 9.14 | 2108.42 c | 5.97 | 1438.23 e | 2.72 | 2017.01 d | 5.84 |
| Serine | 1296.15 a | 2.08 | 546.86 d | 1.71 | 729.03 c | 2.06 | 914.87 b | 1.73 | 726.80 c | 2.10 |
| Glutamine | 9953.29 a | 15.96 | 5992.00 b | 18.70 | 5225.22 c | 14.79 | 3492.97 d | 6.61 | 5069.68 c | 14.67 |
| Histidine (EAA) | 820.70 a | 1.32 | 347.11 c | 1.08 | 371.14 c | 1.05 | 516.37 b | 0.98 | 364.92 c | 1.06 |
| Glycine | 108.47 a | 0.17 | 56.25 d | 0.18 | 65.91 b | 0.19 | 68.65 b | 0.13 | 60.04 c | 0.17 |
| Threonine (EAA) | 1080.68 a | 1.73 | 378.28 cd | 1.18 | 369.27 d | 1.05 | 487.64 b | 0.92 | 401.14 c | 1.16 |
| Citrulline | 106.72 a | 0.17 | 53.04 b | 0.17 | 50.87 b | 0.14 | 32.31 c | 0.06 | 48.20 b | 0.14 |
| Arginine | 925.54 a | 1.48 | 483.77 c | 1.51 | 396.16 d | 1.12 | 630.74 b | 1.19 | 385.01 d | 1.11 |
| Alanine | 607.64 c | 0.97 | 370.36 d | 1.16 | 834.15 b | 2.36 | 1132.56 a | 2.14 | 622.79 c | 1.80 |
| Taurine | 14.65 c | 0.02 | 13.66 c | 0.04 | 25.90 b | 0.07 | 31.97 a | 0.06 | 23.00 b | 0.07 |
| GABA | 6220.85 a | 9.98 | 1906.47 d | 5.95 | 2176.56 c | 6.16 | 4960.61 b | 9.38 | 1449.25 e | 4.19 |
| Tyrosine | 212.93 a | 0.34 | 159.53 b | 0.50 | 77.79 d | 0.22 | 95.15 c | 0.18 | 75.50 d | 0.22 |
| Valine (EAA) | 342.26 a | 0.55 | 116.12 d | 0.36 | 125.43 c | 0.36 | 263.64 b | 0.50 | 120.33 cd | 0.35 |
| Methionine (EAA) | 5.74 b | 0.01 | 10.60 a | 0.03 | 10.17 a | 0.03 | 10.29 a | 0.02 | 10.05 a | 0.03 |
| Tryptophane (EAA) | 366.11 a | 0.59 | 285.02 b | 0.89 | 225.84 c | 0.64 | 282.80 b | 0.53 | 205.82 c | 0.60 |
| Phenylalanine (EAA) | 1420.66 a | 2.28 | 909.42 b | 2.84 | 621.45 e | 1.76 | 876.84 c | 1.66 | 779.57 d | 2.26 |
| Isoleucine (EAA) | 677.53 a | 1.09 | 207.36 c | 0.65 | 174.30 d | 0.49 | 384.94 b | 0.73 | 201.38 c | 0.58 |
| Leucine (EAA) | 640.01 a | 1.03 | 256.19 d | 0.80 | 268.70 c | 0.76 | 385.72 b | 0.73 | 226.90 e | 0.66 |
| Lysine (EAA) | 609.34 a | 0.98 | 437.21 c | 1.36 | 354.76 d | 1.00 | 532.44 b | 1.01 | 366.47 d | 1.06 |
| Proline | 558.91 a | 0.90 | 1130.84 b | 3.53 | 1409.95 b | 3.99 | 3519.51 a | 6.66 | 1233.16 b | 3.57 |
| Total EAA | 5963.04 a | 1.29 | 2947.31 c | 2.60 | 2521.05 d | 3.25 | 3740.67 b | 8.11 | 2676.59 d | 2.84 |
| Total | 62,358.44 a | | 32,047.62 d | | 35,321.97 c | | 52,877.27 b | | 34,558.69 c | |

EAA stands for essential amino acid. The results are shown as the mean ± SD ($n$ = 3), with differing letters in the same raw indicating a significant statistical difference at $p < 0.05$.

Among the individual free amino acids, glutamic acid proved to be the most abundant amino acid across all five cultivars. The content varied from 12.05 g $kg^{-1}$ DW (37.62%) for 'BN Satnolang' to 27.54 g $kg^{-1}$ DW (52.09%) for 'Black Q'. Glutamine was the second most abundant free amino acid, except for 'Black Q', where the contents of aspartic acid and GABA were higher than glutamine. Generally, GABA, asparagine, aspartic acid, glutamine, and glutamic acid emerged as the predominant amino acids in all five cherry tomato cultivars, constituting a range from 80.78% in 'Black Q' to 84.29% in 'Jocheong' of the total free amino acids. Consistent with the findings of this study, Tilahun et al. [5] similarly identified these amino acids as the primary constituents, accounting for 80.65 to 89.98% of the total free amino acids in the 'TY VIP', 'Mamirio', 'Tori', and 'Arya' tomato cultivars at the pink stage of ripening. While the GABA content in tomatoes reduces significantly during the ripening transition, 'Kumato' tomato, which undergoes a color transformation from green to reddish–brown or purple after ripening, has been reported to maintain a high level of GABA [18]. Similarly, in the present study, a high level of GABA (6.22 g $kg^{-1}$ DW) and its precursor, glutamic acid (26.88 g $kg^{-1}$ DW), was recorded in the green-colored 'Jocheong', followed by black-colored 'Black Q' (4.96 g $kg^{-1}$ GABA and 27.54 g $kg^{-1}$ DW glutamic acid). Hence, when considering the amount of TAA, EAA, and GABA, the preferable cultivars of choice would be the green-colored 'Jocheong' and black-colored 'Black Q'.

Phenolic volatiles, which play a crucial role in shaping our perception of the tomato flavor, encompass a range of compounds derived from the amino acid phenylalanine [11]. Additionally, the umami taste of tomatoes is influenced by the ratio of glutamic acid to aspartic acid, which is essential for the overall taste profile [12]. Notably, the contents of phenylalanine, glutamic acid, and aspartic acid varied significantly among the five cherry

tomato cultivars (Table 2), and the ratio of glutamic acid to aspartic acid ranges from 3.54 in 'BN-Satnolang' to 5.61 in 'Snacktom', underscoring their role in imparting a distinct and unique taste to each cultivar. In the current study, taurine, a naturally occurring sulfur-containing amino acid, was also detected in cherry tomatoes and the amount ranged from 13.66 mg $kg^{-1}$ in 'BN-Satnolang' to 31.97 mg $kg^{-1}$ in 'Black Q'. Taurine, primarily sourced from the seafoods, has attracted significant attention in recent years due to its cardiovascular effects, such as regulating blood pressure, improving cardiac fitness, and enhancing vascular health [32]. Hence, cherry tomato cultivars could also be screened based on their taurine content, and the preferable cultivar based on the amount of taurine would be the black-colored 'Black Q'.

### *3.3. Secondary Metabolites*

Secondary metabolites contribute to the plant's interaction with its surroundings, enhancing the aroma and pigmentation to attract seed dispersers while serving as a defense mechanism against both biotic and abiotic stresses [31,33]. The positive health impacts of these secondary metabolites obtained from plant foods become apparent through the consumption of various phytochemicals, exhibiting cumulative or synergistic effects [33].

The distinct vibrant color of tomato is a result of the transformation of chloroplasts into chromoplasts, a process linked to carotenoid synthesis [14]. As tomatoes ripen, they accumulate lycopene (red linear carotene) and β-carotene (orange cyclization pro-vitamin A product) [34]. This study revealed significant variations among the cherry tomato cultivars in terms of the anthocyanins, chlorophylls, lycopene and β-carotene contents (Figures 2 and 3). In addition to the anthocyanins, the colored tomatoes have the underlining carotenoid pigment, including chlorophylls, lycopene and β-carotene, and these pigments could impact consumers' preference by indicating maturity, quality and freshness. The green to red (a* values) and blue to yellow (b* values) observed in Figure 1 are directly proportional to the chlorophylls, lycopene and β-carotene contents (Figures 2 and 3). Chlorophylls, lycopene and β-carotene demonstrate significant antioxidant properties, and dietary anthocyanin consumption has been linked to reduced cardiovascular disease risk factors [14,35,36].

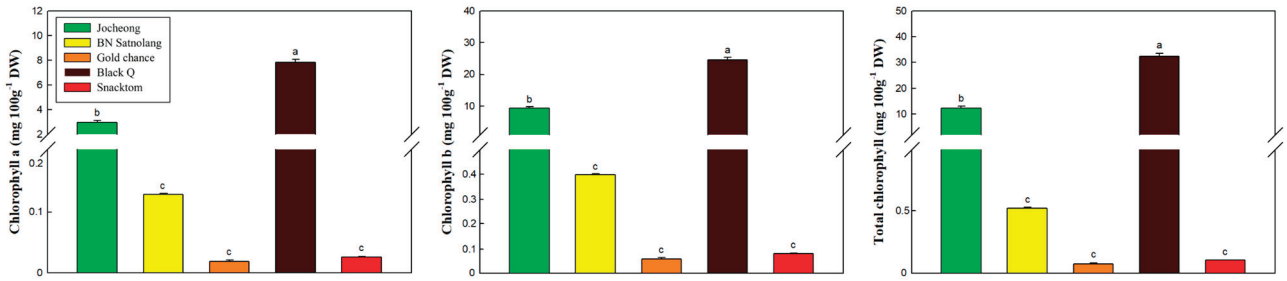


**Figure 2.** Chlorophyll a, chlorophyll b and total chlorophyll content contents of 'Jocheong', 'BN Satnolang', 'Gold chance', 'Black Q', and 'Snacktom' cherry tomato cultivars at harvest. Different letters on the bars indicate a significant difference between the mean values of the cultivars ($n = 3$) at $p < 0.05$.

In this study, the average total Chls content varied between 0.10 mg $g^{-1}$ DW in 'Gold Chance' and 32.40 mg $g^{-1}$ DW in 'Black Q' (Figure 2). The concentrations of chlorophyll a, chlorophyll b, and total chlorophylls exhibited similar patterns, with 'Black Q' and 'Jocheong' showing the highest two scores, respectively. On the other hand, 'Snacktom' (39.40 mg 100 $g^{-1}$ DW) and 'Black Q' (35.96 mg 100 $g^{-1}$ DW) exhibited the highest lycopene content, followed by 'Gold Chance' (16.08 mg 100 $g^{-1}$ DW), 'BN Satnolang' (8.87 mg 100 $g^{-1}$ DW), and 'Jocheong' (6.54 mg 100 $g^{-1}$ DW) (Figure 3). 'Black Q' (17.51 mg 100 $g^{-1}$ DW) had the highest in β-carotene content, followed by 'Gold Chance' (16.62 mg 100 $g^{-1}$ DW), while the lowest (7.23 mg 100 $g^{-1}$ DW) was observed in 'BN Satnolang' (Figure 3). Similar findings of previous works support the variation in the lycopene and β-carotene contents among cultivars [5,14]. However, 'BN Satnolang' and 'Jocheong' showed lower levels of lycopene

and β-carotene compared to the previously reported 'TY Megaton', 'Yureka', Tori', 'TY VIP', 'Mamirio', and 'Arya' tomato cultivars [5,14].

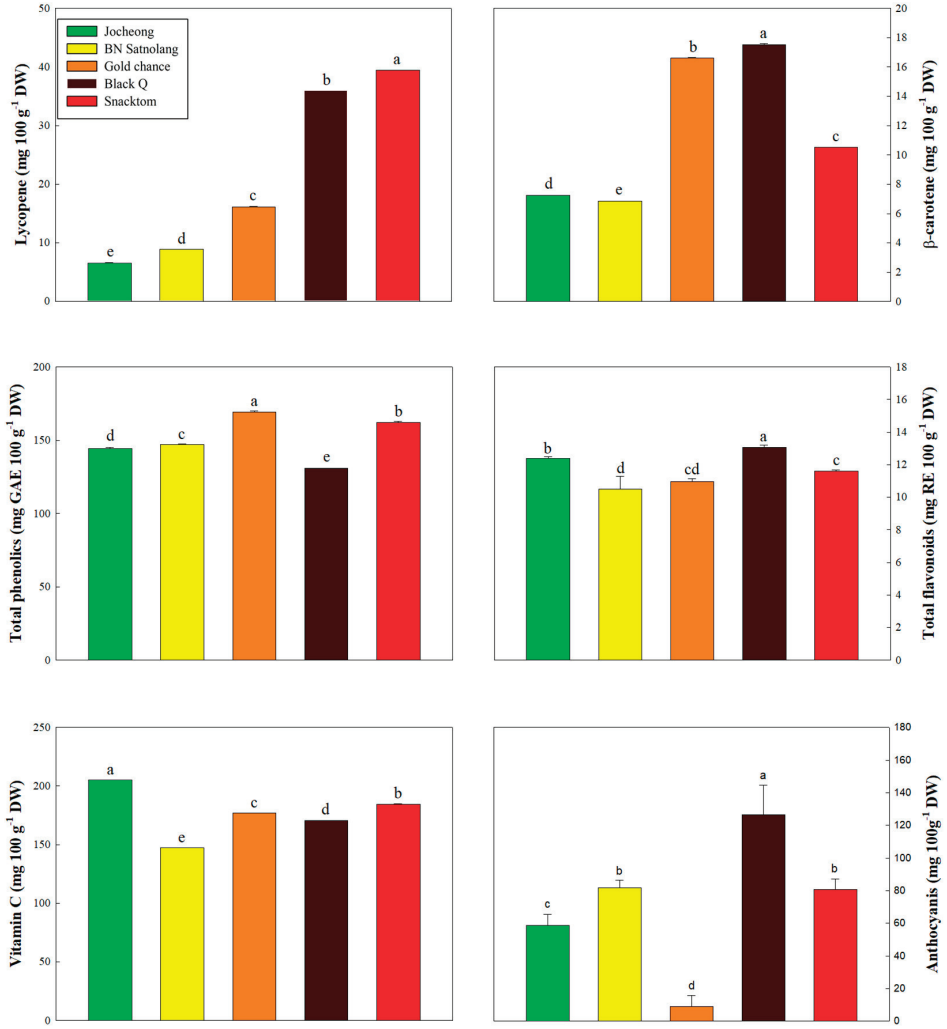


**Figure 3.** Lycopene, β-carotene, total phenolics, total flavonoids, vitamin C and anthocyanins contents of cherry tomato cultivars at harvest. Different letters on the bars indicate a significant difference between the mean values of the cultivars ($n$ = 3) at $p < 0.05$.

Conversely, 'Gold Chance' showed the highest level of total phenolics at 169.36 mg GAE 100 $g^{-1}$ DW, followed by 'Snacktom' and 'BN Satnolang' with 162.15 and 147.03 mg GAE 100 $g^{-1}$ DW, respectively (Figure 3). The total phenolics content in this study ranged from 144.30 to 169.36 mg GAE 100 $g^{-1}$ DW, slightly lower than the range (168.20 to 290.70 mg GAE 100 $g^{-1}$) reported by Bhandari et al. [37] for Korean commercial tomato cultivars.

The black-colored 'Black Q' recorded high levels of total flavonoids (13.05 mg RE 100 $g^{-1}$ DW) and anthocyanins (126.47 mg 100 $g^{-1}$ DW). The green-colored 'Jocheong' had a high level of vitamin C (204.92 mg 100 $g^{-1}$ DW), while the red-colored 'Snacktom' ranked second in the contents of vitamin C (184.42 mg 100 $g^{-1}$ DW) and anthocyanins (80.60 mg 100 $g^{-1}$ DW) (Figure 3).

Tomatoes, one of the most extensively cultivated vegetables worldwide, are a significant source of bioactive compounds, including carotenoids and polyphenols, such as phenolic acids and flavonoids. Nonetheless, the level of flavonoids in tomatoes is deemed less than optimal, primarily due to the lack of anthocyanins [38]. Therefore, breeding attempts were conducted to develop anthocyanin-enriched tomatoes. Anthocyanin-rich tomatoes developed through breeding programs produce fruits with a dark skin color

(purple or black) and high nutraceutical values, combining the health advantages of anthocyanins with those of other tomato phytochemicals, especially carotenoids [39].

Therefore, in the context of secondary metabolites, the preferable cultivar choices would be the black-colored 'Black Q' for chlorophylls, β-carotene, total flavonoids and anthocyanins; the red-colored 'Snacktom' for lycopene; the orange-colored 'Gold Chance' for total phenolics; and the green-colored 'Jocheong' for chlorophylls and vitamin C. This suggests the benefit of choosing cultivars tailored to specific target functional compounds and distributing a blend of differently colored cherry tomatoes through packaging for cumulative health benefits.

### *3.4. Antioxidant Activities*

Free radicals have been indicated as a natural by-product of aerobic metabolism, where approximately 2–3% of the cell's oxygen consumption undergoes conversion into these radicals, contributing to the processes of aging and age-related diseases if their amount becomes excessive over an extended period [15,40]. A high intake of fruits and vegetables exhibiting antioxidant activity is receiving increased attention due to their potential in mitigating the detrimental effects of free radicals [15]. The consumption of fresh and processed products made of tomatoes has been reported as a valuable source of antioxidants, contributing significantly to reducing the risk of various cancers [41,42] and plasma lipid peroxidation [15,43]. Therefore, it has become essential to evaluate the nutritional quality of tomatoes by assessing their antioxidant activity. In this study, variations in the antioxidant activity were observed among the five cultivars of cherry tomatoes using four different assays. In all four assays, 'Gold Chance' exhibited the highest antioxidant activity, with 'Snacktom' ranking second (Table 3). As indicated in Figure 3, 'Gold Chance' demonstrated the highest total phenolics, while 'Snacktom' showed the highest levels in lycopene and β-carotene. The findings of our study valorize the direct correlations of secondary bioactive metabolites with the antioxidant activity of fresh cherry tomatoes.

**Table 3.** Antioxidant activities of cherry tomato cultivars at harvest.

| Cultivars | DPPH (%) | ABTS (%) | FRAP (Absorbance) | RP (Absorbance) |
|---|---|---|---|---|
| Jocheong | 63.69 ± 0.04 c | 14.16 ± 0.68 c | 0.231 ± 0.002 b | 0.222 ± 0.002 c |
| BN Satnolang | 58.55 ± 0.31 d | 13.67 ± 0.67 c | 0.212 ± 0.003 c | 0.221 ± 0.004 c |
| Gold chance | 69.72 ± 0.33 a | 16.32 ± 0.09 a | 0.250 ± 0.000 a | 0.249 ± 0.002 a |
| Black Q | 56.63 ± 0.33 e | 13.27 ± 0.71 c | 0.202 ± 0.002 d | 0.221 ± 0.002 c |
| Snacktom | 69.02 ± 0.31 b | 15.22 ± 0.36 b | 0.253 ± 0.003 a | 0.243 ± 0.001 b |

DPPH, FRAP, ABTS, and RP stand for α-diphenyl-β-picrylhydrazyl, ferric-reducing antioxidant power, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), and reducing power assays, respectively. The results are shown as the mean ± SD ($n$ = 3), with differing letters in the same column indicating a significant statistical difference at $p < 0.05$. All the data were detected at 10 mg $mL^{-1}$ methanol extracts of freeze-dried cherry tomatoes.

### *3.5. Principal Component and Correlation Analysis*

Evaluating the functional properties of tomatoes involves examining their antioxidant activity and determining the key contributors, such as carotenoids, ascorbic acid, phenolics, flavonoids, and anthocyanins. In this study, the different colored cherry tomato cultivars tested were grown under the same agronomic practices based on the hypothesis that the color variation would result in significant differences in their metabolite profiles. In addition, all the parameters were standardized and collected under the same condition for all the cultivars. Consequently, the trends observed in the principal component analysis (PCA) and correlation analysis highlight the parameters that primarily cause variations among the tested cultivars and the relationship between each parameter, respectively. The PCA elucidates the distinctions among treatments and the prominent factors influencing the spatial distribution and correlation of observed parameters. The data collected for cherry tomato cultivars were subjected to the PCA, and the resulting PCA is depicted in Figure 4, illustrating the separation of cultivars based on the observed parameters. The results showed that factor 1 (F1) and factor 2 (F2) together accounted for about 80.42% of the total variance. F1 held the highest variation, elucidating 53.72% of the overall variance, while F2

contributed an additional 26.69% to the total variances. F1 and F2 demonstrated the clear separation of the cherry tomato cultivars, indicating the differences in nutritional quality (metabolites and antioxidant activities) among the five cherry tomato cultivars (Figure 4). Among the five cultivars analyzed, 'Black Q' and 'Snacktom' showed a high level of lycopene and β-carotene, 'Gold Chance' a high level of total phenolics, 'Black Q' a high level of total flavonoids and anthocyanins, and 'Jocheong' a high level of vitamin C. In addition, the heat map in Figure 5 illustrates the comprehensive distinctions in the physiochemical characteristics, free amino acids, secondary metabolites, and antioxidant activities among the tested cherry tomato cultivars. The correlations among the collected parameters are also shown in Figure 6. Significant positive correlation coefficients were observed between the secondary metabolites (lycopene, β-carotene, vitamin C, total phenolics, flavonoids, and anthocyanins) and the antioxidant activities measured with the four assays (DPPH, FRAP, ABTS, RP) (Figure 6). These results prove the contribution of secondary metabolites to the antioxidant capacity of cherry tomatoes. Moreover, the contribution of the total phenolics to the antioxidant activity was clearly indicated in the PCA for 'Gold Chance', 'Snacktom', and 'BN Satnolang'. Our results agree with those of Choi et al. [31], who reported a higher positive correlation of total phenolics with the antioxidant capacity in kiwifruit.

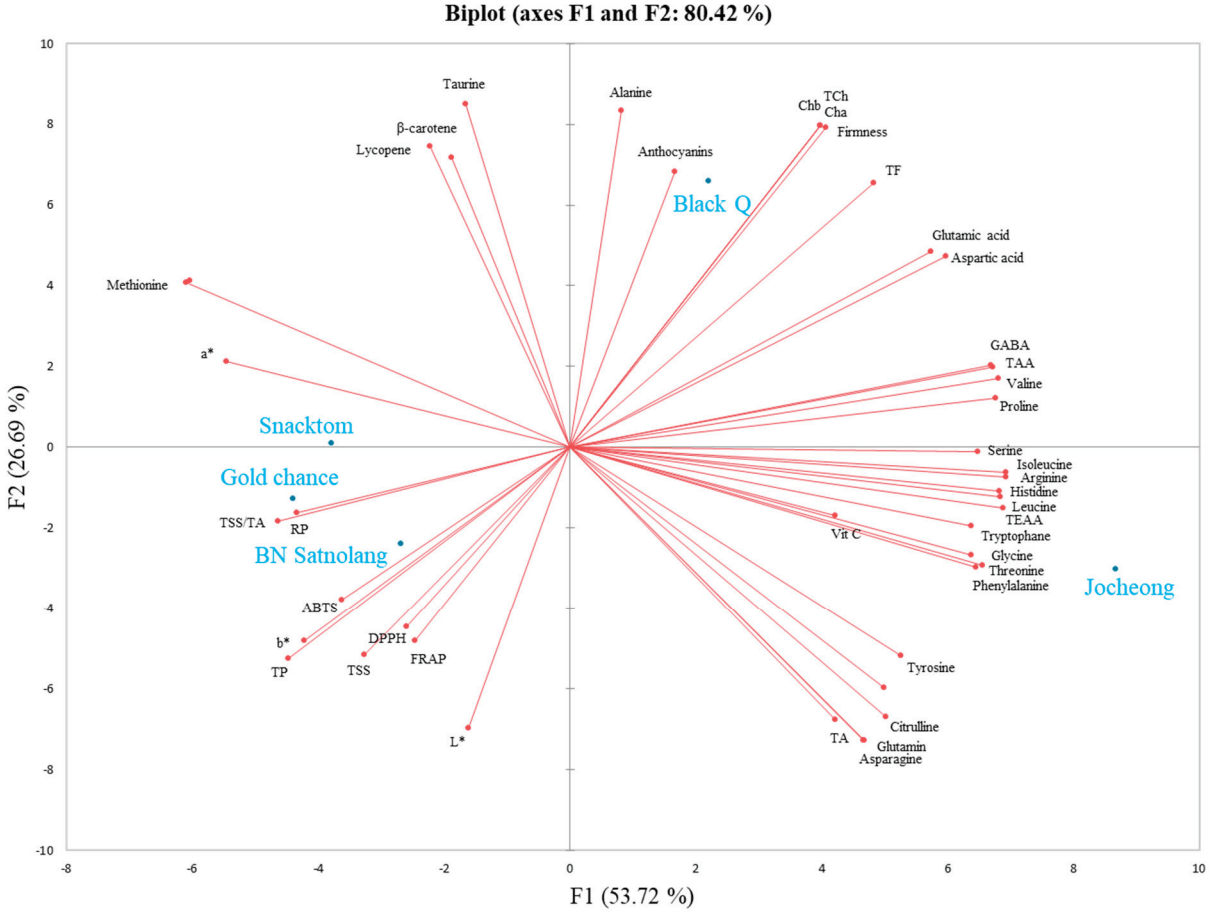


**Figure 4.** Biplot of nutritional quality parameters in five cherry tomato cultivars at harvest. Data normalization was performed using the median combined with autoscaling, and the analysis was conducted using MetaboAnalyst 6.0 software (https://www.metaboanalyst.ca/ (accessed on 20 November 2023)). The parameters include chlorophyll a (Cha), chlorophyll b (Chb), total chlorophyll (TCh), total soluble solids (TSSs), titratable acid (TA), total amino acids (TAAs), total essential amino acids (TEAs), vitamin C (Vit C), total phenolics (TPs), total flavonoids (TFs), Hunter's a* (a*), Hunter's b* (b*), Hunter's L* (L*), α-diphenyl-β-picrylhydrazyl (DPPH), ferric-reducing antioxidant power (FRAP), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and reducing power (RP).

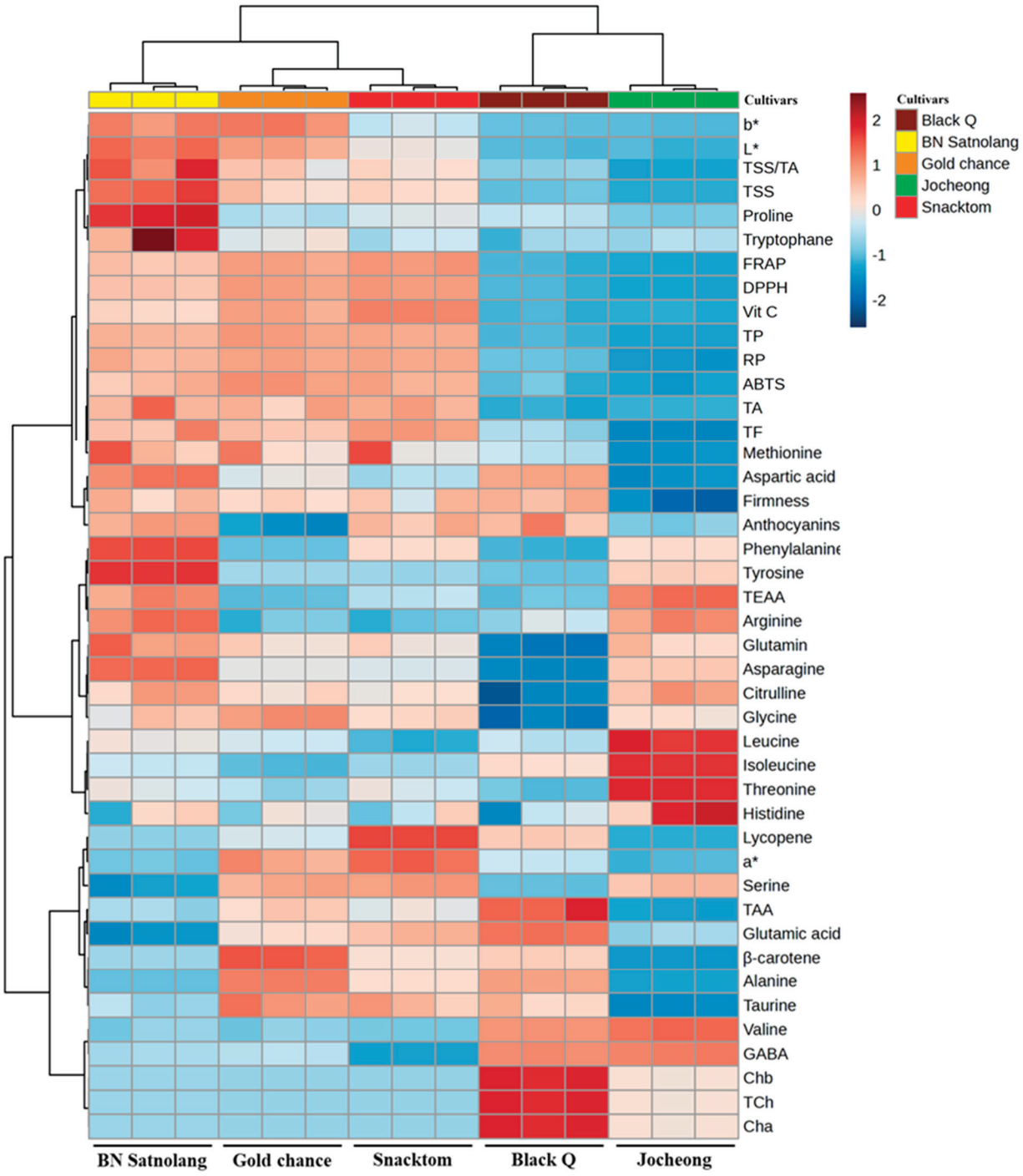


**Figure 5.** Heat map of the collected parameters for the 'Jocheong', 'BN Satnolang', 'Gold chance', 'Black Q', and 'Snacktom' cherry tomato cultivars at harvest. Data normalization was performed using the median combined with autoscaling, and the analysis was conducted using MetaboAnalyst 6.0 software (https://www.metaboanalyst.ca/ (accessed on 20 November 2023)). The parameters include chlorophyll a (Cha), chlorophyll b (Chb), total chlorophyll (TCh), total soluble solids (TSSs), titratable acid (TA), total amino acids (TAAs), total essential amino acids (TEAs), vitamin C (Vit C), total phenolics (TPs), total flavonoids (TFs), Hunter's a* (a*), Hunter's b* (b*), Hunter's L* (L*), α-diphenyl-β-picrylhydrazyl (DPPH), ferric-reducing antioxidant power (FRAP), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and reducing power (RP).

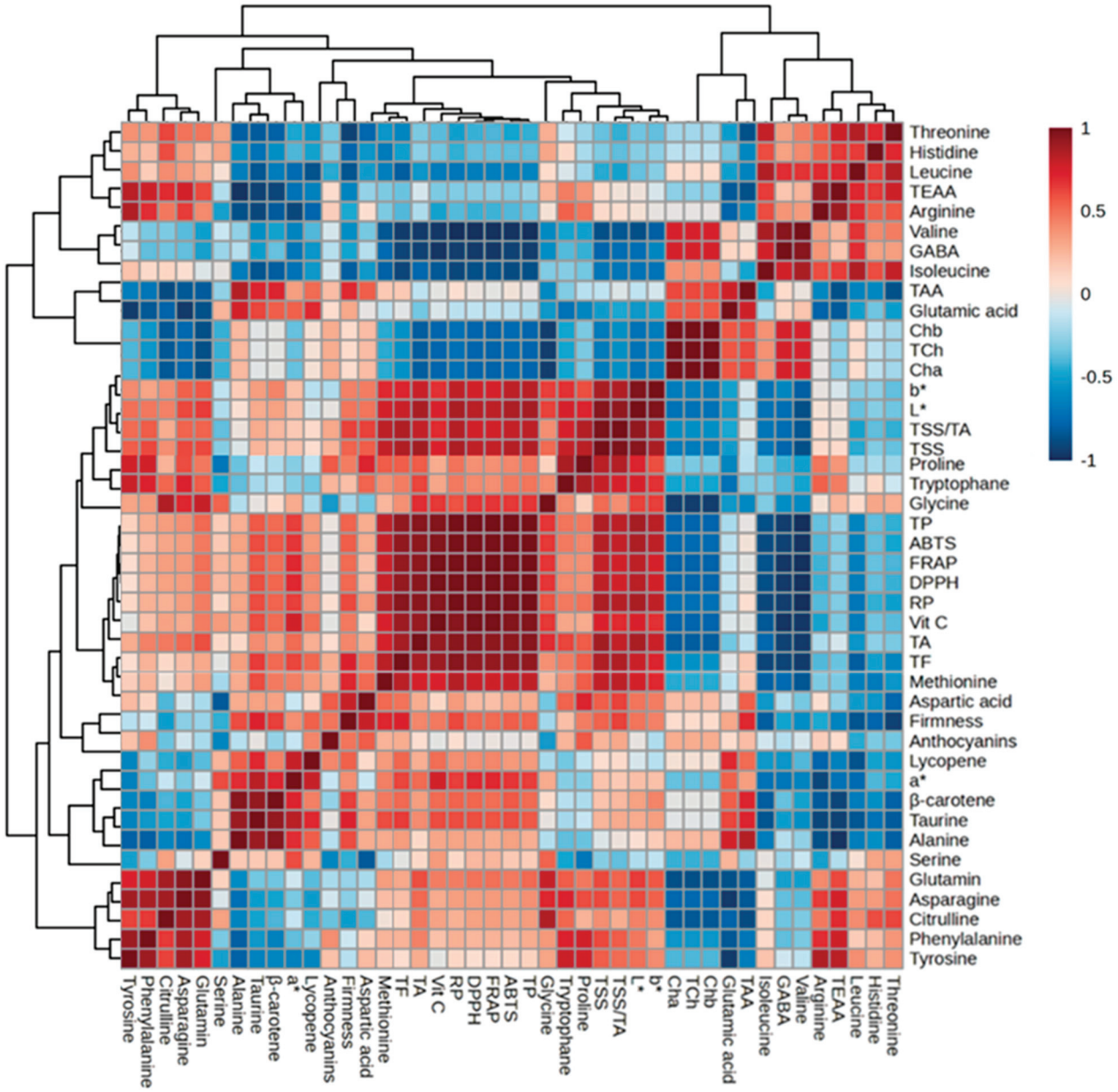


**Figure 6.** Correlation heat map of nutritional quality parameters in five cherry tomato cultivars at harvest. Data normalization was performed using the median combined with autoscaling, and the analysis was conducted using MetaboAnalyst 6.0 software (https://www.metaboanalyst.ca/ (accessed on 20 November 2023)). The parameters include chlorophyll a (Cha), chlorophyll b (Chb), total chlorophyll (TCh), total soluble solids (TSSs), titratable acid (TA), total amino acids (TAAs), total essential amino acids (TEAs), vitamin C (Vit C), total phenolics (TPs), total flavonoids (TFs), Hunter's a* (a*), Hunter's b* (b*), Hunter's L* (L*), α-diphenyl-β-picrylhydrazyl (DPPH), ferric-reducing antioxidant power (FRAP), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and reducing power (RP).

## 4. Conclusions

In this study, five different colored cherry tomato cultivars, namely 'Jocheong', 'BN Satnolang', 'Gold Chance', 'Black Q', and 'Snacktom', were assessed for their firmness, taste characteristics, and nutritional content. The cultivars exhibited sufficient firmness to withstand impacts during harvesting and postharvest operations, making them well-suited for distribution. The BAR ranged from 11.33 in 'BN Satnolang' to 6.84 in 'Jocheong', indicating the differences in the taste characteristics of the tested cherry tomato cultivars. A

higher amount of a given metabolite means that a smaller quantity of tomatoes is needed to meet the daily requirements, which in turn reduces the production, distribution, and consumption costs. Considering the amount of TAA, EAA, and GABA, the preferred choices were the green-colored 'Jocheong' and the black-colored 'Black Q'. For the secondary metabolites, 'Black Q' excelled in β-carotene, total flavonoids, and anthocyanins, while the red-colored 'Snacktom' stood out in lycopene. The orange-colored 'Gold Chance' led in total phenolics, and the green-colored 'Jocheong' topped in vitamin C. The antioxidant activity varied among the cultivars, with 'Gold Chance' consistently exhibiting the highest activity across the four assays, followed by 'Snacktom'. 'Gold Chance' also demonstrated the highest total phenolics, while 'Snacktom' had the highest levels of lycopene and β-carotene, implying the direct correlation of the secondary bioactive metabolites with the antioxidant activity of fresh cherry tomatoes. In addition, this study recommends selecting cultivars based on the target functional compounds and promoting a diverse mix of colored cherry tomatoes in packaging to align with consumers' requirements. This approach encourages the consumption of various cultivars, enhancing the cumulative or synergistic effects of secondary metabolites and offering potential health benefits. Future research could explore the findings of sensory acceptance analysis, the impact of various preharvest and postharvest treatments, and the effects of storage on the quality of different colored cherry tomatoes.

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# Biochemical Characterization of New Sweet Orange Mutants Rich in Lycopene and β-Carotene Antioxidants

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**Abstract:** Carotenoid-rich foods such as citrus fruits have a wide range of functions in human health. They primarily exert antioxidant effects, but individual carotenoids may also act through other health-promoting mechanisms such as β-carotene as pro-vitamin A. Here, we show that red-fleshed sweet oranges grown in tropical climates are 4–9 times richer in carotenoids than their orange-fleshed counterparts, regardless of their maturation stage. The most significant difference observed between both varieties was the presence of lycopene at moderate concentrations (around 8 μg/g FW) in the mature pulp of the red varieties, which was absent in the blond ones. This is because the red-fleshed sweet oranges grown in tropical climates with high temperatures increase lycopene and β-carotene concentrations in their pulp during fruit maturation. Due to lycopene accumulation, red orange juice offers a promising addition to popular blond-orange, with the new varieties Carrancas and Pinhal being perfectly suitable for blending to enhance juice colour. Sao Paulo, one of the world's leading citrus orange juice producers, as well as other tropical citrus regions could benefit from cultivating using such lycopene-rich cultivars and industrially.

**Keywords:** carotenoids; lycopene; β-carotene; red-fleshed oranges; antioxidants; phytonutrients

## 1. Introduction

Carotenoids are a large family of isoprenoid pigments that have essential functions as components of the light-harvesting system and in protecting plant cells against oxidative processes [1]. These pigments are also components of the human diet, with important antioxidant activity and protective effects against carcinogenesis, cardiovascular diseases and degenerative progression [2–6]. Moreover, carotenoids with at least one unsubstituted β-ionone ring like β-carotene or β-cryptoxanthin are the precursors of vitamin A [7–9].

Carotenoids impart attractive colours to many fruits and vegetables, including citrus fruits [10,11]. Citrus fruit and juice are widely consumed, with a total production of 158.5 million tons of fruit per year worldwide [12], positioning them as important sources of carotenoids for the human diet [13,14]. The *Citrus* genus displays a complex carotenoid profile, with more than 110 different carotenes and xanthophylls reported, provide external and internal fruit coloration of the main citrus species and hybrids [15–17]. The carotenoid content and composition impact both the commercial and nutritional quality, these are the major determinants of consumer acceptance, since the external colour of citrus fruit and juice constitutes the consumer's first perception of the product and thus represents a primary quality attribute. Differences in the carotenoid profiles in mature citrus fruits, where content and composition vary depending on the cultivar [17,18], are responsible for the wide range of colorations that can be found among diverse citrus cultivars, from the white of grapefruits, pale yellow of some lemons, the intense orange of certain sweet oranges and mandarins to the red of specific oranges and grapefruits [16]. For example, although the β,β-xanthophyll violaxanthin predominates in mandarin and sweet orange

fruits, a higher β-cryptoxanthin content can be found in the former, providing their distinctive orange-reddish colour [16]. On the other hand, lemons are characterised by an accumulation of linear colourless carotenes (see the review in [17]).

The carotenoid biosynthesis pathway has been well characterised [17,19–22]. Carotenoids are derived in light-grown plants from the precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) from the methyl-D-erythritol 4-phosphate (MEP) pathway, which occurs in the plastids [23,24]. The first step is catalysed by *DXP synthase* (*DXS*) to produce deoxyxylulose 5-phosphate (DXP), which is reduced by *DXP reductoisomerase* (*DXR*) and yields MEP (Figure 1). MEP is converted into the precursors IPP and DAMPP by the sequential action of different enzymes. The condensation of these precursors generates geranylgeranyl pyrophosphate (GGPP). Through the action of *phytoene synthase* (*PSY*), two GGPPs are converted into the colourless carotene named phytoene, which is desaturated to phytofluene by *phytoene desaturase* (PDS). Phytofluene is desaturated by *ζ-carotene desaturase* (ZDS), generating the red-coloured carotene lycopene, which is cyclised by *lycopene ε-cyclase* (*εLCY*) and *lycopene β-cyclase* (*βLCY*) to produce α-carotene or by the enzyme *βLCY* to generate β-carotene. In orange fruits, the accumulation of carotenoids during the maturation process is coordinated by the induction of *βLCY2* expression, concomitantly with a decrease in *εLCY* expression, favouring a shift to β,β-xanthophylls production. The *β-carotene hydroxylase* (*βCHX*) enzyme acts on β-carotene, yielding zeaxanthin via β-cryptoxanthin, followed by the epoxidation reaction of zeaxanthin via *zeaxanthin epoxidase* (*ZEP*) to generate violaxanthin, the most abundant carotenoid in sweet orange juices.

In rare case, citrus fruits accumulate lycopene. This has been reported and characterised in only a few species, such as grapefruits (*Citrus paradisi* Marcf.) and pummelo (*Citrus grandis* Osbeck) [25]. Lycopene is a potent antioxidant carotene which confers major nutritional and nutraceutical value [26,27]. Few sweet oranges have also been reported to be capable of accumulating lycopene, such as Shara orange, a sport of Shamouti [28], the red orange Hong Anliu, a bud mutation of Anliu sweet orange [29] and Cara cara, which originated in Venezuela as a bud mutation from a Navel orange [25,30]. In addition to the nutritional value of red oranges, lycopene accumulation provides an atypical internal colouration that varies from the pink of the Shara orange to the bright red pulp of Cara cara oranges, providing important commercial value to these varieties. Because of that, the main citrus industries have been focused on searching for and selecting sweet orange types containing lycopene pigmentation in the recent years, including Puka Valencia from Argentina or Ruby Valencia from South Africa [31]. Sao Paulo (Brazil), which contributes 70% of worldwide juice production, has incorporated Puka and Cara cara red-oranges as commercial cultivars of interest [32]. More recently, the red orange Mombuca has been also selected, this is a Brazilian red variety that originated as a spontaneous sweet orange mutant [33]. The characterisation of new red-orange cultivars could offer a strategy for the citrus industry to extend the range of colours and increase the nutritional properties of orange juice. Thus, the aim of this work was to characterise the fruit quality and biochemical features of two new red-fleshed sweet orange spontaneous mutants, Carrancas and Pinhal, both originated and selected in Sao Paulo (Brazil). It is presented here that both new mutants are productive, mid-season varieties, showing high accumulation of lycopene and β-carotene phytonutrient carotenoids, with sufficiently high juice quality to consider them as possible new appealing red-flesh additions to the currently existing and highly successful blond-orange varieties.

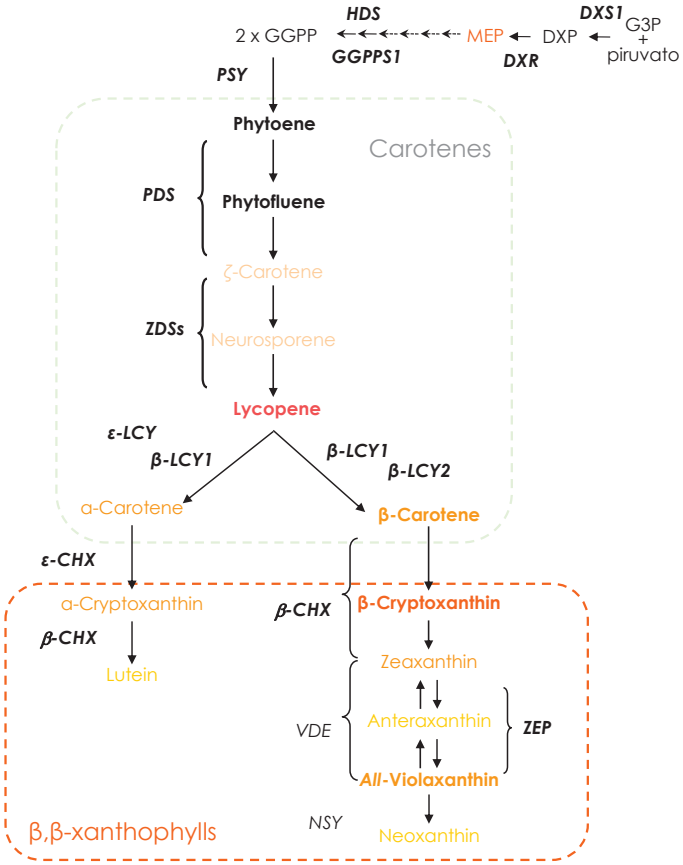


**Figure 1.** Schematic diagram of the biosynthesis pathway of carotenoid in citrus fruit. The carotenoids and genes analysed in this work are bold-lettered. G3P, glyceraldehyde-3-phosphate; DXP, deoxyxylulose 5-phosphate; MEP, methyl-D-erythritol 4-phosphate; GGPP, geranylgeranyl pyrophosphate; DXS, DXP synthase; DXR, DXP reductoisomerase; HDS, HDR, hydroxymethylbutenyl 4-diphosphate reductase; GGPPS, geranylgeranyl pyrophosphate synthase;PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ- carotene desaturase; ε-LCY, lycopene ε-cyclase; β-LCY1, lycopene β-cyclase 1; β-LCY2, lycopene β-cyclase 2; β-CHX, β-carotene hydroxylase; εCHX, ε-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase. Metabolites are coloured according to their colours, whereas black indicates no colour. Dotted rectangles separate different groups of carotenoids.

## 2. Materials and Methods

### *2.1. Plant Material*

In this study, sweet orange (*C. sinensis* L. Osbeck) fruits from cultivars Hamlin (Ha), Pera (Pe) and Valencia (Va), were used as early, mid-season and late-season blond oranges, respectively, Cara cara (Cc), Carrancas (Ca), Mombuca (Mo), Pinhal (Ph) and Puka (Pk) were used as early (Cc), mid-season (Ca, Mo, Ph) and late (Pk) red-fleshed mutants, at different developmental stages: immature-green (IG), mature-green (MG), breaker (BR) and full-coloured (FC). They were harvested at random from adult (4-year-old) trees grown under standard conditions in an experimental area in a commercial orchard (Fazenda Guacho) in Sta. Cruz de Rio Pardo, Sao Paulo State, Brazil (22°53′56″ S–49°37′58″ O) during two consecutive seasons (season 1: 2021/22–season 2: 2022/23). The climatological conditions of the area were monitored (Figure S1). Fruits were uniform in size and colour, and free of damage or defects. Pulp was separated with a scalpel, immediately frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C until analysis. For all analyses, 3 replicate samples of 5 fruits each per developmental stage were used.

*2.2. Analysis of Fruit Quality*

Fruit quality assessment was carried out for every cultivar over two seasons. Measurements of quality parameters were achieved based on fruit samples for every citrus tree from the orchard. A total of 40 fruits (4 samples of 10 fruits each) were harvested annually when the fruit was fully mature. The following fruit quality parameters were measured and averaged for each sample: fruit weight (W), fruit volume (V), fruit diameter (D), fruit height (H), cortex fruit thickness (C), peel fruit thickness (P), number of segments (Sg), number of seeds (S), juice content (JC), total soluble solids (TSS), titratable acidity (TA) and maturity index (MI). The parameters were measured as described in Pons et al. [34]. All measures are presented as the mean ± standard error of each sample.

*2.3. Carotenoid Extraction and UPLC Analysis of Individual Carotenoids*

Carotenoids were extracted as is described in Carmona et al. [35]. The carotenoid composition of each sample was analysed through UPLC with a Nexera X2 Shimadzu liquid chromatography system equipped with a LC-30AD pump and a SPD-M20A photodiode array detector, and LabSolution software (version 5.57 SP1). An Acquity BEH C18 carotenoid column (100 mm × 2.1 mm, 1.8 µm) coupled to a C18 guard column (20 mm × 2.1 mm) (Waters, Milford, MA, USA) was used. The samples were prepared for UPLC by dissolving the dried carotenoid extracts in $CHCl_3$:MeOH:acetone (3:2:1, *v*:*v*:*v*). For carotenoid separation, a binary gradient elution was adapted for red oranges [36] from the ternary described by Alquezar et al. [37] for red-oranges by using the Gradient Method Calculator (Thermo Scientific, Waltham, MA, USA). Carotenoids were identified based on their retention time, absorption and fine spectra [38–41]. The carotenoid peaks were integrated at their individual maxima wavelength and their contents were calculated using calibration curves of β-carotene (Sigma, St. Louis, MO, USA) for α- and β-carotene; β-cryptoxanthin (Extrasynthese, Genay, France) for β-cryptoxanthin; zeaxanthin (Extrasynthese) for zeaxanthin; lutein (Sigma) for violaxanthin isomers, mutatoxanthin and antheraxathin; lycopene (Sigma) for lycopene and phytoene (Sigma) for phytoene and phytofluene. The total carotenoids were calculated as the sum of all quantified individual ones.

*2.4. Total RNA Isolation and Quantitative RT-PCR Analysis*

The total RNA extraction, DNase treatment, cDNA synthesis, quantitative real-time PCR procedure and primers used for the analysed genes followed Carmona et al. [42]. Th primer sequences used for each gene used are detailed in Table S1 [35,37,43–45]. To demonstrate the expression stability of the reference genes *glyceraldehyde-3-phosphate dehydrogenase C2* (*GAPC2*), *ubiquitin-conjugating enzyme 21* (*UBC21*) and *ubiquitin-protein ligase 7* (*UPL7*) under our experimental conditions, the algorithm geNorm was used (https://genorm.cmgg.be/, accessed on 1 February 2021) [46]. The relative expression of genes of interest (GOI) in this study was determined throughout the maturation process as described by Carmona et al., [42]. Values are presented as the mean of at least three independent analyses ± SD.

## 3. Results

*3.1. Fruit Appearance at Different Developmental Stages and Fruit Quality Parameters of New Red-Fleshed Sweet Orange Mutants*

The changes in external and internal appearance during the maturation period for each variety are shown in Figure 2. Peel and pulp colour progressively turned from green to red or orange along the maturation process, with a remarkable treddish coloration occurring in the pulp of red-fleshed cultivars. The differences in pulp coloration between the blond cultivars Hamlim (Ha), Pera (Pe) and Valencia (Va), and the red-fleshed ones Cara cara (Ca), Mombuca (Mo), Carrancas (Ca), Pinhal (Ph) and Puka (Pk) were detected from the initial stages of fruit development. The pulp of immature-green (IG) fruit from blond cultivars displayed a yellow colour while fruit belonging to red mutants already

presented a distinctive reddish-pink colouration at that developmental stage (Figure 2B). Then, during the maturation period, the internal colouration of Ha, Pe and Va fruit turned a characteristic orange colour (Figure 2). The red-fleshed cultivars remained deep red throughout their development, displaying orange shades in advanced mature stages. The intensity of the red colour at full maturity and their progress during maturation were comparable for all red mutants, with small variations depending on the season.

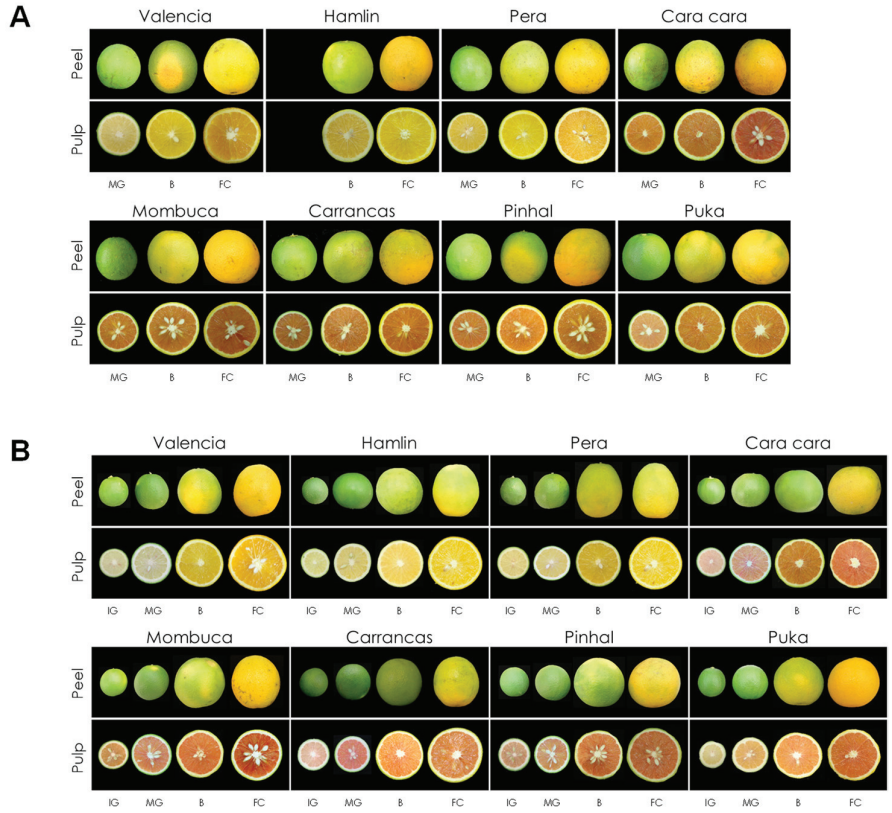


**Figure 2.** External and internal appearance of Valencia, Hamlin, Pera, Cara cara, Mombuca, Carrancas, Pinhal and Puka fruits (*C. sinensis* L. Osbeck) in two different seasons: (**A**) Season 1 (2021/2022) and (**B**) Season 2 (2022/2023). The physiological fruit stages are indicated: IG (Immature-green), MG (Mature-green), B (breaker) and FC (Full-color).

The fruit quality parameters of each sweet orange cultivar at the mature stage are represented in Table 1 The highest total soluble solids (TSS) were found in blond Pe and Va cultivars (12.6° ± 0.2° and 11.8° ± 0.3° Brix, respectively), while Mo and Ca presented the lowest TSS (8.5° ± 0.2° and 8.0° ± 0.4° Brix, respectively) in season 2. In general, TSS values were lower in season 1, although there remained differences among varieties. The titratable acidity (TA) was similar for most cultivars in both seasons, with Ca, Ph and Mo having the lowest acidity (0.4° ± 0.3°, 0.5° ± 0.0° and 0.5° ± 0.2°, respectively in 2022/2023). The Maturity index (MI) expressed as the ratio of TSS/TA and measured for each variety and season showed that blond varieties tended to display lower values (ranging from 14.5° ± 0.2° to 17.9° ± 0.5°, in season 2) due in part to increased acidity, while intermediate concentrations were found in the red-fleshed mutants Mo and Pk, which presented MIs of 17.8° ± 0.4° and 19.7° ± 0.4° in season 2. The red-fleshed Ca and Ph showed the highest MI ratios, which were around 19–20 for both seasons (Table 1).

**Table 1.** Quality parameters from fruits of Valencia (Va), Hamlin (Ha), Pera (Pe), Cara cara (Cc), Mombuca (Mo), Carrancas (Ca), Pinhal (Ph) and Puka (Pk) fruits (*C. sinensis* L. Osbeck) in seasons 1 (2021/2022, up) and 2 (2022/2023, down). The data are means ± SD of at least three independent measurements.

| Variety | Total Soluble Solids (°Brix) (TSS) | Titratable Acidity (g/L) (TA) | Maturity Index (MI) | Juice Content Per Orange (JC) (mL) | Fruit Weight (W) (g) | Percentage Juice Content/Fruit Weight (%) | Fruit Diameter (mm) | Fruit Height (mm) | Cortex (mm) | Peel (mm) | Number of Segments | Number of Seed |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Valencia | 11.7 ± 0.0 | 0.7 ± 0.3 | 13.5 ± 0.3 | 85.0 ± 11.3 | 219.3 ± 20.2 | 38.9 ± 4.6 | 73.7 ± 2.0 | 76.5 ± 3.1 | 4.6 ± 0.2 | 1.7 ± 0.1 | 10.9 ± 0.3 | 56.3 ± 7.4 |
| Hamlim | 10.8 ± 0.3 | 0.8 ± 0.0 | 14.1 ± 0.5 | 61.3 ± 5.3 | 168.8 ± 5.1 | 36.3 ± 3.2 | 68.9 ± 0.9 | 69.4 ± 0.6 | 4.4 ± 0.4 | 1.6 ± 0.1 | 10.5 ± 0.2 | 51.5 ± 6.7 |
| Pera | 12.6 ± 0.1 | 0.7 ± 0.2 | 12.8 ± 0.3 | 88.5 ± 1.5 | 207.7 ± 5.8 | 42.7 ± 1.8 | 71.1 ± 0.7 | 76.1 ± 0.7 | 5.1 ± 0.1 | 1.6 ± 0.1 | 10.6 ± 0.2 | 61.5 ± 6.4 |
| Cara Cara | 9.5 ± 0.0 | 0.5 ± 0.2 | 17.9 ± 0.7 | 38.1 ± 4.5 | 277.0 ± 26.8 | 14.4 ± 2.8 | 86.1 ± 3.8 | 88.9 ± 1.6 | 7.5 ± 0.8 | 2.5 ± 0.4 | 10.7 ± 0.3 | 0.3 ± 0.3 |
| Mombuca | 8.3 ± 0.0 | 0.5 ± 0.0 | 18.0 ± 0.6 | 92.0 ± 5.3 | 188.9 ± 8.1 | 48.6 ± 0.9 | 71.0 ± 1.2 | 72.0 ± 1.0 | 4.6 ± 0.2 | 1.7 ± 0.1 | 10.6 ± 0.2 | 144.3 ± 3.7 |
| Carrancas | 7.8 ± 0.0 | 0.4 ± 0.3 | 20.1 ± 0.7 | 55.9 ± 1.6 | 144.6 ± 7.0 | 38.7 ± 1.8 | 64.9 ± 1.3 | 65.2 ± 1.6 | 3.2 ± 0.2 | 1.8 ± 0.1 | 10.2 ± 0.4 | 69.0 ± 5.9 |
| Pinhal | 8.8 ± 0.3 | 0.5 ± 0.2 | 18.9 ± 0.3 | 54.3 ± 3.1 | 157.2 ± 2.0 | 34.5 ± 1.8 | 65.5 ± 1.0 | 68.1 ± 1.6 | 4.9 ± 0.2 | 2.2 ± 0.2 | 10.2 ± 0.2 | 137.5 ± 3.8 |
| Puka | 9.6 ± 0.2 | 0.5 ± 0.1 | 18.6 ± 0.3 | 109.1 ± 4.4 | 209.2 ± 5.4 | 52.2 ± 2.9 | 73.3 ± 0.7 | 72.9 ± 0.9 | 4.7 ± 0.1 | 1.6 ± 0.2 | 10.4 ± 0.3 | 72.5 ± 9.9 |
| Valencia | 11.8 ± 0.3 | 0.7 ± 0.2 | 16.1 ± 0.2 | 92.3 ± 1.9 | 190.6 ± 4.3 | 48.4 ± 1.1 | 70.4 ± 0.8 | 72.5 ± 0.7 | 6.1 ± 1.3 | 1.1 ± 0.1 | 10.8 ± 0.2 | 45.0 ± 5.3 |
| Hamlim | 9.5 ± 0.1 | 0.7 ± 0.2 | 14.5 ± 0.2 | 80.0 ± 0.6 | 152.7 ± 2.2 | 52.4 ± 0.8 | 66.3 ± 0.7 | 69.3 ± 1.1 | 4.4 ± 0.2 | 2.0 ± 0.0 | 10.2 ± 0.2 | 49.3 ± 7.4 |
| Pera | 12.6 ± 0.2 | 0.7 ± 0.3 | 17.9 ± 0.5 | 93.8 ± 2.1 | 181.7 ± 1.2 | 51.6 ± 0.9 | 68.2 ± 0.1 | 73.9 ± 0.7 | 4.7 ± 0.2 | 1.6 ± 0.2 | 9.7 ± 0.2 | 43.5 ± 6.5 |
| Cara Cara | 9.9 ± 0.1 | 0.5 ± 0.1 | 18.6 ± 0.4 | 53.8 ± 3.1 | 225.7 ± 5.6 | 27.1 ± 0.7 | 75.0 ± 0.4 | 74.9 ± 0.8 | 6.2 ± 0.3 | 1.4 ± 0.1 | 10.4 ± 0.2 | 7.0 ± 0.4 |
| Mombuca | 8.5 ± 0.2 | 0.5 ± 0.2 | 17.8 ± 0.4 | 72.3 ± 1.7 | 141.2 ± 1.5 | 51.2 ± 0.8 | 64.8 ± 0.3 | 66.8 ± 0.3 | 5.1 ± 0.1 | 2.2 ± 0.1 | 10.4 ± 0.1 | 147.5 ± 5.5 |
| Carrancas | 8.0 ± 0.4 | 0.4 ± 0.3 | 20.4 ± 0.8 | 74.3 ± 6.7 | 143.9 ± 2.3 | 51.7 ± 5.2 | 64.3 ± 0.7 | 66.0 ± 0.6 | 4.6 ± 0.2 | 1.6 ± 0.1 | 10.4 ± 0.2 | 89.0 ± 11.7 |
| Pinhal | 9.1 ± 0.0 | 0.5 ± 0.0 | 19.7 ± 0.1 | 62.8 ± 2.8 | 131.0 ± 3.0 | 47.9 ± 1.3 | 62.5 ± 0.7 | 64.5 ± 0.6 | 4.8 ± 0.2 | 2.0 ± 0.1 | 10.4 ± 0.2 | 116.8 ± 7.8 |
| Puka | 9.6 ± 0.3 | 0.6 ± 0.2 | 17.1 ± 0.2 | 66.8 ± 5.6 | 160.6 ± 6.6 | 41.9 ± 4.9 | 74.7 ± 0.6 | 65.7 ± 0.8 | 4.4 ± 0.1 | 1.9 ± 0.1 | 10.6 ± 0.1 | 35.5 ± 4.1 |

### *3.2. Carotenoid Composition of Red-Fleshed Sweet Orange Pulp during Maturation*

The total content and composition of carotenoids was analysed in the pulp of the three blond oranges and five red-fleshed mutants at four developmental stages: immature-green (IM), mature-green (MG), breaker (Br) and full-coloured (FC) (Figures 3, 4 and S2). Seven main carotenoids were measured through UPLC analysis, accounting for more than 90% of the total carotenoids in all samples analysed. The total carotenoid content was calculated as the sum of all carotenoids identified (Figure 3). The changes in total carotenoid content were markedly different in the blond oranges compared to red-fleshed mutants. In the blond cultivars, the total carotenoid content in the pulp was very little until the Br stage, and then increased to reach its maximum values of 4.8–8.9 µg/g FW and 2.0–8.6 µg/g FW in seasons 2 and 1, respectively. The carotenoids accumulation in the pulp of red-fleshed mutants was high from the early stages of development (8.3–15.1 µg/g FW and 4.6–23.9 µg/g FW in seasons 2 and 1, respectively) and progressively increased throughout the whole maturation process (23.0–38.7 µg/g FW and 25.5–40.8 µg/g FW in seasons 2 and 1, respectively). The pulp of the red-fleshed mutants at early stages presented about 10-times higher concentrations of total carotenoids than that of the blond cultivars. From the Br to FC stages, the total carotenoid content in the pulp of the red mutants was 4 to 9 times higher than that in the blond oranges.

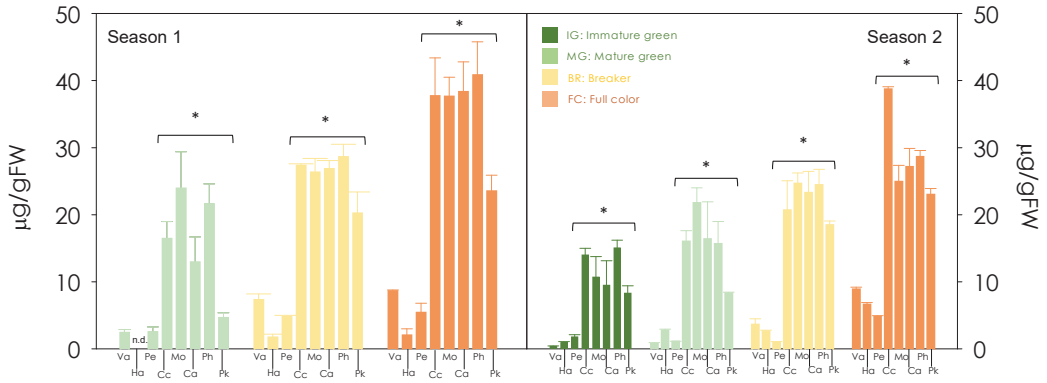


**Figure 3.** Changes in carotenoid contents in the pulp during development and maturation of Valencia (Va), Hamlin (Ha), Pera (Pe), Cara cara (Cc), Mombuca (Mo), Carrancas (Ca), Pinhal (Ph) and Puka (Pk) fruits (*C. sinensis* L. Osbeck) in two different seasons. The 10 samples analysed correspond to the developmental and maturation stages indicated in Figure 2. The physiological fruit stages are indicated: IG (immature-green), MG (mature-green), B (breaker) and FC (full-colour). The data are means ± SD of at least three independent measurements. Statistical analyses were performed using analysis of variance (ANOVA) and an asterisk above the bars indicates significantly different values at $p \leq 0.01$ (n.d.: non detected).

The evolution of carotenoid content and composition in the pulp of blond oranges was markedly different to that of red-fleshed mutants, with differences obvious from the IG stage, well before the beginning of the maturing process (Figure 4 and S2). During the maturation of blond oranges, the pulp accumulated almost exclusively xanthophylls, with *all*-violaxanthin (as the sum of *E*-violaxanthin and 9-*Z*-violaxanthin) as the major carotenoid, which accounted for more than 50% of the total carotenoids in mature fruit. On the contrary, linear carotenoids were predominant carotenoids in the pulp of all the red-fleshed mutants, accounting for more than 70% of total carotenoids in all stages analysed. Phytoene concentration augmented gradually as fruit matured, containing around 3–17 fold (0.8–9.8 fold) more than the pulp of the blond cultivars in season 2. A similar pattern was noticed for the other linear carotene phytofluene, whose content increased 1–9 fold (2.1–8.3 fold) in season 2 during maturation in red oranges. This carotenoid was not detected in blond fruits. For both compounds, Pk was the red-fleshed mutant with the least accumulation. As expected, no lycopene was detected in Ha, Pe and Va throughout all maturation stages and β-carotene only was detected in traces in mature stages. In

the case of red-fleshed mutants, both bioactive carotenes were accumulated in the pulp from the early stages on. The maximum lycopene content was reached in mature fruit in all red-fleshed mutants, with Pk being the cultivar with the lowest content of these pigments in both seasons, 1.5 ± 0.5 and 2.2 ± 0.4 μg/g FW in season 2 and 1, respectively. Instead, the other red-fleshed mutants accumulated higher contents of lycopene, reaching up to 7.2 ± 1.5 μg/g FW or 8.3 ± 1.0 μg/g FW as was the case of the Cc and Ca varieties (Figure 4). There was a higher accumulation of β-carotene in red-fleshed mutants (between 0.6–2.9± 0.1 μg/g FW and 0.5–1.7 ± 0.2 μg/g FW for season 1 and 2, respectively) than blond cultivars (between 0.1–0.4 ± 0.1 μg/g FW and 0.2–0.1 ± 0.2 μg/g FW for season 1 and 2, respectively). In any case, the mutant Ca accumulated the highest quantity of β-carotene in both seasons (Figure 4). Related to carotenoids of the ε,β-branch, only traces of α-carotene and α-cryptoxanthin were detected in all the varieties during the maturation period, while lutein accumulated during the maturation process, with the exception of Va in which it was only was detected in the early stages (Figures 4 and S2). The accumulation of β,β-xanthophylls (as the sum of β-cryptoxanthin, mutatoxanthin, anteraxanthin, *E*-violaxanthin and 9-*Z*-violaxanthin) was increased in the pulp along the maturation process in all varieties, presenting the highest contents in the Va cultivar, 8.5 ± 0.4 μg/g FW and 7.5 ± 0.1 μg/g FW in season 2 and 1, respectively (Figures 4 and S2). At full maturation stage, red-flesh mutants presented a higher content of β,β-xanthophylls, being Cc and Pk showing the highest contents (4.1 ± 0.3 μg/g FW and 3.5 ± 0.3 μg/g FW in season 2 and 1, respectively).

### *3.3. Gene Expression Profile of Carotenogenic Genes during Development and Maturation in the Pulp of Red-Fleshed Sweet Oranges*

In order to determine whether the carotenoid composition in red-fleshed oranges was associated with specific gene expression patterns, the expression of a total of 14 genes involved in carotenoid production was analysis (Figure 5). Four genes of the MEP pathway and eleven genes of the carotenoid pathway were analysed in the pulp of every cultivar at the different developmental stages, as shown in Figure 2. Transcript accumulation of *DXS* and *HDR* presented a maximum at the MG in season 1, although in season 2 it was similar to the FC stage, with the exception of the Ph cultivar which showed the highest expression (3.7 ± 0.3 and 3.7 ± 0.1, relative expression for *DXS1* and *HDR*, respectively) compared with other cultivars at this stage (Figure S3). In general, the *HDS* gene showed the lowest expression of all genes in the MEP pathway, while *GGPPS1* presented no changes during season 1 and only a slight increment in MG and FC stages in the second season (Figure S3).

The expression profile of the carotenogenic genes was different between seasons. While in season 1 no relevant changes were observed between the MG, Br and FC stages, several differences were detected in season 2 (Figures 5 and S4). Among them, the Va cultivar displayed a boost in the expression of *PDS*, *β-LCY2* and *β-CHX* in the MG stage (3.4 ± 0.5, 4.9 ± 0.1 and 4.3 ± 0.2, respectively). On the other hand, Ph was the cultivar with the highest expression of carotenogenic genes in the FC stage with a relatively higher expression of 2.2 ± 0.1, 2.5 ± 0.6, 4.0 ± 0.2, 3.4 ± 0.1 and 2.0 ± 0.2, for *PSY*, *PDS*, *ZDS1*, β-*LCY2* and *β-CHX*, respectively (Figures 5 and S3). In any case, these differences in gene expression did not explain the carotenoid profiles in the pulp in red-fleshed sweet oranges fruits compared to that of blond cultivars.

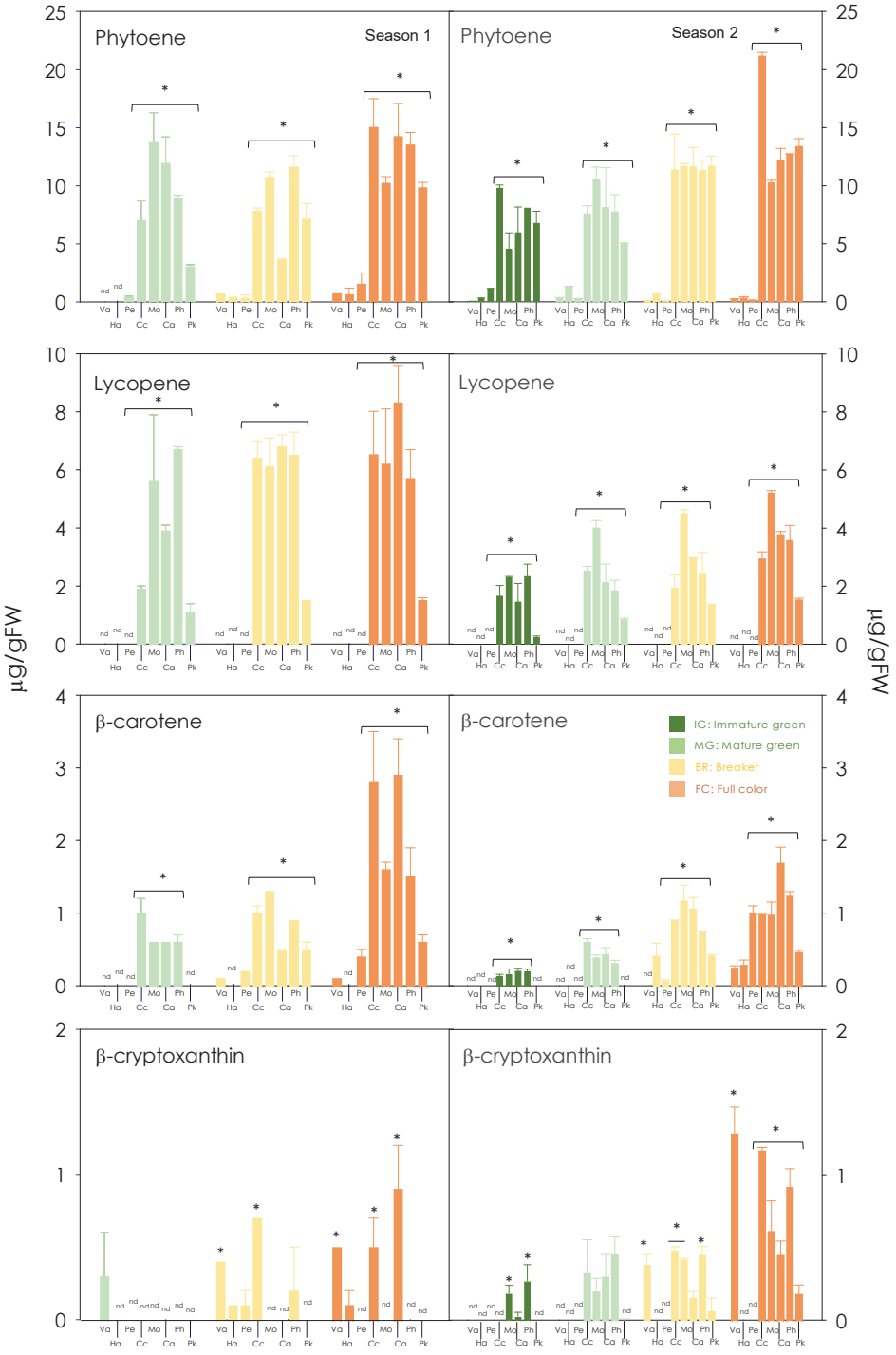


**Figure 4.** Carotenoid content of phytoene, phytofluene, β-carotene and β-cryptoxanthin in the pulp during development and maturation of Valencia (Va), Hamlin (Ha), Pera (Pe), Cara cara (Cc), Mombuca (Mo), Carrancas (Ca), Pinhal (Ph) and Puka (Pk) fruits (*C. sinensis* L. Osbeck) in two different seasons. The samples analysed correspond to the developmental and maturation stages indicated in Figure 2. The physiological fruit stages are indicated: IG (immature-green), MG (mature-green), B (breaker) and FC (full-colour). The data are means ± SD of at least three independent measurements. Statistical analyses were performed using analysis of variance (ANOVA) and an asterisk above the bars indicates significantly different values at $p \leq 0.01$ (n.d.: non detected).


**Figure 5.** Quantitative RT-PCR analysis of the expression of PSY, PDS, β-LCY2 β-CHX and ZEP genes in the pulp during development and maturation of Valencia (Va), Hamlin (Ha), Pera (Pe), Cara cara (Cc), Mombuca (Mo), Carrancas (Ca), Pinhal (Ph) and Puka (Pk) fruits (*C. sinensis* L. Osbeck) in two different seasons. The samples analysed correspond to the developmental and maturation stages indicated in Figure 2. The physiological fruit stages are indicated: IG (immature-green), MG (mature-green), B (breaker) and FC (full-colour). Statistical analyses were performed using analysis of variance (ANOVA) and an asterisk above the bars indicates significantly different values at $p \leq 0.01$.

## 4. Discussion

There is currently an active search in most fruit industries worldwide for citrus fruit mutants with distinctive attractive colours, especially reddish hues. This is not only because of the appealing of the red colour for most human cultures [47,48] but also because it is associated with the accumulation of healthy phytonutrients, namely carotenoids such as lycopene. It is shown here that the juice and pulp from sweet orange varieties producing red-fleshed fruit are four to nine times richer in carotenoids than those from orange-fleshed counterparts when trees are grown under a tropical climate and independently of fruit maturation of early, mid-season and late varieties, reinforcing an association between red colour and the functional attributes of sweet orange fruit and juices. Carotenoid-rich foods have been associated with risk reduction for several chronic diseases, including reduced incidence of obesity, type 2 diabetes, eye diseases, certain types of cancer (especially prostate and digestive tract tumours), and even lower total mortality. In addition, some carotenoids, particularly β-carotene, constitute important vitamin A precursors [49,50]. Carotenoids have been widely recognised as physiological antioxidants because of their ability to quench singlet molecular oxygen [5,6,51].

The carotenoid profiles in the fruit pulp/juice showed that linear carotenoids such as phytoene and phytofluene were the most abundant in all the five red-fleshed varieties, representing more than 70% of total carotenoids in all developmental/maturation stages, while the blond varieties accumulated mainly xanthophylls, with *all*-violaxanthin depicting more than 50% of total carotenoids in mature fruit. Nevertheless, the most remarkable difference was the accumulation of lycopene at moderate concentrations (about 8 µg/g FW) in mature pulp from red fruit, while it was absent in blond varieties. Moreover, red-fleshed varieties accumulated β-carotene in mature fruit for at least one of the seasons while this compound was only detected in traces in blond varieties (Figures 3, 4 and S2). A positive association between the accumulation of linear carotenoids and lycopene has been described before for Cc and other red-fleshed sweet orange varieties [29,37,52,53] when grown under sub-tropical climate conditions, but not in the Chinese Anliu sweet orange variety, which does not accumulate upstream carotenes but has lycopene-rich pulp [29,54]. Such correlation was shown in the case of the five red-fleshed varieties studied here, suggesting that enhanced *PSY* activity may be responsible at least in part for the accumulation of lycopene [37] as it occurs in other fruits such as tomato [55,56]. Together with this or alternatively, a partial blockage in the function of *β-LCY2* has been proposed in other lycopene-rich citrus varieties [57,58]. However, the accumulation of lycopene and β-carotene was not accompanied by a consistent decrease in the production of xanthophylls (Figures 4 and S2) as it has been reported in other works with red-fleshed sweet orange mutants [37,53] and as it may be expected if *β-LCY2* expression were interfered with. Moreover, we did not find alterations of the transcriptional profiles in the pulp of the red-fleshed oranges, neither in the expression of MEP pathway gene precursors of phytoene or in that of *β-LCY2*, when compared with those of the blond orange controls (Figures 5, S3 and S4). Therefore, the molecular features behind the mutation that leads to the accumulation of lycopene in the pulp of these (and other) sweet orange varieties remain to be elucidated, although this mutation seems to be post-transcriptionally regulated at the level of the carotenoid biosynthetic pathway.

Seasonal differences were observed in the accumulation of carotenoids for all the varieties investigated here. For example, highest concentrations of lycopene and β-carotene in the pulp of red-fleshed varieties Cc, Mo, Ca and Ph were linked to higher maximum temperatures during fruit maturation in season 2 vs. season 1. Although arelatively low temperature is required for chlorophylls to degrade and for carotenoids to accumulate during the maturation of citrus fruits, it is well known that high temperatures in the later stages of fruit maturation enhance the accumulation of lycopene and other carotenoids in the pulp of lycopene-rich varieties (review in Alquezar et al. [37]). Our results indicate that growing lycopene-rich varieties in other areas of Sao Paulo state, at the north and centre of the region (Figure S1), closer to the main areas of the citrus belt, where temperatures are

more amenable than in the south, may further improve the accumulation of lycopene and β-carotene.

So far, lycopene and β-carotene are the most investigated carotenoids in search for in vivo human health benefits [50,59]. Apart from the role of β-carotene as provitamin A precursor, recent epidemiological and cellular studies are showing the antioxidant effect of lycopene in inhibiting the development of diseases such as cardiovascular diseases inflammation, obesity, neurodegenerative disorders, type 2 diabetes, metabolic diseases that affect the bone, eye, kidney and liver and ulcerative colitis [26,27,60]. Moreover, lycopene accumulates at high concentrations in the testis, where its antioxidant activity may help to eliminate oxidative damage [59,60].

Sao Paulo state (Brazil) concentrates 70% of the orange juice produced worldwide. Ha, Pe and Va, used here as blond-orange controls, are the most important varieties grown in Sao Paulo, representing about 80% of the total orange juice produced. Lycopene-rich red orange juice may become an appealing addition to the already highly popular blond orange juices. Considering the five red-fleshed mutants investigated, Cc is not a suitable variety for the juice industry, because it accumulates the bitter triterpene compound limonin in its flesh just minutes after processing, like most Navel oranges [25]. Pk is a mutant from Va, and as suchis the only late red-fleshed variety of the group. However, it does accumulated the lowest concentrations of lycopene and β-carotene. From the three mid-season red-fleshed mutants, Mo showed the lowest MI and Ca showed the highest amounts of lycopene/β-carotene. Both Ca and Ph are highly productive (A.R. Violante, Cutrale, personal communication, https://www.cutrale.com.br/, accessed on 1 March 2023) and may be used either alone or for blending to give a reddish orange colour to usually pale-orange juices such as those from Hamlin and other early and mid-season blond varieties. Further agronomic and nutraceutical studies may help to promote the use of these new mutants potentially beneficial red-fleshed cultivars for the global citrus industry.

## 5. Conclusions

Carotenoid profiles in the fruit pulp of five red-fleshed orange varieties revealed that about 70% were linear carotenoids at all stages of development and maturation. In contrast, the blond varieties accumulated primarily xanthophylls, with *all*-violaxanthin comprising more than 50% of total carotenoids in mature fruit. In this study, the most noteworthy distinction between red-fleshed and blond varieties was the presence of lycopene and the accumulation of β-carotene in red-fleshed ones, which was absent or at traces in blond counterparts. In tropical areas, the incorporation of lycopene-rich red orange juice could be a potential practice to enhance the appeal of orange juices. Among the 5 red-fleshed mutants investigated, both the new Ca and Ph mutants could be used to blend for imparting a reddish-orange colour to typically pale-orange juices, potentially benefiting the global citrus industry. Moreover, the increased accumulation of lycopene in these red-fleshed varieties not only enhances the juice colour but also improves its nutritional value, thanks to lycopene antioxidant properties and increase in β-carotene content.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/antiox13080994/s1. Table S1. Primer sequences used for the quantitative RT-PCR analyses. Figure S1. Representative climatological condition parameters: (A) temperature, (B) relative humidity and (C) precipitations in different commercial orchards Fazenda Guacho in Sta. Cruz de Rio Pardo (Sao Paulo State, Brazil) during the seasons 2021/22 (season 1) and 2022/23 (season 2). Figure S2. Carotenoid content of phytofluene, lutein and *all*-violaxanthin in the pulp during development and maturation of Valencia (Va), Hamlin (Ha), Pera (Pe), Cara cara (Cc), Mombuca (Mo), Carrancas (Ca), Pinhal (Ph) and Puka (Pk) fruits (*C. sinensis* L. Osbeck) in two different seasons. The samples analysed correspond to the developmental and maturation stages indicated in Figure 2. The physiological fruit stages are indicated: IG (immature-green), MG (mature-green), B (breaker) and FC (full-colour). The data are means ± SD of at least three independent measurements. Statistical analyses were performed using analysis of variance (ANOVA) and an asterisk above the bars indicates significantly different values at $p \leq 0.01$. Figure S3. Quantitative RT-PCR analysis of the expression of *DXS1*,

HDR, HDS and *GGPPS1* genes in the pulp during development and maturation of Valencia (Va), Hamlin (Ha), Pera (Pe), Cara cara (Cc), Mombuca (Mo), Carrancas (Ca), Pinhal (Ph) and Puka (Pk) fruits (*C. sinensis* L. Osbeck) in two different seasons. The samples analysed correspond to the developmental and maturation stages indicated in Figure 2. The physiological fruit stages are indicated: IG (immature-green), MG (mature-green), B bBreaker) and FC (full-colour). Statistical analyses were performed using analysis of variance (ANOVA) and an asterisk above the bars indicates significantly different values at $p \leq 0.01$. Figure S4. Quantitative RT-PCR analysis of the expression of *ZDS1*, *ZDS2*, *ZDS3*, *ε-LCY2* and *β-LCY1* genes in the pulp during development and maturation of Valencia (Va), Hamlin (Ha), Pera (Pe), Cara cara (Cc), Mombuca (Mo), Carrancas (Ca), Pinhal (Ph) and Puka (Pk) fruits (*C. sinensis* L. Osbeck) in two different seasons. The samples analysed correspond to the developmental and maturation stages indicated in Figure 2. The physiological fruit stages are indicated: IG (immature-green), MG (mature-green), B (breaker) and FC (full-colour). Statistical analyses were performed using analysis of variance (ANOVA) and an asterisk above the bars indicates significantly different values at $p \leq 0.01$.

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# Exploring the Therapeutic Potential of *Theobroma cacao* L.: Insights from In Vitro, In Vivo, and Nanoparticle Studies on Anti-Inflammatory and Anticancer Effects

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**Abstract:** *Theobroma cacao* L., commonly known as cocoa, has been an integral part of human culture and diet for thousands of years. However, recent scientific research has highlighted its potential therapeutic properties, particularly in the treatment of cancer and inflammatory diseases. This comprehensive review analyzes the anti-inflammatory and anticancer effects of *Theobroma cacao* extracts combined with nanoparticles using in vitro and in vivo studies. Its diverse biological activity can be attributed to its rich phytochemical profile, including polyphenols, flavonoids, and alkaloids. In vitro studies have found that cocoa extracts, alone or in combination with nanoparticles, inhibit cancer cell proliferation, induce apoptosis and modulate key signaling pathways in various cancer cell lines. The extracts have also been found to reduce tumor growth and enhance the efficacy of conventional chemotherapeutic agents, potentially reducing their side effects, in vivo. Its anti-inflammatory properties are based on its ability to modulate inflammatory mediators, inhibit NF-κB signaling, and regulate macrophage polarization. These effects have been observed in both cellular and animal models of inflammation. This review opens up new possibilities for future research and therapeutic applications, highlighting the potential of *Theobroma cacao* as a valuable complementary approach in the treatment and prevention of cancer and inflammatory diseases.

**Keywords:** *Theobroma cacao*; anticancer; anti-inflammatory; polyphenols; in vitro studies; in vivo studies; nanoparticles

## 1. Introduction

*Theobroma cacao* L., the botanical name for the cacao tree, means "food of the gods", a fitting title for a plant that has played a significant role in human history for thousands of years. The history of cacao is rich and multifaceted, beginning with the ancient Mesoamerican civilizations and extending to its global prominence today. This paper explores the cultural significance and historical use of *T. cacao*, from its origins, through its introduction to Europe, and ultimately to its worldwide impact. Cacao trees originally grew in the river valleys of South America, and by the seventh century AD, the Mayan Indians had introduced them to Mexico. They were also domesticated and cultivated by other Central

American Indian groups, including the Aztecs and the Toltecs. The word chocolate comes from the Aztec language, derived from inter alia xhocolatl or cacahuatl. Ancient texts describe various cacao mixtures used for ceremonial, medicinal, and culinary purposes [1–3]. The name *Theobroma cacao* was coined by the Swedish botanist Carl Linnaeus in 1753 and published in *Species Plantarum*. *Theobroma* itself is derived from the Greek *broma*, meaning food, and Theo, meaning god, which translates as food of the gods [4–6]. The cacao tree thrives at elevations of 200–400 m in regions receiving 1000–3000 mm of annual rainfall. It needs a humid environment with consistent rainfall and rich soil. *Theobroma cacao* can grow only within 10 degrees north or south of the equator, favoring the moist, shady regions of tropical rainforests [7,8]. One of the greatest individual causes of death is cancer, caused by harmful chemicals, viruses, agents that produce free radicals, certain environmental toxins, and unavoidable everyday factors. Traditional cancer treatments such as chemotherapy, radiotherapy, and surgery typically provide only limited and temporary relief. Therefore, there is great interest in identifying alternative remedies for cancer that are widely accessible, cost-effective, and have minimal side effects [9–12]. *Theobroma cacao* appears to have anticancer and anti-inflammatory properties thanks to its flavonoid, polyphenol, and alkaloid content [13–15]. Cocoa also has cardioprotective, neuroprotective, and hepatoprotective effects, and cocoa leaf and bark extracts have demonstrated protective effects against doxorubicin-induced oxidative stress and organ damage [16–20]. Among promising strategies to enhance the therapeutic potential of *T. cacao* extracts, nanoparticle-based delivery systems have emerged as a particularly interesting approach. Nanoparticles can improve the bioavailability of bioactive compounds, enable targeted delivery to specific tissues, and potentially enhance the therapeutic efficacy of cocoa extracts. The combination of *T. cacao* extracts with nanoparticles represents an innovative direction in developing more effective treatments for cancer and inflammatory diseases [21].

The aim of this study is to comprehensively evaluate the anti-inflammatory and anticancer properties of *T. cacao* extract in vitro and in vivo, alone and in combination with nanoparticles. This research aims to elucidate the potential mechanisms underlying these effects and explore the potential of *T. cacao* as a source of novel bioactive therapeutic compounds for the treatment of cancer and inflammatory diseases.

## 2. Experimental Paper Selection Criteria

This review focuses on in vitro and in vivo studies of plant extracts from *Theobroma cacao* and the potential mechanisms of their anticancer and anti-inflammatory effects. The studies were identified using the PubMed, Google Scholar, and Scopus databases and included data published between 2004 and 2024. The following key words were used in the search: *Theobroma cacao* extract, secondary metabolite, in vitro study, in vivo study, anticancer potential of *Theobroma cacao*, anti-inflammatory potential of *Theobroma cacao*, anticancer potential of *Theobroma cacao* in combination with nanoparticles, anti-inflammatory potential of *Theobroma cacao* in combination with nanoparticles, bioactive compounds in *Theobroma cacao*, therapeutic potential of *Theobroma cacao*, synergistic anticancer effects of Theobroma cacao and nanoparticles. The articles published in languages other than English or abstracts were excluded. Duplicate articles from different databases were also excluded. All the inclusion and exclusion criteria were checked again after these articles had been removed. Finally, after searching the above sources, 203 articles were found, including the following: anticancer in vitro effect of *Theobroma cacao* extract (55 articles ), in vitro anti-inflammatory effect of *Theobroma cacao* extract (49 articles), in vivo anticancer effect of *Theobroma cacao* extract (31 articles), in vivo anti-inflammatory effect of *Theobroma cacao* extract (46 articles), biological effect of nanoparticles with *Theobroma cacao* extract (22 articles). Due to similarities, lack of relevance to the topic, and imprecise information contained within them, 98 records were excluded. As a result, the final analysis includes 105 articles in total, of which 50 are presented in tables.

Each selected scientific paper was analyzed, and the following data were selected and tabulated: scientific name of the plant, plant parts used to prepare the extract or isolate the

pure compound, type of extract, class of compounds or different compounds identified in the extract, cancer cell lines used or animal model/cell line inoculated with carcinogenic compounds, activity or mechanism of action, and reference. Articles explaining the mechanism(s) of action of plant extracts derived from *T. cacao* are discussed in the main text before being presented in tables.

## 3. Agricultural Livelihoods and Socio-Economic Development

The global demand for chocolate and cocoa products drives a robust international market for cacao beans. The chocolate industry is a multi-billion dollar market, with annual global chocolate sales exceeding $100 billion [22,23]. Cocoa cultivation is an important source of income for millions of smallholder farmers in tropical regions, mainly in West Africa, Latin America, and Southeast Asia. Leading producers, including Ivory Coast, Ghana, Indonesia, and Brazil, rely heavily on cocoa cultivation as a key component of their agricultural sectors [24] (Figure 1). In these countries, cocoa exports are crucial, accounting for a significant proportion of their gross domestic product (GDP) and export earnings. For smallholder farmers, cocoa is a stable cash crop that provides an essential income. Cocoa cultivation also has cultural significance in many production regions. It supports traditional farming practices and contributes to the preservation of local heritage. In some areas, cocoa cooperatives play a key role in community organization, empowering farmers through collective bargaining and shared resources. The economic stability provided by cocoa cultivation helps to improve living standards, fund education, and provide health care. In addition, cocoa supports ancillary industries such as local processing, transport, and agricultural supply, further stimulating the rural economy.

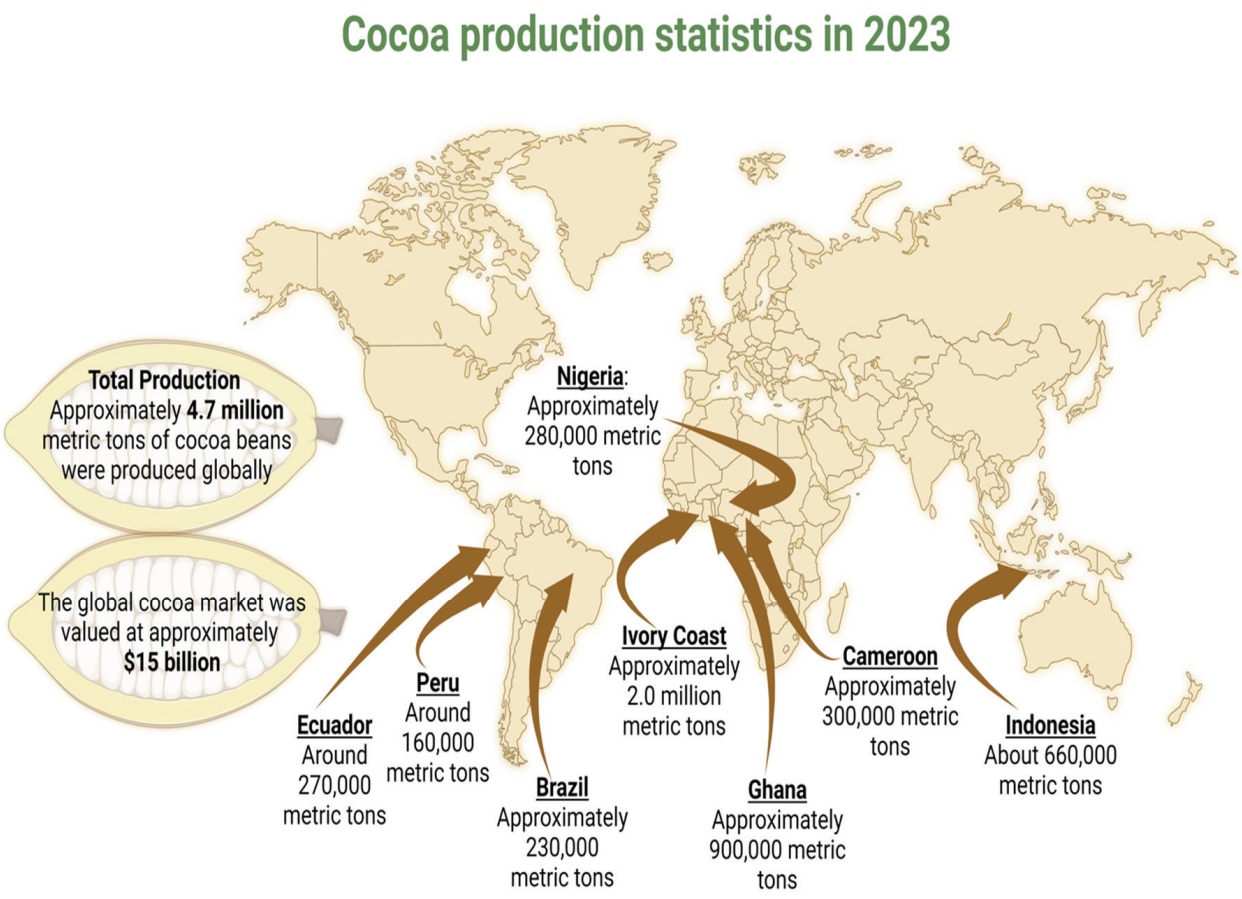


**Figure 1.** Global cocoa production.

## 4. *Theobroma cacao* L.—Characteristics, Secondary Metabolites, and Health Benefits

The *Theobroma* genus (*Theobroma cacao* L.), globally renowned as the basis of chocolate (Figure 2), lies within the family Malvaceae, a large and diverse family of flowering plants. It includes around 244 genera and over 4225 species, encompassing a variety of growth forms, such as trees, shrubs, and herbaceous plants. Its members are distributed worldwide, particularly in tropical and subtropical regions. Among these, *Theobroma cacao*, the cocoa tree, is a particularly desirable crop for its seeds [25]. The cocoa tree thrives exclusively in humid, tropical regions, and cocoa has become a vital agricultural product in numerous developing nations across West Africa, where it accounts for over 70% of global production, as well as in Southeast Asia, Latin America, and the Caribbean [26]. The seeds of *T. cacao*, commonly enjoyed in cocoa drinks and chocolate, are recognized as a rich source of bioactive compounds, particularly polyphenols, alkaloids phytosterols, and fatty acids; these are believed to confer many of the proposed protective effects associated with the consumption of cocoa and chocolate. These secondary metabolites not only contribute to the unique flavor profile of cocoa but also play a significant role in its potential health benefits [9,10].

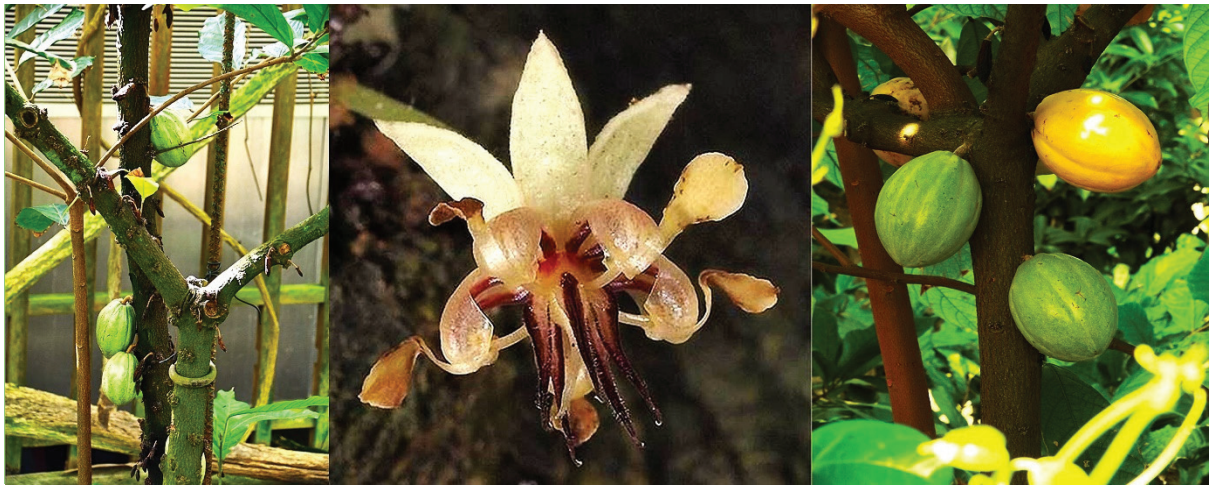

**Figure 2.** *Theobroma cacao* L. (courtesy of the Missouri Botanical Garden and the Royal Botanic Gardens, Kew, ©RBG Kew).

*Theobroma cacao* is particularly rich in polyphenols, a diverse group of plant-based compounds. Cocoa beans contain three main groups of polyphenols: catechins (about 37% of the polyphenol content), anthocyanidins (around 4%), and proanthocyanidins (approximately 58%). The most abundant catechin is (−)-epicatechin, which makes up as much as 35% of the total catechin content, with smaller amounts of (+)-catechin, (+)-gallocatechin, and (−)-epigallocatechin. For anthocyanidins, the primary compounds are cyanidin-3-α-L-arabinoside, and cyanidin-3-β-D-galactoside. In the case of procyanidins, the main components are dimers, trimers, or oligomers of flavan-3,4-diol connected by 4 → 8 or 4 → 6 bonds, with the most significant procyanidins being B1, B2, B3, B4, B5, C1, and D [27,28]. Another group of compounds present in *T. cacao* are alkaloids. Among all structurally-related purine alkaloids found in chocolate (methylxanthines), theobromine stands out as the primary compound. *Theobroma cacao* has two major classes of phenylpropanoids, viz. cinnamic acid and tocopherol derivatives [29]. Jalal and Collin isolated p-coumaric acid and caffeic acid, two common cinnamic acid derivatives, along with p-coumaroylquinic acid and chlorogenic acid, i.e., cinnamic acid derivatives containing quinic acid, from *T. cacao* leaves [30]. The same compounds were detected by UHPLC-MS/MS study in the leaves, seeds, and pericarp of *Theobroma* genus by Tauchen et al. [31].

The seeds of *Theobroma cacao*, also known as cocoa beans, are rich in fats. Approximately 50–57% of the dry weight of whole cocoa beans consists of lipids, commonly referred

to as cocoa butter. This cocoa butter, predominantly found in dark chocolate, typically consists of around 33% oleic acid, 25% palmitic acid, and 33% stearic acid [32]. *Theobroma cacao* contains a wealth of minerals, including potassium, phosphorus, copper, iron, zinc, and magnesium. These minerals play an important role in the proper functioning of the body and are involved in most physiological processes, e.g., maintaining the acid–base balance, transporting some substances, and maintaining healthy bones and teeth. Therefore *T. cacao*, in the form of high-cocoa chocolate rich in nutrients, can be included in a well-rounded diet, when consumed in moderation [33]. Selected secondary metabolites contained in *Theobroma cacao* are shown in Figure 3.

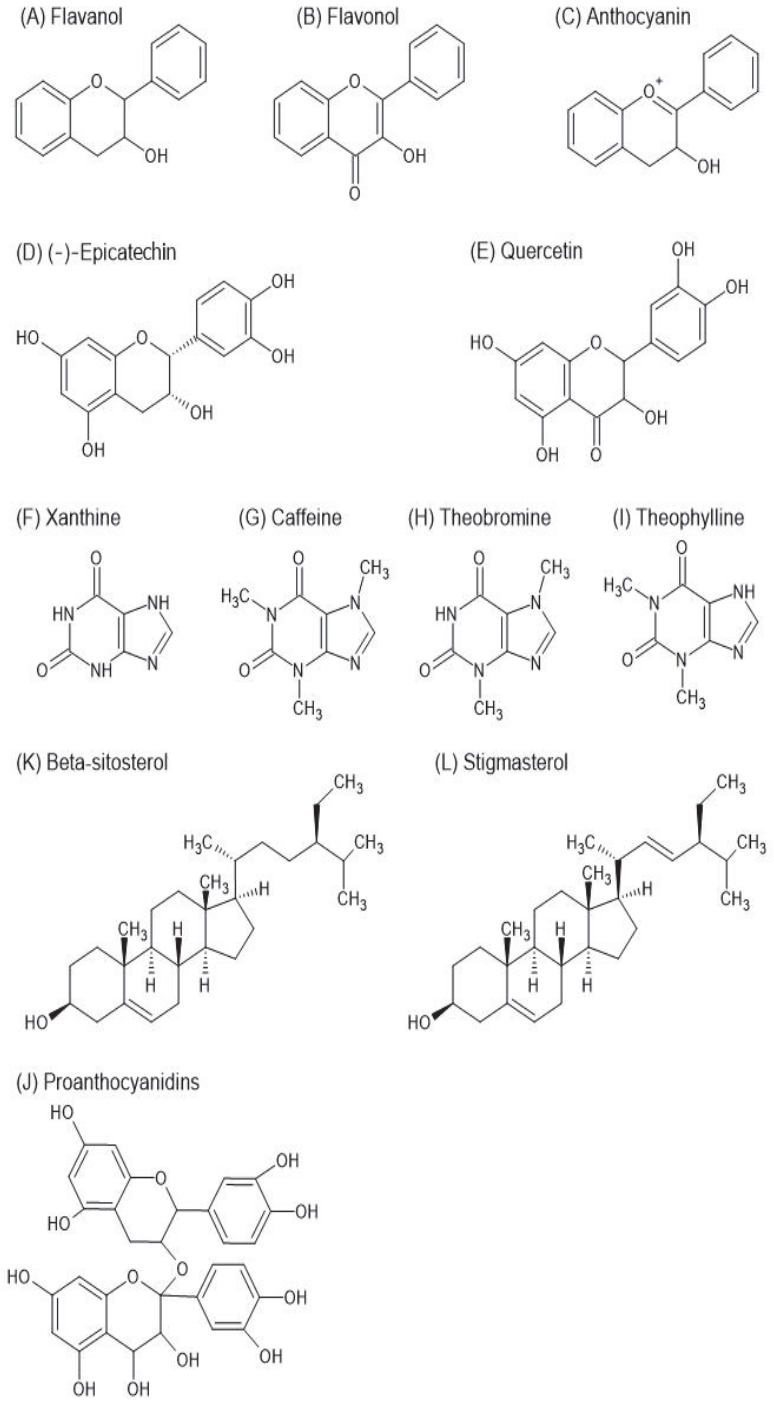


**Figure 3.** Chemical structure of selected secondary metabolites of *Theobroma cacao* L. (ChemSketch (freeware), version 2023.2.4, Advanced Chemistry Development, Inc. (ACD/Labs), Toronto, ON, Canada, www.acdlabs.com accessed on 23 June 2024).

The bioactive compounds in *Theobroma cacao* have been associated with various potential health benefits, including antioxidant properties, metabolic effects, anti-inflammatory potential, and neuroprotective effects, as well as cardiovascular health and anticancer properties [10].

Polyphenols and flavonoids exhibit strong antioxidant properties, acting as free radical scavengers and potentially reducing oxidative stress in the body [34]. Studies have also shown cocoa consumption to have promising metabolic effects. Cocoa has been demonstrated to alleviate obesity. Studies showed that mice fed a high-fat diet supplemented with cocoa demonstrated less weight gain, attenuated insulin resistance, and reduced severity of obesity-related fatty liver disease. Moreover, it has been proven that daily consumption of 40 g/d of dark chocolate rich in cocoa lowers markers of oxidative stress, hepatocyte apoptosis and ALT in the serum compared to the consumption of milk chocolate [35]. Furthermore, cocoa and its procyanidins demonstrate significant anti-inflammatory potential. They may provide anti-inflammatory benefits by modulating cytokine production and regulating NF-κB target gene expression, which could have implications for various inflammatory conditions [36]. Notably, in vitro studies have suggested that cocoa polyphenolic extracts may have neuroprotective effects, which may be significant in neurodegenerative conditions. For example, in Alzheimer's disease, cocoa polyphenolic extracts not only exhibit antioxidant properties but also provide neuroprotective effects. These neuroprotective effects are linked to the activation of the BDNF survival pathway in cells treated with Aβ plaques or Aβ oligomers, resulting in a decrease in neurite dystrophy. Alzheimer's disease is characterized by an increased production of amyloid (A)β oligomers, which activates microglia, leading to the release of inflammatory substances and neuronal death [37]. Cocoa extracts have demonstrated cardioprotective potential by exerting hypoglycemic and hypocholesterolemic effects, with treatment lowering serum glucose levels and improving lipid profiles. The consumption of chocolate and chocolate-containing confections has been found to induce relatively low levels of postprandial glycemia compared to equivalent amounts of carbohydrates in starchy foods such as bread, rice, and potatoes. They may help lower serum glucose levels and improve lipid profiles, potentially contributing to the prevention of cardiovascular diseases and diabetes [38]. Additionally, the phytosterols present in cocoa seeds lower total plasma cholesterol and low-density lipoprotein levels by inhibiting cholesterol absorption in the intestine. Cocoa seeds contain 2–3 mg of plant sterols per gram of fat, mainly β-sitosterol and stigmasterol. The fatty acid profile of cocoa may also contribute to its cardiovascular benefits, comprising primarily triacylglycerols (TAGs) with 2-oleyl glycerides (O) of palmitic (P) and stearic (S) acids (POP, POS, SOS) [39].

Polyphenols are especially renowned for their beneficial impact on cancer. For example, they appear to prevent the progression of cancer and have been found to induce non-apoptotic cell death and block the cell cycle in the G2/M phase, enhancing their antiproliferative properties [28]. In addition, in laboratory studies, cocoa compounds such as epicatechin, catechin, quercetin, and extracts of procyanidins and B-type dimeric procyanidins have been found to decrease the expression or activity of NF-κB and AP-1 in Hodgkin and Reed–Sternberg (HRS), Daudi, human acute monocytic leukemia (THP-1), and Jurkat cancer cell lines. Pentameric procyanidin from *T. cacao* was found to inhibit breast cancer cell growth by inducing mitochondrial effects that lead to growth arrest or trigger apoptotic and non-apoptotic cell death processes. Additionally, research on the novel mitochondriotoxic molecule F16 has shown that compounds causing mitochondrial depolarization can inhibit breast cancer cell growth through G1 arrest, apoptosis, or necrosis, depending on the genetic makeup of the cells [10].

These diverse potential health benefits highlight the growing interest in *Theobroma cacao* as a possible source of nutraceuticals and functional food components. As research in this area continues to expand, it is becoming increasingly clear that the use of *Theobroma cacao* may extend far beyond chocolate production, opening new avenues for health promotion and disease prevention.

## 5. Anticancer and Anti-Inflammatory Effects of *Theobroma cacao* Extract In Vitro and In Vivo

The relationship between inflammation and cancer has been the subject of growing interest in recent years. Chronic inflammation can contribute significantly to the development and progression of cancer through multiple mechanisms. Inflammatory cells, particularly tumor-associated macrophages (TAMs), can promote tumor growth by producing growth factors, cytokines, and chemokines that stimulate cell proliferation, angiogenesis, and metastasis [40–42]. These cells also generate reactive oxygen and nitrogen species, which can induce DNA damage and genomic instability in proliferating cells. Furthermore, the inflammatory microenvironment can facilitate the survival of neoplastic cells by activating transcription factors such as NF-κB and STAT3, which regulate pro-survival genes [43,44]. Chronic inflammation associated with infections, autoimmune diseases, or environmental factors can create a tumorigenic microenvironment that fosters neoplastic transformation and progression. Understanding this intricate relationship between inflammation and cancer provides valuable insights into potential therapeutic targets and underscores the importance of anti-inflammatory strategies in cancer prevention and treatment [45–47]. *Theobroma cacao* extracts exhibit diverse anticancer effects through multiple mechanisms targeting various aspects of cancer cell biology. These extracts, rich in bioactive compounds, demonstrate antiproliferative, pro-apoptotic, and cell cycle regulatory activities across different cancer cell lines. The antiproliferative effects of cocoa extracts are evidenced by their ability to inhibit cancer cell growth in various cell lines, including MCF-7 (breast cancer), HepG2 (liver cancer), and Caco-2 (colon cancer) [34,48]. This growth inhibition is often associated with cell cycle arrest, particularly at the G2/M phase, as observed in Caco-2 cells [49]. The extracts also modulate key enzymes involved in polyamine biosynthesis, such as ornithine decarboxylase and S-adenosylmethionine decarboxylase, potentially contributing to their antiproliferative effects [49]. Cocoa extracts induce apoptosis in cancer cells through multiple pathways. For instance, methanolic leaf extracts have been shown to upregulate pro-apoptotic genes (DDIT3, HRK, GADD45G) and increase the activity of caspases 8 and 9 in MCF-7 cells [50]. Additionally, procyanidin-rich extracts can increase intracellular reactive oxygen species (ROS) levels and promote caspase-3-dependent cell death in ovarian cancer cells [51]. Cocoa extracts may also possess antimetastatic properties. For example, procyanidin-rich extracts have been observed to downregulate matrix metalloprotease-2 (MMP2), an enzyme associated with cancer metastasis [51]. Theobromine, a major alkaloid in cocoa, demonstrates significant anticancer potential. It enhances the cytotoxicity of doxorubicin in LoVo colon cancer cells and modulates proteins associated with proliferative and anti-apoptotic pathways, including PDE4, ERK, NF-κB, and Akt/mTOR [34,52]. Theobromine also activates pro-apoptotic pathways involving JNK and p38-MAPK [52]. It is important to note that the anticancer effects of cocoa extracts can vary depending on the plant part used, extraction method, and specific composition of bioactive compounds. Furthermore, the relationship between antioxidant activity, total phenolic content, and anticancer effects is complex and does not always show a direct correlation [53]. Finally, *Theobroma cacao* extracts exhibit multifaceted anticancer activities, including antiproliferative, pro-apoptotic, and potentially antimetastatic effects. These activities are mediated through various molecular mechanisms, highlighting the potential of cocoa-derived compounds in cancer research and therapy. However, further research, particularly in vivo studies and clinical trials, is needed to fully elucidate the therapeutic potential and mechanisms of action of these extracts. Other studies are presented in Table 1 below.

**Table 1.** Anticancer in vitro effect of *Theobroma cacao* extract.

| Part of the Plant | Class of Compounds | Cell Line | $Ic_{50}$ | Activity/Mechanism/Effects | Ref. |
|---|---|---|---|---|---|
| Cocoa beans | phenylpropenyl-amino acids, hydroxycinnamic-amino acid conjugates, procyanidin compounds, fatty acids and lysophospholipids | MCF-7, Hep-G2, OE19, Caco-2 | Indonesian cocoa beans:<br>MCF-7—254.20 μg/mL<br>Hep-G2—122.00 μg/mL<br>OE19—903.30 μg/mL<br>Caco-2—104.90 μg/mL<br>Peruvian cocoa beans:<br>MCF-7—708.30 μg/mL<br>Hep-G2—199.70 μg/mL<br>OE19—>1000 μg/mL<br>Caco-2—133.90 μg/mL | The extract did not significantly inhibit proliferation of all cancer cell lines. | [48] |
| Cocoa leaf extract | - | MCF-7 | 41.43 μg/mL | The extract did not significantly inhibit proliferation of all cancer cell lines. | [34] |
| Cacao fruit powder | polyphenols<br>flavonoids | HeLa, CaCo2 | HeLa:<br>1810 μg/mL, 2170 μg/mL<br>CaCo2:<br>2650 μg/mL | The extract inhibited proliferation of both cancer cell lines. | [54] |
| Theobromine extract | theobromine | LoVo, LoVo/Dx | - | Theobromine highly enhanced cytotoxic and resistance reversal potency of MAE-TPR. | [55] |
| Cocoa bean phenolic extract | protocatechuic acid, p-hydroxybenzoic acid, ideain, catechin, chlorogenic acid, caffeic acid, epicatechin, cyanidin, quercetin, kaempferol, procyanidin B1, procyanidin B2, clovamide | AML12, MLP29 | - | The cocoa extract inhibited drug-triggered cytotoxicity in liver, possibly by activating autophagy. The phenolic compounds protected the cells from celecoxib-induced viability inhibition. Apoptotic pathways (e.g., Bax) are the main target for cocoa extract. | [50] |
| Edel cocoa bean extract from fermented and unfermented cocoa beans | polyphenols | Human gingival fibroblast cells | 1552.877 μg/mL (from fermented cocoa extract)<br>32.1282 μg/mL (from unfermented cocoa extract) | The extract from fermented cocoa beans did not show a cytotoxic effect, but the extract from unfermented beans showed a cytotoxic effect on fibroblast cells. | [49] |

**Table 1.** *Cont.*

| Part of the Plant | Class of Compounds | Cell Line | $Ic_{50}$ | Activity/Mechanism/Effects | Ref. |
|---|---|---|---|---|---|
| Cocoa leaf extract | methanolic extract | MCF-7 | 6.4 µg/mL (concentration after 48 h) | The compounds present in the methanolic leaf extract induced apoptosis in breast cancer cells by inducing cell shrinkage and membrane blebbing. The bioactive fraction upregulated pro-apoptotic genes (DDIT3, HRK, GADD45G) and increased the activity of caspase 3, caspase 8, and caspase 9. | [56] |
| Cocoa powder | caffeine, theobromine, flavonols, procyanidins | Caco-2 | - | The cocoa extract had an anti-proliferative effect by blocking cells at the G2/M phase. Cocoa extract causes polyamine biosynthesis inhibition. | [52] |
| Cocoa leaf, bark, husk, unfermented cocoa shell, fermented cocoa shell, root, cherelle, pith extracts | methanolic extract | MCF-7, A549, HeLa, HepG2, HT-29, MDA-MB-231, WRL-68 | For MCF-7 cell line:<br>Leaf—41.43 µg/mL<br>Bark—71.97 µg/mL<br>Husk—62.23 µg/mL<br>Unfermented shell—65.03 µg/mL<br>Fermented shell—242.33 µg/mL<br>Pith—329.67 µg/mL<br>Root—76.40 µg/mL<br>Cherelle—68.90 µg/mL | The cocoa leaf extract had strong cytotoxic activity against the MCF-7 cell line. The hexane-partitioned fraction had the highest cytotoxic effect. | [57] |
| Theobromine extract | theobromine | U87-MG | - | Theobromine showed antiproliferative properties on the U87-MG cell line. This activity was mediated by the modulation of proteins associated with proliferative and anti-apoptotic pathways (e.g., PDE4, ERK, NF-κB, Akt/mTOR) and the activation of the pro-apoptotic pathway by JNK, p38-MAPK. | [58] |
| Phenolic cocoa powder extract | theobromine, procyanidin B1, procyanidin B2, catechin, epicatechin | HepG2 | - | The cocoa extract protected cells from oxidative stress. | [59] |

**Table 1.** *Cont.*

| Part of the Plant | Class of Compounds | Cell Line | $Ic_{50}$ | Activity/Mechanism/Effects | Ref. |
|---|---|---|---|---|---|
| Roasted cocoa, unroasted cocoa, roasted fermented cocoa, unroasted fermented cocoa, | phenols | A549 | - | Cocoa bean extracts inhibited cell proliferation, stopped the cell cycle in different phases, and increased apoptosis process in the A549 cell line. | [53] |
| Cocoa seeds | albumin, globulin, prolamin, glutelin | L5178Y | Unfermented cocoa:<br>Albumin—3140 (μg protein/mL)<br>Globulin—2890 (μg protein/mL)<br>Glutelin—580 (μg protein/mL)<br>Semi-fermented cocoa:<br>Albumin—1510 (μg protein/mL)<br>Globulin—2210 (μg protein/mL)<br>Glutelin—220 (μg protein/mL) | Antitumor activity was observed only in the albumin fraction that inhibited the growth of lymphoma cells. It may be associated with sulfur and hydrophobic amino acids. Antioxidant activity was observed in the glutelin and albumin fractions. No correlation was found between antitumor and antioxidant activity. | [51] |
| Cocoa leaf, bark, husk, fermented and unfermented shell, pith, root, cherelle | methanolic extract | MCF-7, MDA-MB-231, HepG2, HT-29, A549, HeLa, WRL-68 | 41.4 μg/mL–857.04 μg/mL | The root extract had the highest antioxidant activity, but only the cherelle extract inhibited lipid peroxidation. The leaf extract had highest antiproliferative potential. A negative correlation was found between antioxidant activity, total phenolic content, and anticancer effect. | [60] |
| Procyanidin rich cocoa powder extract | procyanidins, flavan-3-ol, catechin | OAW42, OVCAR3 | - | The procyanidin-rich extract increased the intracellular level of ROS. The treatment induced caspase-3-dependent death and the downregulation of MMP2 (a matrix metalloprotease associated with metastasis). | [61] |
| Cocoa bean husk | polyphenol, flavonoids | PC3, DU145 | - | Bean husk includes large amounts of phenolic compounds, which demonstrated antioxidant and anticancer activity on prostate cancer cell lines. | [62] |
| Cocoa pod husk | methanolic extract, lupeol, syringaresinol, catechol, squalene | MCF-7, HeLa | MCF—7:<br>161.53 μg/mL, 45.36 μg/mL, 53.91 μg/mL<br>HeLa:<br>272.58 μg/mL, 82.44 μg/mL, 120.71 μg/mL | The ethyl acetate partition derived from cocoa pod husk had moderate activity against MCF-7 cells and low activity against HeLa. The extract demonstrated high levels of lupeol, syringaresinol, catechol and squalene, which showed anticancer activity. | [63] |

The anti-inflammatory properties of *Theobroma cacao* extracts have been extensively studied in various in vitro models, revealing a complex array of mechanisms through which cocoa compounds modulate inflammatory responses. These effects are primarily attributed to the diverse bioactive compounds present in cocoa. Cocoa extracts demonstrate significant modulatory effects on inflammatory mediators. Multiple studies have shown that these extracts can reduce the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in various cell lines, including macrophages and endothelial cells [64–66]. This cytokine modulation is often accompanied by a decrease in the expression of their corresponding mRNAs, suggesting regulation at the transcriptional level [64]. The anti-inflammatory action of cocoa extracts also involves the modulation of key signaling pathways. For instance, cocoa polyphenols have been shown to inhibit the nuclear translocation of NF-κB, a crucial transcription factor in inflammatory responses [67]. Additionally, some studies report the activation of MAPK pathways by theobromine, which can lead to complex immune-modulatory effects [68]. Interestingly, cocoa extracts demonstrate the ability to influence macrophage polarization. Phenolic extracts from cocoa beans have been observed to promote a shift from pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages, thereby altering the overall inflammatory milieu [69]. Moreover, cocoa extracts have demonstrated the ability to maintain gut barrier function and reduce epithelial inflammation [70,71]. Another significant anti-inflammatory mechanism of cocoa extracts is the suppression of nitric oxide (NO) production in activated macrophages [72]. This effect is particularly important given the role of NO in perpetuating inflammatory responses.

Cocoa compounds also show protective effects on cellular structures. Theobromine, for instance, has been reported to protect membrane integrity by reducing levels of inflammatory factors and matrix metalloproteinases [73]. It is worth noting that the anti-inflammatory effects of cocoa extracts can vary depending on the specific composition and preparation method. For example, different fractions of pectin obtained from cocoa pod husks showed varying abilities to modulate macrophage functions and cytokine production [74]. In conclusion, the anti-inflammatory properties of *Theobroma cacao* extracts are mediated through multiple mechanisms, including cytokine modulation, signaling pathway regulation, macrophage polarization, and protection of cellular structures. These diverse effects underscore the potential of cocoa-derived compounds as novel anti-inflammatory agents. However, further research, particularly in vivo studies and clinical trials, is necessary to fully elucidate the therapeutic potential of these extracts in inflammatory conditions. Other studies are presented in Table 2 below.

**Table 2.** In vitro anti-inflammatory effect of *Theobroma cacao* extract.

| Part of the Plant | Class of Compounds | Cell Line | Dose | Activity/Mechanism/Effects | Ref. |
|---|---|---|---|---|---|
| Cocoa pod husk | Pectin | Mice peritoneal macrophages | 25, 50, 100, 200, 400 $\mu g \cdot mL^{-1}$ | Optimized pectin, partially deacetylated pectin, de-esterified pectin, and homogalacturonan pectin obtained from cocoa pod husk are able to modulate some macrophage functions, e.g., the secretion of pro-inflammatory factors (NO, TNF-α, IL-12) and anti-inflammatory IL-10. The optimized pectin fraction showed anti-inflammatory activity, while homogalacturonan pectin increased the number of activated macrophages. | [74] |
| Theobromine extract | Theobromine | CaCo-2 | 10–30 μM | Theobromine protected membrane structure integrity by decreasing the level of specific inflammatory factors such as cytokines and matrix metalloproteinases. | [73] |
| Cocoa powder extract | Flavonoids Polyphenols | NR8383, RAW 264.7 | 5–100 μg/mL | Cocoa extract lowered the level of TNF-α, IL-1α, IL-6, NO and MCP-1. | [64] |
| Aqueous cocoa extract | Polyphenols | J744A.1 | 0.25%, 0.05% | Cocoa extract contains compounds that suppress NO production in macrophages activated by LPS and IFN-γ. | [72] |
| Dried cocoa beans | Polyphenols | HUVEC | 25, 50, 100 ppm | Cocoa extract prevents increases in IL-6 and sVCAM-1 levels in human endothelial cells following induction by plasma from preeclamptic patients. | [65] |
| "Guiana" cocoa pods | Theobromine, Caffeine, Epicatechin, Procyanidin A1, Procyanidin A2, Procyanidin B2, Procyanidin C1 | J774-A1 | 100 μL | Guiana cocoa shows better inhibition of IL-6 production and stimulation of TNF-α secretion. | [66] |

**Table 2.** *Cont.*

| Part of the Plant | Class of Compounds | Cell Line | Dose | Activity/Mechanism/Effects | Ref. |
|---|---|---|---|---|---|
| Cocoa beans | Phenolic extract | THP-1 | 0.1–100 μM | Cocoa extract reduced the inflammatory response in M1 macrophage by increasing the secretion of anti-inflammatory cytokines. It also caused a metabolic switch from the M1 pro-inflammatory to the M2 anti-inflammatory type. | [69] |
| Cocoa bean shell | Theobromine, Caffeine, Protocatechuic acid, Catechin, Epicatechin, Procyanidin B2, Procyanidin B, Procyanidin A, Quercitin-3-O-glycosides | CaCo-2 | 10, 25, 50 μg/mL | The extracts inhibited IL-8 and TLR2, and TLR4, indicating that cocoa could interfere with oxysterol-mediated inflammation. | [67] |
| Theobromine extract | Theobromine | RAW 264.7 | 1–500 μg/mL | Theobromine activated MAPK and NF-κB signaling pathways, which enhance immune effects. Theobromine increases production of inflammatory factors by p38, JNK, and NF-κB pathways. It also upregulates the expression of iNOS. Higher expression of COX-2 causes increased PGE2 production. | [68] |
| Procyanidin-rich cocoa extract | Flavanols, Catechin, Epicatechin | CaCo-2, HT-29 | Max concetration—100 μg/mL or 10–25 μg/mL | Cocoa extract prevents loss of gut barrier function and epithelial inflammation. | [70] |
| Cocoa beans | Polyphenols | GMSM-K | 31.25, 62.5, 125, 250, 500 μg/mL | Cocoa extract inhibited *F. nucleatum*-induced inflammatory response in monocytes and oral epithelial cells. The extract improves the barrier function of oral epithelial cells. | [71] |
| Cocoa powder | Aqueous and ethanolic extract | PBMC, THP-1 | 0.5–10 μg/mL | Cocoa extracts suppressed mitogen-induced degradation of tryptophan. IFN-γ and neopterin production were strongly inhibited by extracts. | [75] |
| Cocoa extract | - | HUVEC | 6.25, 12.5, 25, 50, 100 μg/mL | The cocoa extract inhibited angiotensin-covering enzyme activity and increased the NO level. | [76] |

The in vivo anticancer effects of *Theobroma cacao* extracts have been investigated in various animal models, providing crucial insights into their potential therapeutic applications. These studies reveal a complex interplay of mechanisms through which cocoa-derived compounds exert their anticancer activities while also demonstrating protective effects against chemotherapy-induced toxicity. One of the most notable findings is the ability of cocoa extracts to enhance the efficacy of conventional chemotherapeutic agents. For instance, the combination of cocoa extract with doxorubicin has been shown to increase the overall anticancer effect, albeit with a partial increase in systemic toxicity [77]. This synergistic action suggests that cocoa compounds may potentiate the cytotoxic effects of chemotherapy on cancer cells. Furthermore, cocoa powder has demonstrated protective efficacy against vital organ damage induced by doxorubicin, without compromising its chemotherapeutic effect in BALB/c mice with Ehrlich ascites carcinoma [17]. This dual action of organ protection and maintenance of anticancer efficacy is particularly noteworthy, as it addresses one of the major challenges in cancer therapy—reducing side effects while maintaining treatment effectiveness.

The anticancer potential of cocoa extracts is further evidenced by their ability to act as carcinogenic inhibitors. This is achieved through the down-regulation of heat shock protein 90 (Hsp90) and the normalization of heat shock protein expression [78]. Given the role of Hsp90 in stabilizing various oncogenic proteins, its downregulation represents a significant mechanism by which cocoa compounds may inhibit cancer progression. Importantly, cocoa extracts have demonstrated protective effects against chemotherapy-induced organ toxicity. Studies in female BALB/c mice have shown that cocoa extract can protect vital organs such as the heart, liver, and kidney from doxorubicin-induced damage [79]. This protective effect, combined with the synergistic anticancer activity, suggests a potential role for cocoa extracts in improving the therapeutic index of conventional chemotherapy. Moreover, cocoa extracts demonstrated significant antioxidant and hepatoprotective properties in vivo. Studies using BALB/cN mice have shown that cocoa polyphenols favorably modulate cellular redox state and molecular signaling pathways associated with oxidative stress [78]. These extracts have been observed to decrease hepatic cell necrosis and suppress Pkm2, a key enzyme in cancer metabolism. Furthermore, they reduce hepatotoxicity by lowering the activity of serum transaminases and phosphatic phosphatase, indicating a protective effect on liver function [78]. The anticancer effects of cocoa are not limited to direct cytotoxicity or chemotherapy enhancement. Daily consumption of cocoa products has been shown to increase plasma flavonol concentrations in Wistar–Unilever rats, thereby enhancing the overall antioxidant potential of plasma [80]. This systemic increase in antioxidant capacity may contribute to cancer prevention by reducing oxidative stress and DNA damage. In conclusion, the in vivo anticancer effects of *Theobroma cacao* extracts are multifaceted, involving enhancement of chemotherapy efficacy, modulation of oxidative stress and cellular signaling pathways, carcinogenic inhibition, and protection against chemotherapy-induced organ toxicity. These diverse mechanisms highlight the potential of cocoa-derived compounds as adjuvants in cancer therapy, possibly improving treatment outcomes while mitigating side effects. However, further research, including clinical trials, is necessary to fully elucidate the therapeutic potential and safety profile of these extracts in human cancer treatment. Other studies are presented in Table 3 below.

**Table 3.** In vivo anticancer effect of *Theobroma cacao* extract.

| Part of the Plant | Class of Compounds | Organism | Dose | N | Exposure Time | Activity/Mechanism/Effects | Ref. |
|---|---|---|---|---|---|---|---|
| Cocoa seeds glycolic extract | polyphenols | Mice bone marrow | Genotoxicity test: 0.5, 1.0, 1.5, and 2.0 g/kg of theobroma extract Antigenotoxicity test: 2.0 g/kg of theobroma extract and 5.0 mg/kg of doxorubicin | 5 male mice and 5 female mice | 24 h 48 h | The combination of cocoa extract (2 g/kg) with doxorubicin (5 mg/kg) partially increases systemic toxicity. The extract enhances the toxic effects of doxorubicin. | [77] |
| Cocoa cake extract | polyphenols, flavonoids, gallic acid, procyanidin B1, epigallocatechin, catechin, procyanidin B2, epicatechin, epigallocatechin gallate, vanillin, p-coumaric acid, m-coumaric acid, quercetin | BALB/cN mice liver and blood samples | Cocoa extract—34.5 mg/kg Epicatechin extract—2.24 mg/kg | 4 groups of 8 female BALB/cN mice | 2 weeks | Cocoa polyphenols improved cellular redox state and molecular signaling pathways associated with oxidative stress. They also decreased hepatic cell necrosis and suppressed Pkm2. Hepatotoxicity was decreased by lowering the activity of serum transaminases and phosphatic phosphatase activity. The extract acted as a carcinogenic inhibitor by down-regulating Hsp 90 and returning the expression of Hsp70 to normal levels. | [78] |
| Cocoa nibs powder | polyphenols, alkaloids, flavonoids | Female BALB/c mice vital organs (heart, liver, kidney) and blood samples | Doxorubicin-treated mice—4.91 mg/kg Cocoa extract-treated mice—200 mg/kg Doxorubicin- and cocoa-treated mice—4.91 mg/kg of doxorubicin and 200 mg/kg of cocoa extract | 5 groups of 16 mice | 21 days | Cocoa extract protects organs against doxorubicin-induced intoxication (heart, liver, kidney). It also has a synergistic anticancer effect, enhancing doxorubicin activity. | [79] |

**Table 3.** *Cont.*

| Part of the Plant | Class of Compounds | Organism | Dose | N | Exposure Time | Activity/Mechanism/Effects | Ref. |
|---|---|---|---|---|---|---|---|
| Acticoa powder | flavonoids, flavonol, epicatechin, procyanidins | Wistar–Unilever rat prostate | Acticoa powder 24 mg/kg, Acticoa powder 48 mg/kg | 4 groups of 15 rats | 9 months | Daily consumption of cocoa products influence plasma flavonol concentration, enhancing the antioxidant potential of plasma. Treatment with Acticoa powder protects rats from CI prostate tumor. | [80] |
| Cocoa pods powder | polyphenols, flavonoids, alkaloids | BALB/c mice Ehrlich ascites carcinoma tumor cells | Doxorubicin-treated mice—4.91 mg/kg Cocoa extract-treated mice—200 mg/kg Doxorubicin and cocoa-treated mice—4.91 mg/kg of doxorubicin and 200 mg/kg of cocoa extract Pretreated mice—200 mg/kg of cocoa extract for 21 days before cancer induction. After cancer induction, the mice received 200 mg/kg of cocoa extract and 4.91 mg/kg of doxorubicin | 6 groups of 10 mice | 21 days | Cocoa powder protects against vital organ damage induced by doxorubicin without compromising chemotherapeutic effects. Moreover, it can neutralize free radicals. | [17] |

The in vivo anti-inflammatory effects of *T. cacao* extracts have been extensively studied in various animal models, revealing a wide array of mechanisms through which cocoa-derived compounds modulate inflammatory responses. These studies provide crucial insights into the potential therapeutic applications of cocoa extracts in inflammatory conditions. Cocoa polyphenols demonstrate significant anti-inflammatory activity through multiple pathways. In ICR mice, cocoa polyphenols have been shown to lower the expression of cyclooxygenase-2 (COX-2), a key enzyme in the inflammatory process. Furthermore, they inhibit the nuclear translocation of p65, a subunit of NF-κB, and prevent the degradation of IκBα, thus suppressing the NF-κB signaling pathway, which is central to inflammatory responses [81]. Studies in C57BL/6 mice have revealed that cocoa supplementation decreases the mRNA levels of pro-inflammatory cytokines such as TNF-α and IL-6, as well as nitric oxide synthase. Additionally, cocoa extracts inhibit the activity of enzymes associated with inflammation, including phospholipase A2 and cyclooxygenase-2 [82]. These effects collectively contribute to a comprehensive anti-inflammatory action. The anti-inflammatory properties of cocoa extracts extend to wound healing. In rabbits, cocoa pod extract has been shown to accelerate wound healing, likely due to its rich content of polyphenols, flavonoids, and tannins [83]. This suggests potential applications in dermatological conditions and post-surgical recovery. Cocoa extracts also demonstrate significant metabolic effects that indirectly contribute to their anti-inflammatory action. In Wistar rats, a diet enriched with cocoa bean phenolic extract led to a decrease in fat tissue, inhibition of protein tyrosine phosphatase 1B (PTP1B), attenuation of hepatic steatosis, and improved serum lipid profiles [84]. These metabolic improvements can reduce systemic inflammation associated with obesity and metabolic disorders. The immunomodulatory effects of cocoa are further evidenced by studies in Wistar rats, where cocoa-enriched diets caused a decrease in prostaglandin E2 (PGE2) production and prevented imbalances in T-cell proportions [85]. This suggests that cocoa compounds can modulate both innate and adaptive immune responses. Cocoa extracts have also shown promise in specific inflammatory conditions. In a rat model of periodontitis, cocoa pod husk extract increased alveolar bone regeneration by enhancing osteoblast numbers and bone morphogenetic protein-2 (BMP-2) expression [86]. This indicates potential applications in inflammatory bone disorders. Ethanol extracts of cocoa stem bark have been shown to reduce inflammation in Wistar rats by lowering neutrophil migration and reducing inflammatory mediator production [85]. Cocoa extracts also demonstrate antioxidant properties that complement their anti-inflammatory effects. Polyphenol-enriched cocoa leaf extracts inhibit xanthine oxidase and angiotensin-converting enzymes in male Wistar rats, indicating potential in preventing oxidative stress [87]. Finally, dietary studies in female Wistar rats have shown that cocoa-containing diets lower serum levels of TNF-α and inducible nitric oxide synthase (iNOS) activity, while also decreasing colon cell infiltration [88]. This suggests potential applications in inflammatory bowel conditions. In conclusion, the in vivo anti-inflammatory effects of Theobroma cacao extracts are mediated through multiple mechanisms, including the modulation of inflammatory enzymes, suppression of pro-inflammatory cytokines, inhibition of NF-κB signaling, improvement of metabolic parameters, and enhancement of wound healing. These diverse effects underscore the potential of cocoa-derived compounds as novel anti-inflammatory agents with wide-ranging therapeutic applications. However, further research, including clinical trials, is necessary to fully elucidate the efficacy and safety of these extracts in human inflammatory conditions. Other studies are presented in Table 4 below.

**Table 4.** In vivo anti-inflammatory effect of *Theobroma cacao* extract.

| Part of the Plant | Class of Compounds | Organism | Dose | N | Exposure Time | Activity/Mechanism/Effects | Ref. |
|---|---|---|---|---|---|---|---|
| Cocoa polyphenols from cocoa powder | Polyphenols, Gallic acid, Epicatechin. | Skin from ears of ICR mice | With 12-O-tetradecanoylphorbol-13-acetate:<br>4 mg/kg<br>20 mg/kg<br>40 mg/kg<br>200 mg/kg<br>Without 12-O-tetradecanoylphorbol-13-acetate:<br>200 mg/kg | Groups of 6 female mice | 5 h | Cocoa polyphenols lower the activity of COX-2 expression, inhibit the activation of MAPK and NF-κB pathways. | [84] |
| Unsweetened cocoa powder | Polyphenols. | Male C57BL/6 mice | 80 mg/g of unsweetened cocoa powder | Low-fat diet 23 mice<br>High-fat diet 21 mice<br>High-fat diet cocoa treated 24 mice | 18 weeks | Cocoa supplementation decreases adipose tissue inflammation by downregulating genes associated with NF-κB. | [82] |
| Cocoa pod extract | Polyphenols, Flavonoids, Tannins. | Rabbit | Concentration of cocoa:<br>0%, 5%, 10%, 15% | 36 male rabbits | 3 days<br>5 days<br>7 days | Cocoa pod extract can accelerate the speed of wound healing. | [83] |
| Cocoa bean phenolic extract | Phenols | Wistar rats | Raw cocoa bean: 2.25% of diet;<br>Roasted cocoa bean: 2.45% of diet;<br>Flavan-3-ol: 0.114% of diet | 5 groups of 8 male rats | 4 weeks | A diet enriched with cocoa bean extract causes fat tissue reduction, PTP1B inhibition, hepatic steatosis attenuation, ROS protection, and improved serum lipid profile, and increases serum ACl. It shows anti-obesity properties. | [89] |
| Leaf extracts | Tannins, Phenols, Saponins, Terpenoids, Flavonoids, Glycosides. | African earthworms (*Pheretima posthuma*) | 10, 25, 50 mg/mL | - | - | Cocoa leaf extracts have antioxidant and anthelmintic activity. | [90] |

**Table 4.** *Cont.*

| Part of the Plant | Class of Compounds | Organism | Dose | N | Exposure Time | Activity/Mechanism/Effects | Ref. |
|---|---|---|---|---|---|---|---|
| Cocoa pods powder | Flavonoids, Alkaloids, Tannins, Saponins. | Wistar rats | Fermented cocoa: 150 mg/kg 300 mg/kg | 35 rats | 21 days | Anti-hyperglycemia compounds were discovered in fermented cocoa polyphenol extracts. | [85] |
| Cocoa pod husk extract | Polyphenols | Wistar rats | Cocoa pod husk extract 100 mg/mL | 24 male rats | 7 days 14 days | Cocoa pod husk extract increases alveolar bone regeneration in periodontitis by increasing osteoblast numbers and BMP-2 expression. | [86] |
| Stem bark extract | - | Wistar rats | Cocoa stem bark: Extract 250 mg/kg Ethylacetate fraction 65, 125 and 250 mg/kg | 7 groups of 10 rats | 72 h | Ethanol extracts of cocoa stem bark reduce inflammation by decreasing inflammatory mediator production (TNF-$\alpha$). | [81] |
| Cocoa leaves | Polyphenols | Male Wistar rats | 10, 20, 30, 40 µg/mL | 3 male rats | 7 days acclimatization | Polyphenol-enriched cocoa leaf extract inhibits xanthine oxidase and angiotensin 1-converting enzyme. Leaf extract may be useful in preventing oxidative stress. | [87] |
| Cocoa | Procyanidin B2 Catechin, Epicatechin, Isoquercetin, Quercetin | Female Wistar rats | 5% cocoa diet | 4 groups of 12 rats | 3 weeks | Cocoa-containing diet shows anti-inflammatory potential. In addition, lower colon cell infiltration was observed. | [88] |

While numerous in vitro and in vivo studies have demonstrated the anticancer and anti-inflammatory potential of Theobroma cacao, clinical evidence has been more limited. However, several important clinical trials have specifically investigated these properties in human subjects.

A particularly significant clinical investigation examining the anticancer effects of Theobroma cacao is the "Effects of Chocolate Consumption in Elderly Patients With Cancer in Palliative Care" trial (NCT04367493). This randomized intervention study specifically focused on elderly cancer patients receiving palliative care, providing direct clinical evidence of cocoa's effects in cancer patients. The study design involved three groups of 15 subjects each: a control group, a 55% cocoa intervention group, and a white chocolate intervention group. The trial measured multiple key parameters, including inflammatory markers through serum interleukin-6 levels, oxidative stress parameters via reduced glutathione (GSH) levels and ascorbic acid quantification, lipid peroxidation through malonaldehyde measurements, and DNA damage assessment using 8-hydroxy-2′-deoxyguanosine levels. Additionally, the study evaluated quality of life measures using the EORTC-QLQ-C30 questionnaire.

The COcoa Supplement and Multivitamin Outcomes Study (COSMOS; NCT02422745) represents a large-scale investigation into cocoa's potential anticancer properties. This randomized clinical trial examined whether daily supplementation with cocoa extract (containing 500 mg/day of flavanols, including 80 mg of (−)-epicatechin) could reduce cancer risk in older adults. The scale and design of this study provide valuable insights into the potential role of cocoa flavanols in cancer prevention.

An ancillary study to COSMOS (NCT05510375) specifically investigated the anti-inflammatory aspects of cocoa supplementation through the examination of inflammaging—the chronic, low-grade inflammation associated with aging. This study is particularly relevant, as it bridges the gap between laboratory findings on cocoa's anti-inflammatory properties and their clinical manifestation in human subjects.

These clinical trials represent critical steps in validating the anticancer and anti-inflammatory properties of Theobroma cacao observed in preclinical studies. They provide important insights into the translation of laboratory findings to human applications and suggest potential therapeutic roles for cocoa compounds in cancer care and inflammation management.

## 6. Antitumor and Anti-Inflammatory Effects of *Theobroma cacao* Extract in an In Vitro and In Vivo Model in Combination with Nanoparticles

Nanoparticles have emerged as a promising platform for enhancing cancer therapy due to their unique physicochemical properties and ability to be functionalized with various targeting moieties and therapeutic agents. Their small size allows them to penetrate biological barriers and accumulate preferentially in tumor tissues through the enhanced permeability and retention (EPR) effect [91,92]. Nanoparticles can be engineered to carry multiple drugs simultaneously, enabling combination therapies and overcoming drug resistance mechanisms. Moreover, stimuli-responsive nanoparticles can be designed to release their payload in response to specific tumor microenvironment conditions, such as pH or enzyme activity, thereby improving the therapeutic index and reducing systemic toxicity. The surface of nanoparticles can also be modified with imaging agents, allowing for theranostic applications that combine therapy and diagnostics [93–95].

In the context of inflammatory disorders, nanoparticles offer several advantages for targeted drug delivery and the modulation of the inflammatory response. Their ability to encapsulate and protect anti-inflammatory compounds can enhance drug stability and prolong circulation time. Nanoparticles can be designed to target specific cells or tissues involved in inflammation, such as activated endothelial cells or macrophages, through the incorporation of appropriate ligands. This targeted approach can improve the efficacy of anti-inflammatory treatments while minimizing off-target effects [96–98]. Additionally, certain types of nanoparticles, such as metal nanoparticles, have inherent anti-inflammatory

properties that can complement the effects of loaded drugs. The versatility of nanoparticle platforms allows for the development of multifunctional systems that can simultaneously deliver therapeutics, modulate the immune response, and provide real-time monitoring of treatment efficacy [99,100].

The literature is limited in relation to biological studies on the anticancer and anti-inflammatory effects of *Theobroma cacao* extract in combination with nanoparticles, which is why further work confirming this synergistic effect is so important. Table 5 below shows current studies that demonstrate combinations of *Theobroma cacao* extract in combination with nanoparticles in the context of biological research.

**Table 5.** Biological effect of nanoparticles with *Theobroma cacao* extract.

| Part of the Plant | Nanoparticles | Dose | Activity/Mechanism/Effects | Ref. |
|---|---|---|---|---|
| Cocoa powder | Silver nanoparticles | 25, 50 mg/mL | An insignificant cytotoxic effect on human dermal fibroblast cells has been demonstrated. | [101] |
| Cocoa seeds | Palladium/Copper (II) oxide nanoparticles | 15 mL of cocoa seed extract | The chemical components of cocoa (e.g., catechin, phenolic acids) were identified as stabilizing, reducing and capping agents. This method of obtaining nanoparticles shows lower toxicity and is environmentally friendly. | [102] |
| Cocoa powder | Gold nanoparticles | 0.1, 1, 2.5, 10, 50 mg/mL | Gold nanoparticles are not toxic for human dermal fibroblast. | [103] |
| Cocoa seed extract | Gold nanoparticles | 200, 250, 270, 300 μL | Anisotropic b-AuNPs (derived from *T. cacao* [cocoa] seed extract) demonstrated excellent photothermal properties in A431 epidermal cancer cells using a laser power density of 6 W/cm$^2$. The b-AuNP nanoparticles exhibited near-infrared absorbance at 700–1000 nm, facilitating an effective photothermal therapy against A431 cancer cells. | [104] |
| Cocoa bean extract | Graphene nanoparticles | $IC_{50}$—31.2 μg/mL | The cytotoxicity studies showed that the synthesized CSE-GQDs exhibited dose-dependent toxicity on human breast cancer (MCF-7) cell lines. | [105] |

## 7. Conclusions and Future Perspectives

In conclusion, *Theobroma cacao* appears to offer complex and promising therapeutic potential based on its anti-inflammatory and anticancer properties, which can be attributed to its polyphenol, flavonoid, or alkaloid content. The extracts have demonstrated significant antiproliferative, proapoptotic and potentially antimetastatic properties in various cancer cell lines and animal models, both alone and in combination with nanoparticles. An important aspect of *T. cacao* extracts is that they are able to demonstrate potential synergistic effects with conventional chemotherapeutic agents and protect against chemotherapy-induced organ toxicity. This dual action suggests a unique role for cacao-derived compounds in enhancing the efficacy of cancer treatment, while mitigating its side effects.

Further research into combination therapies, particularly the synergistic effects between *Theobroma cacao* extracts and conventional treatments, may open up new avenues in cancer therapy and the treatment of chronic inflammatory diseases. Although several mechanisms of action have been identified, a deeper understanding of the molecular pathways involved in the anticancer and anti-inflammatory effects of *Theobroma cacao* extracts is needed. The integration of nanotechnology with *T. cacao* extracts represents a particularly promising direction for future research. Nanoparticle-based delivery systems offer several

advantages, including enhanced bioavailability, targeted delivery, and controlled release of bioactive compounds. Future studies should focus on optimizing nanoformulations, investigating their biodistribution and safety profiles, and evaluating their long-term efficacy in various therapeutic applications. The development of new formulations and delivery systems may enhance the bioavailability and targeted delivery of bioactive cacao compounds, potentially improving their therapeutic efficacy. Long-term research into the effects of cocoa consumption on the prevention of cancer and chronic inflammation is warranted, as is research into how individual genetic variants may influence the response to *Theobroma cacao* extracts, potentially paving the way for personalized therapeutic strategies. The potential mechanism of action of *Theobroma cocoa* extract is shown in Figure 4.

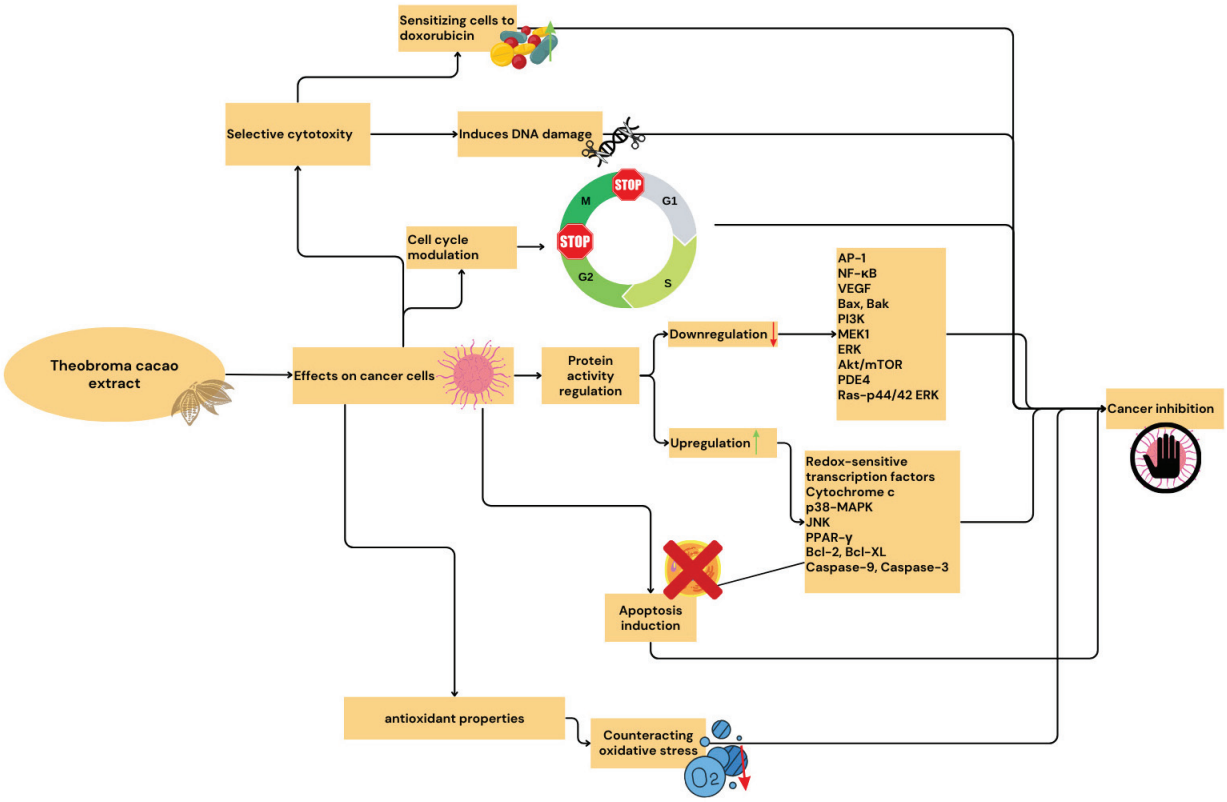


**Figure 4.** The potential mechanism of action of cocoa extract (Canva, version 1.97.0.0, Canva Inc., Sydney, Australia, https://www.canva.com accessed on 26 June 2024).

In summary, *Theobroma cacao* has long played a key role in the global economy. Recent research shows that it can play a key role in health protection. The economic importance of cocoa is obvious in the food industry, and its medicinal properties, especially its anti-inflammatory and anticancer properties, offer promising opportunities for health. Continuous research and development in the cultivation and practical use of cocoa may further increase its share in both the global economy and biomedicine in the future. Its multifaceted biological activities, combined with a long history of human consumption and enhanced effects when combined with nanoparticles, make it an attractive candidate for further research.

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## Abbreviations

| | |
|---|---|
| GDP | Gross domestic product |
| ALT | Alanine transaminase |
| BDNF | Brain-derived neurotrophic factor |
| HRS | Hodgkin and Reed-Sternberg |
| THP-1 | Human acute monocytic leukemia |
| TAMs | Tumor-associated macrophages |
| ROS | Reactive oxygen species |
| MMP2 | Matrix mettaloproteinase-2 |
| MAPK | Mitogen activated protein kinases |
| NO | Nitric oxide |
| TNF | Tumor necrosis factor |
| COX-2 | Cyclo-oxygenase-2 |
| PTP1B | Protein tyrosine phosphatase 1B |
| PGE2 | Prostaglandin E2 |
| BMP-2 | Bone morphogenetic protein-2 |
| EPR | Permeability and retention effect |

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*Review*

# Flavonoids: Antioxidant Powerhouses and Their Role in Nanomedicine

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**Abstract:** This study emphasizes the critical role of antioxidants in protecting human health by counteracting the detrimental effects of oxidative stress induced by free radicals. Antioxidants—found in various forms such as vitamins, minerals, and the phytochemicals abundant in fruits and vegetables—neutralize free radicals by stabilizing them through electron donation. Specifically, flavonoid compounds are highlighted as robust defenders, addressing oxidative stress and inflammation to avert chronic illnesses like cancer, cardiovascular diseases, and neurodegenerative diseases. This research explores the bioactive potential of flavonoids, shedding light on their role not only in safeguarding health, but also in managing conditions such as diabetes, cancer, cardiovascular diseases, and neurodegenerative diseases. This review highlights the novel integration of South African-origin flavonoids with nanotechnology, presenting a cutting-edge strategy to improve drug delivery and therapeutic outcomes. This interdisciplinary approach, blending traditional wisdom with contemporary techniques, propels the exploration of flavonoid-mediated nanoparticles toward groundbreaking pharmaceutical applications, promising revolutionary advancements in healthcare. This collaborative synergy between traditional knowledge and modern science not only contributes to human health, but also underscores a significant step toward sustainable and impactful biomedical innovations, aligning with principles of environmental conservation.

**Keywords:** antioxidant; anti-inflammatory; flavonoids; natural products; nanoparticles; South Africa

## 1. Introduction

Antioxidants are a class of compounds that play a crucial role in maintaining human health by protecting the body from oxidative stress and the damage caused by harmful molecules known as free radicals. These molecules are highly reactive and can lead to cellular damage, DNA mutations, and various diseases if not properly controlled [1]. Antioxidants work by neutralizing free radicals, preventing them from causing harm to cells and tissues. They do this by donating electrons to stabilize these highly reactive molecules, thereby reducing their potential for damage [2]. There are numerous types of antioxidants, including vitamins (such as vitamin C and vitamin E), minerals (like selenium and zinc), and the phytochemicals found in fruits, vegetables, and other plant-based foods. Each type of antioxidant has its unique mechanism of action and may be more effective against specific types of free radicals [3]. Consuming antioxidant-rich foods is associated with a lowered risk of chronic diseases, such as heart disease, cancer, and neurodegenerative disorders [4].

Antioxidants are believed to help combat inflammation, support the immune system, and slow the aging process by reducing oxidative stress [4]. However, it is important to maintain a balanced intake of antioxidants as excessive supplementation can have negative effects and potentially disrupt the delicate balance of oxidative reactions within the body. A well-rounded diet that includes a variety of fruits, vegetables, and whole grains is typically the best way to ensure a healthy intake of antioxidants [5]. Antioxidants are essential

components of a healthy diet and play a significant role in protecting the body from the damaging effects of oxidative stress and free radicals, ultimately promoting overall human health and well-being [3].

The Phyllanthaceae family, a large group of flowering plants with approximately 1301 species, includes the genus Phyllanthus. This genus is widely distributed across the tropical and subtropical regions of Africa, America, Asia, and Australia [6,7]. Historically, Phyllanthus *Cicca* and *P. Kirganelia* have been the most significant species within the Phyllanthaceae family for treating various human ailments [8]. Phyllanthus plants are rich in phytochemical components important in pharmacology, including terpenoids, alkaloids, and polyphenolic substances such as phenolic acids, flavonoids, coumarins, lignins, stilbenes, and anthocyanins [9]. In South African flora, many plant species are rich sources of flavonoids, and traditional medicine systems have utilized these bioactive compounds for centuries [10]. Flavonoids are well known for their potent antioxidant properties, which play a crucial role in protecting cells and tissues from oxidative stress. This is essential for maintaining overall health and preventing various chronic diseases [11]. A high consumption of fruits and vegetables has been shown to correlate with a reduced incidence and mortality rate of various degenerative diseases, including cancer, cardiovascular disease, and immune dysfunction, according to multiple human cohort and case–control studies [12–14]. Alongside the vitamins and minerals found in fruits and vegetables, phytochemicals such as flavonoids and other phenolics may contribute to these protective benefits. Estimating flavonoid intake is an essential step toward understanding their protective effects against chronic diseases [15]. Although flavonoids are significant dietary antioxidants, the limited data on their comprehensive food composition has hindered the assessment of dietary intake in populations. A study by Chun et al. indicated that the highest daily mean intake of flavonoids came from tea (157 mg), citrus fruit juices (8 mg), wine (4 mg), and citrus fruits (3 mg) [16]. The efficacy of flavonoids is highly dependent on their bioavailability and metabolism. The concentration needed to achieve therapeutic effects can vary based on the form of flavonoid, how it is consumed, and individual differences in metabolism [11]. Flavonoid supplements are associated with numerous health benefits, including antioxidant, anti-inflammatory, and anticancer properties [17]. Regular consumption of flavonoid-rich supplements can help in reducing the risk of chronic diseases such as cardiovascular diseases, diabetes, and certain types of cancer [18]. Common flavonoid supplements include quercetin, EGCG, and genistein, which are available in various forms such as capsules, tablets, and powders. These supplements are often combined with other vitamins and minerals to enhance their health effects [19]. Kaempferol, also known as 3,4′,5,7-tetrahydroxyflavone, is a flavonol with hydroxyl groups at positions 3, 5, 7, and 4′ [20]. It can be found in various sources such as broccoli, cabbage, gooseberries, grapes, kale, strawberries, tomatoes, citrus fruits, brussels sprouts, and several medicinal plants. Kaempferol has demonstrated strong binding affinities with EGFR, surpassing those of the native ligand and even gefitinib [21]. Inflammation is a key factor in the development of many diseases, including cardiovascular diseases and cancer. Flavonoids have demonstrated anti-inflammatory properties that can help mitigate the inflammatory processes in the body [20]. The antioxidant and anti-inflammatory activities of flavonoids have also been associated with a reduced risk of cancer development. These compounds may inhibit the growth of cancer cells and promote apoptosis, making them a potential component in cancer prevention [22]. Flavonoids have also been linked to improving cardiovascular health due to their ability to reduce oxidative stress and inflammation in blood vessels. This can help prevent atherosclerosis and reduce the risk of heart diseases [23]. They have also shown promise in protecting the brain from oxidative damage and inflammation, potentially reducing the risk of neurodegenerative diseases such as Alzheimer's and Parkinson's [24]. Many studies have indicated that flavonoids may help manage diabetes by improving insulin sensitivity and reducing inflammation, making them valuable in the management of this chronic condition [25].

The study of flavonoids' bioactive properties may lead to the development of new pharmaceuticals or nutraceuticals for various health conditions, offering alternatives or complementary approaches to traditional therapies. By elucidating and harnessing the antioxidant and anti-inflammatory potential of flavonoids, researchers can contribute to the development of strategies and products that have a positive impact on human health and well-being [26]. Nanomedicine employs biocompatible nanomaterials for therapeutic interventions in the treatment of diverse diseases. Flavonoids derived from plant materials serve a dual role as reducing and electrostatic stabilizing agents, facilitating the green synthesis of metal nanomaterials. These synthesized nanomaterials demonstrate effective applications in treating cancer cells and combating pathogenic microbes [27]. Flavonoid-loaded nanoparticles have demonstrated effectiveness in preventing bacterial cell damage in oxidizing environments. The prolonged antioxidant effect observed in the oxidizing medium can be attributed to the gradual release of flavonoids from the nanoparticles. This is in contrast to free flavonoids, which are largely depleted in the initial phase due to their rapid consumption [28]. Encapsulating flavonoids in nanoparticles enhances their effects on cytokine production in vitro and in vivo. This is due to improved cellular uptake as nanoparticles, with their small size and surface traits, facilitate membrane interaction, resulting in higher intracellular flavonoid concentrations. Additionally, encapsulation protects flavonoids, increasing their stability and bioavailability, thereby preventing degradation and improving absorption and circulation [29]. Understanding the interaction between cell membranes and nanoparticles is crucial for evaluating the efficiency of flavonoid-loaded nanoparticles. Hydrophobic interactions between polyphenols and liposome membranes, for instance, enhance the encapsulation and delivery of polyphenols. This interaction facilitates efficient loading into liposomes, protecting the polyphenols from degradation and enhancing their stability during storage and transport [29].

This review focuses on novel studies about the treatment potential of flavonoids and their fusion with nanotechnology, presenting a cutting-edge approach that could revolutionize drug delivery and improve therapeutic outcomes. Integrating traditional wisdom with contemporary techniques not only enriches our comprehension of these compounds, but it also facilitates their efficient utilization in nanomedicine.

## 2. South African Flora: A Rich Source of Flavonoids

The geographical distribution of key plants in South Africa is remarkably diverse, and this diversity extends to the presence of various flavonoids within these plants (Table 1). Among these botanical treasures, *Rooibos* stands out as a beloved South African plant, thriving in the Western and Northern Cape regions. *Rooibos* is renowned for its rich flavonoid composition, and it is particularly characterized by flavonols like quercetin and aspalathin [30]. Indigenous South African communities have traditionally used *rooibos*, such as the Khoi and San, for centuries, as shown in Table 1. It has been consumed as an herbal infusion for its refreshing taste and potential health benefits [31]. Rooibos tea is abundant in flavonoids like aspalathin and nothofagin, which are known for their potent antioxidant properties. These flavonoids help neutralize free radicals, reducing oxidative stress and preventing cellular damage [31]. Research indicates that the flavonoids in rooibos tea can enhance lipid profiles and lower blood pressure, contributing to cardiovascular health. Regular consumption of rooibos tea may aid in the prevention of cardiovascular diseases. Aspalathin, a distinctive flavonoid in rooibos, has shown promise in regulating blood sugar levels by improving insulin sensitivity and reducing fasting glucose levels, making it a beneficial dietary supplement for diabetes management [32]. *Cyclopia intermedia* is indigenous to South Africa, and it particularly thrives in the fynbos biome spanning the Western and Eastern Cape provinces. This area boasts diverse flora and rich biodiversity, making it a prime habitat for the *Cyclopia* species, which are widely cultivated for the production of honeybush tea [33]. This plant is also utilized to create a sweet herbal infusion known for its therapeutic effects, including soothing coughs and easing respiratory issues like tuberculosis, pneumonia, and catarrh [33]. In the arid landscapes of South

Africa, another notable indigenous species, *Hoodia gordonii*, finds its habitat. While *Hoodia gordonii* is primarily recognized for its appetite-suppressing properties, it also possesses flavonoids such as quercetin and kaempferol [34]. *Hoodia gordonii* has a history of use by San Bushmen in South Africa as an appetite suppressant during long hunting trips [35]. *Hoodia* contains flavonoid compounds that might contribute to weight management by reducing caloric intake and supporting weight loss. Although mainly recognized for its appetite suppression, it is also thought to boost energy levels, though scientific evidence for this effect is limited [36]. The South African region is also home to various cycad species, and these plants often harbor flavonoids among their diverse phytochemical constituents. Traditional uses of cycads among South African indigenous communities include the consumption of certain species as a starchy food source, although this practice has raised conservation concerns [37]. Notably, *Pelargonium sidoides*, commonly referred to as *Umckaloabo*, is a medicinal plant native to South Africa. It is celebrated for its flavonoid-rich composition, particularly in the form of quercetin derivatives [38]. *Pelargonium sidoides* has been traditionally used by South African communities, including the Zulu and Xhosa, for its medicinal properties in the treatment of respiratory infections [38]. Additionally, the South African region is host to *Sceletium tortuosum*, a plant with a historical and cultural significance. *Sceletium tortuosum*, which is native to this region, contains mesembrine alkaloids, which are a distinctive type of flavonoid [39]. *Sceletium tortuosum* has a long history of traditional use among indigenous South African communities, such as the San and Khoi, as a mood-enhancing and stress-reducing herb [40]. These diverse plant species and their flavonoid-rich compositions underscore the unique botanical wealth of South Africa and its importance in the study of flavonoids within a variety of ecological and pharmacological contexts.

**Table 1.** Diversity of South African plant species and their flavonoid-rich compositions.

| Plant Species | Flavonoids | Traditional Uses | References |
|---|---|---|---|
| *Rooibos* (*Aspalathus linearis*) | Flavonols like quercetin and aspalathin | A traditional herbal infusion that is consumed for its refreshing taste and potential health benefits | [30,31] |
| *Cyclopia intermedia* (Honey bush) | Various flavonoids | Soothing coughs and easing respiratory issues like tuberculosis, pneumonia, and catarrh | [33] |
| *Hoodia gordonii* | Quercetin, kaempferol | Appetite-suppressing properties | [34,35] |
| *Cycads* (Various species) | Various flavonoids among diverse phytochemical constituents | Consumption as a starchy food source, raising conservation concerns | [37] |
| *Pelargonium sidoides* (*Umckaloabo*) | Quercetin derivatives | Treating respiratory infections | [38] |
| *Sceletium tortuosum* | Mesembrine alkaloids (distinctive flavonoid type) | Mood enhancement and stress reduction | [40] |

## 3. Antioxidant Potential of Flavonoids

Antioxidants, such as flavonoids, in preventing oxidative stress-related diseases, are supported by various studies that highlight their impact on mitigating cellular damage and disease development. Free radicals, or ROS, are inherently unstable molecules generated by living organisms as part of routine cellular metabolism. When there is a tilt in the equilibrium between oxidants and antioxidants in favor of oxidants, this condition is referred to as oxidative stress [41]. Flavonoids are potent antioxidants with multiple mechanisms that contribute to their effectiveness in neutralizing free radicals and preventing oxidative damage in the body. Flavonoids act as free radical scavengers, neutralizing reactive oxygen species (ROS) through their ability to donate hydrogen atoms or electrons [42]. In cardiovascular diseases, flavonoids help to improve endothelial function and reduce inflammation by scavenging free radicals [43]. Some flavonoids exhibit metal chelation properties, binding

to transition metals such as iron and copper, which can otherwise catalyze the generation of free radicals. This chelation inhibits metal-induced oxidative stress [44]. Flavonoids possess the capability to hinder the activity of enzymes that generate reactive species like xanthine oxidase and NADPH oxidase, thereby diminishing the production of ROS [45]. Additionally, flavonoids have the ability to restore other antioxidants such as vitamin C and vitamin E, thus bolstering the body's overall antioxidant defense system [46]. In vitro studies focusing on South African flavonoids have unveiled their robust antioxidant capabilities through various experimental setups and assessments. Research conducted by Oyedemi et al. examined the antioxidant potential of South African plant extracts rich in flavonoids using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assays, demonstrating significant radical scavenging activity [47]. Studies by Firuzi et al. further explored the antioxidant activity of South African flavonoids through ferric-reducing antioxidant power (FRAP) assays, indicating their capacity to reduce ferric ions, a marker of antioxidant potential [48]. In an investigation by Aderogba et al., South African plant extracts, particularly flavonoid-rich ones, were tested for their total antioxidant capacity, demonstrating significant antioxidant properties through various in vitro assays [49]. The fermented leaves and stems of *Cyclopia intermedia* are utilized to create Honeybush tea, a traditional herbal infusion native to South Africa. This plant is also processed into a sweet herbal drink known for its therapeutic properties, including soothing coughs and easing respiratory conditions like tuberculosis, pneumonia, and catarrh. Honeybush tea is reputed to be low in tannins and being caffeine-free while also containing a range of antioxidants [33]. Marnewick et al. explored the antioxidant characteristics of several South African herbal teas, notably *rooibos* (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.), both of which contain distinctive flavonoids like aspalathin and mangiferin [31]. Another study indicated that honeybush tea, brewed from *Cyclopia intermedia*, contains a substantial quantity of polyphenols, which contribute to its various physiological benefits. Traditionally used as a medicinal drink, honeybush tea is now gaining recognition for its health-promoting properties, including its antioxidant activity and low caffeine content [50]. Several roles and the functional significance of flavonoids are illustrated in Figure 1.

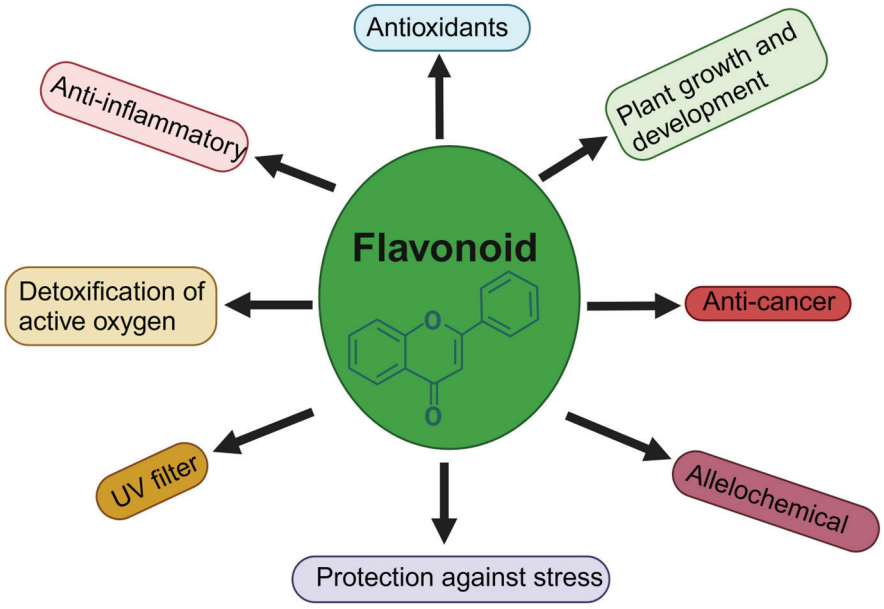


**Figure 1.** The role and functional significance of flavonoids: an illustrated overview.

### *3.1. Anti-Inflammatory and Anti-Oxidative Flavonoids*

Oxidative stress stands as the primary underlying factor for conditions like cancer, diabetes, arthritis, rheumatoid arthritis, neurodegenerative disorders, hypertension, atherosclerosis, and chronic inflammatory ailments [51]. Research by Ciumarnean et al. suggested

a protective association between flavonoid intake and a reduced risk of cardiovascular diseases, attributing this effect to the antioxidant properties of flavonoids, particularly their ability to reduce oxidative stress in the cardiovascular system [52]. As shown in Figure 2, in the development of cardiovascular disease, a crucial inflammatory process occurs. Various research studies have connected inflammatory and immune responses to the vascular damage linked with atherosclerosis [53]. Oxidative stress triggers the elevation of enzymes like cyclooxygenase (COX) and lipoxygenase (LPO), which contribute to the release of chemokine factors such as interleukins [54,55]. Notably, certain polyphenols, especially quercetin, have demonstrated the ability to inhibit COX and LPO enzymes, potentially curbing this inflammatory cascade [56]. Polyphenols exhibit anti-inflammatory characteristics and can regulate inflammatory mediators in individuals at a heightened risk of cardiac disease (Figure 2) [57]. Flavonoids have become a focal point for specialists due to their diverse potential benefits. Research on these compounds is intricate and time-consuming because of their varied molecular structures. However, several studies have proposed that dietary polyphenols could be advantageous as a supplementary approach in preventing and treating chronic inflammatory conditions [57,58]. Studies, such as those by Meng-zhen, demonstrate the potential of flavonoids in mitigating oxidative stress and inflammation in neurodegenerative diseases, thereby suggesting a role in preventing conditions like Alzheimer's and Parkinson's [59]. Flavonoids have shown promise in cancer prevention due to their antioxidative and anti-inflammatory properties. Flavonoids function as signaling molecules that regulate cell growth, trigger apoptosis, and decrease the production of reactive oxygen species. These capabilities offer potential alternative strategies for both treating and preventing cancer [60]. Quercetin, a naturally occurring flavonoid present in numerous fruits and vegetables, has demonstrated diverse biological effects in experimental models. Among its observed effects is the alleviation of key symptoms associated with asthma, including bronchial hyperactivity, mucus production, and airway inflammation (Figure 2) [61]. The impact of flavonoids on oxidative stress and diabetes has been explored in previous studies, indicating that these antioxidants can help reduce oxidative stress in diabetes by scavenging free radicals and modulating key signaling pathways [62]. The World Health Organization identified cardiovascular disease (CVD) as the leading cause of death worldwide in 2019. Previous research has indicated differences in the composition of the gut microbiome between individuals with and without CVD, and it has been suggested that flavonoids may reduce the risk of heart disease [63].

The body initiates inflammation as a normal biological response to tissue injury, microbial pathogen infection, and chemical irritation. This process involves the migration of immune cells from blood vessels, releasing mediators at the site of damage. Following this, inflammatory cells are recruited, and there is a release of ROS, reactive nitrogen species (RNS), and proinflammatory cytokines to eliminate foreign pathogens and aid in tissue repair. Typically, normal inflammation is a quick and self-limiting process, but if resolution is aberrant and inflammation persists, it can contribute to the development of various chronic disorders [64]. Persistent inflammation and oxidative stress play crucial roles in the development of obesity, cancer, and neurodegenerative diseases. Flavonoids are gaining recognition as promising therapeutic agents for these conditions, given their anti-inflammatory and antioxidant properties [60]. Several flavonoids, including hesperidin, apigenin, luteolin, and quercetin, have been documented for their anti-inflammatory and analgesic properties. These flavonoids may specifically impact the function of enzyme systems that play a crucial role in the initiation of inflammatory processes, particularly tyrosine and serine-threonine protein kinases [65]. Flavonoids exert their kinase inhibition by competitively binding with ATP at the catalytic sites of these enzymes. The targeted enzymes play crucial roles in signal transduction and cell activation processes, particularly within the immune system's cells. Research indicates that flavonoids have the capability to inhibit the expression of isoforms of inducible nitric oxide synthase, cyclooxygenase, and lipoxygenase. These enzymes are responsible for generating significant amounts of nitric oxide, prostanoids, leukotrienes, and other inflammatory mediators, including cytokines,

chemokines, and adhesion molecules [66]. Elisha et al. [67] conducted an in vitro study on acetone leaf extracts from nine selected plants, revealing significant antioxidant, anti-inflammatory, and anti-arthritic properties. This research aligns with traditional assertions regarding the efficacy of these South African medicinal plants in addressing conditions such as arthritis, infections, rheumatism, and inflammation. The identified plants exhibit potential for becoming therapeutic agents in the treatment of inflammation and other autoimmune disorders.

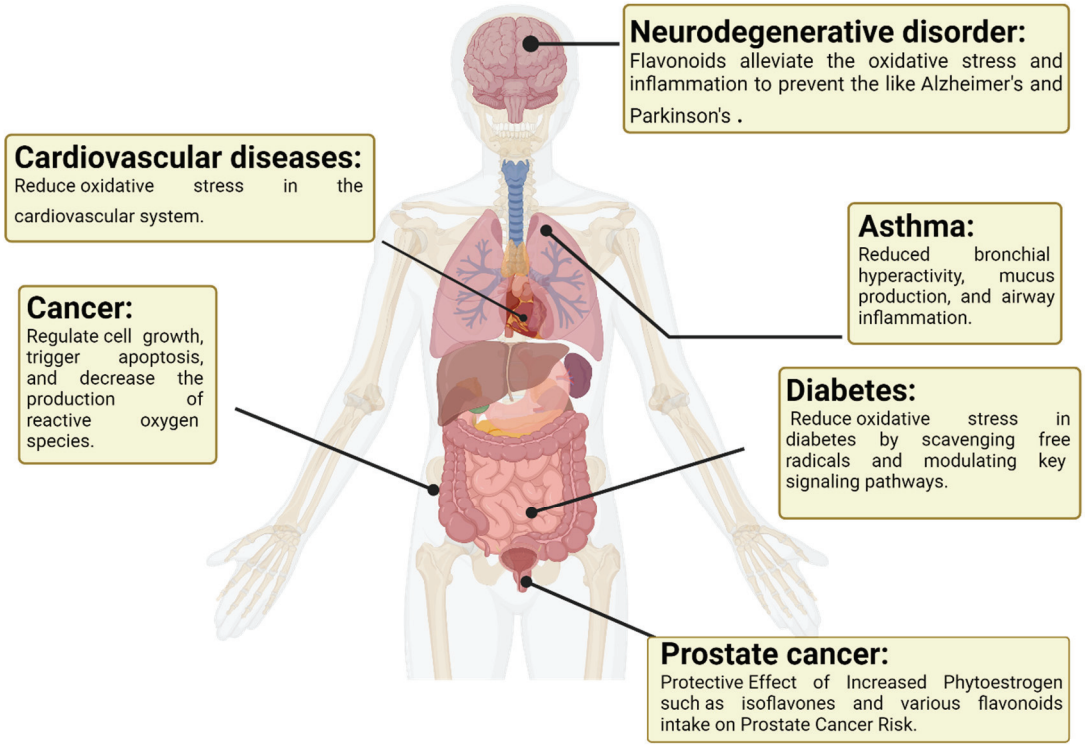


**Figure 2.** Flavonoids prevent oxidative stress-related diseases.

Natural compounds, including flavonoids, exhibit neuroprotective capabilities, likely attributed to their capacity to regulate the inflammatory responses implicated in neurodegenerative diseases [68]. Numerous studies have proposed a potential neuroprotective and anti-inflammatory function of plant extracts that are rich in flavonoids. For instance, the anti-inflammatory potential of honey flavonoid extract (HFE) was evaluated using N13 microglia cells stimulated by lipopolysaccharide. In this model, HFE (at flavonoid concentrations of 0.5 and 1 mg/mL) significantly reduced the generation of pro-inflammatory mediators, specifically inhibiting the expression of inducible nitric oxide synthase (iNOS) mRNA and protein levels. Additionally, HFE demonstrated a notable suppression in the production of both tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [69]. As shown in Figure 3, flavonoids have been demonstrated to exhibit anti-inflammatory effects by modulating immune cell function, reducing chemokine and COX-2 expression, suppressing cytokine release, and inhibiting pro-inflammatory transcription factors such as PI3K/Akt and IKK/JNK [42,70]. A recent study highlighted the potential of encapsulated flavonoids in modulating cytokine activity in various in vitro and in vivo models. Encapsulated flavonoids can influence cytokine activity differently, depending on the flavonoid type, cell line, concentration, and treatment duration. Some studies have shown the downregulation of cytokines like IL-1β, IL-6, TNF-α, and IL-8, while others have noted the upregulation of IL-10. Encapsulated flavonoids often have a more significant impact on cytokine activity compared to their non-encapsulated counterparts [29]. Flavonoids can modulate various cell signaling pathways involved in oxidative stress, inflammation, and apoptosis. These pathways can be differentially regulated in cancer and non-cancerous

conditions. For example, in non-cancerous cells, flavonoids can activate the Nrf2 pathway, leading to the upregulation of antioxidant defenses. In cancer cells, flavonoids might inhibit the NF-κB pathway, reducing cell survival and proliferation [71]. The therapeutic effects of flavonoids, particularly their modulation of ROS, differ between cancerous and non-cancerous conditions due to variations in cellular redox states, metabolic requirements, and signaling pathways [72]. In cancerous cells, the redox balance is often disrupted, leading to elevated levels of ROS [2,73]. These elevated ROS levels can promote cancer cell proliferation, survival, and metastasis through various signaling pathways, such as the MAPK, PI3K/Akt, and NF-κB pathways [74]. Flavonoids can modulate these pathways by reducing ROS levels, thereby inhibiting cancer cell growth and inducing apoptosis [75]. For example, quercetin has been shown to induce apoptosis in cancer cells by modulating the PI3K/Akt pathway and reducing ROS levels [76]. On the other hand, in non-cancerous cells, flavonoids can help maintain redox homeostasis and protect against oxidative stress-induced damage. This protective effect is particularly beneficial in conditions such as cardiovascular diseases, neurodegenerative disorders, and aging, where oxidative stress plays a key role [77]. Flavonoids like kaempferol and epicatechin have been found to enhance antioxidant defenses and reduce ROS levels in non-cancerous cells, thereby preventing cellular damage and promoting overall health [75]. Despite the promising therapeutic potential, several challenges need addressing for the effective utilization of South African flavonoids in clinical settings. The standardization of extraction processes, identification of optimal dosages, and clarification of potential interactions with conventional medications are crucial areas that require attention. Future research should focus on unraveling the specific mechanisms of action of different South African flavonoids, considering their interactions within complex cellular pathways. Moreover, the exploration of synergistic effects among flavonoids and with other bioactive compounds present in South African plants is a promising avenue for future investigations.

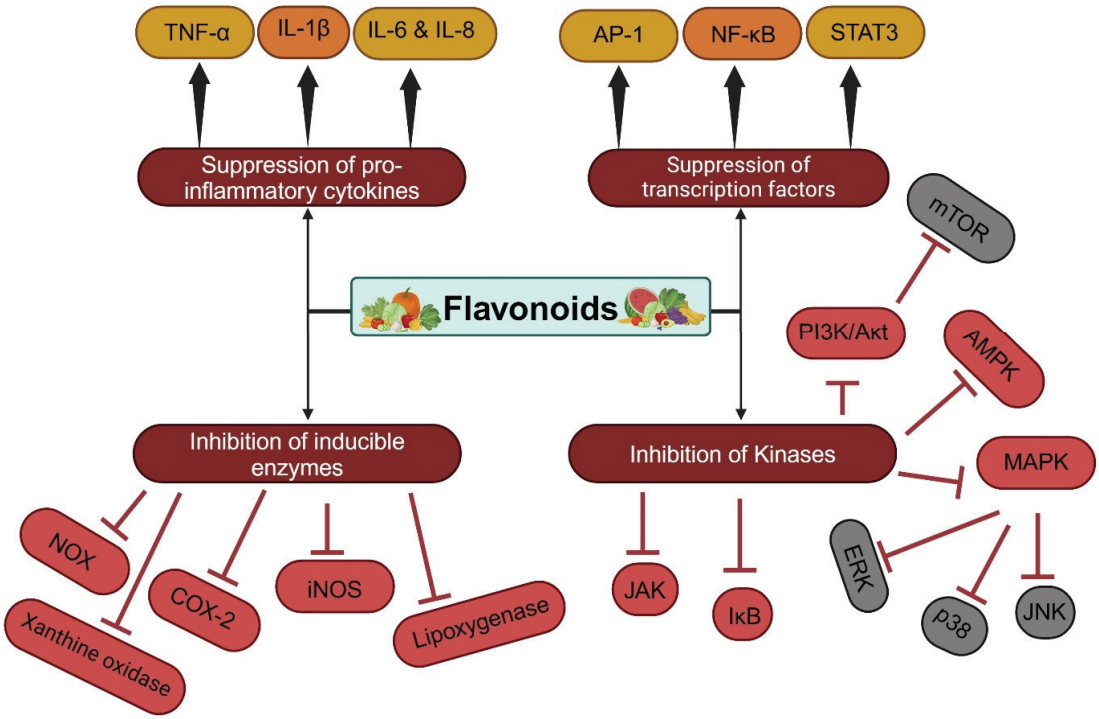


**Figure 3.** The targets of flavonoids in inflammatory processes.

### *3.2. Anti-Cancer Effects of Flavonoids*

Flavonoids have been consistently shown in various studies to possess the capacity to neutralize free radicals, modulate cellular metabolism, and mitigate the risk of diseases associated with oxidative stress [78]. Numerous studies have suggested that a variety of

flavonoids demonstrate anticancer properties. Despite this, the precise molecular mechanisms underlying these effects remain incompletely understood. Cancer originates from internal factors such as oxidative stress, hypoxia, genetic mutations, and deficient apoptotic function [70]. Studies by Kopustinskiene et al. [75] have emphasized the role of flavonoids in reducing oxidative stress and preventing DNA damage, which are critical factors in cancer development. The role of oxidative stress in initiating cancer is well established, and potent antioxidants have the potential to impede the progression of carcinogenesis. The effectiveness of an antioxidant compound to act as an anticancer agent relies on its ability to neutralize oxygen radicals and act as an inhibitor. Diets abundant in radical scavengers have the capacity to reduce the cancer-promoting effects of certain radicals [79]. Among the most common genetic abnormalities in human cancers are mutations in the p53 gene. Inhibiting the expression of p53 may halt the progression of cancer cells in the G2-M phase of the cell cycle. In human breast cancer cell lines, flavonoids have been shown to effectively decrease the expression of the mutant p53 protein, bringing it down to nearly undetectable levels [80]. External factors contributing to its development include exposure to stress, pollution, smoking, radiation, and ultraviolet rays [81]. Cancer cells exhibit distinctive traits, including modified metabolism, disrupted cell cycles, frequent genetic mutations, resilience against immune responses, persistent inflammation, development of metastases, and stimulation of angiogenesis [82]. External factors contributing to its development include exposure to stress, pollution, smoking, radiation, and ultraviolet rays [81,82]. Flavonoids display a diverse range of anticancer properties, influencing the activities of enzymes involved in scavenging ROS. They contribute to cell cycle arrest, promote apoptosis and autophagy, and inhibit the proliferation and invasiveness of cancer cells [75,83,84]. When the balance of pro-oxidant activities and the antioxidant defense within cells is disrupted, there is a rise in ROS production, resulting in the accumulation of free radicals [83]. ROS primarily originate in the mitochondrial electron transport chain as byproducts of the oxidative phosphorylation in cells [85]. The increased levels of ROS contribute to oxidative stress, which plays a role in the onset of inflammatory processes associated with various degenerative diseases and cancer. Flavonoids exhibit a dual role in maintaining ROS homeostasis: they function as antioxidants under normal conditions and serve as potent pro-oxidants in cancer cells, activating apoptotic pathways [86,87]. Flavonoids exhibit direct ROS-scavenging capabilities and can chelate metal ions by stabilizing free radicals through the presence of phenolic hydroxyl groups [88,89]. The indirect antioxidant effects of flavonoids are associated with the activation of antioxidant enzymes, as well as the inhibition of pro-oxidant enzymes and phase II detoxification enzymes. Both antioxidant and pro-oxidant activities contribute to the anticancer effects of flavonoids [2]. A recent in vivo study suggested that a specific polyphenolic compound found in green tea, which consists mainly of flavanol and proanthocyanidin components, effectively prevents the formation of skin tumors in mice. Additionally, it showed that, for the first time, certain fractions that are extracted from rooibos and honeybush, using a method involving ethyl acetate, also protect against the tumor promotion induced by TPA (a chemical compound) in mouse skin. Their findings indicate a significant reduction in the number of tumors, a decrease in their size, and a delay in their formation [90]. Another study suggested that rooibos and honeybush extracts, like green tea extracts, disrupt cell growth by affecting mitochondrial function, causing mitochondrial membrane depolarization. They inhibit cell proliferation at lower concentrations and induce apoptosis at higher concentrations, targeting precancerous cells. The anticancer effects of green tea and rooibos extracts primarily involve monomeric flavonoids, while honeybush extracts, rich in polymeric proanthocyanidins, alter cell growth parameters and often exhibit protective effects with their monomeric polyphenols. These herbal teas' ability to prevent cancer is influenced by specific interactions between polyphenols and cells, which vary between extracts and contribute to their unique anticancer properties when compared to green tea [91]. Genistein, an isoflavone, facilitates the arrest of breast cancer MDA-MB-231 and MCF-7 cells at the G2/M phase, leading to a subsequent apoptosis dependent on ROS [92]. As shown in Table 2, daidzein

induces apoptosis in MCF-7 breast cancer cells by generating ROS [93]. Hesperetin, a flavanone, triggers apoptosis in gall bladder carcinoma [94], esophageal cancer [95], hepatocellular carcinoma [96], and human breast carcinoma MCF-7 cells [97]. This effect is achieved by activating the mitochondrial apoptotic pathway through increased ROS production. Naringenin, another flavanone, exhibits anticancer effects on choriocarcinoma JAR and JEG 3 cell lines by inducing ROS generation and activating signaling pathways [98]. Additionally, it initiates an apoptotic cascade in human epidermoid carcinoma A431 cells [99]. In prostate cancer PC3 and LNCaP cell lines, naringenin suppresses proliferation and migration, induces apoptosis, and generates ROS (Table 2) [100]. An increased intake of phytoestrogens, such as isoflavones and various flavonoids, demonstrates a protective effect against the risk of prostate cancer [101]. Moreover, naringenin has demonstrated the ability to decrease the generation of ROS while augmenting the activity of superoxide dismutase, catalase, and glutathione in chronic diseases and cancer [102]. The pro-oxidant properties of cocoa catechins and procyanidins have been observed to induce apoptotic morphological changes and DNA damage, leading to apoptosis in epithelial the ovarian cancer cells OAW42 and OVCAR3 [103]. Flavonoids appear to be significant anti-cancer agents by targeting receptor tyrosine kinases (RTKs) and influencing their downstream signaling pathways, such as MAPK, PI3K/Akt, and JAK/STAT [104]. The development of various cancers may be linked to the activation of specific proto-oncogenes, such as EGFR. EGFR is widely distributed in mammalian epithelial cells and binds to epidermal growth factor (EGF) or tumor necrosis factor (TNF) [105,106]. A study by Chen et al. found that quercetin reduces cervical cancer cell viability, promotes G2/M phase cell cycle arrest, induces apoptosis, and inhibits cell migration and invasion. The activation of the EGFR and ERK pathways has been observed, with both kinases markedly activated by quercetin. Inhibition of these pathways using afatinib (EGFR inhibitor) and U0126 (ERK inhibitor) enhances apoptosis and cell cycle arrest, suggesting that EGFR and ERK activation may counteract the anticancer effects of quercetin [107]. In HepG2 cells, cocoa polyphenolic extract activates the ERK1/2 pathway, enhancing the activities of glutathione peroxidase and reductase [108]. Additionally, cocoa catechins and procyanidins provides protection to Caco2 cells against induced oxidative stress, reducing ROS production and preventing cellular death [109]. The antioxidant properties of cocoa flavanols have demonstrated beneficial effects in safeguarding against colon cancer [110]. Recent studies have indicated that quercetin reduces the proliferation of hepatocellular carcinoma HepG2 cells by decreasing intracellular ROS levels [111]. Moreover, it has been shown to increase ROS production and the number of apoptotic cells in human gastric cancer AGS and human breast cancer MCF-7 cells [112]. Kaempferol, a flavonol, impedes the growth of bladder cancer EJ cells by inducing apoptosis and causing S phase arrest through the modulation of ROS levels [113]. In colorectal cancer cell lines (HCT116, HCT15, and SW480), kaempferol activates caspases through ROS generation, leading to apoptosis [114]. Additionally, kaempferol exhibits cytotoxic effects on rat hepatocellular carcinoma cells by targeting mitochondria through ROS mediation [115]. The anticancer properties of flavones apigenin and luteolin in ovarian cancer cell lines (A2780, OVCAR-3, and SKOV-3) have been associated with alterations in ROS signaling and the promotion of apoptosis [116]. Apigenin also induces apoptosis in human cervical cancer-derived cell lines, including HeLa, SiHa, CaSki, and C33A, through increased ROS generation and the initiation of mitochondrial apoptotic pathways [117]. Flavone chrysin increases ROS and lipid peroxidation levels, leading to the death of choriocarcinoma (JAR and JEG3), bladder, and ovarian cancer (ES2 and OV90) cells [118,119]. The antioxidant activity of flavonoids has been explored in human studies, revealing a correlation between serum total antioxidant capacity and dietary anthocyanin consumption [120]. Cyanidin induces cell death in DU145 and LnCap human prostatic cancer cells through ROS modulation [121]. Cyanidin and delphinidin accelerates cellular ROS accumulation, suppresses glutathione reductase, and depletes glutathione, resulting in cytotoxicity in metastatic colorectal cancer cells (LoVo and LoVo/ADR) [122]. Quercetin and daidzin are two natural compounds currently receiving significant attention

for their anticancer properties [123]. Quercetin, a flavonoid primarily found in vegetables and fruits such as capers, lovage, dill, cilantro, and onions, has been reported to inhibit HeLa cells by blocking the phosphatidylinositol 3-kinase (PI3K)-Akt/PKB (protein kinase B) pathway. It also promotes apoptosis through the activation of the intrinsic apoptotic pathway, which includes the upregulation of Bax, Bad, Bid, caspase-9, and caspase-3, as well as the downregulation of Bcl-2 and Bcl-xL. Additionally, quercetin triggers cytochrome c release and inhibits NF-κB, PKC-δ, and ERK1/2 while activating AMPK and reducing uPA/uPAR, MMP-9, and MMP-2 to hinder cell migration and invasion [124–126]. Daidzin, a glycoside form of the flavonoid daidzein and found in soy isoflavones, has been noted for its anticancer effects in the early stages of prostate cancer development and for reducing the risk of postmenopausal breast cancer [127]. It is also known to inhibit telomerase activity by forming hydrogen bonds with the base of the G-quadruplex [128,129]. A recent study by Zubair et al. revealed that daidzin is more cytotoxic and selective on T47D and HeLa cell lines than quercetin, likely due to its glucose units forming hydrogen bonds with specific amino acid residues. Docking results have supported this, showing lower binding energy for daidzin compared to quercetin [130].

**Table 2.** Anticancer effects and the mechanism of action of various flavonoids.

| Flavonoids | Cancer Type | Cell Line | Effect | Mechanism | References |
|---|---|---|---|---|---|
| Genistein | Breast cancer | MDA-MB-231 and MCF-7 | G2/M phase arrest, Apoptosis | ROS dependency | [92] |
| Daidzein | Breast cancer | MCF-7 | Apoptosis induction | ROS generation | [93] |
| Hesperetin | Gall bladder, esophageal, hepatocellular, and breast cancer | MCF-7 | Apoptosis trigger | Mitochondrial pathway, ROS production | [94–97] |
| Naringenin | Choriocarcinoma, epidermoid carcinoma, and prostate cancer | JAR, JEG 3 A431, PC3 and LNCaP | Anticancer effects | ROS generation, apoptotic pathways | [98–100] |
| Cocoa Catechins/Procyanidins | Ovarian cancer | OAW42 and OVCAR3 | Apoptosis | DNA damage, apoptotic changes | [103] |
| Cocoa Catechins/Procyanidins | Adenocarcinoma | Caco2 | Oxidative Stress Protection | Reduced ROS production | [109] |
| Cocoa Polyphenolic Extract | Liver cancer | HepG2 | ERK1/2 pathway activation | Increased antioxidant enzyme activity | [108] |
| Quercetin | Liver cancer | HepG2 | Chemo preventive properties | Reduced proliferation, decreased ROS levels | [111] |
| Quercetin | Gastric adenocarcinoma and breast cancer | AGS and MCF-7 | Apoptosis induction | Increased ROS production | [112] |
| Kaempferol | Bladder cancer | EJ | Growth inhibition | Apoptosis, S phase arrest, ROS modulation | [113] |
| Kaempferol | Colorectal cancer | HCT116, HCT15, and SW480 | Apoptosis activation | Caspase activation, ROS generation | [114] |
| Kaempferol | Hepatocellular carcinoma | HepG2 | Cytotoxic effects | Mitochondrial targeting, ROS mediation | [115] |

**Table 2.** *Cont.*

| Flavonoids | Cancer Type | Cell Line | Effect | Mechanism | References |
|---|---|---|---|---|---|
| Apigenin | Ovarian cancer | A2780, OVCAR-3, and SKOV-3 | Apoptosis promotion | ROS signaling alteration | [116] |
| Apigenin | Cervical cancer | HeLa, SiHa, CaSki, and C33A | Apoptosis activation | ROS generation, mitochondrial pathway | [117] |
| Chrysin | Choriocarcinoma, bladder cancer, and ovarian cancer | JAR, JEG3, ES2 and OV90 | Induction of death | Increased ROS, lipid peroxidation | [118,119] |
| Cyanidin | Prostate cancer | DU145 and LnCap | Induced cell death | ROS modulation | [121] |
| Cyanidin and Delphinidin | Colorectal cancer | LoVo and LoVo/ADR | Cytotoxic effects | ROS accumulation | [122] |

Cancer cells often display resistance to apoptosis, a cellular process typically initiated by various signal transduction pathways and involves proapoptotic proteins such as caspases and the Bcl-2 family [131,132]. As shown in Figure 4, there are two primary signaling pathways of apoptosis: the extrinsic pathway, which is associated with the tumor necrosis factor (TNF) superfamily and is predominantly mediated by caspase 8; and the intrinsic pathway, also known as the mitochondrial pathway, which involves the Bcl-2 family proteins triggering the activation of caspases 9, 3, and 7 (Figure 4) [70]. In cancer cells, there is often an upregulation of oncogenic genes like c-Myc, promoting cellular proliferation and suppressing p53 function. Additionally, antiapoptotic proteins from the Bcl-2 family are activated, while proapoptotic proteins and caspases may be downregulated [133]. Flavonoids have the potential to intervene in apoptotic signaling cascades, thereby activating cell death pathways [75].

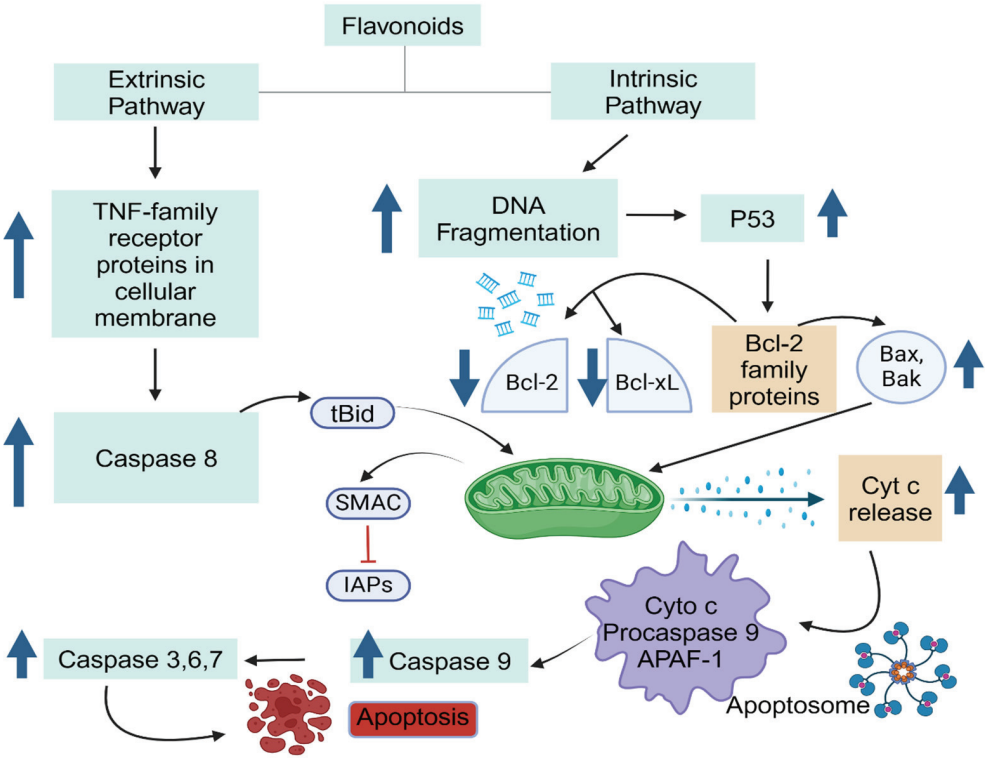


**Figure 4.** Flavonoid targets in the extrinsic and intrinsic apoptosis pathways: key molecules and signaling cascades.

### *3.3. Anti-Hypertensive Effects of Flavonoids*

Cardiovascular disease is the leading cause of death worldwide. Several key risk factors contribute to these conditions, including high blood pressure, aging, obesity, dyslipidemia, a sedentary lifestyle, smoking, stress, and poor lifestyle choices [134]. Nitric oxide released from endothelium plays a vital role in controlling vascular tone and blood pressure. Nitric oxide exerts its effects by triggering the cGMP-protein kinase G pathway in vascular smooth muscle cells. The activation of this pathway stimulates potassium channels, leading to membrane hyperpolarization and reduced intracellular calcium influx, which ultimately results in vasodilation. Protein kinase G achieves this effect by phosphorylating myosin light chains, thereby reducing the contractility of smooth muscles in blood vessels [135,136].

Flavones, a subgroup of flavonoids rich in luteolin, lower blood pressure by initiating and activating the cAMP/protein kinase A pathway. This pathway subsequently activates nitric oxide synthase, leading to an elevated concentration of endothelial nitric oxide. The resulting vasodilation is regulated by potassium and calcium channels [92,137]. The blood pressure-lowering effects of flavonols, such as kaempferol and quercetin, are achieved through modulation of the renin–angiotensin–aldosterone system, enhancement of the endothelial function, and regulation of smooth muscle contraction in blood vessels [138,139]. These mechanisms are attributed to their capability to activate nitric oxide-synthase 3, leading to increased plasma levels of nitric oxide. The enhancement of endothelial function is achieved by inhibiting the response of smooth muscle cells in blood vessels to endothelin-1 [140,141]. The dietary intake of quercetin varies across different countries. Estimated flavonoid consumption ranges from 50 to 800 mg per day, with quercetin making up approximately 75% of this intake. This variation largely depends on the consumption of fruits, vegetables, and tea. In vitro studies involving endothelial denudation have shown that the antihypertensive effects of quercetin and kaempferol rely on nitric oxide synthesized in the endothelium. This conclusion was drawn from the significant reduction in the vasodilator activity that was observed of these two flavonols when the endothelium was removed [142,143]. An in vivo study by Manach et al. [144] demonstrated that dietary flavonols, specifically quercetin and rutin, are absorbed and present in rat plasma as conjugated metabolites at concentrations around 115 μmol/L, and they also exhibit significant antioxidant properties, particularly in inhibiting LDL oxidation. Another study by Edwards et al. showed that quercetin administered for 4 weeks at a dose of 730 mg/day to individuals with prehypertension and stage 1 hypertension resulted in a reduction in blood pressure, but it did not affect the oxidative stress parameters in blood and urine [145].

In contrast to quercetin, naringenin, a member of the flavanone class, demonstrates vasodilatory effects even in the absence of the endothelium. This is due to its ability to activate potassium channels, especially those that are calcium-activated and voltage-dependent. Naringenin's blood pressure-lowering effects are a result of membrane hyperpolarization and the relaxation of vascular smooth muscle, which are both influenced by calcium-activated potassium channels [146]. A study on hypertensive rats demonstrated that epicatechin's antihypertensive mechanism involves decreasing superoxide production in the aorta and left ventricle, as well as in enhancing nitric oxide-synthase activity [147].

Soy isoflavones are structurally like human estrogen, which is believed to contribute to their beneficial role in preventing hypertension in menopausal women. Daidzein induces vasodilation through mechanisms similar to other flavonoids, but it also uniquely stimulates the production of prostaglandins [148]. Genistein's antihypertensive effects are achieved by inhibiting the tyrosine kinase Pyk2, which regulates calcium ion channels and activates signaling pathways [149]. Additionally, genistein helps reduce pulmonary hypertension by decreasing smooth muscle hypertrophy in pulmonary arteries [150].

### *3.4. Anti-Microbial Effects of Flavonoids*

Flavonoids demonstrate broad-spectrum antimicrobial activity against bacteria, fungi, and viruses [151]. They achieve this by disrupting microbial cell membranes, inhibiting nucleic acid synthesis, and interfering with microbial metabolism [152]. A recent study

identified four compounds—pectolinaringenin (1), (±)-4′,5,7-trimethoxy flavanone (2), 5-hydroxy-3,7,4′-trimethoxyflavone (3), and 3,5,7-trihydroxy-4′-methoxyflavone (4)—from the South African weed *Chromolaena odorata*. Compounds 2 and 3 exhibited promising antimicrobial activity against *E. coli*, *S. aureus*, *K. pneumoniae*, *A. fumigatus*, and *C. neoformans*, with minimum inhibitory concentrations (MIC) between 0.016 and 0.125 mg/mL, which is comparable to gentamicin, ciprofloxacin, and amphotericin B. These compounds also demonstrated good anti-biofilm and metabolic inhibition activities but weak anti-adhesion effects. They showed low toxicity to human and animal cells, with selectivity indexes between 1 and 12.625, indicating higher toxicity to microbial strains. These findings suggest that Compounds 2 and 3 could be potential leads for developing prophylactic treatments and anti-infective drugs against urinary tract infection [153].

To comprehend the mechanisms through which dietary flavonoids exert their effects in the body, it is essential to identify the specific chemical forms of their various metabolites that are present in systemic circulation, as these are the physiologically active forms [154]. Dietary flavonoids are primarily found in their glycoside forms. However, in plasma, glycosides are rare due to the deglycosylation process that occurs in both the small and large intestines, which vary based on the type of sugar moiety [155–157]. In the small intestine, two enzymes act as β-glucosidases against flavonoid monoglucosides [158]. Lactase-phlorizin hydrolase (LPH), a brush border-associated enzyme, hydrolyzes lactose into glucose and galactose. Day et al. discovered that LPH also hydrolyzes quercetin 3-O-glucoside (Q3G), quercetin 4′-O-glucoside (Q4′G), and the monoglucosides of genistein and daidzein to produce corresponding aglycons in vitro [159]. The other enzyme, cytosolic β-glucosidase (CBG), located in enterocytes, shows broad specificity and hydrolyzes Q4′G, genistein 7-O-glucoside (genistin), and daidzein 7-O-glucoside (daidzin), but not quercetin 3,4′-O-diglucoside, Q3G, or quercetin 3-O-rhamnoglucoside (rutin), using cell-free extracts from the human intestine and liver [160]. Before CBG can hydrolyze these glucosides, they are taken up into the cell via the sodium-glucose co-transporter type 1 (SGLT1), as evidenced by studies using human Caco-2 cells and SGLT1-transfected rodent G6D3 cells [161]. In individuals with lactase deficiency, plasma levels of isoflavone metabolites are initially lower but eventually become like those in lactase-sufficient individuals, likely due to compensation by the intestinal microbiota. Isoflavone glucosides are primarily hydrolyzed by CBG in cell-free extracts of the human intestine and liver, with LPH playing a lesser role compared to rats [162]. Recently, calycosin-7-O-glucoside, a methylated isoflavone, was identified as a novel substrate for SGLT1 but not for LPH in rats [163]. Therefore, deglycosylation in the small intestine is crucial for enhancing the bioavailability of flavonoid monoglucosides. Nevertheless, the intestinal microbiota can often compensate for the absence of this process, ensuring sufficient hydrolysis and absorption of these compounds [164]. In recent years, numerous studies have highlighted the dual role of the gut microbiota in maintaining host health. Gut-resident bacteria produce various metabolites and bioproducts essential for protecting host and gut homeostasis. Conversely, during pathological dysbiosis, certain microbiota subpopulations can expand and produce high levels of toxins, leading to inflammation and tumorigenesis [165]. Flavonoids such as quercetin, epigallocatechin gallate (EGCG), and genistein have been demonstrated to alter the composition of the microbiota within the tumor microenvironment, resulting in changes in immune response and tumor growth [166]. A study by Maheswari et al. demonstrated that titanium dioxide nanoparticles modified with extracts from *Plectranthus amboinicus* (*Karpooravalli*), *Phyllanthus niruri* (*Keezhanelli*), and *Euphorbia hirta* (*Amman Pacharisi*) were synthesized using the hydrothermal method. X-ray diffraction revealed the anatase nature of the samples, and TEM analysis showed an increase in particle size for the bio-modified samples. These nanoparticles exhibited significant antibacterial activity against both Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus* mutans), with *Plectranthus amboinicus*-modified titanium dioxide nanoparticles showing the best results [167].

## 4. Flavonoid-Mediated Nanoparticles and Their Efficacy

South Africa is home to approximately 10% of the world's plant species, with over 3000 species recognized for their significant medicinal properties [23]. Medicinal plants have a longstanding role in traditional medicine, with over 80% of the South African population depending on them for primary health care [168]. Medicinal plants provide diverse therapeutic benefits due to their rich array of secondary metabolites, including flavonoids, alkaloids, phenolics, terpenoids, tannins, glycosides, quinones, steroids, and saponins [169]. Flavonoids are commonly formulated into various food supplements to leverage their health benefits, enhancing both their bioavailability and efficacy for consumers. Formulating flavonoids into these supplements can improve their bioavailability, which is often low due to poor solubility and rapid metabolism [19]. Techniques such as nanoparticle encapsulation, liposomal delivery, and complexation with cyclodextrins are used to increase the absorption and stability of flavonoids [170]. Several phytochemical and pharmacological studies of these plants and their derivatives have shown impressive in vitro activity but less in vivo efficacy. To enhance their effectiveness, a growing body of literature is suggesting combining traditional medicinal plants with nanotechnology [171].

Despite this rich biodiversity and the potential advantages of plant-mediated metallic nanoparticles (MNPs), the use of South African medicinal plants for MNP synthesis remains largely underexplored [172]. Recently, several indigenous South African plants, including *Salvia africana-lutea*, *Sutherlandia frutescens*, *Galenia Africana*, *Catharanthus roseus*, *Hypoxis hemerocallidea*, *Cotyledon orbiculata*, and *Aspalathus linearis*, have been employed in the synthesis of MNPs [173]. These nanoparticles, with sizes ranging from 5 to 50 nm, have demonstrated significantly higher antibacterial activities compared to their corresponding plant extracts [174,175]. Given the substantial potential of plants as alternative sources of reducing agents with enhanced bioactivity, ongoing research into the rich medicinal plant reserves of South Africa is crucial [176]. Nanoparticles can exploit the enhanced permeability and retention (EPR) effect, which is a phenomenon where nanoparticles tend to accumulate in tumor tissue much more than in normal tissues due to the leaky vasculature and poor lymphatic drainage in tumors. Quercetin nanoparticles have shown higher accumulation in tumor sites compared to free quercetin [177]. Nanoparticles can be engineered with surface modifications to target specific tissues or cells, enhancing their distribution to desired sites within the body. Flavonoid nanoparticles functionalized with antibodies or ligands can target cancer cells specifically, improving therapeutic outcomes [178]. They can also increase the solubility of flavonoids that are poorly soluble in water, leading to better absorption in the gastrointestinal tract. Nanoparticles can shield flavonoids from the degradation caused by enzymes and acidic conditions in the gastrointestinal tract, enhancing their stability and bioavailability. Encapsulating flavonoids in polymeric nanoparticles has proven effective in preventing early degradation and boosting their therapeutic efficacy [179]. Nanoparticles not only protect flavonoids from degradation in the gastrointestinal tract, but also enable controlled release, maintaining therapeutic levels in the bloodstream over extended periods. For example, the sustained release of EGCG from nanoparticles has demonstrated enhanced bioavailability and prolonged antioxidant effects [180].

Green nanobiotechnology involves the creation of nanoparticles or nanomaterials using biological methods, harnessing the abilities of microorganisms, plants, viruses, or their components, like proteins and lipids, aided by biotechnological techniques [181]. Plants have served as natural remedies for various physiological disorders in traditional Eastern medicine, notably in Indian and Chinese practices, since ancient times. There is existing literature on the 'Green' synthesis of nanoparticles such as copper (Cu), gold (Au), nickel (Ni), platinum (Pt), titanium (Ti), selenium (Se), silver (Ag), and zinc (Zi) using plant resources [182]. These plant-based metal nanoparticles have demonstrated remarkable antimicrobial, anticancer, antidiabetic, anti-inflammatory, antioxidant, and immunomodulatory activities, as reported in previous studies [183,184]. Previous research has consistently indicated that the reduction and stabilization of metal ions during synthesis are attributed

to the presence of phytochemicals in plant materials. These phytochemicals encompass alkaloids, flavonoids, phenols, terpenoids, alcohols, sugars, and proteins [185,186]. While synthesizing MNPs using a singular active substance from plant extracts can aid in nanoparticle purification, additional research is necessary to explore the biomedical applications of such MNPs for treating specific diseases. Currently, there is limited literature available on the use of a single substance from plant extracts for MNP synthesis. Recent findings have emphasized the significant role of flavonoids, which are widely present in plant extracts, in the bio reduction of metal ions leading to nanoparticle formation [187,188]. A recent innovative study found that *Karpooravalli–Keezhanelli*-modified *titanium dioxide nanoparticles* demonstrated minimal cell viability at a concentration of 50 $\mu g\ mL^{-1}$, indicating excellent anticancer activity. Subsequent MTT assays on normal L929 cells showed these nanoparticles to be safer and less toxic. Therefore, *Karpooravalli–Keezhanelli*-modified titanium dioxide nanoparticles have potential as future anticancer drugs [167]. Table 3 provides a comprehensive overview of various nanoparticles (NPs) synthesized using different flavonoids, each exhibiting unique properties and potential applications. Quercetin-based silver and selenium nanoparticles (Ag-SeNPs) have a size range of 30–35 nm [189]. The selection of the nanocarrier is based on the structure of quercetin, specifically its 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one configuration [29]. Quercetin can scavenge free radicals that are generated by metals. Mittal et al. proved quercetin is mainly responsible for the reduction and stabilization of metal ions. Interestingly, the aforementioned study confirmed quercetin-mediated bimetallic Ag-SeNPs possess higher therapeutic efficacy in terms of antioxidant, antimicrobial, and anticancer activities [189]. Kaempferol, a flavanol widely found in various plant sources, has been investigated for its involvement in the synthesis of gold nanoparticles (AuNPs). In a study conducted by Raghavan et al. [190], it was revealed that kaempferol plays a crucial role in the formation of AuNPs. Furthermore, the resulting nanoparticles exhibit considerable potential in the realm of cancer treatment, particularly showing promise in combating human breast cancer [190]. Apiin-coated AuNPs with a size of 21 nm display notable anticancer activity [191]. Proanthocyanidin-coated AuNPs, sized between 17 and 29 nm, exhibit efficient cardio-protective potential along with good biocompatibility [192]. Luteolin-conjugated silver nanoparticles (AgNPs) at size of 13 nm exhibit significant antimicrobial activity against Bacillus subtilis [193]. Baicalein, a major bioactive flavonoid, demonstrates significant synergistic effects when combined with tetracycline and β-lactams in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA). Additionally, it has been noted for its ability to mitigate quorum sensing-controlled virulence factors, particularly biofilm formation, in *Pseudomonas aeruginosa* [194]. In a study by Rajkumari et al. the synthesis of AuNPs mediated by baicalein was reported. Baicalein-based AuNPs at a size of 39 nm demonstrate antibiofilm activity against *Pseudomonas aeruginosa* [195]. Flavonoids from *Dalbergia spinosa*, when used in the synthesis of silver nanoparticles (AgNPs) at a size of 18 nm, exhibit both anti-inflammatory and antibacterial activities against *Escherichia coli*, *P. aeruginosa*, *Staphylococcus aureus*, and *B. subtilis* [196]. These findings highlight the versatility and diverse applications of bioactive compounds in nanoparticle synthesis. The nanoparticles show potential in areas such as cancer treatment, antimicrobial applications, and anti-inflammatory responses. The varied sizes and compositions of the nanoparticles also contribute to their unique functionalities, demonstrating the broad spectrum of possibilities in the field of nanomedicine and nanotechnology.

**Table 3.** Flavonoid-mediated nanomaterials and their sizes with applications in biomedicine.

| Flavonoids | Nanomaterials | Size (nm) | Biomedical Applications | References |
|---|---|---|---|---|
| Quercetin | Ag-SeNPs | 30–35 nm | Exhibit antioxidant, antimicrobial, and anticancer activities. | [189] |
| Proanthocyanidin | AuNPs | 17–29 nm | Efficient cardio-protective potential with good biocompatibility | [192] |
| Luteolin | AgNPs | 13 nm | Antimicrobial activity against *B. subtilus* | [193] |
| Kaempferol | AuNPs | 16.5 nm | Anticancer activity against human breast cancer. | [190] |
| Apiin | AuNPs | 21 nm | Anticancer activity | [191] |
| Baicalein | AuNPs | 39 nm | Antibiofilm activity against *P. aeruginosa* | [195] |
| Flavonoids (*Dalbergia spinosa*) | AgNPs | 18 nm | Anti-inflammatory and antibacterial (*E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis)* activities | [196] |

Numerous studies have explored the interactions between flavonoids and metal nanoparticles, particularly those involving Ag and Au, as well as the oxides of Fe, Zn, and Ti, resulting in materials with intriguing physicochemical and biological properties. Silver nanoparticles have shown promising antioxidant, antibacterial, antifungal, antiparasitic, antiviral, and anticancer properties. To investigate potential medical applications, silver nanoparticles have been functionalized with various flavonoids such as apigenin, catechin, EGCG, kaempferol, myricetin, 4′,7-dihydroxyflavone, dihydromyricetin, hesperidin, and quercetin. Moreover, silver@quercetin nanoparticles have been studied as biocompatible and photostable aggregation-induced emission luminogens for the in situ and real-time monitoring of biomolecules and biological processes. In biological studies, silver nanoparticles combined with quercetin have been examined for their potential anti-inflammatory effects. Additionally, silver nanoparticles loaded with isoorientin have been investigated for their potential toxicity and their activity on enzymes related to type II diabetes and obesity [197]. In a 2021 study, Kollur et al. investigated the anticancer properties of luteolin-functionalized zinc oxide nanoparticles (L-ZnONPs). These nanoparticles were synthesized by combining an aqueous solution of $Zn(OAc)_2$ with luteolin, followed by filtration of the resulting white precipitate, ethanol washing to eliminate impurities, and calcination of the final product [198]. Results suggest that the nanohybrid induces intrinsic cellular mechanisms leading to apoptosis and halts the cell cycle. Moreover, by downregulating MMP-2 and VE-cadherin expression, the nanoparticles suppress the migration and invasion of A549 cells [198]. In another investigation, Salaheldin et al. explored the potential application of three novel nano formulations of (-)-epigallocatechin-3-gallate (EGCG) as natural chemo preventive agents against DNA damage induced by ultraviolet beam (UVB) radiation in keratinocytes [199].

Gold nanoparticles (AuNPs), much like silver nanoparticles (AgNPs), have garnered significant attention since their discovery. This is primarily attributed to their straightforward synthesis, easy surface modification, and their broad spectrum of potential applications, particularly in the field of medical sciences [200]. Gold nanoparticles are frequently functionalized with flavonoid compounds to enhance their properties and expand their range of applications [201]. Gold nanorods integrated with metal–phenolic networks, which include EGCG, procyanidins (OPC), and tannic acid (TA), have demonstrated significantly accelerated wound healing and strong bactericidal effects when used with NIR irradiation. Similarly, quercetin-conjugated gold nanoparticles have shown both antibacterial and antioxidant activities. Additionally, gold nanoparticles coated with CHY, kaempferol, and quercetin have exhibited enhanced antibacterial efficacy against Gram-negative bacteria [202,203]. In a study by Sivakumar et al. [204], suggested silver nanoparticles (AgNPs) were phytosynthesized using leaf extract from *Phyllanthus urinaria L*. They proposed that polyols like flavones and catechins play a role in reducing silver ions to form AgNPs,

and they are supported by the disappearance of the C-O band at 1226 $cm^{-1}$ in IR spectra. The nanoparticles range in shape from cuboidal to orthorhombic, with sizes varying from 15 nm to 80 nm. Antimicrobial tests against *Escherichia coli*, *Salmonella typhi*, *Vibrio cholera*, *Pseudomonas aeruginosa*, and *Proteus mirabilis* showed strong bactericidal activity across all tested concentrations (100 to 400 μg/mL). Maximum inhibition zones of 18 mm were observed against *V. cholera* at 400 μg/mL, while the lowest activity was 8 mm against *P. mirabilis* at 100 μg/mL. Further research is needed to determine the minimum inhibitory concentration (MIC) values for comprehensive efficacy assessment [204].

Flavonoid-modified metal nanoparticles exhibit their usefulness starting from the initial synthesis stage to advanced investigations in medical and pharmaceutical domains. Understanding various methods for synthesizing flavonoid–metal nanoparticle conjugates and hybrids, along with their characterization, biological attributes, and medical uses, offers insights into numerous possibilities. Numerous research outcomes have indicated that combining flavonoids with metal nanoparticles enhances their individual characteristics and potential applications.

## 5. Conclusions and Suggestions

In conclusion, this review highlights the significant potential of flavonoid-mediated nanoparticles, especially those derived from South African plant species, as promising therapeutic agents owing to their potent antioxidant and anti-inflammatory properties. The diverse flora in South Africa provide a rich source of these compounds, opening avenues for future research and development, particularly in the context of nanomedicine. Exploring the unique aspects of flavonoid-mediated nanoparticles, such as their size, structure, and interactions, presents novel dimensions for enhancing therapeutic efficacy.

Further investigations into the distinctive mechanisms of action, bioavailability, and potential synergistic effects of flavonoid-loaded nanoparticles across various plant species offer exciting prospects for the development of innovative therapies. Understanding these nanoparticles behavior and leveraging traditional knowledge with modern scientific approaches are crucial for unlocking their full potential in treating a spectrum of diseases.

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# Extra Virgin Olive Oil Polyphenol-Enriched Extracts Exert Antioxidant and Anti-Inflammatory Effects on Peripheral Blood Mononuclear Cells from Rheumatoid Arthritis Patients

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**Abstract:** Rheumatoid arthritis (RA) is a long-term systemic autoimmune disorder that causes joint inflammation, swelling, pain, bone erosion, and deformities. Recent findings emphasize the anti-inflammatory and antioxidant properties of bioactive natural compounds, such as polyphenols extracted from plants and fruits, and their possible synergistic effect when used in combination with current therapies to improve the prognosis and symptoms of inflammatory rheumatic diseases. Here, we report that Sicilian extra virgin olive oil polyphenol-enriched extracts (PE-EVOOs) reduce intracellular reactive oxygen species (ROS) and pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 β (IL-1β), in peripheral mononuclear cells (PBMCs) obtained from both RA patients and healthy subjects (HSs) treated with lipopolysaccharides (LPS) as a control. HPLC-ESI-MS analysis highlighted that PE-EVOOs are rich in different polyphenolic compounds responsible for many of the observed biological effects. At molecular levels, Western blotting analyses revealed that PE-EVOO treatment is associated with the downregulation of the phosphorylated and active form of the inflammatory transcription factor NF-κB and the pro-inflammatory enzyme cyclooxygenase 2 (COX2). In addition, PE-EVOOs upregulated the transcription factor Nrf2 and its target antioxidant enzyme catalase and manganese superoxide dismutase (MnSOD). Collectively, these results suggest a possible use of PE-EVOOs as potential adjuvants for the treatment of RA.

**Keywords:** rheumatoid arthritis; PBMCs; extra virgin olive oil polyphenols; IL-1β; TNF-α; Nrf2

## 1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease primarily causing synovial joint inflammation, cartilage devastation, bone erosion, pain, deformities, and progressive disability with a reduction in an individual's quality of life [1]. Although its etiopathogenesis is still unclear, it is estimated that RA affects 0.5–1% of the global population [2]. Current management strategy primarily focuses on the reduction in symptoms at the joints and the slowdown of its progression towards disability through the administration of disease-modifying anti-rheumatic drugs (DMARDs) either alone or in association with nonsteroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids [3]. DMARDs are immunosuppressive and immunomodulatory agents classified as conventional DMARDs, such as methotrexate, leflunomide, hydroxychloroquine, and sulfasalazine, biologic DMARDs, introduced in the early nineties and, more recently, the synthetic target molecules usually prescribed after the failure of conventional DMARD therapy. They are monoclonal, chimeric humanized fusion antibodies, receptors fused to a part of the human immunoglobulin, or small molecules, like Janus kinase (JAK) inhibitors, highly specific and targeting a specific pathway of the immune system [4]. Although the availability of biological DMARDs has significantly improved the course of the disease in patients suffering from RA, patients often do not adequately respond to current treatment regimens due to tolerance development or severe side effects. Thus, over the past few decades, researchers have investigated if natural phytochemicals are effective in relieving RA-associated symptoms. Several phytochemicals, such as alkaloids, flavonoids, steroids, terpenoids, and polyphenols have shown anti-inflammatory and immunomodulatory activity against RA [5–7]. However, their use is often limited because of their high molecular weight, poor water solubility, permeability, and stability affecting their absorption and bioavailability, which can be currently implemented by phenolic-enabled nanotechnology (PEN) [8,9]. The Mediterranean diet is a healthy eating plan characterized by the high consumption of fruits, vegetables, and whole grains and reduced consumption of red meat. Extra virgin olive oil (EVOO), the main source of Mediterranean diet lipids, is obtained via mechanical extraction from the olive fruit under conditions that do not alter its composition. The consumption of EVOO has been shown to exert beneficial effects against several immune-inflammatory chronic diseases, including RA, systemic lupus erythematosus (SLE), and inflammatory bowel disease (IBD) [10–12].

In the past, the health-promoting properties of EVOO have been largely correlated to the high content of monounsaturated fatty acids, in particular oleic acid. However, more recently, attention has been focused on the presence of a variety of phenolic compounds, such as hydroxytyrosol (HTy), tyrosol, and oleuropein [13], on which the pharmacological properties of the olive tree seem to depend [14,15]. Thus, EVOO quality depends not only on the content of free fatty acids but also its content in polyphenols. Polyphenols are a class of compounds characterized by the presence of one or more aromatic rings and two or more hydroxyl groups. They are found in different parts of many plants including roots, stems, leaves, fruit, and flowers and are produced for defense against pathogens (microbes and fungi) and to discourage leaf-eating insects [16]. Due to their structure, they possess antioxidant and anti-inflammatory properties and are able to modulate abnormal cellular signaling induced by pro-inflammatory stimuli and oxidative stress, such as those related to the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) and NF-E2-related factor 2 (Nrf2) [17].

Several recent studies [18] demonstrated the ability of EVOO polyphenols to activate the signaling pathway of Nrf2, a transcription factor that regulates the expression of phase II detoxifying enzymes, including NAD(P)H quinone oxidoreductase 1, glutathione per-

oxidase, ferritin, heme oxygenase-1 (HO-1), and antioxidant genes, which protect cells from different injuries via their anti-inflammatory effects, thus influencing the course of several diseases characterized by inflammation, such as RA, asthma, IBD, and Helicobacter pylori infection-induced gastritis [19]. Furthermore, EVOO polyphenols exert their anti-inflammatory activity also by preventing the activation of NF-κB, a protein complex responsible for the transcription of pro-inflammatory mediators, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), interleukin-1 β (IL-1β), and cyclooxygenase 2 (COX2), in almost all types of animal cells and are involved in different processes such as inflammation, oxidative stress, apoptosis, immune response, cell growth, and development [17].

The consumption of EVOO or EVOO polyphenol-enriched extracts (PE-EVOOs) exerts beneficial effects against RA [20]. In collagen-induced arthritis (CIA), DBA/1 mice treated with EVOO or PE-EVOO have been shown to reduce joint edema and cartilage degradation, as well as decrease the levels of pro-inflammatory cytokines, preventing arthritis development [20,21]. In addition, in IL-1β-activated human synovial SW982 fibroblasts PE-EVOOs inhibited the production of pro-inflammatory mediators, like matrix metalloproteinases 1 (MMP-1) and IL-6, and this protective effect seems to be related to the inhibition of mitogen-activated protein kinases (MAPKs) (JNK and p38), which are involved in NF-κB activation in the cytoplasm and the modulation of its transactivating potential in the nucleus [22–24]. Here, we investigated the modulation of the inflammatory response in peripheral blood mononuclear cells (PBMCs) isolated from RA by treatment with PE-EVOOs. Our results provided evidence that PE-EVOOs exhibit significant anti-inflammatory properties by inhibiting NF-κB activation and downregulating the levels of COX-2 and the pro-inflammatory cytokines TNF-α and IL-1β. Furthermore, PE-EVOOs displayed potent antioxidant effects, which appear to be mediated by the activation of Nrf2, the master regulator of cellular antioxidant defenses, and its targets manganese superoxide dismutase (MnSOD) and catalase.

## 2. Materials and Methods

### *2.1. Chemical Extraction of Polyphenols from EVOO*

EVOO was purchased from Azienda Agricola Signorello, Campobello di Mazzara, Sicily, Italy. PE-EVOOs were obtained through repeated centrifugations in a mixture containing ethanol and water at an 8:2 ratio, along with an immiscible n-hexane at a 1:1 ratio with the initial quantity of EVOO. Subsequently, the extracted polyphenol content was assessed using the Folin–Chiocalteu protocol [25] by determining the equivalent amount of gallic acid, one of the most represented polyphenols in EVOO [25]. To remove the ethanol, which can be toxic to cells, a freeze-drying process was employed, and the product obtained was resuspended in dimethyl sulfoxide (DMSO).

### *2.2. HPLC/MS/ESI/Q-Tof Analysis*

Previously reported methods were adapted for performing HPLC/MS analysis [26,27]. A solution of the freeze-dried sample in ethanol (1 mg/mL $v/v$) was prepared. The ethanolic solution was directly injected without further treatment. Water and acetonitrile were of HPLC/MS grade. Formic acid was of analytical quality. A reversed-phase Phenomenex Luna Omega Polar C18 100 column, (150 mm × 4.6 mm, particle size 5 μm) with a Phenomenex (Torrance, CA 90501-1430, USA) C18 security guard column (4 × 3 mm) was used. The injection volume was 25 μL. The eluate was monitored with Mass Total Ion Count (MS TIC) and UV (530 nm). Mass spectra were obtained on an Agilent 6540 UHD, Santa Clara, CA 95051, USA, accurate-mass quadrupole time-of-flight (Q-TOF) spectrometer

equipped with a dual AJS Electrospray Ionization (ESI) source working in negative mode. Nitrogen N2 was used as desolvation gas at 300 °C and a flow rate of 8 L $min^{-1}$. The nebulizer was set to 45 psig. The sheath gas temperature was set at 400 °C and a flow of 12 L $min^{-1}$. A potential 2.6 kV was used on the capillary. The fragmentor was set to 75 V. MS spectra were recorded in the 100–1500 m/z range. Quality control was performed prior to analysis using mass calibration in the range of 100–3000 Dalton (Q-TOF calibration mix) and solvent delay calibration for retention time. An in-house quality check mix containing known compounds (phenylalanine, saccharose, benzoic acid, and rutin) was injected during the batch of analysis. Mass spectrum data were analyzed for metabolite annotation using MassHunter Qualitative Analysis B.06.00 and the Metabolomics Workbench database (https://www.metabolomicsworkbench.org/) (accessed on 1 December 2024).

### *2.3. Patients*

For our study, 22 RA naïve patients were recruited at the Rheumatology Unit of Policlinico "Paolo Giaccone", University of Palermo. All patients recruited met the ACR/EULAR 2010 classification criteria for RA. They had active diseases and had never received DMARD treatment. Patients had previously been treated with a stable dose of NSAIDs. All patients presented active diseases, defined as a disease activity score of 28 C-reactive protein (DAS28CRP) > 5.1. In addition, 15 healthy subjects (HSs) were recruited as controls, paired by age and sex. The baseline demographic and clinical features of the patients are shown in Table 1. This study was approved by the local Ethics Committee of the University of Palermo and complied with the provisions of the Declaration of Helsinki. Informed consent was obtained from all patients and control.

**Table 1.** Clinical characteristics of RA patients and HSs.

| | **RA (*n* = 22)** | **HSs (*n* = 15)** |
|---|---|---|
| Age mean, years (range) | 54.8 (32–70) | 47.2 (31–60) |
| Female sex, *n* (%) | 14 (63.6) | 11 (73.3) |
| Disease duration, years (range) | 3 (0.5–5.7) | - |
| Anti-CCP, *n* (%) | 10 (45.5) | - |
| RF, *n* (%) | 13 (59) | - |
| CRP mg/l, mean (range) | 6.2 (0.2–7.6) | - |
| ESR, mean (range) | 27.7 (1–89) | - |
| DAS28CRP score, mean (range) | 3.4 (0.9–5.7) | - |

Anti-CCP: cyclic citrullinated peptide; CRP: C-reactive protein; DAS28CRP: disease activity score 28CRP; ESR: erythrocyte sedimentation rate; HSs: healthy subjects; RA: rheumatoid arthritis; RF: rheumatoid factor.

### *2.4. MTS Assay*

The MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, MTS, Promega Corporation, Madison, WI, USA) was used to evaluate the metabolic activity and viability of PBMCs. The assay involves the conversion of the tetrazolium salt MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) to a purple formazan in the presence of phenazine methosulfate. The enzymes responsible are NADPH-dependent dehydrogenases, which are active only in viable cells. The absorbance of the resulting formazan solution is proportional to the number of viable cells and can be quantified using a spectrophotometer (Abs measured at 490–500 nm). To this end, PBMCs of HSs were incubated in a 96 multiwell plate ($100 \times 10^3$/well/100 μL) for 48 h with different doses (0.5–50 μg/mL) of PE-EVOOs. Then, 20 μL of MTS solution was added

to each well at a final concentration of 0.33 mg/mL, incubated for 2 h at 37 °C, and the absorbance was recorded at 490 nm.

### *2.5. Radical Scavenging Activity by DPPH*

Radical scavenging activity of PE-EVOOs was determined by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical (Sigma, Merck KGaA, Darmstadt, Germany) [28]. Different amounts of PE-EVOOs (0.5–50 μg/mL) were added to 1 mL of ethanol DPPH solution (100 μM) and incubated for half an hour at room temperature and in the dark. DPPH radicals are stable molecules, characterized by a deep-violet color with an absorption maximum of 517, which is reduced by the addition of antioxidant compounds. Discoloration was measured spectrophotometrically. Ethanol DPPH solution was used to prepare the negative control (A0), and a blank sample (A2) containing ethanol was used as a reference. The radical scavenging activity (% of DPPH radical inhibition) was calculated using the following equation:

$$\text{Inhibition (\%)} = 1 - (A1 - A2/A0 - A2) \times 100$$

### *2.6. Isolation of PBMCs*

Peripheral blood samples were obtained from healthy subjects (HSs) and RA patients and collected in ethylenediaminetetraacetic acid (EDTA) tubes. PBMCs were isolated from patients and HSs by density gradient centrifugation. Peripheral blood was diluted at a 1:2 ratio with PBS and then centrifuged on a Ficoll gradient (Lympholyte for human cell separation from Cederlane, Burlington, Ontario, Canada) in a 15 mL polystyrene conical centrifuge tube for 20 min at 770× *g* at room temperature (RT). Subsequently, PBMCs were carefully aspirated from the plasma–Ficoll interface and washed twice (at 500× *g* for 5 min) with fresh RPMI 1640 medium (Euroclone, Pero, Italy) supplemented with 20 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin (Euroclone). Finally, PBMCs were resuspended in 1 mL of complete RPMI medium, containing 10% heat-inactivated FCS and 2 mM L-glutamine (Euroclone).

### *2.7. Cytofluorimetric Analysis*

Following the Ficoll–Hypaque gradient separation, freshly isolated PBMCs from HSs and RA patients were cultured in a complete medium alone or in the presence of 10 μg/mL of PE-EVOO for 48 h. LPS (Sigma) (5 μg/mL) was added only to HS PBMCs cultured in complete medium alone or in the presence of PE-EVOOs (10 μg/mL) after the first 24 h of PE-EVOO treatment. In addition, 10 μg/mL of Monensin (BioLegend, San Diego, CA, USA) was added to all the conditions after the first hour of incubation as a Golgi blocker to ensure the intracellular retention of cytokines. At the end of the incubation period, PBMCs were stained with a Zombie NIR Fixable Viability Kit (BioLegend), a fluorescent dye to assess their viability, and anti-human monoclonal antibodies (mAbs) specific for surface molecules, such as CD3 PerCP clone REA613, CD4 PECy7 clone REA623, and CD14 APC clone REA599 (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously reported [29,30]. Following surface molecule detection, PBMCs were fixed, permeabilized (Inside Stain Kit, Miltenyi Biotec), and incubated with mAbs directed against TNF-α FITC clone REA656 and IL-1β PE clone REA1172 (Miltenyi Biotec). Flow cytometry acquisition of stained PBMCs was carried out using the FACS Lyric cytometer (BD Biosciences, San Diego, CA, USA), and a minimum of 200,000 total events were acquired. Subsequently, sample analysis was performed using FlowJo v10 software (BD Biosciences).

*2.8. Intracellular Detection of Reactive Oxygen Species (ROS) Through $H_2$DCFDA Staining*

Intracellular levels of reactive oxygen species (ROS) were assessed by quantifying the oxidation of the cell-permeable dye 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) (Molecular Probe, Life Technologies, Eugene, OR, USA) [28,31]. PBMCs obtained from RA patients and HSs were treated as previously described for cytofluorimetric analysis but in the absence of Golgi blocker Monensin. Subsequently, the cells, after washing in PBS, were incubated in the dark at 37 °C with 5% $CO_2$ in the presence of 0.5 μM $H_2$DCFDA dye for 5 min. Following the incubation, excess fluorochrome was removed through PBS washing, and the intracellularly produced fluorescent 2′,7′-dichlorofluorescein (DCF), resulting from oxidation, was quantified using flow cytometric assays. These assays employed excitation and emission wavelengths suitable for green fluorescence, with an FITC filter having an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

*2.9. Western Blotting Analysis*

Western blot analyses were performed as previously reported [32–34]. Following treatment with LPS and/or PE-EVOOs, cells were rinsed with PBS and subsequently lysed at 4 °C for 30 min in an ice-cold lysis buffer containing 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate in PBS and supplemented with a protease inhibitor cocktail. The lysate was then sonicated in three 10 s bursts. Protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories S.r.l., Segrate, Milan, Italy). Subsequently, an equivalent amount of proteins (30 μg/lane) was loaded and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The gels were then transferred onto a nitrocellulose membrane (Bio-Rad). Immunodetection was carried out by incubating the membranes with specific primary antibodies: phosphorylated p65 NF-κB (Ser536, 93H1, Cell Signaling (Danvers, MA, USA)), p65 NF-κB (sc-8008, Santa Cruz, CA, USA), phosphorylated Nrf2 (pSer40, NBP2–67465, Santa Cruz, CA, USA), Nrf2 (NBP1-32822, Novus Biologicals Bio-Techne SRL, Milan, Italy), MnSOD (sc-133254, Santa Cruz, CA, USA), catalase (sc-271358, Santa Cruz, CA, USA), and COX2 (sc-7951, Santa Cruz, CA, USA). Finally, immunoreactive signals, developed using HRP-conjugated secondary antibodies (Amersham, GE Healthcare Life Science, Milan, Italy), were visualized with enhanced chemiluminescence (ECL) reagents (Cyanagen, Bologna, Italy) and captured with ChemiDoc XRS (Bio-Rad). Signal quantification was performed using Quantity One 1-D software. The intensity of the protein of interest was quantified via densitometric analysis using SMX Image software (Bio-Rad). Protein expression was normalized with GAPDH (AM4300, Invitrogen-TermoFisher Scientific, Milan, Italy) as a control.

*2.10. Statistical Analysis*

Multiple unpaired Student's *t*-tests by GraphPad Prism, version 9.0 (GraphPad Software, San Diego, CA, USA), were used to perform statistical analyses reported here, and all the data were expressed as mean ± SD. A *p*-value of $<0.05$ was considered statistically significant for each test. When not specified, the data are not significant with respect to the related control. All experiments were conducted in triplicate.

## 3. Results

*3.1. Characterization of Phytochemical Compounds Present in Sicilian EVOO Extracts*

The phenolic composition of EVOO is profoundly influenced by agronomic, technological, and storage conditions. Factors such as olive cultivar, ripening stage, environmental conditions, harvesting methods, and milling techniques contribute to the diversity and concentration of phenolic compounds. In order to assess the composition of EVOO cultivated in

the Mediterranean area (Sicily, Italy), ethanol extracts prepared as reported in the Section 2 were analyzed by an HPLC/ESI/Q-TOF in negative mode analysis. The analysis permitted the identification of 31 metabolites in ethanolic extracts of EVOO (Table 2). Approximately half of these metabolites belong to the saponifiable fraction, including carboxylic acids (mandelic acid and azelaic acid), fatty acids (oleic, linoleic, stearic, and palmitic acids), and fatty acid derivatives. The remaining metabolites were part of the unsaponifiable fraction, with derivatives of 3-hydroxytyrosol (3,4-dihydroxyphenylethanol, 3,4-DHPEA) emerging as the predominant compounds in the polar phenol extract. Ligstroside, formed by ester bonds between 3,4-DHPEA and oleanolic acid or its demethylated form, is one of two secoiridoids dominant in polar phenol extracts.

**Table 2.** The bioactive natural compounds in the phenolic extracts of Sicilian EVOO. Metabolites were identified using HPLC-ESI-MS analysis.

| | Compound | Rt (min) | ESI$^+$ [M-H]$^-$ (m/z) (*Exp.*) | ESI$^-$ [M-H]$^-$ (m/z) (*Teor.*) | Molecular Formula | Metabolite Classes |
|---|---|---|---|---|---|---|
| 1 | Mandelic acid | 3.17 | 151.0417 | 151.0401 | $C_8H_8O_3$ | Carboxylic acid |
| 2 | Abscisic acid | 7.59 | 309.1368 [a] | 309.1344 | $C_{15}H_{20}O_4$ | Phytohormone |
| 3 | Azelaic acid | 9.19 | 187.1010 | 187.0976 | $C_9H_{16}O_4$ | Carboxylic acid |
| 4 | 3-hydroxytyrosol 4-glucoside | 9.95 | 351.0801 | 351.0852 | $C_{14}H_{20}O_8$ | Phenolic compound |
| 5 | Abscisic acid isomer | 10.45 | 309.1368 [a] | 309.1344 | $C_{15}H_{20}O_4$ | Phytohormone |
| 6 | Erythritol phosphate | 13.82 | 201.0218 | 201.0170 | $C_4H_{11}O_7P$ | Sugar derivative |
| 7 | Oleuropein aglycon | 15.98 | 377.1276 | 377.1242 | $C_{19}H_{22}O_8$ | Secoiridoid |
| 8 | 5,6′-dihydroxy-6,7-dimethoxyflavone-2′-O-glucoside | 16.69 | 491.1220 | 491.1195 | $C_{23}H_{24}O_{12}$ | Flavone |
| 9 | Trihydroxyoctadecenoic acid | 17.17 | 329.2379 | 329.2334 | $C_{18}H_{34}O_5$ | Fatty acid derivative |
| 10 | Deoxygeniposidic acid | 17.33 | 357.1155 | 357.1191 | $C_{16}H_{22}O_9$ | Iridoid glucoside |
| 11 | Coniferyl ferulate | 17.64 | 355.1225 | 355.1187 | $C_{20}H_{20}O_6$ | Monolignol cinnamate |
| 12 | Methyltyrosine | 19.48 | 194.0854 | 194.0823 | $C_{10}H_{13}NO_3$ | Amino acid derivative |
| 13 | 3-hydrossityrosol heptanoate | 21.58 | 265.1513 | 265.1445 | $C_{15}H_{22}O_4$ | Phenolic compound |
| 14 | Decyl gallate | 25.41 | 309.1775 | 309.1708 | $C_{17}H_{26}O_5$ | Phenolic compound |
| 15 | Hydroxyoctadecadienoic acid | 25.89 | 295.2321 | 295.2279 | $C_{18}H_{32}O_3$ | Fatty acid derivative |
| 16 | Hydroxylinolenic acid | 26.20 | 293.2165 | 293.2122 | $C_{18}H_{30}O_3$ | Fatty acid derivative |
| 17 | Dihydroxyoctadecadienoic acid | 26.20 | 311.2273 | 311.2228 | $C_{18}H_{32}O_4$ | Fatty acid derivative |
| 18 | Dihydroxyoctadecenoic acid | 26.76 | 313.2421 | 313.2384 | $C_{18}H_{34}O_4$ | Fatty acid derivative |
| 19 | Gingerol | 27.06 | 293.1826 | 293.1758 | $C_{17}H_{26}O_4$ | Phenolic compound |
| 20 | Dihydroxypropyl tetradecanoate | 27.81 | 337.2091 [b] | 337.2151 | $C_{17}H_{34}O_4$ | Fatty acid derivative |
| 21 | Olean-12-ene-3,7,15,21,23,28-hexol | 27.89 | 551.3639 [a] | 551.3590 | $C_{30}H_{50}O_6$ | Triterpenoid |
| 22 | Linoleic acid | 28.34 | 279.2361 | 279.2330 | $C_{18}H_{32}O_2$ | Fatty acid |
| 23 | Linoleyl 3-hydroxytyrosol | 28.87 | 415.2906 | 415.2854 | $C_{26}H_{40}O_4$ | Phenolic compound |
| 24 | Oleic acid | 29.22 | 281.2521 | 281.2486 | $C_{18}H_{34}O_2$ | Fatty acid |
| 25 | Palmitoyl 3-hydroxytyrosol | 29.48 | 391.2895 | 391.2854 | $C_{24}H_{40}O_4$ | Phenolic compound |
| 26 | Oleyl 3-hydroxytyrosol | 29.67 | 417.3054 | 417.3010 | $C_{26}H_{42}O_4$ | Phenolic compound |
| 27 | Oleanene-tetrol | 30.18 | 519.3732 [a] | 519.3691 | $C_{30}H_{50}O_4$ | Triterpenoid |
| 28 | Stearic acid | 30.58 | 283.2676 | 283.2643 | $C_{18}H_{36}O_2$ | Fatty acid |
| 29 | Palmitic acid | 30.79 | 255.2369 | 255.2330 | $C_{16}H_{32}O_2$ | Fatty acid |
| 30 | Octadecanoyl-glycerol | 32.08 | 393.2829 [b] | 393.2777 | $C_{21}H_{42}O_4$ | Fatty acid derivative |
| 31 | Palmitoyl 3-hydroxytyrosol isomer | 32.25 | 391.2906 | 391.2854 | $C_{24}H_{40}O_4$ | Phenolic compound |

[a] formiate; [b] chloride.

### *3.2. PE-EVOOs Possess Radical Scavenging Activity*

To ascertain the potential radical scavenging activity of PE-EVOOs, different concentrations of the extracts (0.5–50 μg/mL) were incubated with DPPH, as reported in the Section 2. As shown in Figure 1, our data demonstrated that PE-EVOOs own a high and dose-dependent radical scavenging activity. Indeed, at the lower tested concentration (0.5 μg/mL), PE-EVOOs inhibited DPPH radicals by only 15%. This effect rises by increasing the dose of PE-EVOOs, reaching an inhibition value of 90% at 50 μg/mL.

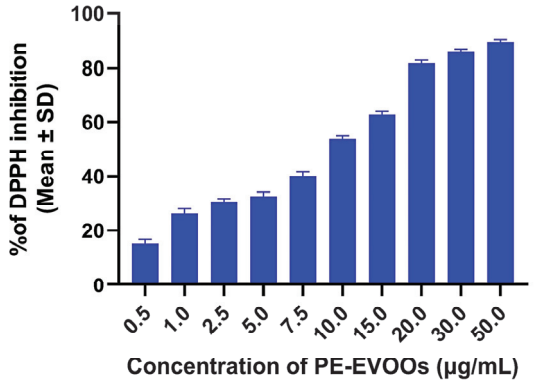


**Figure 1.** PE-EVOOs exert a high dose-dependent radical scavenging activity. The antioxidant activity of Sicilian PE-EVOOs was evaluated by a DPPH radical scavenging assay. Different concentrations of PE-EVOOs (from 0.5 to 50 μg/mL) were added to an ethanol DPPH• solution, and the relative absorption was measured at 517 nm spectrophotometrically. The bar graphs represent the mean of three independent experiments ± SD. *p*-value summary < 0.001 compared to the only vehicle control (ethanol).

### *3.3. Effects of PE-EVOOs on PBMC Viability*

The present study aimed to investigate whether PE-EVOOs were capable of counteracting oxidative stress and inflammatory pathways in RA patients' PBMCs. To rule out potential cytotoxic effects of PE-EVOOs on PBMCs, preliminary experiments were performed by incubating HS PBMCs with different doses (0.5–50 μg/mL) of PE-EVOOs for 48 h. Then, the viability was evaluated by an MTS assay, as reported in the Section 2. The results reported in Figure 2 indicate that PE-EVOOs at 50 μg/mL reduced cell viability by 67%. However, doses of PE-EVOOs lower than 25 μg/mL did not affect PBMC viability.

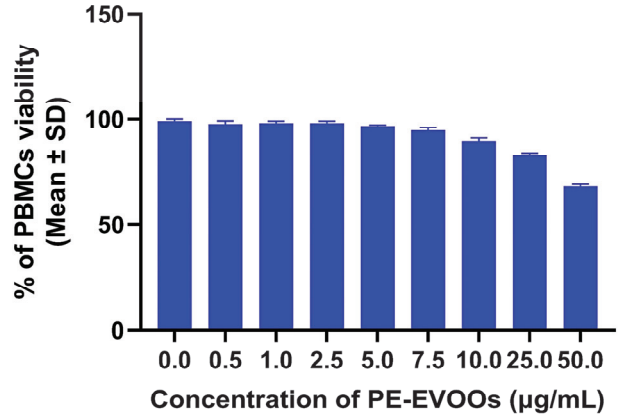


**Figure 2.** PE-EVOO effects on PBMC viability. PBMCs ($100 \times 10^3$/100 μL/well) were incubated with different doses (0.5–50 μg/mL) of PE-EVOOs for 48 h. Then, the percentage of viable cells was assessed by an MTS assay. The values reported are the mean ± SD of three independent experiments. *p*-value summary < 0.05 with respect to cells cultured in medium alone (RPMI).

Based on these preliminary data, the concentration of PE-EVOOs chosen for further experiments was 10 μg/mL, exhibiting 55% radical scavenging activity and a very low cytotoxic effect on PBMCs (about 10%).

### *3.4. PE-EVOOs Exert Anti-Inflammatory Effects in RA Patients and HS LPS-Stimulated PBMCs*

Then, to investigate whether PE-EVOOs exert anti-inflammatory effects on RA patients' PBMCs, we isolated them from RA patients, as described in the Section 2, and treated them in vitro for 48 h with 10 μg/mL of PE-EVOOs. After treatments, cytofluorimetric analyses were performed to evaluate the production by PBMCs of TNF-α and IL-1β, the main cytokines playing an important role in the development and progression of inflammatory rheumatic disease (IRDs). As shown by cumulative data of cytofluorimetric analyses obtained from RA patients and reported in Figure 3A, PE-EVOO treatment reduced the percentage of RA PBMCs producing both TNF-α and IL-1β with respect to untreated RA PBMCs. In fact, the mean percentage of TNF-$\alpha^+$ RA PBMCs lowered from 4.73 (SD ± 0.681) in untreated cells to 3.40 (SD ± 0.490) in PE-EVOO-treated ones. In addition, the percentage of IL-1$\beta^+$ RA PBMCs decreased from 3.31 (SD ± 0.375) in untreated cells to 2.75 (SD ± 0.558) in PBMCs treated for 48 h with PE-EVOOs.

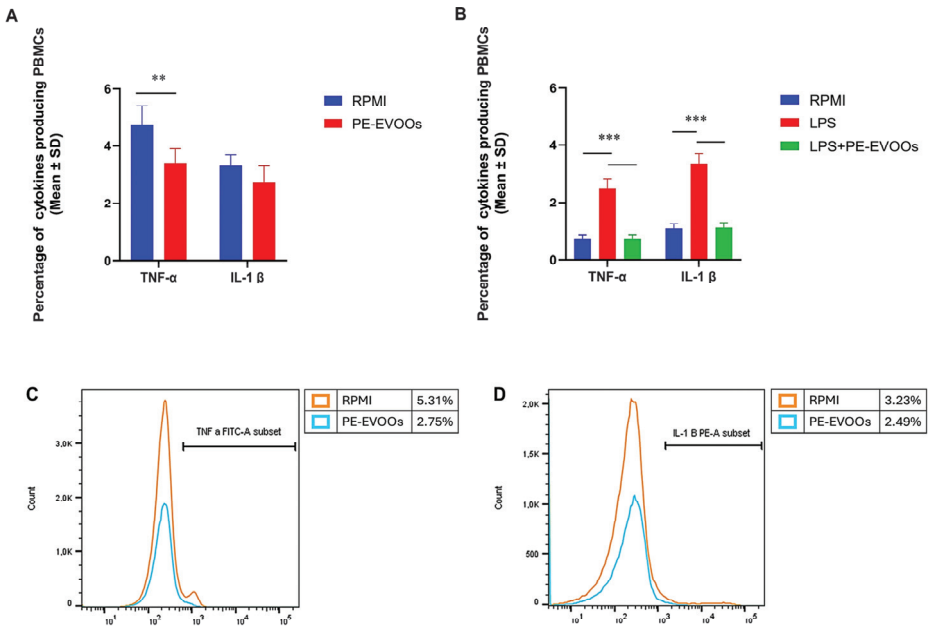


**Figure 3.** PE-EVOOs reduce the production of pro-inflammatory cytokines from RA patients and HS LPS-stimulated PBMCs. Cumulative data obtained from flow cytometric analyses of RA patients (**A**) and HS LPS-stimulated (**B**) PBMCs intracellularly stained for TNF-α and IL-1β show that both cytokines decreased after 48 h of 10 μg/mL PE-EVOO treatment. Histogram overlays of the production of TNF-α (**C**) and IL-1β (**D**) from an RA representative patient. ** $p < 0.01$ and *** $p < 0.001$ with respect to cells cultured in medium alone (RPMI) or with LPS (LPS).

We also evaluated the effects of PE-EVOOs treated on HS PBMCs stimulated with 5 μg/mL of LPS, as reported in the Section 2, to simulate in vitro the inflammatory conditions that characterize RA patients. Interestingly, the anti-inflammatory effect of PE-EVOOs was also observed in LPS-treated HS PBMCs. In fact, in HS PBMCs, LPS treatment increased the production of both TNF-α and IL-1β, and these cytokines were both reduced in a statistically significant manner when the cells were pretreated with PE-EVOOs. In fact, as the same Figure 3B shows, the mean percentage for TNF-$\alpha^+$ cells was reduced from 2.49 (SD ± 0.347) in PBMCs stimulated with LPS alone to 0.73 (SD ± 0.129) in PE-

EVOO-pretreated cells and for IL-1β$^+$ cells from 3.35 (SD ± 0.334) in PBMCs stimulated with LPS alone to 1.11 (SD ± 0.174) in PE-EVOO-pretreated cells. Figure 3C,D show histogram overlays of the production of TNF-α and IL-1β, respectively, obtained from an RA-representative patient.

In addition, to understand which cell subset was more susceptible to the anti-inflammatory PE-EVOO effect, cytofluorimetric analyses were performed on RA patients and HSs to evaluate the effect of PE-EVOO treatment on TNF-α production from T helper cells and monocytes, characterized by different cell surface markers (CD3$^+$/CD4$^+$ for T helper subset and CD3$^-$/CD14$^+$ for monocytes). Our data reported in Figure 4A demonstrated that 48 h of treatment with 10 μg/mL of PE-EVOOs reduced the percentage of monocytes producing TNF-α in RA patients, with a mean percentage going from 2.91 (SD ± 0.130) in untreated PBMCs to 2.18 (SD ± 0.356) in PE-EVOO-treated PBMCs. The same effect was observed in HSs, where the percentage of monocytes producing TNF-α lowered from 0.79 (SD ± 0.065) in LPS-stimulated PBMCs to 0.54 (SD ± 0.097) in PE-EVOO-pretreated LPS-stimulated PBMCs (Figure 4B). Instead, the production of TNF-α from T helper cells remained unvaried in both RA and LPS-stimulated HS PBMCs.

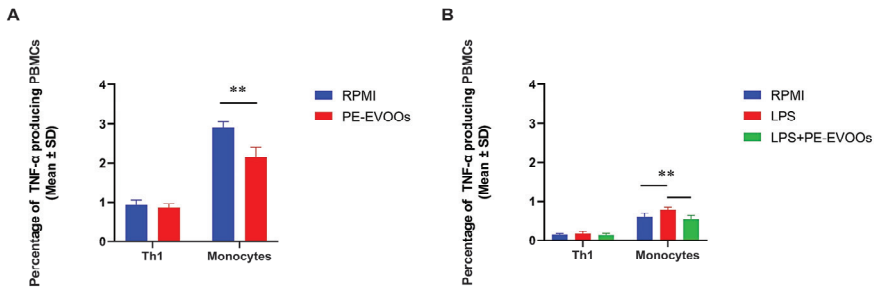


**Figure 4.** PE-EVOOs reduce TNF-α production only from monocytes and not from T helper cells. Cumulative data obtained from flow cytometric analyses of RA patients (**A**) and HS LPS-stimulated (**B**) PBMCs intracellularly stained for TNF-α production from Th1 cells and monocytes show that only monocytes producing TNF-α decreased after 48 h of 10 μg/mL PE-EVOO treatment. ** $p < 0.01$ with respect to cells cultured in medium alone (RPMI) or with LPS.

### *3.5. PE-EVOO Treatment Reduces the Activation of NF-κB in RA Patients and HS LPS-Stimulated PBMCs*

NF-κB is identified as one of the main transcription factors involved in the transcription of inflammatory cytokines, like IL-1β, TNF-α, and COX2, whose increased levels in RA are associated with bone erosion, pain hypersensitivity, and disease progression [35].

In mammals, the NF-κB family consists of five members: RelA (p65), RelB, c-Rel, NF-kB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100), which form different homodimers and heterodimers, and each of them activates its own characteristic target genes [36–38]. It has been reported that TNF-α induces via IKK the phosphorylation of p65 RelA on Ser-536, leading to the activation of NF-κB signaling and enhanced inflammation [39]. For this reason, we investigated the ability of PE-EVOOs to modulate the activity of phospo-NF-κB p65 (Ser536) in RA patients and HS LPS-stimulated PBMCs. The expression levels of both NF-κB and its phosphorylated p65 subunit were evaluated by Western blotting analysis in both RA patients and HS LPS-stimulated PBMCs alone or after 48 h of treatment with 10 μg/mL of PE-EVOOs. Our data demonstrated that the PE-EVOO treatment reduced the levels of phosphorylated NF-κB p65 in both RA patients and HS LPS-stimulated PBMCs, whereas the expression levels of total NF-κB did not change in both cases (Figure 5). Thus, the reduced activity of NF-κB could, in part, mediate the anti-inflammatory effect of PE-EVOOs.

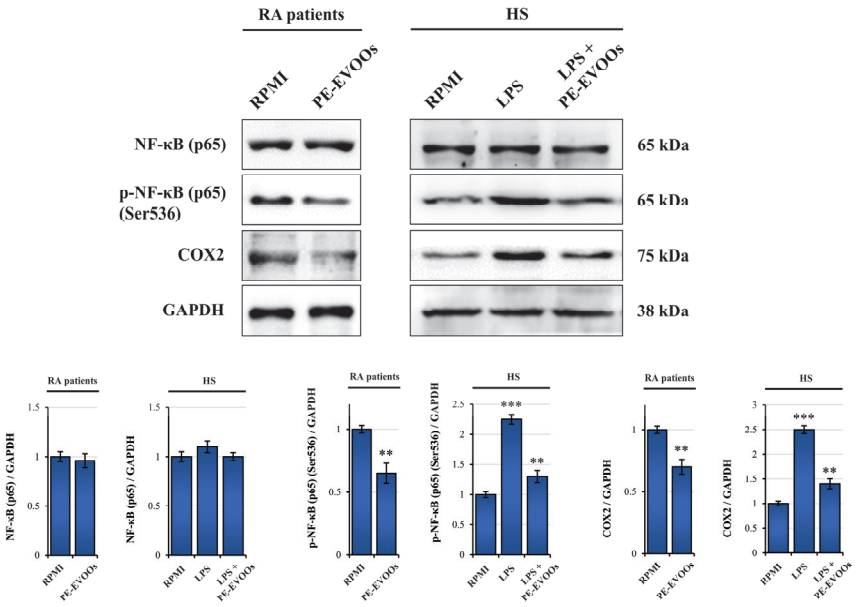


**Figure 5.** PE-EVOOs exert anti-inflammatory effects by reducing the expression of the active form of NF-κB and COX2. Western blotting analysis of NF-κB, phosphorylated-NF-κB, and COX2 in RA patients and HS LPS-stimulated PBMCs treated for 48 h with 10 μg/mL of PE-EVOOs. Equal loading of proteins was verified by immunoblotting for GAPDH. The bar graphs represent the mean of three independent experiments. ** $p < 0.01$ and *** $p < 0.001$ with respect to cells cultured in medium alone (RPMI).

Moreover, in RA patients and HS LPS-stimulated PBMCs, PE-EVOO treatment also reduced the expression levels of COX2, a pro-inflammatory enzyme whose expression is upregulated by the increased IL-1β secretion via NF-kB [40] (Figure 5).

### *3.6. PE-EVOOs Exert Antioxidative Effects in RA Patients and HS LPS-Stimulated PBMCs*

It has been reported that ROS generation plays a key role in the pathogenesis of several IRDs, including RA [41]. It is now well established that elevated ROS production amplifies the inflammatory response by upregulating genes associated with immune and inflammatory cytokines through the activation of NF-κB, a nuclear transcription factor sensitive to redox signaling [42].

Therefore, we investigated the ability of PE-EVOOs to reduce ROS production in both RA and LPS-stimulated HS PBMCs. Cumulative cytofluorimetric analyses, obtained by the oxidation of the H2DCFDA dye, showed that 48 h of treatment with 10 μg/mL of PE-EVOOs caused a reduction in intracellular ROS in RA patients' PBMCs, from a mean percentage of 47.14 (SD ± 8.322) $H_2DCFDA^+$ untreated PBMCs to 33.40 (SD ± 6.542) $H2DCFDA^+$ PE-EVOOs treated PBMCs (Figure 6A). A similar effect was observed in HS PBMCs, where PE-EVOO treatment significantly reduced ROS production induced by LPS treatment (5 μg/mL) (Figure 6B). The mean percentage of $H_2DCFDA^+$ cells decreased from 36.68 (SD ± 14.268) in LPS-stimulated PBMCs to 14.60 (SD ± 4.771) in PE-EVOO-pretreated LPS-stimulated PBMCs (Figure 6B). Figure 6C,D show histogram overlays of intracellular ROS production obtained from PBMCs of a representative RA patient and HSs, respectively.

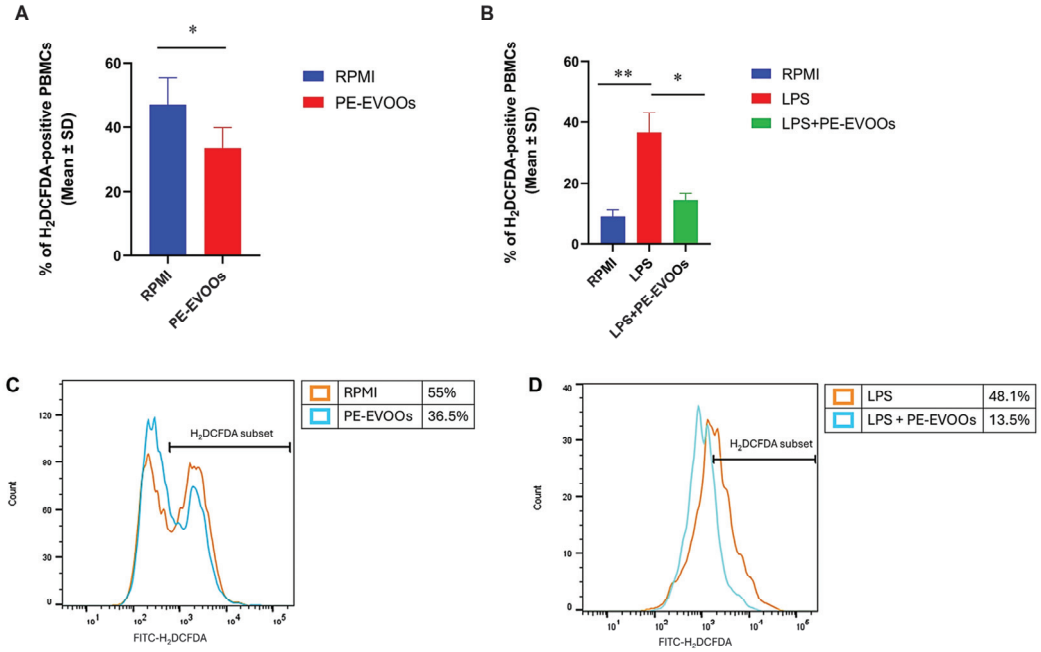


**Figure 6.** PE-EVOOs exert antioxidant effects in RA patients and HS LPS-stimulated PBMCs. Cumulative data obtained from flow cytometric analysis of RA patients (**A**) and HS LPS-stimulated (**B**) PBMCs treated for 48 h with 10 µg/mL of PE-EVOOs stained for intracellular ROS using the redox-sensitive fluorochrome H2-DCFDA, as reported in the Section 2. Histogram overlays of intracellular ROS production obtained from PBMCs of a representative RA patient (**C**) and HSs (**D**). * $p < 0.05$ and ** $p < 0.01$ with respect to cells cultured in medium alone (RPMI) or with LPS.

### *3.7. PE-EVOO Treatment Upregulates the Antioxidant Nrf2-Mediated Pathway in RA Patients and HS LPS-Stimulated PBMCs*

To deeply investigate the molecular mechanisms responsible for PE-EVOO antioxidant effect on RA patients and HSs, we performed Western blotting experiments to evaluate the ability of PE-EVOOs to upregulate Nrf2, a transcription factor that controls the expression of several antioxidant genes, regulating the physiological and pathophysiological outcomes of oxidant exposure [43]. We demonstrated that PE-EVOO treatment increased the levels of phosphorylated and active forms of Nrf2 in RA patients and HS LPS-stimulated PBMCs, while the levels of total Nrf2 did not change (Figure 7).

According to these data, we further demonstrated by Western blotting analysis that the expression levels of catalase and MnSOD, two important ROS scavenger enzymes regulated transcriptionally by Nrf2 [44], markedly increased in PBMCs from RA patients and HSs stimulated with LPS after 48 h of PE-EVOO treatment.

Taken together, these results seem to indicate that the activation of Nrf2 and antioxidant enzymes, catalase and MnSOD, could mediate the antioxidant behavior of PE-EVOOs in both RA patients and HS LPS-stimulated PBMCs.

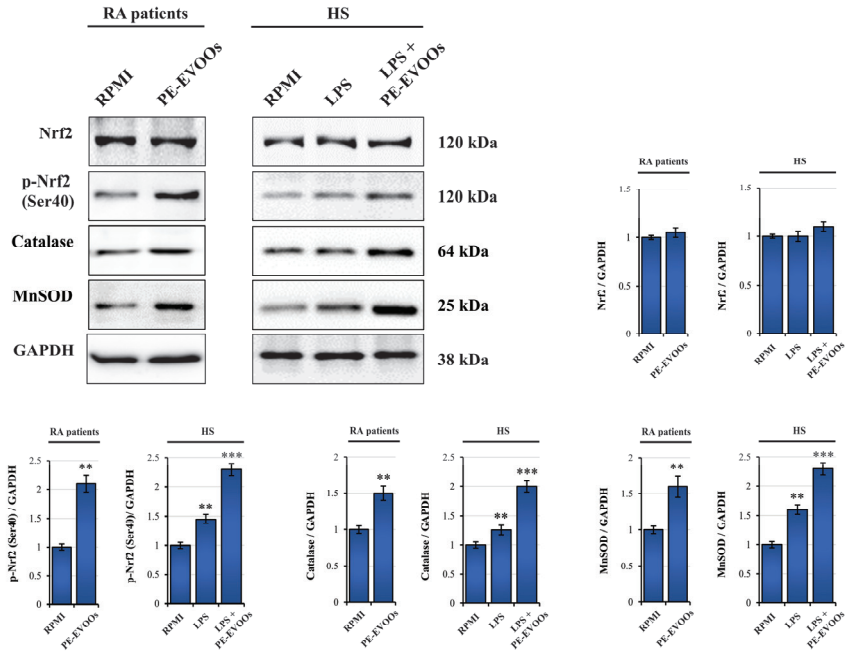


**Figure 7.** PE-EVOOs exert antioxidant effects upregulating the active form of Nrf2 and its transcriptional targets, catalase and MnSOD. Western blotting analysis of Nrf2, p-Nrf2 (Ser 40), catalase, and MnSOD in RA patients and HS LPS-stimulated PBMCs treated for 48 h with 10 μg/mL of PE-EVOOs. Equal loading of proteins was verified by immunoblotting for GAPDH. The bar graphs represent the mean of three independent experiments ± SD. ** $p < 0.01$ and *** $p < 0.001$ with respect to cells cultured in medium alone (RPMI).

## 4. Discussion

RA is one of the most prevalent complex, chronic, inflammatory diseases, manifested by elevated oxidative stress and inflammatory biomarkers [45]. While current RA therapies, including conventional DMARDs, biologics, and corticosteroids, have significantly improved the course of the disease, there is an urgent need for novel approaches that can better target the underlying pathogenetic mechanisms, reduce side effects, prevent joint damage, and improve long-term quality of life [46]. Recently, several natural compounds have been shown to exert a supportive role in managing RA, helping to reduce inflammation, relieve pain, and potentially improve joint health [21].

As part of the ongoing quest to identify natural compounds for the treatment of RA, our study focused on investigating the anti-inflammatory and antioxidant properties of polyphenol-rich extracts derived from extra virgin olive oil produced in Sicily, Italy. This region, located in the Mediterranean basin, is characterized by an optimal climate, traditional olive harvesting techniques, and the immediate milling of olives after harvest, all of which contribute to producing high-quality EVOO with a superior nutritional and chemical profile. Notably, the DPPH assay evidenced that ethanol extracts of Sicilian EVOO possess strong ROS scavenging properties. The characterization of the polyphenolic profile of these extracts using HPLC/MS provided evidence that they are rich in polyphenols. In particular, we demonstrated the presence of 3,4-DHPEA and its derivatives. The high prevalence of 3,4-DHPEA and its derivatives underscores the significant role these compounds play in EVOO's biological effects. The potent antioxidant activity of 3,4-DHPEA is well documented, with mechanisms such as free radical scavenging and metal ion chelation supporting its cardioprotective, neuroprotective, and anticancer effects [47,48]. While the antioxidant activity forms the foundation of its health benefits, the molecular pathways underlying its diverse biological effects warrant further exploration [15].

TNF-α, IL-1β, and IL-6 are pro-inflammatory cytokines that play a critical role in the pathogenesis and progression of RA, with growth-promoting properties directed towards synovial fibroblasts whose hyperproliferation is responsible for the alteration of joint structures and destruction of connective tissue and subchondral bone [49]. Therefore, elevated serum levels of these cytokines are commonly observed in individuals with active RA and are associated with disease severity and joint damage [50]. In line with this observation, we demonstrated that RA PBMCs expressed elevated levels of both TNF-α and IL-1β. Notably, an increased expression of both pro-inflammatory cytokines was observed in healthy-subject PBMCs stimulated with lipopolysaccharide LPS to mimic an inflammatory condition. Notably, RA PBMCs were not stimulated with LPS because it is widely reported that the high basal secretion of pro-inflammatory cytokines characterizing RA patients causes a decrease in the ability to secrete increased amounts of cytokines in response to pro-inflammatory stimulation, such as LPS [51].

High levels of circulating TNF-α, produced primarily by activated macrophages and T cells and, to a lesser extent, by neutrophils, mast cells, and endothelial cells [52], lead to fibroblast activation in RA patients, resulting in the recruitment of inflammatory cells into the lesion and the NF-κB-mediated secretion of inflammatory cytokines, cathepsins, matrix metalloproteinases, and other inflammatory mediators, which cause destructive changes in the joints [53]. TNF-α binds to the ubiquitously expressed TNF receptor 1 (TNFR1/TNFR-55) or the tissue-restricted TNFR2 (TNFR-75) [54]. TNFR1 binding activates canonical NF-κB, the MAP kinases p38 and JNK, and due to the presence of a death domain, apical caspases. Signaling via TNFR2, which does not contain a death domain, activates MAP kinases and also leads to noncanonical NF-κB activation [54,55], causing the differentiation of multiple Th subsets towards inflammatory phenotypes, like Th1 and Th17.

IL-1β is a member of the interleukin-1 family, produced by activated macrophages, monocytes, and a subpopulation of dendritic cells called slanDC, as a proprotein, which is proteolytically processed into its active form by caspase 1 (CASP1/ICE) [56]. IL-1β is a central mediator of innate immunity and inflammation, leading to fever onset and immune activation upon binding to IL-1 receptor 1, and its secretion is tightly regulated.

The activation of Toll-like receptors (TLRs), TNF-α receptors, or IL-1 receptors upon binding of mature IL-1α or IL-1β, induces the transcription of the biologically inactive pro-form, although for its secretion, inflammasome activation is requested [56,57]. IL-1β, together with TNF-α and IL-6 serum levels, often correlate with disease severity, including joint swelling and bone erosion [58,59]. For this reason, a reduction in the synthesis of both TNF-α and IL-1 β using bioactive natural compounds may represent an effective method to manage inflammation in IRDs, including RA [60].

Interestingly, we found that exposure to PE-EVOOs significantly reduced the production of TNF-α and IL-1β, both in RA PBMCs and in HS PBMCs stimulated with LPS, thus suggesting an anti-inflammatory potential of EVOO extracts. In addition, the observation that the treatment with PE-EVOOs efficiently reduces specifically monocytic TNF-α production confirms that their use could be beneficial to RA symptoms, given its role in cartilage and bone infiltration and destruction characterizing RA progression. Our findings in fact support the literature data reporting that TNF-α-producing macrophages are the most abundant immune cells found in RA synovium, where they produce also other pro-inflammatory cytokines (IL-1β and IL-6), chemoattractant factors (CCL2 and IL-8), and metalloproteinases (MMP-3 and MMP-12) [61–63], inducing cellular proliferation and increased vascular permeability, which contribute to the pathogenic features of tissue proliferation and neovascularization characterizing rheumatoid synovium, along with cartilage and bone infiltration and destruction [63].

It has been known that IL-1β upregulates the expression of COX2, an inducible enzyme that converts arachidonic acid into prostaglandins, amplifying the inflammatory response [64]. In turn, COX2-derived prostaglandins, like PGE2, can enhance the release of IL-1β from immune cells, creating a feedback loop that sustains inflammation [65]. In line with these suggestions, the reduction in COX2 production observed in RA PBMC treated with PE-EVOOs may contribute to reduced inflammation in RA patients.

Our data also demonstrated that PE-EVOOs reduced the active phosphorylated form of NF-κB in both RA and healthy LPS-stimulated PBMCs. NF-κB is a critical transcription factor that controls the expression of a wide array of pro-inflammatory genes [65]. Its activation is promoted by different pro-inflammatory stimuli, like TNF-α or IL-1β, or TLR activation by pathogens [66]. When activated, NF-κB binds to specific DNA response elements in the nucleus, initiating the transcription of pro-inflammatory genes, including TNF-α and other cytokines, such as IL-1β and IL-6, in a positive feedback loop [67]. Thus, the reduction in NF-κB activation may explain the anti-inflammatory role exerted by PE-EVOOs in RA PBMCs.

A link between chronic inflammation and oxidative stress has been observed, with free radicals being the cause of many chronic diseases, such as RA [68]. Sustained ROS production can lead to chronic NF-κB activation, contributing to inflammation and tissue damage [68]. ROS act as signaling molecules activating NF-κB by activating the IκB kinase (IKK) complex, which phosphorylates IκB (inhibitor of NF-κB), marking it for ubiquitination and proteasome 26S degradation [68]. This releases NF-κB, allowing it to translocate to the nucleus and activate target genes [68]. ROS can also directly oxidize specific cysteine residues on IKK or other upstream signaling proteins, modulating their activity [69]. Thus, the reduction in ROS level can contribute to inactivating NF-κB and inflammation. Notably, our data provided evidence that PE-EVOOs reduce the production of ROS both in RA PBMCs and HS LPS-stimulated PBMCs. The antioxidant effects of PE-EVOOs could be explained by the ability of these extracts to upregulate the transcriptional factor Nrf2 and its antioxidant target enzymes MnSOD and catalase in RA PBMCs and healthy LPS-stimulated PBMCs.

In line with our hypothesis, it has been shown that Nrf2 prevents LPS-induced transcriptional upregulation of several pro-inflammatory cytokines, such as IL-6 and IL-1β [70], whose production is increased in Nrf2−/− mice with dextran sulfate-induced colitis [70]. Nrf2 also inhibits the production of IL-17 and other Th1 cytokines, reducing disease progression in autoimmune encephalitis, an experimental model of multiple sclerosis [71]. Furthermore, Nrf2-dependent antioxidant genes, such as HO-1, NQO-1, Gclc, and Gclm, block the production of TNF-α, IL-6, monocyte chemo-attractant protein-1 (MCP1), macrophage inflammatory protein-2 (MIP2), and other inflammatory mediators [70,72]. Our results, therefore, underline how the marked anti-inflammatory activity of PE-EVOOs is strongly correlated to the reduction in oxidative stress through the increased expression of the active form of Nrf2 and its target antioxidant genes, MnSOD and catalase.

## 5. Conclusions

Our findings confirm the dual anti-inflammatory and antioxidant efficacy of PE-EVOOs, underscoring their potential as natural therapeutic agents for managing RA. The anti-inflammatory activity of PE-EVOOs seems to be mediated by the inhibition of the NF-κB signaling pathway. This suppression significantly reduces the expression of COX2, a pivotal enzyme in the inflammatory process, and decreases the synthesis of critical pro-inflammatory cytokines, such as TNF-α and IL-1β. This mechanism may help to mitigate the chronic inflammation associated with RA and other degenerative diseases.

Simultaneously, PE-EVOOs exhibit robust antioxidant properties by neutralizing ROS and upregulating the expression of essential antioxidant enzymes, including catalase and MnSOD. This effect seems to involve the activation of Nrf2. By protecting cells from oxidative damage, which often exacerbates chronic inflammation, PE-EVOOs provide a comprehensive therapeutic benefit.

These results highlight the potential of PE-EVOOs not only to modulate inflammatory responses but also to enhance antioxidant defense mechanisms, suggesting a valuable role in the management of chronic inflammatory conditions, such as RA. By leveraging their natural bioactive properties, PE-EVOOs could serve as an adjunct to conventional RA therapies, contributing to pain relief, inflammation reduction, and improved overall treatment outcomes. This dual action underscores their promise as a complementary natural intervention for enhancing the quality of life in individuals with RA. Comprehensive clinical trials are needed to confirm the efficacy and safety of PE-EVOOs in diverse patient populations and to explore optimal dosing regimens. Such research will be crucial to translate these preliminary findings into viable therapeutic strategies, paving the way for their integration into evidence-based management plans for RA and other chronic inflammatory diseases.

**Author Contributions:** Conceptualization, B.T., D.D.L., M.L. and G.G.; methodology, D.C. and A.D.; investigation, B.T., D.D.L., G.P., A.M., A.P.P., C.R. and L.L.B.; data curation, D.D.L. and G.P.; software, G.P. and D.C.; writing—original draft preparation, D.D.L. and M.L.; review and editing, all authors; supervision N.C. and G.G.; funding acquisition, N.C. and G.G. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study involves human participants and was approved by the local Ethics Committee of the University of Palermo and complied with the provisions of the Declaration of Helsinki.

**Informed Consent Statement:** Informed consent was obtained from all patients and control.

**Data Availability Statement:** Data presented in this study are available in this manuscript.

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## Abbreviations

| | |
|---|---|
| COX2 | Cyclooxygenase 2 |
| CIA | Collagen-induced arthritis |
| DMARDs | Disease-modifying anti-rheumatic drugs |
| DPPH | 1,1-diphenyl-2-picrylhydrazyl |
| EVOO | Extra virgin olive oil |
| $H_2$DCFDA | 2′,7′-dichlorodihydrofluorescein diacetate |
| IL-1β | Interleukin-1β |
| IRD | Inflammatory rheumatic disease |
| IκB | Inhibitor of NF-κB |
| IKK | IκB kinase |
| LPS | Lipopolysaccharide |

| | |
|---|---|
| MnSOD | Manganese superoxide dismutase |
| MTS | 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium |
| NF-κB | Nuclear factor kappa-light-chain enhancer of activated B cells |
| Nrf2 | NF-E2-related factor 2 |
| PBMCs | Peripheral blood mononuclear cells |
| PE-EVOOs | Polyphenol-enriched extracts |
| ROS | Reactive oxygen species |
| Th1 | T helper 1 |
| Th17 | T helper17 |
| TNF-α | Tumor necrosis factor-α |

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antioxidants

*Article*

# Antioxidant Capacity of Free and Bound Phenolics from Olive Leaves: In Vitro and In Vivo Responses

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**Abstract:** Olive leaves are rich in phenolic compounds. This study explored the chemical profiles and contents of free phenolics (FPs) and bound phenolics (BPs) in olive leaves, and further investigated and compared the antioxidant properties of FPs and BPs using chemical assays, cellular antioxidant evaluation systems, and in vivo mouse models. The results showed that FPs and BPs have different phenolic profiles; 24 free and 14 bound phenolics were identified in FPs and BPs, respectively. Higher levels of phenolic acid (i.e., sinapinic acid, 4-coumaric acid, ferulic acid, and caffeic acid) and hydroxytyrosol were detected in the BPs, while flavonoids, triterpenoid acids, and iridoids were more concentrated in the free form. FPs showed a significantly higher total flavonoid content (TFC), total phenolic content (TPC), and chemical antioxidant properties than those of BPs ($p < 0.05$). Within the range of doses (20–250 μg/mL), both FPs and BPs protected HepG2 cells from $H_2O_2$-induced oxidative stress injury, and there was no significant difference in cellular antioxidant activity between FPs and BPs. The in vivo experiments suggested that FP and BP treatment inhibited malondialdehyde (MDA) levels in a D-galactose-induced oxidation model in mice, and significantly increased antioxidant enzyme activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and the total antioxidant capacity (T-AOC). Mechanistically, FPs and BPs exert their antioxidant activity in distinct ways; FPs ameliorated D-galactose-induced oxidative stress injury partly via the activation of nuclear factor erythroid-2-related factor 2 (Nrf2) signaling pathway, while the BP mechanisms need further study.

**Keywords:** olive leaves; free phenolics; bound phenolics; antioxidant properties; oxidative stress

## 1. Introduction

Oxidative stress is a destructive state of signaling caused by excessive production of reactive oxygen/nitrogen species (ROS/RNS), resulting in imbalanced redox control between oxidants and antioxidants that favors the oxidants [1,2]. Oxidative stress has been considered the key trigger of many chronic diseases, such as cardiovascular disease, inflammation, diabetes, and cancer [3,4]. Therefore, supplementation with external antioxidants is required to resist oxidative stress and maintain oxidative balance. In this context, natural antioxidants, such as polyphenols, carotenoids, phytosterols, ascorbic acid, and polyunsaturated fatty acids, with safe and efficient antioxidant effects, have attracted considerable interest.

Although antioxidant activity can be assessed in many ways on the basis of different mechanisms, several studies on antioxidant foods and components only use in vitro chemical antioxidant methods to characterize their activities, due to the low cost and easy implementation [5–7]. It is necessary to realize that in vitro antioxidant evaluations, such as 2,2′-azinobis-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition, and ferric reducing antioxidant power (FRAP) assay, usually have low biological significance. Antioxidant defense is a complex and multicomponent enzymatic defense system, which typically includes GSH-Px, CAT, SOD, and different exogenous antioxidants that may have selective action on specific antioxidant enzymes [8]. In vitro chemical antioxidant methods have limitations for assessing absorption, bioavailability, distribution, and metabolism [9]. As a result, inconsistent results are often observed between the in vitro antioxidant potential and the in vivo responses of antioxidant foods and components [10,11], indicating that in vitro antioxidant potential is sometimes not reproduced in vivo [12]. In order to obtain an accurate antioxidant capacity and avoid one-sided or even contradictory results of tested antioxidants, a combination of multiple methods, such as cellular antioxidant activity (CAA) assays and animal models, are thus needed to evaluate the antioxidant activity of foods and components.

Olive leaves, which are low-value byproducts generated from olive tree cultivation and olive oil processing, contain a considerable quantity of antioxidant-active substances. Phenolic acids, flavonoids, secoiridoids, hydroxycinnamic acids, simple phenols, and triterpenic acids, which have been shown to be have the potential to scavenge reactive oxygen radicals and resist oxidative stress in the body, are the main sources of the antioxidant properties of olive leaves [13,14]. However, phenolic compounds in plants are usually present in free and bound forms [15,16]. In general, BPs are difficult to directly extract by traditional maceration methods, because they are covalently bound to cell wall structural components (e.g., cellulose, hemicellulose, and pectin) [17]. Nevertheless, numerous in vitro antioxidant assays demonstrated that the insoluble BPs in some plants possess a significantly higher antioxidant capacity compared to FPs [18–20]. However, the BPs in olive leaves and their contributions to total phenolics and antioxidant activity have received little attention, as most studies on olive leaves have only emphasized the antioxidant activity of FPs. Furthermore, no investigations have been performed to assess the antioxidant capacity of olive leaves both in vitro and in vivo.

Thus, the objectives of this study were to (i) characterize the composition and determine the content of FPs and BPs in olive leaves; (ii) investigate and compare the antioxidant capacities and possible mechanisms of FPs and BPs in olive leaves using chemical antioxidant methods (DPPH, ABTS, and FRAP), a cellular antioxidant assay, and in vivo animal models. This work will provide a theoretical basis for the application of olive leaves as antioxidants in products.

## 2. Materials and Methods

### *2.1. Plant Materials*

Fresh olive leaves of the Nocellara del belice cultivar were obtained from the research garden of Zhejiang Academy of Agricultural Sciences (30.28° N, 120.15° E), Zhejiang Province, China. The mature green leaves were randomly picked from different parts of olive trees (7-year-old) in the middle of April 2022. After harvesting, fresh olive leaves were immediately transported to our lab and dried at 105 °C for 90 min in a hot air oven (DHG-9070A, Jinghong, Shanghai, China). The dried leaves were then ground using a grinder (BJ-200, Baijie, Hangzhou, China), passed through a 60-mesh (250 μm) sieve to obtain a fine powder, and then stored in vacuum-sealed bags at −20 °C until extraction.

### *2.2. Chemicals and Reagents*

Trolox, Folin–Ciocalteu reagent, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), α,α′-azodiisobutyramidine dihydrochloride (ABAP), and 3-(4,5-dimethylythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained

from Sigma-Aldrich (St. Louis, MO, USA). Reduced glutathione (GSH), SOD, GSH-Px, CAT, MDA, and T-AOC test kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Other reagents were of analytical grade.

### *2.3. Extraction of Free and Bound Phenolic Fractions from Olive Leaves*

The FP and BP fractions of the olive leaves were extracted following a previously reported method [21]. Briefly, 1 g of dry olive leaf powder was extracted using 10 mL of a 70% ethanol solution in an ultrasonic bath for 30 min at 50 °C. The extraction was replicated three times, and the filtrates were combined and served as the FP fraction. Then, the BP fraction was extracted from the residue by alkali extraction. First, the residues were mixed with 40 mL NaOH (2 M), and hydrolyzed at room temperature for 4 h on a shaker under nitrogen. After alkaline hydrolysis, the mixture was acidified to pH 2 with 6 M HCl and centrifuged for 5 min with 5000 rpm. The obtained supernatants were extracted five times with ethyl acetate (1:1, *v*/*v*), then the pooled ethyl acetate extracts were evaporated, dissolved in 10 mL of methanol, and labeled as BP fractions. The extraction yield of FP and BP were 315.3 mg/g dry weight (DW) and 73.9 mg/g DW, respectively. The FP and BP fractions extracted from the olive leaves were stored at −20 °C before analysis.

### *2.4. Identification and Quantification of Free and Bound Antioxidants in Olive Leaves*

Chemical profiles in the FP and BP fraction of olive leaves were analyzed by a UPLC-Q-Exactive Orbitrap MS (Ultimate 3000, Thermo Fisher Scientific, San Jose, CA, USA) equipped with an ACQUITY UPLC T3 (2.1 × 100 mm, 1.8 μm, Waters, Milford, MA, USA) column. The UPLC-Q-Exactive Orbitrap MS analysis, including gradient elution conditions and mass spectrometer parameters, were described in our previous study [14]. The identification of FPs and BPs in olive leaves was carried out by matching their high-accuracy quasi-molecular ions and ion fragmentation patterns with the built-in database.

The quantification of individual phenolics (i.e., simple phenols, phenolic acids, flavonoids, iridoids, and triterpenic acids) identified by the UPLC-Q-Exactive Orbitrap-MS were analyzed by using a HPLC system (LC-2030C, Shimazu, Kyoto, Japan) equipped with a Diode Array Detector (DAD). The detection wavelengths were 210, 280, and 320 nm [14,22]. An external standard method was used, and the standards hydroxytyrosol, rutin, oleuropein, luteolin, kaempferol, luteolin-7-*O*-glucoside, rhoifolin, apigenin-7-*O*-glucoside, oxalic, maslinic, asiatic, oleanolic, corosolic, ursolic, ferulic, sinapinic, 4-coumaric, caffeic, and chlorogenic acid were used for calculation of calibration curves (Table S1). The results were expressed as μg/g DW.

### *2.5. Determination of TPC and TFC*

The TPC and TFC were determined according to the Folin–Ciocalteu and aluminum chloride colorimetric methods, respectively. The specifics of the method are described in detail in our previous research [14].

### *2.6. Chemical Antioxidant Activity Evaluation*

The chemical antioxidant activity of the FP and BP fractions from olive leaves were determined using DPPH, ABTS, and FRAP assays according to previously reported methods [23], and the absorbances at 517 nm, 734 nm, and 593 nm were measured using a spectrophotometer (UV-2600, UNICO, Shanghai, China). Trolox was used as the control [24], and the results were expressed as mg Trolox equivalent/gram of dry olive leaves (μmol TE/g DW).

### *2.7. Cellular Antioxidant Activity Evaluation*

#### 2.7.1. Cell Culture and Cytotoxicity Assay

The HepG2 cell at passages 5–10 was obtained from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). HepG2 cells were cultured in DMEM medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% $CO_2$ incubator at 37 °C. Cytotoxicity was measured using MTT assay, with slight modifications based on a previous study [25]. Briefly, 190 μL of HepG2 cells were seeded

in 96-well plates at a density of $1 \times 10^5$ cells/mL, and 10 μL of different concentrations (0, 5, 10, 20, 50, 100, 200, 250, and 500 μg/mL) of olive leaf FP or BP extracts were added to the cells for 24 h. After that, an MTT solution (5 mg/mL) was added to each well, and the absorbance values were measured at 490 nm.

#### 2.7.2. $H_2O_2$-Induced Oxidative Stress in HepG2 Cells

The cells were inoculated into 96-well plates (190 μL) at a density of $1 \times 10^5$ cells/mL for 24 h, and 10 μL of $H_2O_2$ (0, 100, 200, 400, 800, 1200, 1600, and 3200 μmol/L) were added to the cells for 1, 2, 4, 6, and 8 h, respectively. Then, the cell survival rate was measured by the MTT assay to screen the optimal $H_2O_2$ treatment concentration and incubation time for constructing the oxidative stress HepG2 cell model. As shown in Tables S2 and S3, the optimal concentration and incubation time for $H_2O_2$ treatment was found to be 800 μmol/L for 6 h according to the half-inhibitory concentration of the cells [26].

HepG2 cells seeded in 96-well plates were incubated for 24 h, and then the cells were grouped as follows: (i) normal group, cells treated with medium but without $H_2O_2$ treatment; (ii) experimental group, cells treated with olive leaf FP or BP extracts and $H_2O_2$ treatment; and (iii) damage group, cells treated with medium and $H_2O_2$ treatment. After the cells were pretreated with 10 μL of different concentrations (20, 50, 100, 200, 250, and 500 μg/mL) of olive leaf FP or BP extracts for 24 h, the medium was removed and 190 μL of fresh medium and 10 μL of 800 μmol/L $H_2O_2$ were added to each well for 6 h of incubation. Finally, the cell survival rate was measured by the MTT assay as described above.

#### 2.7.3. CAA Assay

The CAA of the FP and BP fractions in the olive leaves were determined as described by Wolfe and Liu [27]. HepG2 cells were plated with a density of $6 \times 10^4$ cells/well in 96-well black microplates and incubated for 24 h; after that, the growth medium was removed and the cells were washed with PBS to remove the non-adherent and dead cells. Then, the cells were cultivated for 1 h with 100 μL of FP and BP extracts or quercetin with 25 μM DCFH-DA dissolved in medium. After that, the culture medium was removed, and HBSS solution (with 600 μM ABAP) was added to each well. Finally, the black microplates were placed into a fluorescent microplate reader (Spectra MAX 190, Molecular Devices, San Jose, CA, USA). The real-time fluorescence was read every 5 min for 1 h with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

### *2.8. In Vivo Antioxidant Activity Evaluation*

#### 2.8.1. Animals and Experimental Design

Male ICR mice (six weeks, 20 ± 2 g) were purchased from Hangsi Laboratory Animal Co., Ltd. (Hangzhou, China). All animal procedures were conducted in compliance with institutional guidelines for the care and use of laboratory animals at the Zhejiang Academy of Agricultural Sciences (Certificate NO.2023ZAASLA80). After 7 days of adaptive feeding, the mice were randomly divided into seven groups ($n$ = 8): (1) control group (administrated 0.1 mL/10 g body weight (b.w.) saline); (2) model group (administrated 0.1 mL/10 g b.w. saline); (3) Vitamin C (Vc) group (administrated 100 mg/kg b.w. Vc); (4) FP-L (administrated 125 mg/kg b.w. FPs); (5) FP-H (administrated 250 mg/kg b.w. FPs); (6) BP-L (administrated 125 mg/kg b.w. BPs); and (7) BP-H (administrated 250 mg/kg b.w. BPs).

All solutions were administered once daily by gastric gavage for 8 weeks. In addition, all mice except those in the control group received subcutaneous injections of D-galactose at a dose of 150 mg/kg b.w. daily, and the control group received injections of saline (Figure 1). After 8 weeks, mice were fasted for 12 h before being euthanized with $CO_2$ and sacrificed by cervical dislocation to obtain blood samples and heart, kidney, and liver tissues.

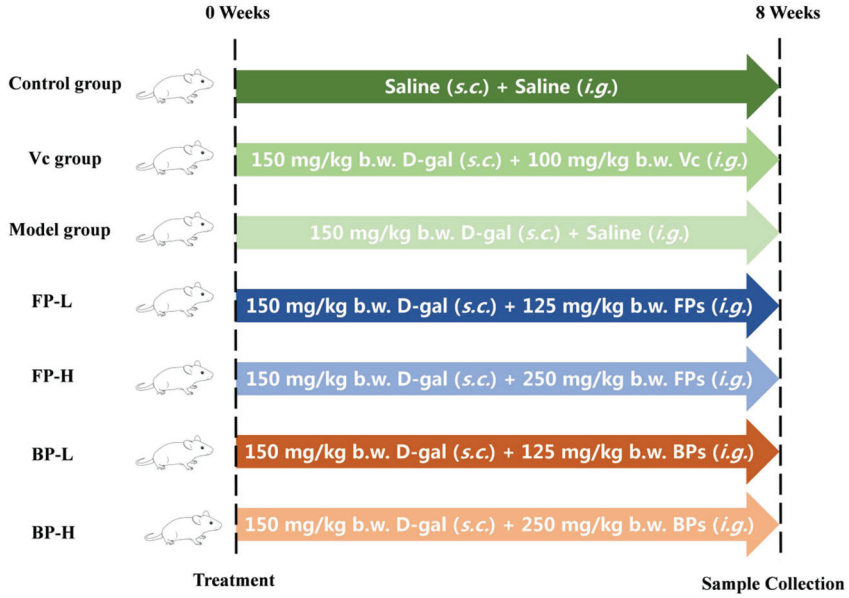


**Figure 1.** The schematic diagram of the experimental design for evaluating the in vivo antioxidant ability of FPs and BPs in olive leaves.

#### 2.8.2. Histological Analysis

The fixed liver tissues were embedded in paraffin, then sectioned (5-mm thick) and stained with hematoxylin and eosin (H&E) for histopathology. After installation, the slides were viewed under a microscope (DXIT 1200, Nikon, Tokyo, Japan).

#### 2.8.3. Biochemical Assays

The in vivo antioxidant activity in the serum, liver, and kidney was evaluated by measuring MDA, GSH-Px, SOD, CAT, GSH, and T-AOC using commercial ELISA kits according to the instructions.

#### 2.8.4. RT-qPCR Analysis

Hepatic total RNA was isolated using Trizol reagent, followed by reverse transcription of total RNA to cDNA. The mRNA expression levels were measured by an RT-qPCR system (ABI PRISM 7300, Applied Biosystems, Foster City, CA, USA), and the results were calculated by the $2^{-\Delta\Delta CT}$ method. GAPDH was used as an internal reference control, and primer sequences used in this study are shown in Table S4.

### *2.9. Statistical Analysis*

All results are expressed as mean ± SD. The data were processed by one-way ANOVA, followed by Tukey's post hoc tests using SPSS Statistics software (V21.0, SPSS Inc., Chicago, IL, USA), with the significant difference set at $p < 0.05$. Origin software (2019b, Originlab Inc., Massachusetts, MA, USA) was used for $EC_{50}$ analysis.

## 3. Results and Discussion

### *3.1. Identification and Quantification of Free and Bound Antioxidants in Olive Leaves*

Phenolic compounds in plants exist in free and bound forms [28]. Although BPs are usually found at lower levels compared to FPs, numerous studies have demonstrated that BPs in some food matrices possess a significantly higher antioxidant capacity than FPs [18,19]. Previously, the profiles of BPs in olive leaves and their contributions to antioxidant activity were not studied. Therefore, the chemical profiles of FP and BP fractions in olive leaves were investigated in this research, and their in vitro and in vivo antioxidant capacity and possible mechanism were studied comprehensively.

The chemical profiles of FP and BP fractions in olive leaves were preliminarily identified by using a UPLC-Q-Exactive Orbitrap-MS, and the identified 28 compounds along with their retention time (Rt), mass data, MS/MS fragments, and molecular formula are described in Table 1 and Figure S1. These compounds were classified into five different families according to their structural skeletons, and included one simple phenol, five phenolic acids, fifteen flavonoids, two iridoids, and five triterpenoid acids. As shown in Table 1, 24 compounds were identified in the FP fractions of olive leaves, with flavonoids, iridoids, and triterpenic acids being the primary class. The chemical profiles of FP fractions are in accordance with previous findings; Contreras et al. [29] and Dias et al. [30] analyzed FP fractions of olive leaves by liquid chromatography coupled to mass spectrometry techniques and also revealed their richness in flavonoids (luteolin, quercetin, rutin, luteolin-7-*O*-glucoside, etc), iridoids (oleuropein and secoxyloganin), and simple phenols (hydroxytyrosol). In addition, a total of 14 phenolics were detected in the BP fractions, consisting of flavonoids (6), phenolic acids (5), triterpenic acids (2), and simple phenol (1). As far as we know, this is the first time that phytochemical profiles of BP fractions were identified in olive leaves.

The individual phenolic compounds were further quantified, and our results demonstrated that the FP and BP fractions had different phenolic profiles, as indicated in Table 2. The highest phenolic content detected in the FP fraction was that of oleuropein (17.52 mg/g DW), followed by luteolin-7-*O*-glucoside (2.80 mg/g DW) and kaempferol-7-*O*-glucoside (2.07 mg/g DW), which was consistent with the previously reported findings of the FP composition in olive leaves [14]. In the BP fraction, the content of sinapinic acid was highest at 0.39 mg/g DW, followed by apigenin-7-*O*-neohesperidoside (0.31 mg/g DW), 4-coumaric acid (0.21 mg/g DW), ferulic acid (0.20 mg/g DW), hydroxytyrosol (0.19 mg/g DW), and caffeic acid (0.16 mg/g DW). It was noteworthy that higher levels of phenolic acid (i.e., sinapinic acid, 4-coumaric acid, ferulic acid, and caffeic acid) and hydroxytyrosol were detected in the BP fraction; however, flavonoids, triterpenoid acids, and iridoids were more concentrated in the free form.

Oleuropein, secoxyloganin, maslinic acid, and glycosylated flavonoids are usually the most abundant phenolic compound in olive leaves, and are easily and efficiently extracted by organic aqueous mixtures [14]. However, phenolic acids are mostly covalently linked to structural components of the cell walls forming cross-links in plants, and thus cannot be easily extracted directly by solvent extraction; this may explain why phenolic acids are typically found in bound forms, with free forms being extremely rare [31,32]. Most phenolic acids, such as gallic, *p*-coumaric, ferulic, and caffeic acid, are also found at higher levels in their bound forms than in their corresponding free forms in sorghum and mango leaves [19,31]. Phenolic acids, with many phenolic hydroxyl groups in the molecules, are excellent hydrogen or electron donors that quench free radicals [33,34]. Thus, phenolic acids are probably important contributors to the antioxidant capacity of the BP fraction in olive leaves. Further research aims to release FPs in olive leaves through food processing, such as ultra-high pressure, alkaline or enzymatic hydrolysis, and fermentation.

**Table 1.** Compounds identified by UPLC-Q-Exactive Orbitrap-MS in free and bound phenolic fraction of olive leaves.

| No | Rt (min) | Measured *m*/*z* | MS/MS Fragments | Molecular Formula | CAS | Compounds | Class | FP | BP |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 3.912 | 153.0550 $[M-H]^-$ | 123.04, 153.05 | C8H10O3 | 10597-60-1 | Hydroxytyrosol | Simple phenols | X | X |
| 2 | 5.296 | 353.0880 $[M-H]^-$ | 191.06, 209.66 | $C_{16}H_{18}O_9$ | 327-97-9 | Chlorogenic acid | Phenolic acids | X | X |
| 3 | 5.790 | 163.0390 $[M+H]^+$ | 163.04, 145.03, 135.04 | $C_9H_8O_4$ | 331-39-5 | Caffeic acid | Phenolic acids | | X |
| 4 | 6.605 | 147.0440 $[M+H]^+$ | 147.04, 119.05 | $C_9H_8O_3$ | 7400-08-0 | 4-Coumaric acid | Phenolic acids | | X |
| 5 | 7.110 | 223.0610 $[M-H]^-$ | 164.05, 208.04, 223.06 | $C_{11}H_{12}O_5$ | 530-59-6 | Sinapinic acid | Phenolic acids | | X |
| 6 | 7.155 | 177.0646 $[M+H]^+$ | 177.05, 145.03 | $C_{10}H_{10}O_4$ | 537-98-4 | Ferulic acid | Phenolic acids | | X |
| 7 | 5.698 | 403.1250 $[M-H]^-$ | 59.01, 71.01, 89.02 | C17H24O11 | 58822-47-2 | Secoxyloganin | Iridoids | X | |
| 8 | 7.525 | 539.1776 $[M-H]^-$ | 539.18, 377.12, 275.09 | $C_{25}H_{32}O_{13}$ | 32619-42-4 | Oleuropein | Iridoids | X | |
| 9 | 5.916 | 609.1469 $[M-H]^-$ | 447.09, 609.15, 285.04 | $C_{27}H_{30}O_{16}$ | 52187-80-1 | Luteolin-3′,7-di-*O*-glucoside | Flavonoids | X | |
| 10 | 6.523 | 609.1470 $[M-H]^-$ | 609.15, 300.03, 301.04 | $C_{27}H_{30}O_{16}$ | 153-18-4 | Rutin | Flavonoids | X | X |
| 11 | 6.528 | 611.1608 $[M+H]^+$ | 303.05, 466.11, 85.03 | $C_{27}H_{30}O_{16}$ | 18016-58-5 | Quercetin 3-*O*-glucoside-7-*O*-rhamnoside | Flavonoids | X | |
| 12 | 6.753 | 465.1026 $[M+H]^+$ | 303.05, 85.03 | $C_{21}H_{20}O_{12}$ | 482-35-9 | Quercetin-3β-D-glucoside | Flavonoids | X | |
| 13 | 6.758 | 449.1077 $[M+H]^+$ | 449.11, 287.05 | $C_{21}H_{20}O_{11}$ | 5373/11/5 | Luteolin-7-*O*-glucoside | Flavonoids | X | X |
| 14 | 6.941 | 577.1570 $[M-H]^-$ | 269.05, 577.16 | $C_{27}H_{30}O_{14}$ | 17306-46-6 | Apigenin-7-*O*-neohesperidoside | Flavonoids | X | X |
| 15 | 7.143 | 609.1815 $[M+H]^+$ | 301.07, 609.18 | $C_{28}H_{32}O_{15}$ | 38665-01-9 | Neodiosmin | Flavonoids | X | |
| 16 | 7.214 | 477.1043 $[M-H]^-$ | 431.10, 268.04, 269.05 | $C_{21}H_{20}O_{10}$ | 578-74-5 | Apigenin-7-*O*-glucoside | Flavonoids | X | X |
| 17 | 7.236 | 447.0937 $[M-H]^-$ | 285.04, 447.09 | $C_{21}H_{20}O_{11}$ | 16290-07-6 | Kaempferol-7-*O*-glucoside | Flavonoids | X | X |
| 18 | 7.390 | 447.0939 $[M-H]^-$ | 210.04, 285.04 | C21H20O11 | 6920-38-3 | Luteolin-4′-*O*-glucoside | Flavonoids | X | |
| 19 | 8.919 | 285.0407 $[M-H]^-$ | 285.04, 299.46 | $C_{15}H_{10}O_6$ | 491-70-3 | Luteolin | Flavonoids | X | X |
| 20 | 8.922 | 287.0548 $[M+H]^+$ | 287.05, 269.08 | $C_{15}H_{10}O_6$ | 520-18-3 | Kaempferol | Flavonoids | X | |
| 21 | 9.012 | 303.0496 $[M-H]^-$ | 303.05, 285.15 | $C_{15}H_{10}O_7$ | 117-39-5 | Quercetin | Flavonoids | X | |
| 22 | 9.882 | 301.0703 $[M+H]^+$ | 301.07, 286.05 | $C_{16}H_{12}O_6$ | 520-34-3 | Diosmetin | Flavonoids | X | |
| 23 | 10.001 | 315.0514 $[M-H]^-$ | 315.05, 300.03 | $C_{16}H_{12}O_7$ | 1486-70-0 | 3-*O*-Methylquercetin | Flavonoids | X | |
| 24 | 11.579 | 487.3434 $[M-H]^-$ | 487.34, 469.33 | $C30H_{48}O_5$ | 464-92-6 | Asiatic acid | Triterpenoid acids | X | |
| 25 | 13.414 | 471.3483 $[M-H]^-$ | 471.35, 428.24 | $C_{30}H_{48}O_4$ | 4373-41-5 | Maslinic acid | Triterpenoid acids | X | |
| 26 | 13.434 | 455.3517 $[M+H]^+$ | 205.16, 189.16, 203.18 | $C_{30}H_{48}O_4$ | 4547-24-4 | Corosolic acid | Triterpenoid acids | X | X |
| 27 | 12.759 | 457.3673 $[M+H]^+$ | 457.37, 203.18, 191.18 | $C_{30}H_{48}O_3$ | 508-02-1 | Oleanolic acid | Triterpenoid acids | X | |
| 28 | 16.492 | 439.3567 $[M+H]^+$ | 411.36, 439.36, 203.18 | $C_{30}H_{48}O_3$ | 77-52-1 | Ursolic acid | Triterpenoid acids | X | X |

X: presence of the compound. FP and BP represent free phenolic and bound phenolic, respectively.

**Table 2.** Quantification of phenolics compounds in free and bound phenolic fraction of olive leaves.

| No. | Compounds | Content (mg/g DW) | | No. | Compounds | Content (mg/g DW) | |
|---|---|---|---|---|---|---|---|
| | | FP Fraction | BP Fraction | | | FP Fraction | BP Fraction |
| 1 | Hydroxytyrosol | 0.11 ± 0.01 [b] | 0.19 ± 0.09 [a] | 15 | Apigenin-7-*O*-glucoside | 0.47 ± 0.03 [a] | 0.05 ± 0.01 [b] |
| 2 | Chlorogenic acid | 0.13 ± 0.02 [a] | 0.07 ± 0.01 [b] | 16 | Neodiosmin | 0.54 ± 0.03 | ND |
| 3 | Caffeic acid | ND | 0.16 ± 0.01 | 17 | Kaempferol-7-*O*-glucoside | 2.07 ± 0.15 [a] | 0.14 ± 0.03 [b] |
| 4 | 4-Coumaric acid | ND | 0.21 ± 0.03 | 18 | Luteolin-4′-*O*-glucoside | 0.61 ± 0.04 | ND |
| 5 | Sinapinic acid | ND | 0.39 ± 0.03 | 19 | Luteolin | 0.38 ± 0.03 [a] | 0.03 ± 0.01 [b] |
| 6 | Ferulic acid | ND | 0.20 ± 0.08 | 20 | Kaempferol | 0.03 ± 0.01 | ND |
| 7 | Secoxyloganin | 2.11 ± 0.18 | ND | 21 | Quercetin | 0.12 ± 0.01 | ND |
| 8 | Oleuropein | 17.52 ± 2.61 | ND | 22 | Diosmetin | 0.03 ± 0.00 | ND |
| 9 | Luteolin-3′,7-di-*O*-glucoside | 0.44 ± 0.03 | ND | 23 | 3-*O*-Methylquercetin | 0.02 ± 0.00 | ND |
| 10 | Rutin | 1.31 ± 0.10 [a] | 0.12 ± 0.01 [b] | 24 | Asiatic acid | 0.26 ± 0.01 [a] | ND |
| 11 | Quercetin 3-*O*-glucoside-7-*O*-rhamnoside | 0.52 ± 0.03 | ND | 25 | Maslinic acid | 1.31 ± 0.02 [a] | 0.01 ± 0.00 [b] |
| 12 | Quercetin-3β-D-glucoside | 0.78 ± 0.06 | ND | 26 | Corosolic acid | 0.39 ± 0.08 | ND |
| 13 | Luteolin-7-*O*-glucoside | 2.80 ± 0.27 [a] | 0.12 ± 0.04 [b] | 27 | Oleanolic acid | 1.61 ± 0.07 [a] | 0.06 ± 0.00 [b] |
| 14 | Apigenin-7-*O*-neohesperidoside | 0.71 ± 0.04 [a] | 0.31 ± 0.02 [b] | 28 | Ursolic acid | ND | 0.02 ± 0.01 |

Means followed by different letters ([a], [b]) are significant differences ($p < 0.05$); FP and BP represent free phenolic and bound phenolic, respectively; ND, not detected.

### *3.2. Chemical Antioxidant Activities of FP and BP Fractions in Olive Leaves*

Chemical antioxidant methods are widely used for preliminary screening of oxidation/reduction potentials of antioxidants due to their low cost and easy implementation. TPC and TFC were found to be directly associated with chemical antioxidant activities [6,23]. In this study, the TPC and TFC of the FP and BP fractions of olive leaves were evaluated prior to the chemical antioxidant assay. As shown in Table 3, the TPC and TFC levels in the FP fraction were statistically higher than those in the BP fraction (26.39 vs. 0.65 mg GAE/g DW for TPC, and 145.30 vs. 1.73 mg RE/g DW for TFC, $p < 0.05$). The bound phenolics contributed to only 2.4% of the total phenolics content, suggesting that phenolic compounds in olive leaves were largely present in a free form. The result was in agreement with previous findings reporting that most phenolics in vegetables and fruits were found in free forms, and those in insoluble bound forms generally accounted for no more than 24% of the total phenolic content [17,35].

**Table 3.** TPC, TFC and the chemical antioxidant activity of free and bound phenolic fraction from olive leaves.

| | TPC (mg GAE/g DW) | TFC (mg RE/g DW) | DPPH (μmol TE/gDW) | ABTS (μmol TE/gDW) | FRAP (μmol TE/gDW) |
|---|---|---|---|---|---|
| FP fraction | 26.39 ± 1.26 [a] | 145.30 ± 9.11 [a] | 26.09 ± 0.03 [a] | 249.49 ± 0.29 [a] | 13.79 ± 0.03 [a] |
| BP fraction | 0.65 ± 0.05 [b] | 1.73 ± 0.29 [b] | 10.44 ± 0.01 [b] | 6.66 ± 0.01 [b] | 0.37 ± 0.00 [b] |

Means followed by different letters ([a], [b]) are significant differences ($p < 0.05$); FP and BP represent free phenolic and bound phenolic, respectively.

Furthermore, three chemical methods, including DPPH and ABTS for evaluating the scavenging ability of radicals and the FRAP method for evaluating the iron reduction capacity, were used to estimate the antioxidant activities of FP and BP fractions in olive leaves. As shown in Table 3, the DPPH and ABTS+. free radical scavenging ability of the FP fraction was significantly higher than that of the BP fraction, with values of 26.09 vs. 10.44 μmol TE/g DW with the DPPH assay, and 249.49 vs. 6.66 μmol TE/g DW with the ABTS assay. The FRAP assay also showed a significant order of magnitude difference between the FPs and BPs, with values of 13.79 vs. 0.37 μmol TE/g DW, respectively. The presence of rich polyphenols, such as flavonoids, triterpenoid acids, and particularly oleuropein, of which the levels were very high, could be the reason for the high DPPH and ABTS+. radical-scavenging property of the FP fraction [36]. In conclusion, the chemical antioxidant activity indicated that the BP fraction in olive leaves had a weak DPPH and ABTS+. radical-scavenging ability.

### *3.3. Cellular Antioxidant Activities of FP and BP Fractions in Olive Leaves*

Currently, chemical antioxidant activity assays have a major role in testing antioxidant effects; however, their ability to predict in vivo activity has been questioned due to the limitations on absorption, bioavailability, distribution, and metabolism assessment [9]. Therefore, we established a cellular antioxidant system to compare and investigate the difference between the biological and chemical antioxidant ability of the FP and BP fractions in olive leaves in this study.

#### 3.3.1. FP and BP Fractions in Olive Leaves Resist $H_2O_2$-Induced Oxidative Stress in HepG2 Cells

First, the cytotoxicity of FPs and BPs in HepG2 cells was investigated to screen for a concentration that did not exhibit significant toxicity in cells. As shown in Figure 2A, FPs up to 250 μg/mL did not impact cell viability, but at 500 μg/mL, the cell survival rate decreased to 60.39 ± 12.17%. The BPs at concentrations of 5–500 μg/mL were nontoxic to HepG2 cells, with the cell survival rate between 106.35% and 136.01% (Figure 2B). The subsequent concentrations of FPs and BPs for cellular antioxidant activity assays were set as 0–250 μg/mL.

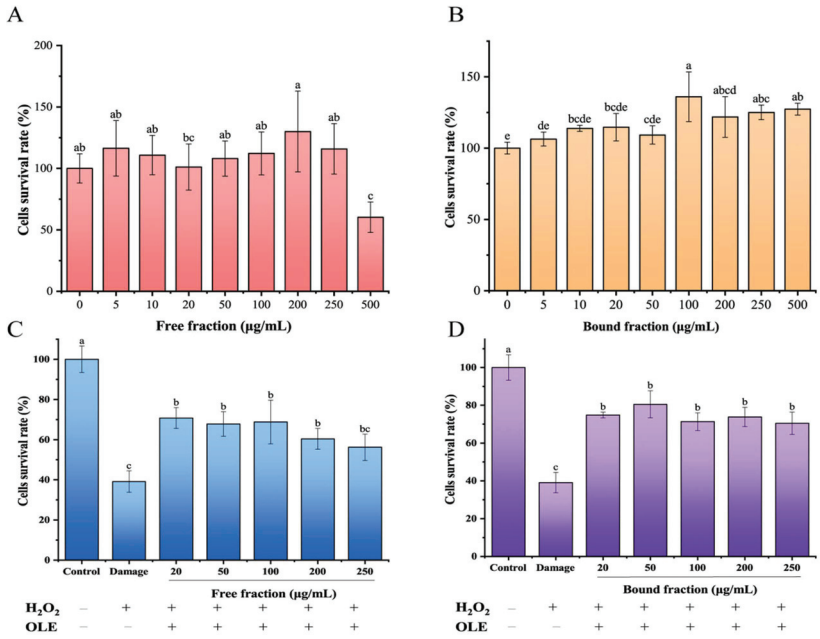


**Figure 2.** Protective effect of FPs and BPs in olive leaves against $H_2O_2$-induced oxidative stress in HepG2 cells. (**A**,**B**) The effect of FPs and BPs on the cell survival rate of HepG2 cells. (**C**,**D**) The effect of FPs and BPs on the cell survival rate of HepG2 cells induced by $H_2O_2$. Different letters indicate significant differences ($p < 0.05$).

$H_2O_2$ is a well-recognized oxidative stress inducer, which can penetrate the cell membrane and generate excessive ROS, leading to oxidative stress and eventually injuring the cell [37]. Next, the protection of FPs and BPs against $H_2O_2$-induced oxidative stress was evaluated in the study. As presented in Figure 2C,D, the cell viability of the $H_2O_2$-damaged group was remarkably decreased to 39.10% in comparison with the normal group ($p < 0.05$). Encouragingly, pretreatment with both the FPs and BPs effectively inhibited the decrease in $H_2O_2$-induced cell viability. The cell viability of HepG2 cells was 70.75% and 74.81% with pretreatment of FPs and BPs at 20 mg/mL, respectively, and was almost unchanged as the FP and BP concentration increased from 20 to 250 μg/mL. The protective capacities of FP and BP on $H_2O_2$-induced oxidative stress are not surprising and can be associated with the presence of phenolic compounds. For example, Goncalves et al. [38] reported phenolic-enriched fractions from sweet cherries had a significant ability to prevent HepG2 cells against $H_2O_2$-induced oxidative injury. Furthermore, many studies have reported that hydroethanolic extracts from vegetal leaf parts were rich in phenolic and flavonoid compounds (e.g., *Apios americana* Medik, *Vaccinium dunalianum* Wight and *Hemerocallis fulva*), and also showed strong ability to inhibit the $H_2O_2$-induced cells damage [39–41]. The results in this study suggested that both FPs and BPs in olive leaves protected HepG2 cells from $H_2O_2$-induced oxidative stress injury, and that BPs showed better antioxidant activity for rescuing $H_2O_2$-induced cell viability losses than did FPs.

### 3.3.2. FP and BP Fractions in Olive Leaves Exhibited Cellular Antioxidant Activity

The CAA assay, which measures the ability of antioxidants to inhibit the formation of fluorescent DCF by ABAP-generated peroxyl radicals in the HepG2 cell model, was used to assess the biological antioxidant capacity of FPs and BPs in olive leaves. As presented in Figure 3, the fluorescence from DCF formation in HepG2 cells decreased with increased quercetin, FP, and BP concentrations, suggesting that they have great antioxidant capacity. The $EC_{50}$ values of CAA for FPs and BPs was 1.58 μg/mL and 1.66 μg/mL, respectively, suggesting that there was no significant difference in the CAA value between the FP and BP fraction ($p > 0.05$). The observations on CAA were inconsistent with the conclusion by the chemical antioxidant evaluation, which found that the BP fraction had a weaker

ability to eliminate DPPH and ABTS+. free radicals, and a significant order of magnitude difference was found between FPs and BPs. Perhaps this difference is related to the higher content of phenolic acids (i.e., sinapinic acid, ferulic acid, and caffeic acid) in BPs, which can increase the activity of antioxidant enzymes, such as CAT, GSH-Px, SOD, and heme oxygenase-1 (HO-1), and thereby activate the intracellular antioxidant defense system [12]. The contradictory conclusions between the chemical antioxidant and cellular antioxidant responses has been previously observed with certain antioxidant foods and components, such as inulin, tangeretin, and citrus flavanones [11,42]. The cell-based antioxidant assay is more biologically relevant than chemical tests due to its sensitivity for cell uptake, metabolism, and intracellular location [9]. Thus, antioxidant assays chosen in future studies should be further extended to obtain a more complete panorama of the antioxidant activity, and should not be limited to an in vitro chemical antioxidant assessment.

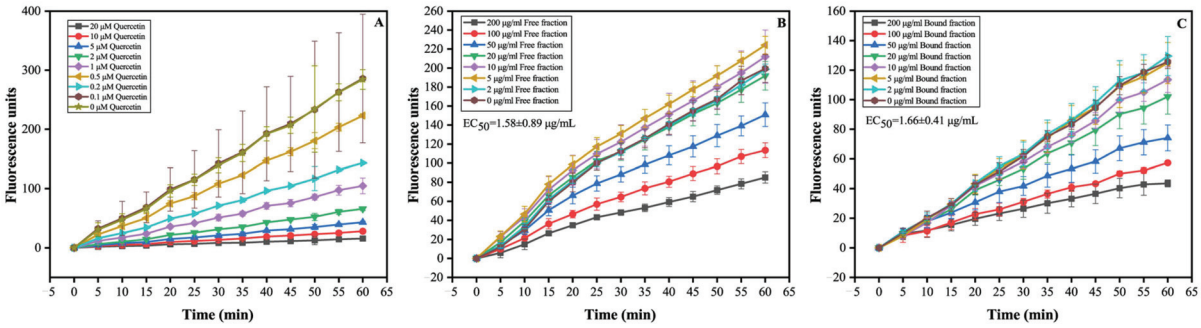


**Figure 3.** Kinetic curves of peroxyl radical-induced DCF fluorescence and the inhibition of oxidation by quercetin (**A**), FPs (**B**) and BPs (**C**) on the fluorescence in HepG2 cells over time.

### *3.4. In Vivo Antioxidant Activities of FP and BP Fractions in Olive Leaves*

D-galactose is widely used for establishing an oxidation model in mice [43]. Excessive D-galactose in the body will produce aldose, hydrogen peroxide, and other oxygen free radicals, causing the accumulation of reactive oxygen species, and ultimately leading to oxidative stress [44]. In this research, D-galactose was chosen to establish the oxidation model for evaluating the in vivo antioxidant capacity and possible mechanisms of FPs and BPs in olive leaves (Figure 1). As shown in Figure 4A, the body weight of mice in all groups exhibited an upward trend without differences among groups during the 8-week experiments. The index results of the kidney, liver, and heart demonstrated that D-galactose injections slightly decreased the organ indexes of mice without statistically significant differences (Figure 4B), which was consistent with previous reports [45].

#### 3.4.1. FP and BP Fractions in Olive Leaves Protect Liver Tissue in Aging Mice

The liver is highly sensitivity to the oxidative damage caused by D-galactose [46]. According to the hepatic H&E staining results (Figure 4C), the liver structure of mice in the control group had a normal cell morphology and was neatly arranged around the central vein, with prominent nuclei and abundant cytoplasm with a uniform distribution. Compared with the control group, the model group exhibited apparent liver damage, which was characterized by vacuolar degeneration, necrosis, and structural disorder, indicating that the D-galactose injections induced liver damage in mice. Treatment with Vc, FPs, and BPs significantly ameliorated liver damage, as evidenced by cells arranged in a more orderly manner, and the structures tended to be complete, indicating the protective effect of FPs and BPs on liver tissue.

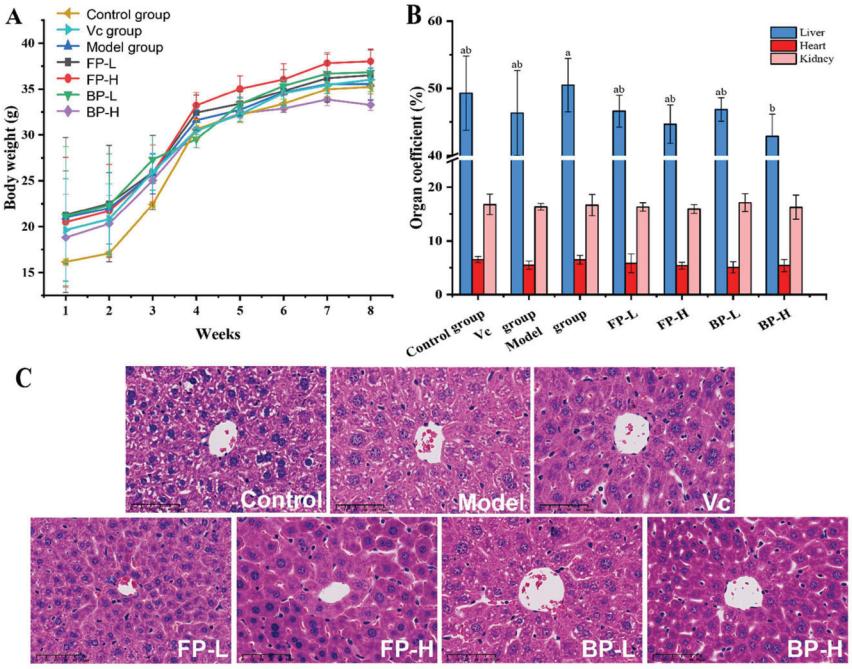


**Figure 4.** Effects of FPs and BPs in olive leaves on body weight (**A**), organ coefficients (**B**), and H&E staining (**C**) in D-galactose-induced aging mice. Scale bar = 50 μm. Different letters indicate significant differences ($p < 0.05$).

#### 3.4.2. FP and BP Fractions in Olive Leaves Relieve Oxidative Stress in Aging Mice

The body has several natural defense systems to counteract oxidative stress, including the production of antioxidant enzymes (SOD, CAT, and GSH-Px) and the action of non-enzymatic antioxidants, such as GSH and Vitamin E [47]. However, endogenous and exogenous oxidants disrupt this antioxidant system, causing accumulation of reactive oxygen species and producing peroxidation products, and ultimately causing oxidative stress [48]. MDA is one of the products of lipid peroxidation, which can reflect the severity of a free radical attack on cells [49]. As shown in Figure 5, the mice in the model group that received subcutaneous injections of D-galactose for 8 weeks displayed 3.9-fold, 2.2-fold, 2.3-fold, and 1.7-fold higher MDA levels in the serum than those of the control group, kidney, heart, and liver, respectively. The FP and BP extract treatments significantly reversed the increased MDA content compared with the model group, indicating that FP and BP extracts effectively regulated the redox status in D-galactose-induced aging mice. Furthermore, the antioxidant enzymes (SOD, CAT, and GSH-Px), the T-AOC, and the non-enzymatic GSH levels in the serum, kidney, heart, and liver of mice were measured to evaluate the repair effect of FPs and BPs on oxidative stress in aging mice. As shown in Figure 5A,B, the levels of CAT, SOD, and GSH-Px in the serum, kidney, and heart of mice in the model group were lower than those in the control group ($p < 0.05$). Each dose of FP and BP increased the activities of SOD, GSH-Px, and CAT in aging mice without a dose-response relationship. In addition, we observed that both FPs and BPs effectively improved the T-AOC and the non-enzymatic GSH level in aging mice. In conclusion, the data from this work showed that the FP and BP fractions were effective in mitigating oxidative damage by restoring SOD, CAT, and GSH-Px activities, and the content of MDA in aging mice.

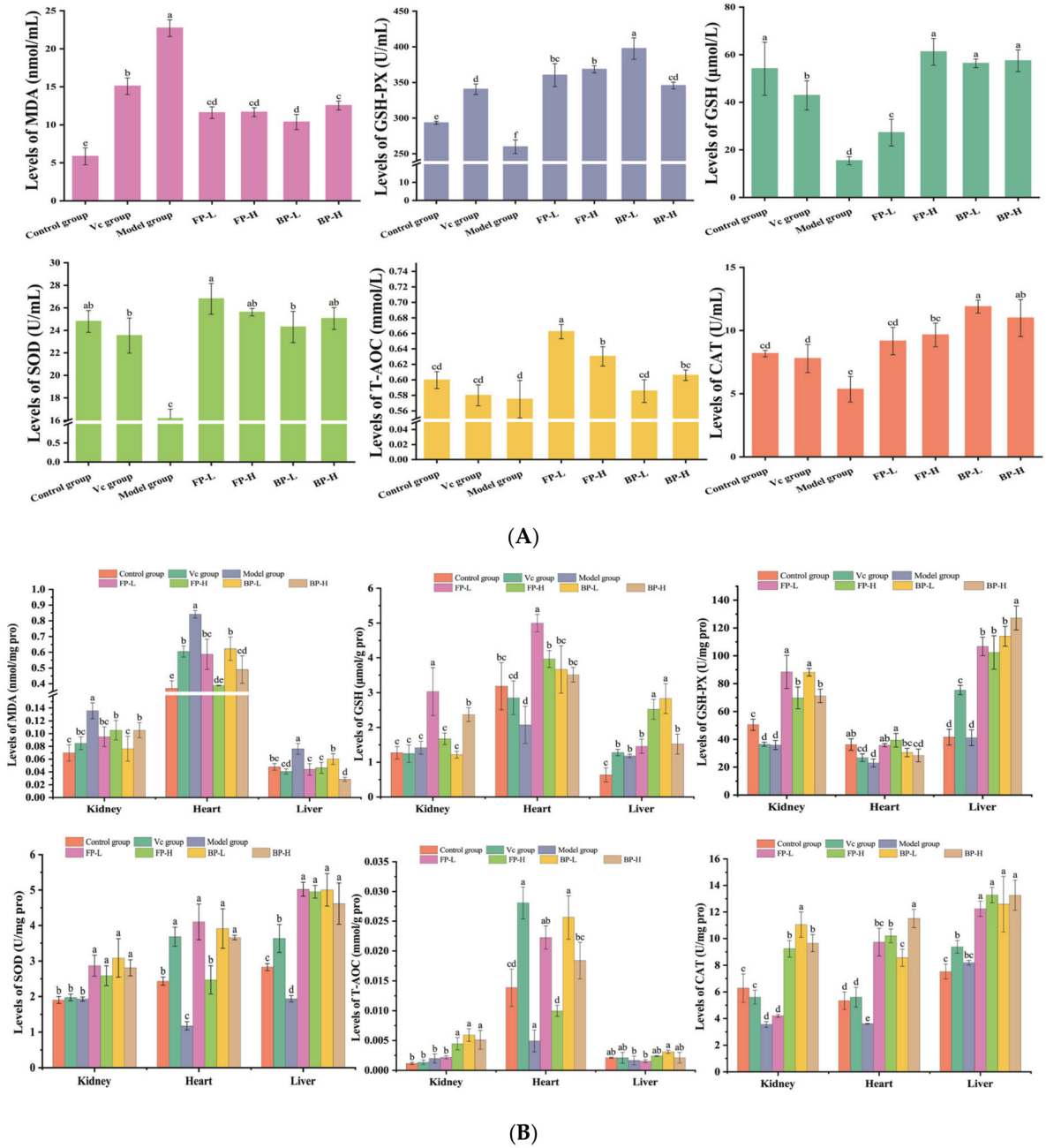


**Figure 5.** Effect of FPs and BPs in olive leaves on antioxidant properties in D-galactose-induced aging mice: (**A**) serum; and (**B**) kidney, heart, and liver tissues. Different letters indicate significant differences ($p < 0.05$).

### 3.4.3. FP Fractions in Olive Leaves Activate Nrf2 Signaling in Aging Mice

Nrf2 has been recognized as a key antioxidant transcription factor involved in redox homeostasis by targeting many antioxidant genes, including NAD(P)H dehydrogenase quinone 1 (NQO1), HO-1, glutamate-cysteine ligase catalytic subunit (GCLC), CAT, and SOD [50,51]. Thus, the expression levels of Nrf2, HO-1, NQO1, GCLC, and Glutathione S-transferase alpha 2(GSTA2) were determined in this research. The results showed that D-galactose administration decreased the expression of Nrf2 compared to the control group (Figure 6A), which was consistent with a previous study that reported that the D-galactose treatment inhibited the translocation of Nrf2 [45]. FP treatment reversed the effect of D-galactose on the expression of Nrf2 in mice; in particular, 125 mg/kg of FPs resulted in a

2.84-fold increase in the expression of Nrf2 compared to the model group. This result indicated that there was not a dose-response relationship between Nrf2 expression and the FP concentration. In contrast, the BP-treated groups, both at 125 and 250 mg/kg, did not affect the expression of Nrf2. As shown in Figure 6B–E, the increase of Nrf2 in the FP-treated group eventually led to the upregulation of the targeted genes HO-1, NQO1, GCLC, and GSTA2 in mouse livers, while BP-treated groups did not have increased expression of these genes. This shows that even though both FPs and BPs effectively increased antioxidant enzyme activity and prevented oxidative damage in vivo, there was a great difference in their mechanisms. Perhaps this difference is related to the different chemical profiles of FPs and BPs, with the polyphenols in FPs protecting against oxidative stress by elevating gene expression of Nrf2. FPs ameliorate D-galactose-induced oxidative stress injury partly via the activation of the Nrf2 signaling pathway, while the mechanism of BPs need further study.

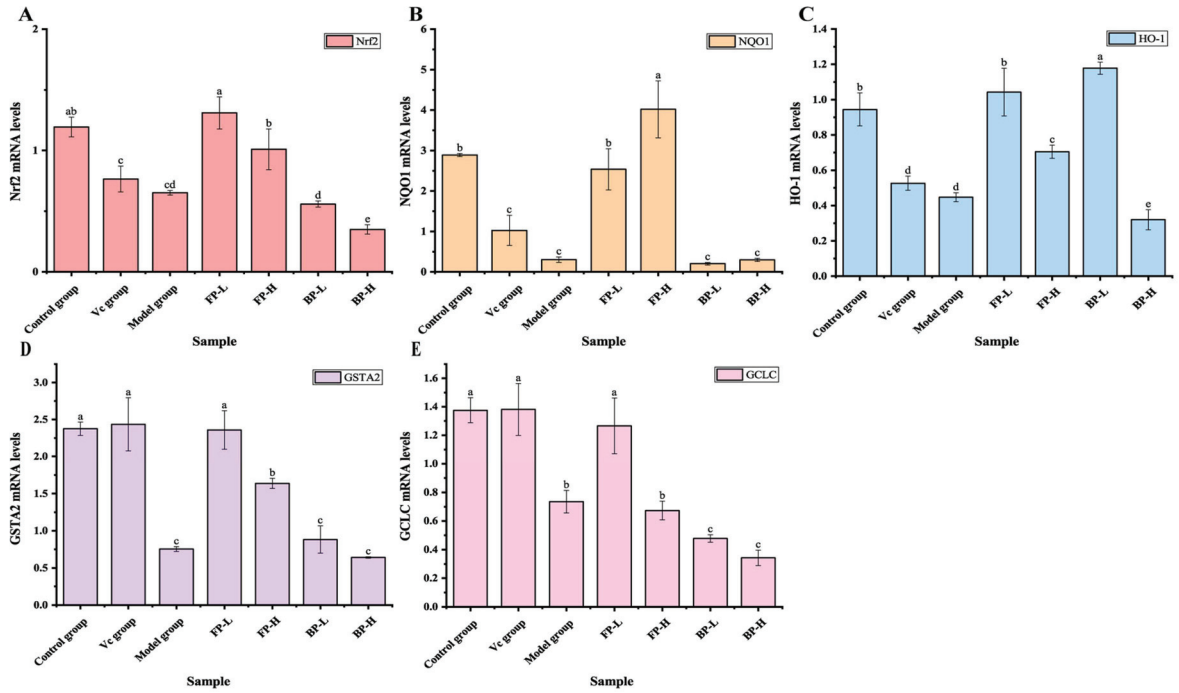


**Figure 6.** Effect of FPs and BPs in olive leaves on the expression of Nrf2 signaling in D-galactose-induced aging mice: (**A**) Nrf2; (**B**) NQO1; (**C**) HO-1; (**D**) GSTA2; and (**E**) GCLC. Different letters indicate significant differences ($p < 0.05$).

## 4. Conclusions

The present study investigated and compared the chemical profiles, contents, and in vitro and in vivo antioxidant capacities of FPs and BPs in olive leaves. The results showed that FPs and BPs exhibited different antioxidant effects in the chemical antioxidant evaluation systems compared to the biological antioxidant evaluation systems. The chemical antioxidant evaluation systems (DPPH, ABTS, and FRAP assays) indicated that FPs exerted significantly higher antioxidant activity than did the BP fraction, and BPs had a weak ability to directly scavenging DPPH and ABTS+. free radicals. In biological antioxidant evaluation systems (cellular and in vivo mouse models), both FPs and BPs exhibited similar antioxidant effects by effectively protecting HepG2 cells from $H_2O_2$-induced oxidative stress injury, and were effective in mitigating oxidative damage by restoring SOD, CAT, and GSH-Px activities in aging mice. Further exploration showed that FPs ameliorated D-gal-induced oxidative stress injury partly via the activation of the Nrf2 signaling pathway, while the mechanism of BPs requires further study (Figure 7). The different manner by which FPs and BPs exert antioxidant activity is probably related to the

different chemical profiles of FPs and BPs. In conclusion, it could be stated that the in vitro chemical antioxidant assessment of antioxidant foods and components is not sufficient to estimate their antioxidant effects in vivo. To obtain a more complete panorama of the antioxidant activity, the antioxidant assay chosen should be further extended to cellular and/or in vivo animal systems in future studies.

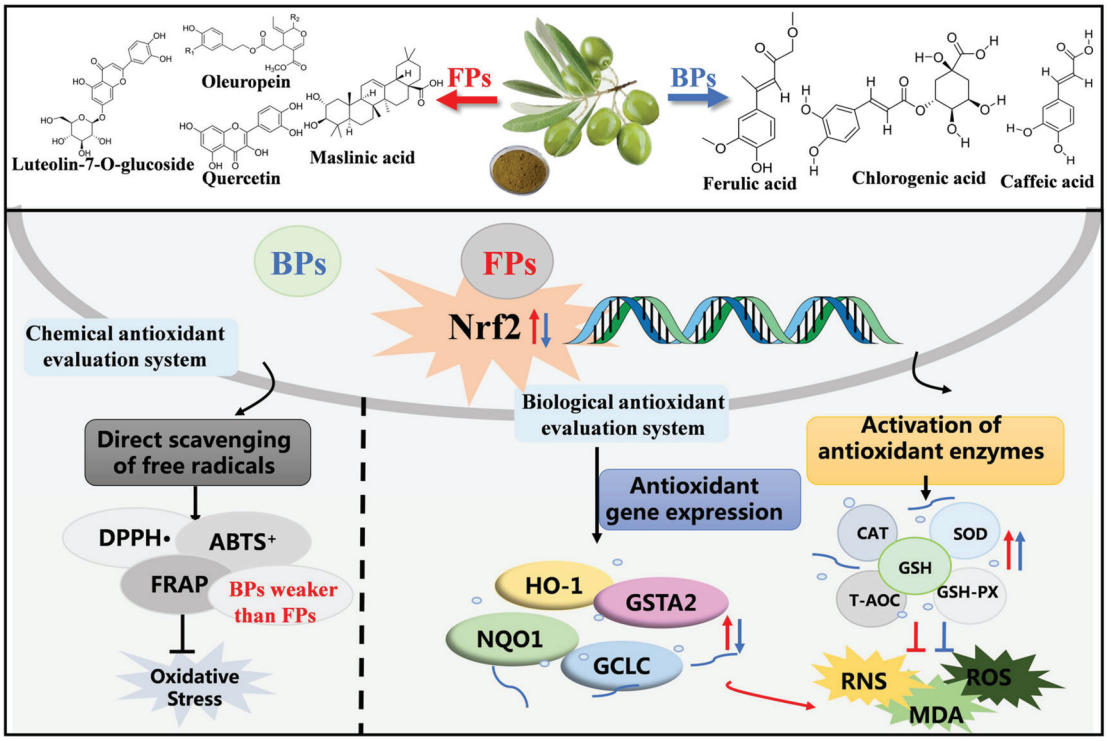


**Figure 7.** Potential molecular mechanisms of antioxidant properties by FPs and BPs in olive leaves. The red arrows represent FPs, and the blue arrows represent BPs.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox12122033/s1, Figure S1. Total ion chromatogram (TIC) of free and bound phenolics fractions in olive leaves for negative ion and positive ion mode UPLC-Q-Exactive Orbitrap-MS. (A,B) FPs in negative and positive ion mode; (C,D) BPs in negative and positive ion mode. Table S1. Calibration curves used for HPLC quantification. Table S2. Effect of different concentrations of $H_2O_2$ on the viability of HepG2 cells. Table S3. Effect of the incubation time of $H_2O_2$ on the viability of HepG2 cells. Table S4. Primer sequences for RT-qPCR.

**Author Contributions:** Conceptualization, T.L., C.J., C.Z. and D.L.; methodology, T.L., W.W., J.Z., Q.W. and S.W.; software, W.W. and J.Z.; investigation, S.Z., E.N. and C.J.; resources, S.Z. and E.N.; data curation, T.L., J.Z. and S.W.; writing—original draft preparation, T.L., W.W., Q.W., S.Z. and E.N.; writing—review and editing, C.J., C.Z. and D.L.; supervision, C.Z. and D.L.; funding acquisition, C.Z. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted according to the Guide for the Care and Use of Laboratory Animals, and was approved by the Animal Care and Use Committee of Zhejiang Academy of Agricultural Sciences (protocol code 2023ZAASLA80).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data are contained within the article and Supplementary Materials.

**Conflicts of Interest:** The authors declare no conflict of interest.

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antioxidants

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# A Comparative Study on UHPLC-HRMS Profiles and Biological Activities of *Inula sarana* Different Extracts and Its Beta-Cyclodextrin Complex: Effective Insights for Novel Applications

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**Abstract:** Within this particular framework, the extracts obtained from *Inula sarana* using a variety of solvents, included n-hexane, ethyl acetate, dichloromethane (DCM), 70% ethanol, ethanol, and water. The extracts obtained from n-hexane, ethyl acetate, and DCM were then subjected to a specific method for their incorporation into β-cyclodextrin (β-CD). The establishment of complex formation was validated through the utilization of scanning electron microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR). The identification of phytochemical components was executed using UHPLC-HRMS. Furthermore, the total phenolic and flavonoid content was evaluated using the Folin–Ciocalteu assay and the $AlCl_3$ method. Subsequently, the determination of antioxidant capacity was conducted utilizing DPPH, ABTS, CUPRAC, Frap, PBD, and MCA assays. The enzyme inhibitory activities of the samples (extracts and β-CD complexes) were also examined by AChE, BChE, tyrosinase, α-glucosidase, and α-amylase. The findings indicated that water and 70% ethanol extracts contained the highest phenolic content. One hundred and fourteen bioactive compounds were identified by UHPLC-HRMS analysis. This study unveiled a substantial array of flavonoids, phenolic acid-hexosides and caffeoylhexaric acids within *I. sarana*, marking their initial identification in this context. Among the various extracts tested, the 70% ethanol extract stood out due to its high flavonoid content (jaceosidin, cirsiliol, and eupatilin) and hydroxybenzoic and hydroxycinnamic acid hexosides. This extract also displayed notably enhanced antioxidant activity, with ABTS, CUPRAC, and FRAP test values of 106.50 mg TE/g dry extract, 224.31 mg TE/g dry extract, and 110.40 mg TE/g, respectively. However, the antioxidant values of the complex extracts with β-CD were generally lower than those of the pure extracts, an observation warranting significant consideration. In terms of enzyme inhibition activity, the ethanol and 70% ethanol extracts exhibited higher inhibitory effects on AChE, tyrosinase, and α-glucosidase. Conversely, n-hexane displayed stronger inhibitory activity against BChE. The ethyl acetate extract demonstrated elevated amylase inhibitory activity. However, the antioxidant values of the complex extracts with β-CD were generally lower than those of the pure extracts, a noteworthy observation, while water and extracts from the *I. sarana* complex with β-CD exhibited minimal or negatable inhibitory activity against specific enzymes.

**Keywords:** *Inula sarana*; β-cyclodextrin; chemical characterization; SEM; enzyme inhibitory

## 1. Introduction

The Asteraceae family, comprising the largest group of flowering plants, encompasses over 24,000 to 30,000 species and 1600 to 1700 genera [1]. Within this family are found essential botanical resources, including medicinal oil-yielding plants, horticultural resources, and economically significant species. Noteworthy phytochemical constituents like polyphenols, diterpenoids, and flavonoids are prevalent among the constituents of this botanical family [2]. Therefore, species of this family typically stand out for their notable antioxidant, anti-inflammatory, antiparasitic, and anticancer properties [3,4]. The genus *Inula* L. encompasses approximately 25 species, with nine endemic to Turkey, is predominantly distributed in Asia, Africa, Europe, and is notably abundant in the Mediterranean region [5–7].

The limited solubility of many drugs in water represents one of the most significant challenges preventing the advancement of various pharmaceuticals. A notable observation reveals that approximately 40% of newly developed drugs display poor water solubility, which prevents their efficient clinical application [8]. This challenge has led to the exploration of various precise drug encapsulation techniques. One particular strategic possibility involves the encapsulation of such drugs using various methods. This approach has the potential to increase dissolution rates, increase membrane permeability, and, ultimately, improve the bioavailability of nutraceuticals with low solubility. Generally, cyclodextrins (CDs) are low-cost starch derivatives that have been manufactured and enzymatically modified. These nontoxic starch derivatives have minimal absorption in the upper gastrointestinal tract and are completely metabolized by the gut microbes [9]. The unique hollow molecular structure of β-cyclodextrin (β-CD) allows it to form inclusion complexes with a diverse range of organic compounds. These compounds partially or completely accommodated within its relatively hydrophobic cavity, eliminating a limited number of high-energy water molecules [9,10]. The pharmaceutical industry frequently utilizes cyclodextrins due to their ability to form inclusion complexes with various molecules, serving as a means to enhance the water solubility and consequently, the bioavailability of lipophilic drugs. This strategy, though, is less effective for hydrophilic or moderately polar drugs, leading to the exploration of cyclodextrin derivatives. However, these challenges can be effectively addressed through chemical modifications. β-CD, categorized as a polysaccharide, has some limitations, including insufficient water solubility and possible toxicities when ingested intravenously [11]. These challenges, however, can be effectively addressed through chemical modifications. The approach of synthesizing nanosponges based on cyclodextrins appears to be a viable technique to bolster and prolong the stability of drugs.

Curcumin's clinical applicability is limited due to challenges like low bioavailability, minimal solubility in aqueous settings, and susceptibility to chemical degradation at physiological pH levels; however, studies have demonstrated that its solubility can be enhanced by up to 2.34 times through complexation with β-CD [12]. In a study recently published, a remarkable enhancement of 206-fold in curcumin solubility was documented, demonstrating the sustained release of curcumin from the resultant inclusion complexes [13]. Recently, a number of studies have reported the successful encapsulation of extracts and essential oils derived from the Asteraceae family. This encapsulation process has been designed to enhance the bioavailability of bioactive compounds contained within these extracts and oils. The ultimate goal is to significantly boost their efficacy in various applications. This development represents a notable advancement in the field of natural product encapsulation and opens up new possibilities for harnessing the therapeutic potential of these bioactive substances [14–16]. Nevertheless, scientific exploration into the characteristics of *Inula sarana* remains limited. Hence, due to the unexplored nature of *I. sarana*, we undertook a comprehensive study of this particular species.

Beyond their pharmaceutical applications, cyclodextrins (CDs) provide a number of benefits with broader implications. Their ability to extend the shelf life of food products has sparked interest, demonstrating their significance beyond the realm of medicine. Moreover, CDs have the ability to mask or eliminate unpleasant tastes and odours, addressing

concerns about the flavour of certain goods [17]. The process of encapsulating plant extract within β-CD at the molecular level holds numerous potentials for several benefits. As study claimed that olive leaf encapsulation in β-CD demonstrates the potential for various enhancements, including increased solubility in aqueous solutions, improved compatibility within oil/water systems, enhanced protection against oxidative degradation during storage, and the possibility of improving its bioavailability [10,18].

In this context, the study aimed to determine the chemical composition (by using UHPLC-HRMS) of *I. sarana* extracts and their biological activities Additionally, we investigated the changes in chemical profiles and biological properties in β-CD incorporation of its ethyl acetate, n-hexane, and DCM. This aspect can provide significant information for preparing formulations using *I. sarana* for further pharmaceutical, nutraceutical, and cosmeceutical applications.

## 2. Materials and Methods

### *2.1. Chemicals*

Acetonitrile, formic acid (for LC–MS) and methanol (analytical grade) were provided from Merck (Merck, Sofia, Bulgaria). The reference standards, using for UHPLC-HRMS protocatechuic, *p*-coumaric, *m*-coumaric, *o*-coumaric, vanillic, gentisic and salicylic acids, luteolin 7-*O*-glucoside, kaempferol-3-*O*-rutinoside, kaempferol 3-*O*-glucoside, isorhamnetin 3-*O*-glucoside, nepetin 7-*O*-glucoside, hyperoside, apigenin, quercetin, kaempferol, hispidulin, chrysoeriol, isorhamnetin, jseocidine, and luteolin were obtained from Extrasynthese (Genay, France). Chlorogenic, neochlorogenic, caffeic, 3,4-dicaffeoylquinic, 3,5-dicaffeylquinic and 1,5-dicaffeoylquinic acids, as well as dehydrocostus lactone, isoalantolactone, alantolactone, and partenolide, were supplied from Phytolab (Vesten-Bergsgreuth, Germany).

### *2.2. Plant Material*

In the summer of 2021, the aerial parts of *I. sarana* were gathered in Konya (Turkey: Seydisehir-Antalya road, Alacabel location, 1640 m) by the botanist Evren Yildiztugay. After collecting the plants, one specimen was deposited at the Selcuk University herbarium (EY-3212). The plant materials underwent a thorough washing process with tap and distilled water to eliminate any soil and contaminants prior to extraction. The aerial parts were then air-dried for 10 days in shade at room temperature and powdered.

### *2.3. Sample Preparation*

The n-hexane, ethyl acetate, DCM, 100% ethanol, and 70% ethanol extracts were obtained through the maceration method, wherein 10 g of plant material was mixed with 200 mL of each solvent and steeped for 24 h at room temperature. We used Whatman 1 filter paper to filter the mixtures and a rotary-evaporator to remove the solvents. To make the water extract (100%), we boiled 200 milliliters of water and infused 10 g of plant material for 15 min. After filtration, we lyophilized the extract for 48 h. Subsequently, all extracts were stored at 4 °C until analysis. The extraction yields were 3.32% (for n-hexane), 4.33% (for DCM), 5.03% (for ethyl acetate), 5.90% (for ethanol), 17.03% (for 70% ethanol) and 6.63% (for water).

### *2.4. Total Phenolic and Flavonoids Content*

Total phenolic (Folin–Ciocalteu assay) and flavonoids ($AlCl_3$ assay) contents of the extracts/complexes by colorimetric method using the describe method for phenolic by Acquaviva et al. [19] and for flavonoids by Llorent-Martínez et al. [20]. Results were expressed as gallic acid equivalents (mg GAE/g dry extract or complex) and rutin equivalents (mg RE/g dry extract or complex) for respective assays.

*2.5. UHPLC-HRMS Analysis*

The UHPLC-HRMS analyses were performed as previously described [21]. Q Exactive Plus mass spectrometer with a heated electrospray ionization (HESI-II) probe (ThermoFisher Scientific, Inc., Waltham, MA, USA), operated in negative and positive ion modes (*m/z* range from 100 to 1500 at a resolution of 70,000) was used for the LC-MS analyses. The tune parameters were as follows: 2.5 kV spray voltage, 38 AU sheath gas flow rate, 12 AU auxiliary gas flow rate, 320 °C capillary temperature, 320 °C probe heater temperature, and level 50 S-lens RF. Acquisition was developed at the full-scan MS and Data Dependent-MS2 modes (DD-MS2). Other parameters for Full MS mode were as follows: maximum injection time (IT) 100 ms, number of scan ranges 1, and automatic gain control (AGC) target $3 \times 10^6$. For the DD-MS2 mode, the instrument parameters were maximum IT 50ms, microscans 1, resolution 17,500, AGC target $1 \times 10^5$, MSX count 1, Top5, and isolation window 2.0 *m/z*, and the stepped normalized collision energy was 10, 20, and 60 eV. The separation was performed on a C18 column Kromasil EternityXT (1.8 µm, 2.1 × 100 mm) (Bohus, Ale, Sweden) at 40 °C. The mobile phase contained 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The run time was 33 min, and the flow rate was 0.3 mL/min. The gradient elution program was as follows: 0–1 min, 5% B; 1–20 min, 5–30% B; 20–25 min, 30–50% B; 25–30 min, 50–70% B; 30–33 min, 70–95%; and 33–34 min, 95–5% B. Equilibration time was 4 min. The injection volume was set to 1 µL, and the flow rate was 300 µL/min. Data acquisition was performed using Xcalibur 4.2 software (Thermo Scientific). MZmine 2.53 software was applied to the UHPLC–HRMS raw files of the *I. sarana* extract for further analysis.

*2.6. Preparation and Characterization of β-CD Complexes*

The inclusions for n-hexane, ethyl acetate and dicholoromethane (DCM) extracts were prepared in a 1:1 ratio. Fifty mg of extracts were dissolved in 50 mL of methanol and these extracts were carefully filtered. Then, almost 50 mg β-CD (in 50 mL of water) was added and the mixture was stirred at room temperature for 24 h. After that, the mixtures were filtered, and the methanol was removed using a rotary evaporator. The final mixtures were lyophilized at −40 °C to remove all water and the dry samples were stored in the dark condition at 4 °C. The obtained inclusions were named hexane/β-CD, ethyl acetate/β-CD and DCM/β-CD.

The samples were directly placed on aluminum stabs; then, the shape and surface characteristics were observed by micrographs of the samples taken by scanning electron microscopy (SEM) (Zeiss EVO LS10; Oberkochen, Germany) using the gold sputter technique. The zone magnification for the images were kept around 20,000×. Observations were performed under 1 and 15 kV. Fourier transform infrared spectrum (FTIR) was recorded on a Vertex 70 Bruker FTIR spectrometer (Bruker, Leipzig, Germany) with attenuated total reflectance (ATR) module. All spectra were captured in the 400 and 4000 $cm^{-1}$ spectral region at a scan rate of 180 scans and a spectral resolution of 4 $cm^{-1}$. The FTIR spectrum was used in the transmittance mode.

*2.7. In Vitro Antioxidant Assays*

We evaluated the antioxidant potential of the extracts through the execution of six complementary in vitro spectrophotometric tests, as described in reference [22]. These tests included examinations of the ABTS and DPPH assays, which probe the extracts' ability to neutralize free radicals. The extract's ability to reduce (FRAP and CUPRAC assays) and its metal chelating capacity (MCA) were evaluated. Total antioxidant ability was measured by the phosphomolybdenum (PBD) assay. We utilized the Trolox standard for assessing all assays except MCA, for which we compared the results in terms of equivalent EDTA per gram of extract.

### *2.8. Enzyme Inhibitory Activity*

The inhibitory effects of extracts and inclusions were investigated against different enzymes acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase, α-amylase and α-glucosidase. The enzyme inhibitory assays were performed as reported by Zengin [23]. The inhibition of AChE and BChE was quantified as milligrams of galanthamine equivalents (GALAE) per gram of extract, while tyrosinase inhibition was measured as milligrams of kojic acid equivalents (KAE) per gram of extract. In addition, α-amylase and α-glucosidase inhibition were assessed in terms of millimoles of acarbose equivalents (ACAE) per gram of extract.

### *2.9. Statistical Analysis*

Statistical analysis was performed using Xl Stat (Version 16). All analyses were conducted in triplicates ($n$ = 3) and presented as mean values with their standard deviation (mean value ± std). Differences between samples were examined using one-way analysis of variance (ANOVA) and Tukey's post hoc test with significance level set at $p < 0.05$. Veen diagrams were designed using an online platform (http://bioinformatics.psb.ugent.be/webtools/Venn/; accessed on 20 August 2023). Circle heat map was also obtained from an online platform (https://www.chiplot.online/; accessed on 20 August 2023). Graph Pad Prism (version 9.2) was used for Pearson correlation analysis.

## 3. Results and Discussion

### *3.1. Characterization of β-CD Complexes*

As because polar solvent extracts are typically not encapsulated by β-CD due to the strong hydrophilic interactions between polar compounds and water, which limit their inclusion within the hydrophobic cavity of β-CD [24]. So, water, 70% ethanol, and water extracts was not encapsulated with β-CD. The n-hexane, ethyl acetate and DCM extracts were incorporated into β-CD, and were characterized.

#### 3.1.1. SEM Analysis

When it comes to scrutinizing the microscopic morphology and structure of solids, especially crystalline materials, scanning electron microscopy (SEM) serves as an indispensable tool [25]. A concentrated electron beam is used to scan a sample's surface in order to create images of it. By interacting with the atoms in the sample, the electrons create signals that provide information about the sample's surface topography and chemical composition. The morphologies of pure β-CD, ethyl acetate/β-CD, hexane/ β-CD and DCM/β-CD were characterized by SEM and the images are shown in Figure 1. The structural differences observed among the related complexes imply that additional crystalline phases may have emerged following complexation. In general, the β-CD particles (Figure 1) displayed irregular and parallelogram shapes under various inclusion complexes.

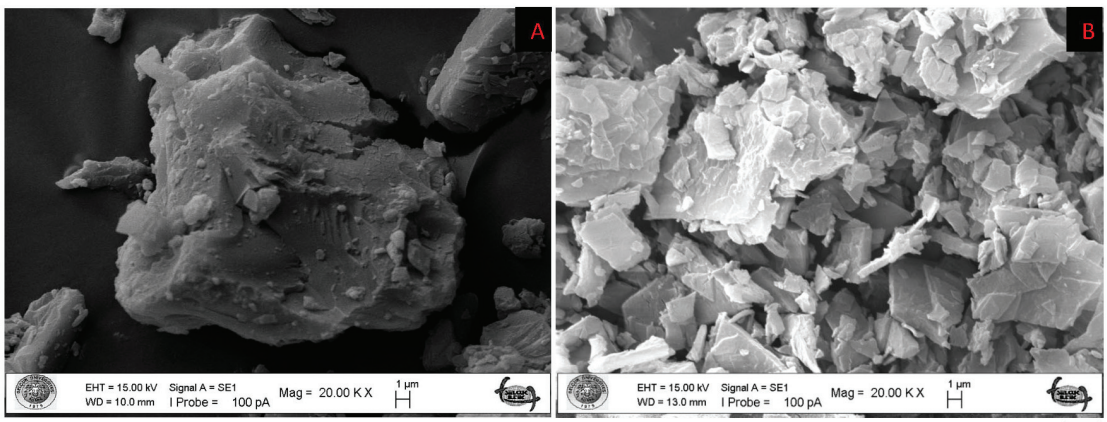


**Figure 1.** *Cont.*

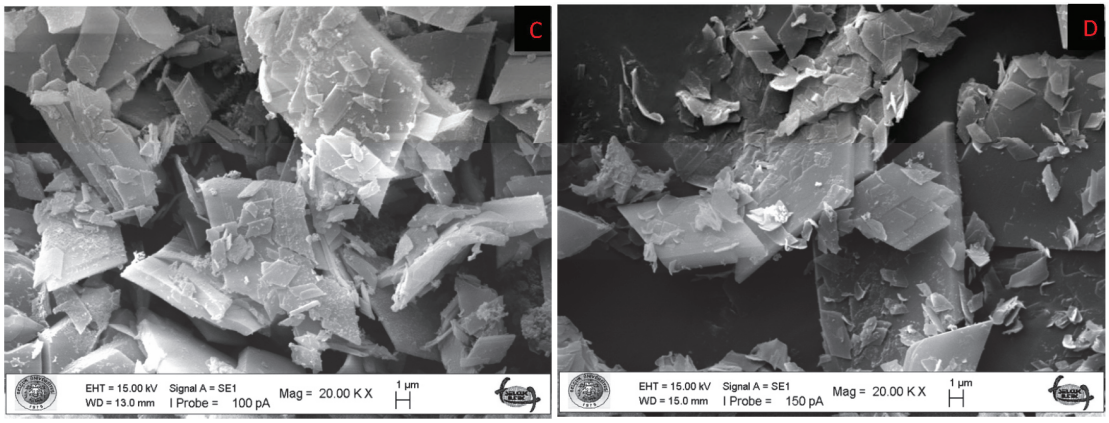


**Figure 1.** SEM images of pure β-cyclodextrin (**A**); Ethyl acetate/β-cyclodextrin (**B**); Hexane/β-cyclodextrin (**C**); DCM/β-cyclodextrin (**D**) inclusion complex.

### 3.1.2. Fourier Transform Infrared Spectroscopic Analysis

The infrared spectrum's position and intensity correspond to the features of molecular structures. The production of inclusion complex and the interaction of ethyl acetate, n-hexane, DCM, and β-CD can be inferred from changes in FTIR spectra. The FTIR spectra of ethyl acetate/β-CD, n-hexane/β-CD, and DCM/β-CD inclusion complex were shown in Figure 2A. In addition, FTIR spectra of pure β-cyclodextrin and each extract without the presence of β-cyclodextrin with enlarged form between 1550 $cm^{-1}$ and 400 $cm^{-1}$ are shown in Figure 2B The typical absorption frequency of β-CDs spans the full 400–3800 $cm^{-1}$ range. Organic small molecules can only constitute a maximum of 25% (*w*/*w*) of the inclusion complex [26]. Their distinctive peaks are obscured and hard to distinguish by the absorption peaks of β-CDs. Tensile vibration absorption occurs about 1700 $cm^{-1}$ if the guest molecule has a group like –COOH, –COOR, or C=O. The stretching (–OH) vibration of hydroxyl groups causes the broad peak around 3380 $cm^{-1}$. According to Figure 2, the stretching (C-H) vibration is responsible for the peaks at 2970 and 2900 $cm^{-1}$ in the FTIR spectrum of all samples, while the (C–H) deformation vibration is responsible for the peak at 1390 $cm^{-1}$. The FTIR spectrum exhibits two peaks as a result of the stretching (C–O) vibration. The stretching (C–O) vibration of the co-carbon carboxyl group causes one peak to occur at 1210 $cm^{-1}$, and the stretching (C–O) vibration of the $–O–CH_2CH_3$ group causes another comparably strong peak to appear at 1040 $cm^{-1}$ [27].

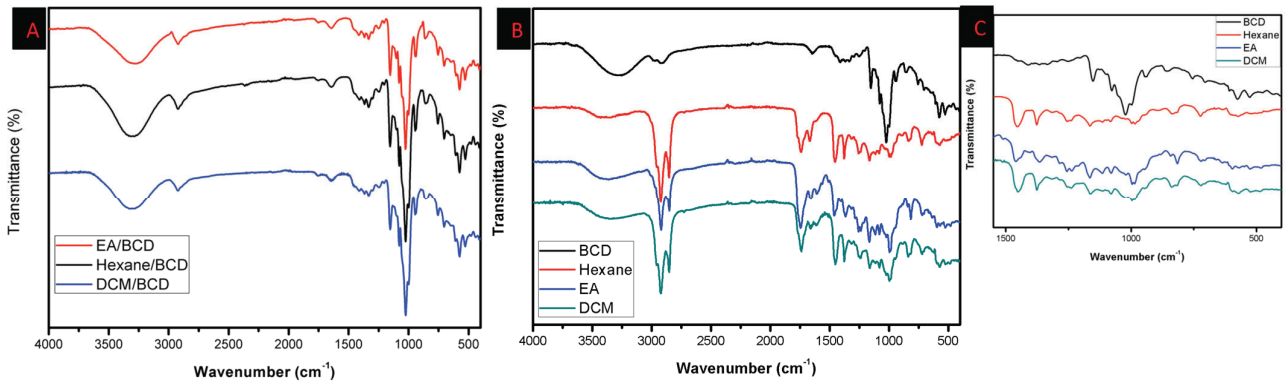


**Figure 2.** FT–IR spectra of Ethyl acetate/β–cyclodextrin, Hexane/β–cyclodextrin, DCM/β–cyclodextrin inclusion complex (**A**), FT–IR spectra of pure β–cyclodextrin and each extract without the presence of β–cyclodextrin (**B**), with enlarged form between 1550 $cm^{-1}$ and 400 $cm^{-1}$ (**C**).

The composition of different extracts of *I. sarana* and its complex with β-CD has been studied in terms of total phenolic content and total flavonoid content. The results have been categorized among various solvents and different extraction conditions.

### *3.2. Total Phenolic and Flavonoid Content*

Among the various extracts obtained, the highest total phenolic content was found in the extract obtained from water, with a value of 55.55 mg GAE/g dry extract (Table 1). On the other hand, the lowest total phenolic content was observed in the n-hexane extract, which had a value of 12.45 mg GAE/g dry extract. In the middle range, the ethanol extract had a total phenolic content of 38.42 mg GAE/g dry extract. In terms of the total flavonoid content, the highest value was recorded for the extract obtained from ethyl acetate, with 22.60 mg RE/g dry extract. Conversely, the lowest total flavonoid content was found in the water extract, which had a value of 9.11 mg RE/g dry extract. In the middle range, the DCM extract exhibited a total flavonoid content of 16.57 mg RE/g dry extract. When considering the extracts obtained from the complex of *I. sarana* with β-CD, the highest total phenolic content was observed in the dichloromethane/β-CD (DCM/β-CD), with a value of 4.54 mg GAE/g dry complex, followed by the ethyl acetate/β-CD had a total phenolic content of 4.17 mg GAE/g dry complex. The lowest total phenolic content in this group was found in the n-hexane/β-CD, which had a value of 3.00 mg GAE/g dry complex. In terms of total flavonoid content in the β-CD complex extracts, the highest value was recorded for the ethyl acetate/β-CD, with 3.82 mg RE/g dry complex. However, n-hexane/β-CD had lowest total flavonoid content 1.96 ± 0.26 mg RE/g dry complex.

**Table 1.** Total phenolic and flavonoid content of the tested samples *.

| Extracts | Total Phenolic Content (mg GAE/g Dry Extract or Complex) | Total Flavonoid Content (mg RE/g Dry Extract or Complex) |
|---|---|---|
| n-Hexane | $12.45 \pm 0.44^{f}$ | $4.60 \pm 0.18^{e}$ |
| Ethyl acetate | $23.77 \pm 0.15^{d}$ | $22.60 \pm 0.44^{a}$ |
| Dicholoromethane | $20.19 \pm 0.78^{e}$ | $16.57 \pm 0.32^{b,c}$ |
| Ethanol | $38.42 \pm 0.10^{c}$ | $17.23 \pm 0.48^{b}$ |
| 70% Ethanol | $46.36 \pm 0.12^{b}$ | $15.93 \pm 0.30^{c}$ |
| Water | $55.55 \pm 0.42^{a}$ | $9.11 \pm 0.14^{d}$ |
| n-Hexane/β-CD | $3.00 \pm 0.03^{h}$ | $1.96 \pm 0.26^{f}$ |
| Ethyl acetate/β-CD | $4.17 \pm 0.11^{g}$ | $3.82 \pm 0.41^{e}$ |
| DCM/-β-CD | $4.54 \pm 0.09^{g}$ | $1.81 \pm 0.07^{f}$ |

* Values are reported as mean ± SD of three parallel measurements. GAE: Gallic acid equivalents; RE: Rutin equivalents. Different letters ("a" indicates the highest level) in the same column indicate significant differences in the tested extracts and complexes ($p < 0.05$).

These results highlight variations in the levels of total phenolic and flavonoid compounds, depending on the solvents and extraction conditions as well as the effect of complexation with β-CD on these levels.

The results of this study on the extracts of *I. sarana* as well as its complex with β-CD provide significant insights into the phenolic and flavonoid composition of this medicinal plant and its complex. Recognized for their antioxidative features, these compounds may have positive implications for human health.

The variations observed in the total phenolic and flavonoid contents based on the extraction solvents indicate that different solvents have varying capabilities to extract these compounds from the plant. For instance, extracts obtained from water and 70% ethanol appear to have the highest contents of phenolic and flavonoid compounds. This suggests that these solvents are more effective in extracting these compounds from the plant [28–30]. In our study, the polar solvents, namely ethanol, 70% ethanol, and water, were generally rich in terms of total phenolic content. This can be attributed to the enhanced solvation of antioxidant compounds in the extracts, which is caused by the interactions (especially hydrogen bonds) between the polar sites of the antioxidant molecules and these solvents.

In addition, both lipophilic and hydrophilic phenolics can be extracted by the solvents and thus can result in high values in the spectrophotometric assays. This fact was also confirmed by UHPLC-HRMS analysis, which showed more components in the polar solvents when compared to nonpolar solvents.

The results of the *I. sarana*/β-CD complex extracts generally showed lower total phenolic and flavonoid contents compared to the classical extracts. Understanding these observations is important for product formulation involving extracts, where a reduction in phenolic and flavonoid compounds might impact their potential effectiveness. Therefore, further research and experimentation are imperative to identify the precise reasons behind this decrease. It is important to acknowledge that the explanation may vary depending on the specific plant extract, extract amount, and experimental conditions. Studies prove the concentration of β-CD and the ratio at which it forms complexes with the relevant extracts playing a significant role. The not optimized ratio may lead to reduced antioxidant activity [31]. Additional studies are necessary to gain deeper insights and validate these findings. This unexpected decrease in phenolic and flavonoid content when using β-CD challenges conventional assumptions, encouraging researchers to explore novel mechanisms and applications. The deviation from the typical enhancement effect of β-CD on phenolic and flavonoid content may complicate the formulation of products reliant on these extracts, potentially affecting their intended effectiveness.

### *3.3. UHPLC-HRMS Analysis*

The compound identification/annotation approach was based on the fragmentation rules and indicative ions for different types of compounds, reference standards and literature data [21]. The main points in the peak's annotation and dereplication are accurate masses in Full MS, MS/MS fragmentation, relative abundance of deprotonated/protonated molecules and fragment ions, elemental composition, correspondence to the simulated monoisotopic profiles, and comparison with the retention times, MS/MS spectra and chromatographic behavior of reference standards. The total ion chromatograms (TIC) are given in Figure 3.

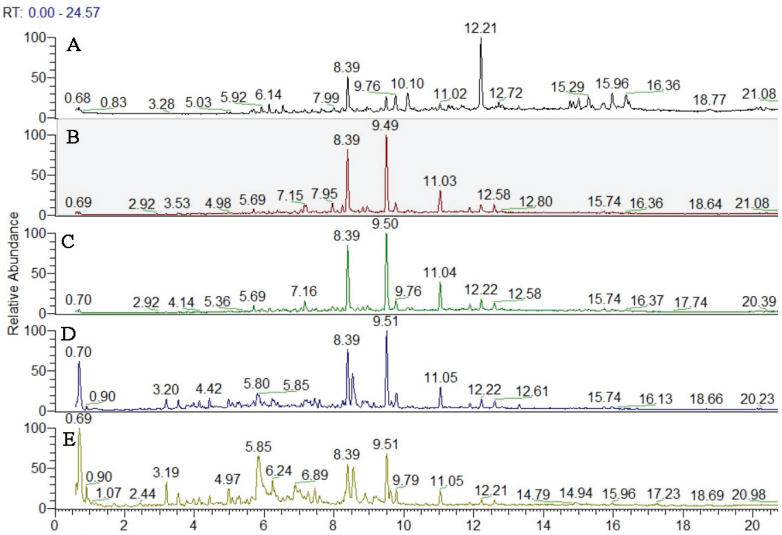


**Figure 3.** *Cont.*

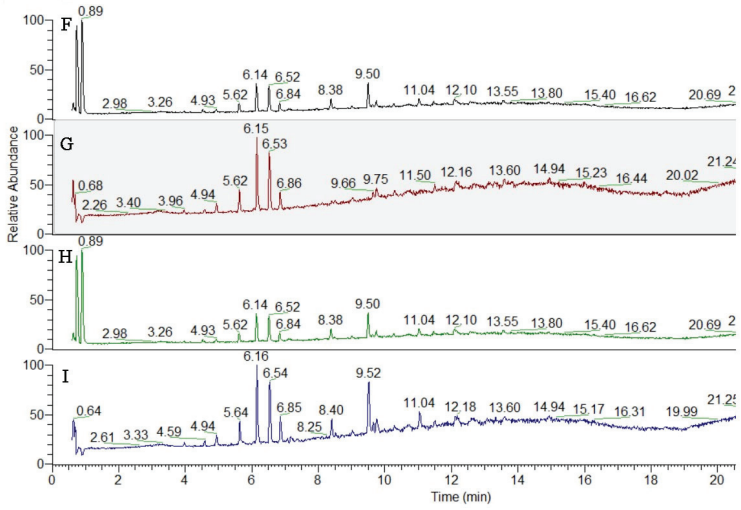


**Figure 3.** TIC of studied *Inula sarana* extracts in negative ion mode; (**A**) n-hexane extract; (**B**) ethyl acetate extract; (**C**) DCM extract; (**D**) ethanol extract; (**E**) 70% ethanol extract; (**F**) water extract; (**G**) n-hexane/β-CD; (**H**) ethyl acetate/β-CD; (**I**) DCM/β-CD.

To compare the number of identified compounds in the tested extracts, we constructed Venn diagrams, and the results are presented in Figure 4. As can be seen from Figure 4A, most compounds were identified in the 70% ethanol extract with 106 compounds, followed by ethanol (83) and water (62). The n-hexane extract contained the lowest number of chemicals (31). In this sense, the polar solvents can be suggested for further application with *I. sarana*. Furthermore, we compared the number of identified compounds in the extracts and their complex. From Figure 4B, it can be seen that almost 45–50% of the identified compounds were protected by β-CD complexation.

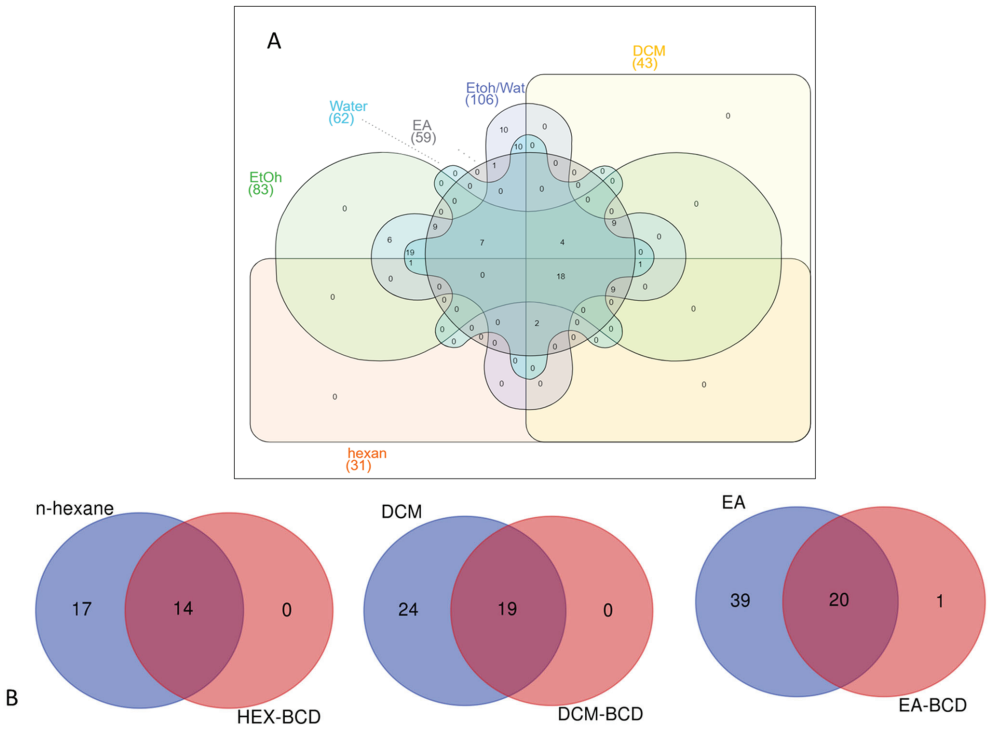


**Figure 4.** Venn diagrams showing number of compounds identified from the tested extracts (**A**). Comparison the identified compounds in the extracts and theirs complexes (**B**). EA: Ethyl acetate; DCM: Dichloromethane; EtOh: Ethanol; EtOh/Water: 70% Ethanol; BCD: β-cyclodextrin.

3.3.1. Hydroxybenzoic and Hydroxycinnamic Acids and Their Derivatives

Several glycosides of hydroxybenzoic and hydroxycinnamic acids, including hexosides 1–4, 6, 8–11 and 17 were tentatively identified (Tables S1 and S2, Figure S1). A variety of the phenylpropanoid glycosides were also evidenced. Thus, compounds 31 and 33 demonstrated the same deprotonated molecules $[M–H]^-$ at *m/z* 521.130 (calc. for $C_{24}H_{25}O_{13}$). The occurrence of caffeoyl and syringyl residues was deduced from the transitions 521.130→323.077 $[M–H–C_9H_{10}O_5]^-$ and 323.077→161.023 $[M–H–C_6H_{10}O_5]^-$, supported by the indicative fragment ions at *m/z* 179.034 [caffeic acid–H]$^-$ and 197.045 [syringic acid–H]$^-$. Additionally, the syringic acid was evidenced by the fragment ions at *m/z* 182.021 [syringic acid–H–$CH_3$]$^-$, 166.998 [syringic acid–H–2$CH_3$]$^-$ and 123.008 [syringic acid–H–2$CH_3$–$CO_2$]$^-$. Accordingly, 31 and 33 were assigned to syringic acid-(caffeoyl)-hexosides. In the same manner, the transitions 441.140→323.077 $[M–H–C_5H_{10}O_3]^-$ and 323.077→161.023 $[M–H–C_6H_{10}O_5]^-$ indicated both hydroxyisovaleryl and caffeoyl moieties in 35 (caffeic acid-(hydroxyisovaleryl(-hexoside). In addition, coumaric acid at *m/z* 163.060, gentisic acid at *m/z* 153.018, hydroxybenzoic acid at *m/z* 137.023 and vanillic acid at *m/z* 167.034 were dedicated in 32, 34, 36/43 and 37, respectively. Thus, they were ascribed as hydroxyisopropanoic acid-(coumaroyl)-hexoside, gentisic acid-(caffeoyl)-hexoside, caffeic acid-(hydroxybenzoyl)-hexoside and vanillic acid-(caffeoyl)-hexoside, respectively.

Six hydroxybenzoic acids (5, 12, 15, 16, 25 and 42) and 3 hydroxycinnamic acids (22, 24, and 30) together with quinic acid (20) and two caffeoylgluconic ester isomers (13 and 18) were identified in the extracts on the basis of comparison with the reference standards, respectively (Table S1, Figure S1).

Although hydroxybenzoic and hydroxycinnamic acids were found in their free form, herein, a great number of their hexosides and phenylpropanoid glycosides were evidenced in *I. sarana* for the first time. Quinic acid (21) was the main compound in this group of compounds in the studied extract, together with 10 and 15 (Figure S1).

3.3.2. Acylquinic Acids (AQAs)

Herein, 5 *mono*AQA and 7 *di*AQA and 1 *tri*AQA acids were annotated/dereplicated in the *I. sarana* extracts (Tables S1 and S2, Figure S2). The AQAs recognition was based on the fragment ions and their relative abundances corresponding to each subclass AQAs. The base peaks at *m/z* 191.055 was indicative for a substitution at C-5 of quinic acid skeleton. Thus, 21, 26 and 28 were assigned to 5-caffeoyl-, 5-*p*-coumaroyl and 5-feruloylquinic acid, respectively.

*di*AQA consisted of the following subclasses: dicaffeoylquinic acids (*di*CQA) (38–41), *p*-coumaroyl-caffeoylquinic acids (*p*-CoCQA) (44, 45) and hydroxydihydrocaffeoyl-caffeoylquinic acids (HC-CQA) (29).

Compounds 40 and 44 gave diagnostic ions at *m/z* 353.088, indicating a loss of caffeoyl (40) and *p*-coumaroyl (44) moiety. Moreover, both compounds gave base peaks at *m/z* 191.055 together with the prominent ion at *m/z* 179.034 [caffeic acid–H]$^-$ (40) and 163.039 (44), as was observed in 3-*mono*AQA (Table S1). Thus, 40 and 44 were ascribed as 3, 5-*di*CQA and 3C-5-*p*-CoQA, respectively.

Vicinal *di*CQA 3, 4-*di*CQA (38), 4,5-*di*CQA (41) and 4*p*-Co-5CQA (45) were evidenced by the diagnostic "dehydrated" quinic acid ion at *m/z* 173.045 (Table S1).

Peak 46 yielded a precursor ion at *m/z* 677.152 (calc. for $C_{34}H_{29}O_{15}$), along with the transitions at *m/z* 677.152→515.120→353.088→191.055, resulting from the losses of three caffeoyl residues. 4,5-*di*substituted quinic acid skeleton was deduced from the fragment ions at *m/z* 191.055 (48%), 179. 034 (68%), 173.045 (92%) and 135.044 (81%). Thus, 46 was assigned to 3,4,5-*tri*caffeoylquinic acid.

3.3.3. Caffeoylhexaric Acids (CHAs)

Overall, 4 *mono*CHA, 5 *di*CHA, 3 *tri*CHA and 2 *tetra*CHA were annotated together with their esters with aliphatic acids and hexosides. The starting points in the CHAs recognition

were the diagnostic fragment ions resulting from the subsequent losses of one (47–50), two (51–55), three (57–59) and four (56 and 60) caffeoyl residues (Table S1, Figure S3). Accordingly, the base peak at *m*/*z* 209.030 ($C_6H_9O_8$) [hexaric acid (HA)–H]$^-$ was observed along with a number of fragment ions at *m*/*z* 191.019 [HA–H–$H_2O$]$^-$, 173.009 [HA–H–$2H_2O$]$^-$, 147.029 [HA–H–$H_2O$–$CO_2$]$^-$, 129.018 [HA–H–$2H_2O$–$CO_2$]$^-$, 111.007 [HA–H–$3H_2O$–$CO_2$]$^-$, 85.028 [HA–H–$2H_2O$–$2CO_2$]$^-$ (47) (Table S1) [21,32]. Compounds 62 and 63 shared the same [M–H]$^-$ at *m*/*z* 617.153 (calc. for $C_{29}H_{29}O_{15}$). They afforded the indicative fragment ions at *m*/*z* 293.088 [M–H–2caffeoyl]$^-$ and 191.019 [M–H–2caffeoyl–$C_5H_{10}O_2$]$^-$ resulting from the losses of caffeoyl residues and a subsequent loss of 102.069 Da (calc. for $C_5H_{10}O_2$) or 2-methylbutiric acid/isovaleric acid (62). Caffeoyl moiety was deduced from the fragment ions at *m*/*z* 179.034 [caffeic acid (CA)–H]$^-$, [(CA–H)–$H_2O$]$^-$ at *m*/*z* 161.023 and 135.044 [(CA–H)–$CO_2$]$^-$. Accordingly, 62 and 63 were ascribed as isomeric 2-methylbutanyl/isovaleryl-dicaffeoylhexaric acids. In the same manner, 66 and 67 were annotated as 2-methylbutanyl/isovaleryl-tricaffeoylhexaric acid. Similarly, the assignment of 65 ([M–H]$^-$ at *m*/*z* 765.169, $C_{37}H_{33}O_{18}$) was suggested by the transition 279.072→191.019, resulting from the loss of 88.054 Da (calc. for $C_4H_8O_2$) or isobutyric acid. Concerning compounds 61 and 64, they afforded the indicative fragment ions at *m*/*z* 279.072 [M–H–3caffeoyl-Hex]$^-$ and 293.088, respectively, before the loss of the corresponding aliphatic acid. Accordingly, 64 was assigned to isobutanyl-tricaffeoylhexaric acid-hexoside (61) and 2-methylbutanyl/isovaleryl-tricaffeoylhexaric acid-hexoside (64).

3.3.4. Flavonoids

Overall, 6 flavone-, 10-flavonol- and 1 flavanone-glycosides were found. The dereplication/annotation of flavonoids was based on the indicative ions and their relative abundances in MS/MS spectra, corresponding to the respective flavonoid's subclass reported elsewhere [21].

The sugar chain of 68–70, 72, 73, 78 and 80 was consistent with rutinoside (308.112 Da, $C_{12}H_{20}O_9$) (Tables S1 and S2, Figure S4). Exemplifying this consistency, in (−)ESI-MS/MS the precursor ions at *m/z* 623.163 (80) generated deprotonated molecule of the aglycone ($Y_0^-$) at *m/z* 315.051. The aglycone was apparent by the prominent fragment at *m/z* 300.028 [$Y_0$–H–$^\bullet CH_3$]$^-$, supported by a series of neutral and radical losses and RDA ions at *m/z* 271.025 [$Y_0$–H–$CH_3$–$CHO^\bullet$]$^-$, 243.030 [$Y_0$-H–$CH_3$–$CHO^\bullet$–CO]$^-$, 227.034 [$Y_0$–H–$CH_3$–$CHO^\bullet$–$CO_2$]$^-$ and 215.035 [$Y_0$–H–$CH_3$–$CHO^\bullet$–2CO]$^-$ (Table S1). Methoxylation at a ring A of the 6-hydroxyluteolin was evident by the RDA ions at *m/z* 165.990 ($^{1,3}A^-$–$CH_3$), 133.028 ($^{1,3}B^-$), as was seen in patuletin. Accordingly, 80 was ascribed as nepetin *O*-rutinoside. Quercetin (68, 69, 71, 74 and 77) and luteolin (72 and 75) were proved by the base peaks and RDA ions at 151.002 ($^{1,3}A^-$) and 107.012 ($^{0,4}A^-$), 178.998 ($^{1,2}A^-$), 121.029 ($^{1,2}B^-$) (quercetin), and 133.028 ($^{1,3}B^-$) (luteolin). Compounds 68 and 69 revealed the same [M–H]$^-$ at *m/z* 609.147. The aglycone quercetin was evidenced in both flavonoids: 68 afforded a base peak ($Y_0^-$) at 301.036, while 69 gave ($Y_0^-$) at *m/z* 301.035 together with an abundant fragment ion of the radical aglycone [$Y_0$–H]$^{\bullet-}$ at *m/z* 300.028 (68.6%), suggesting the 3-*O*-glycosidic bond [33]. Accordingly, 68 was identified as rutin, additionally confirmed with the standard reference, while 69 was annotated as quercetin 7-*O*-rutinoside. Isoquercitrin (71), hyperoside (74), luteolin 7-*O*-glucoside (75), kaempferol 3-*O*-glucoside (81) and isorhamnetin 3-*O*-glucoside (82) were clearly identified by comparison with reference standards.

The approach for recognition of 6-methoxylated flavonoids was delineated elsewhere [21]. Among them, 7 methoxylated derivatives of quercetagetin (85, 88, 90, 94, 101, 102 and 105), four 6-hydroxyluteolin derivatives (89, 91, 100 and 104), and two scutelarein derivatives (96 and 103) were described.

As an example, isomers 94, 101 and 102 ([M–H]$^-$ at *m/z* 359.077, $C_{18}H_{15}O_8$) demonstrated the fragmentation pathway of 6-methoxylated quercetagetin derivatives (Table S1, Figure S5). In (−)ESI-MS/MS 94, 101, and 102 yielded radical losses at *m/z* 344.054 [M–H–$^\bullet CH_3$]$^-$, 329.031 [M–H–2$^\bullet CH_3$]$^-$ and 314.009 [M–H–3$^\bullet CH_3$]$^-$. A series of neutral losses were also recorded at *m/z* 301.036 [M–H–2$^\bullet CH_3$–CO]$^-$, 286.012 [M–H–3$^\bullet CH_3$–CO]$^-$,

258.071 [M–H–3•$CH_3$–2CO]$^-$ and 230.022 [M–H–3•$CH_3$–3CO]$^-$. In addition, a series of RDA ions were observed at *m/z* 165.990 ($^{1,3}A^-$–•$CH_3$), 136.987 ($^{1,3}A^-$–CO–$CH_4$) and 109.999 ($^{1,3}A^-$–CO–CHO–$CH_3$) indicating methoxylation in the A-ring. A dimethoxylated RDA ion $^{1,3}B$ was discernable by prominent fragment ions at *m/z* 163.040 ($^{1,3}B^-$–$CH_2$) and 148.016 ($^{1,3}B^-$–•$CH_3$–$CH_2$) (Table S1). Thus, 94, 101 and 102 were ascribed as quercetagetin-3,6,3′(4′)-trimethyl ether isomers.

3.3.5. Sesquiterpene Lactones and Derivatives

The dereplication of sesquiterpene lactones and derivatives was performed by UHPLC-ESI/HRMS of *I. sarana* extracts in positive ion mode as more relevant for the analysis of this class of specialized metabolites [34]. Based on accurate masse in Full MS, MS/MS fragmentation pathways, relative abundance of precursor and fragment ions, elemental composition, and comparison with reference standards and literature data, six sesquiterpene lactones and three sesquiterpenes were identified or tentatively annotated in *I. sarana* extracts. Previous investigation revealed MS/MS fragmentation patterns of sesquiterpene lactones with characteristic ions corresponding to the loss of $H_2O$ (−18 Da), CO (−28 Da), 2x$H_2O$ (−36 Da), and $CO_2$ (−44 Da), $CH_3COOH$ (−60 Da), as well as concomitant loss of $H_2O$ + CO (−46 Da), 2x$H_2O$ + CO (−64 Da), and $H_2O$ + $CO_2$ (−62 Da) [35].

Two peaks shared the same 108 and 113 protonated ion $[M+H]^+$ at *m/z* 233.153. The abundant fragments at *m/z* 215.143 (55.6% for 108) and (60.4% for 113) $[M+H–H_2O]^+$ are in agreement with a presence of OH group. The base peak at *m/z* 187.148 $[M+H\text{-}H_2O–CO]^+$, together with diagnostic fragments at *m/z* 161.132 $[M+H–CO–CO_2]^+$, *m/z* 117.070 $[C_9H_8C_9H_9]^+$ and *m/z* 105.070 $[C_8H_8C_9H_9]^+$, as well as comparison to the reference standards allowed the identification of 108 and 113 as isoalantolactone and alantolactone, respectively. Similarly, 107 and 110 were related to hydroxyalantolactone and dehydroalantolactone, respectively. In addition, based on a comparison with reference standards, 106 was related to dehydrocostus lactone, while 109 to partenolide.

*3.4. Antioxidant Properties*

The thorough analysis of the antioxidant activity of *I. sarana* extracts and its complex with β-CD revealed intriguing diversity in results. The DPPH, ABTS, CUPRAC, FRAP, PBD, and MCA tests provided essential insights into the antioxidant capacities of each sample, uncovering significant trends. The ethanol extract displayed high values in the ABTS (73.61 mg TE/g dry extract), CUPRAC (159.00 mg TE/g dry extract), and FRAP (74.18 mg TE/g dry extract) tests, reflecting substantial antioxidant activity. Interestingly, the 70% ethanol extract exhibited even more impressive antioxidant activity, with values of 106.5 mg TE/g dry extract in the ABTS test, 224.31 mg TE/g dry extract in the CUPRAC test, and 110.40 mg TE/g dry extract in the FRAP test (Table 2). The equations, range and R2 values of standard compounds in the biological activity assays are presented in Table S3.

**Table 2.** Antioxidant properties of the tested samples *.

| Extracts | DPPH (mg TE/g Dry Extract/Complex) | ABTS (mg TE/g Dry Extract/Complex) | CUPRAC (mg TE/g Dry Extract/Complex) | FRAP (mg TE/g Dry Extract/Complex) | PBD (mmol TE/g Dry Extract/Complex) | MCA (mg EDTAE/g Dry Extract/Complex) |
|---|---|---|---|---|---|---|
| n-Hexane | 17.95 ± 0.24 $^{e}$ | 12.02 ± 0.62 $^{e}$ | 46.56 ± 0.71 $^{e}$ | 24.50 ± 1.87 $^{d}$ | 1.49 ± 0.03 $^{b}$ | 13.85 ± 0.56 $^{c}$ |
| Ethyl acetate | 27.95 ± 0.30 $^{c}$ | 53.24 ± 0.10 $^{c}$ | 71.51 ± 2.43 $^{d}$ | 33.25 ± 0.21 $^{c}$ | 2.07 ± 0.14 $^{a}$ | 15.62 ± 0.97 $^{c}$ |
| Dicholoromethane | 25.90 ± 0.24 $^{d}$ | 48.29 ± 0.37 $^{d}$ | 64.50 ± 2.94 $^{d}$ | 31.39 ± 0.36 $^{c,d}$ | 2.01 ± 0.23 $^{a}$ | 15.44 ± 1.09 $^{c}$ |
| Ethanol | 45.84 ± 0.19 $^{b}$ | 73.61 ± 0.74 $^{b}$ | 159.00 ± 1.30 $^{c}$ | 74.18 ± 0.39 $^{b}$ | 1.97 ± 0.02 $^{a}$ | 10.53 ± 0.41 $^{d}$ |
| 70% Ethanol | 47.97 ± 0.02 $^{a}$ | 106.50 ± 0.09 $^{a}$ | 224.31 ± 0.02 $^{a}$ | 110.40 ± 3.96 $^{a}$ | 1.61 ± 0.06 $^{b}$ | 19.22 ± 0.82 $^{b}$ |
| Water | 47.63 ± 0.04 $^{a}$ | 106.41 ± 0.01 $^{a}$ | 193.85 ± 8.08 $^{b}$ | 113.88 ± 7.43 $^{a}$ | 1.28 ± 0.03 $^{c}$ | 22.99 ± 0.08 $^{a}$ |
| n-Hexane/β-CD | 15.17 ± 0.53 $^{f}$ | na | 9.98 ± 0.22 $^{f}$ | 7.47 ± 0.04 $^{e}$ | 0.12 ± 0.02 $^{e}$ | 0.96 ± 0.25 $^{g}$ |
| Ethyl acetate/β-CD | 13.98 ± 0.36 $^{g}$ | 1.52 ± 0.04 $^{f}$ | 10.67 ± 0.42 $^{f}$ | 8.11 ± 0.18 $^{e}$ | 0.15 ± 0.01 $^{e}$ | 1.87 ± 0.08 $^{f}$ |
| DCM/β-CD | 14.30 ± 0.55 $^{f,g}$ | 2.01 ± 0.36 $^{f}$ | 11.82 ± 0.20 $^{f}$ | 7.97 ± 0.05 $^{e}$ | 0.26 ± 0.02 $^{d}$ | 4.08 ± 0.53 $^{e}$ |

* Values are reported as mean ± SD of three parallel measurements. PBD: Phosphomolybdenum; MCA: Metal chelating Activity; TE: Trolox Equivalent; EDTAE: EDTA equivalent. Different letters ("$^{a}$" indicates the strongest ability) in the same column indicate significant differences in the tested extracts and complexes ($p < 0.05$).

However, the extracts form the complex with β-CD generally showed lower antioxidant values compared to the pure relevant extracts. For instance, the n-hexane extract exhibited DPPH and ABTS scavenging ability as 17.95 mg TE/g dry complex and 12.02 mg TE/g dry complex, respectively; however, the incorporation of the extracts in β-CD negatively affects its ability (DPPH: 15.17 mg TE/g; ABTS: no active). A similar case was also noted for ethyl acetate and DCM. These observations suggest that complexation with β-CD might influence the availability and reactivity of antioxidant compounds.

First and foremost, the observation of significant differences in antioxidant capacities among the various extracts underscores the critical influence of solvent choice in the extraction process. Ethanol-based extracts particularly excelled in multiple tests, illustrating ethanol's efficacy in solubilizing antioxidant compounds. The outstanding outcomes obtained with the 70% ethanol extract underscore the potential in extracting active compounds, which could be attributed to the increased polarity properties [36–38].

Regarding the extracts from the complex with β-CD, the trend of displaying reduced antioxidant values deserves particular attention. This observation suggests that the formation of the complex might have consequences on the release, stability, or accessibility of antioxidant compounds, potentially due to their encapsulation within the β-CD cavity [39,40]. This intricate interaction between the complex and active compounds could hold significant implications for the design and utilization of formulations based on these extracts. Previous studies have indicated that in vitro approaches using antioxidants demonstrate superior efficacy compared to drug complexes with CDs [41]. This difference can be attributed to the potential formation of stable phenoxy radicals through intermolecular hydrogen bonding between the hydroxyl groups of CDs and hydroxyl-containing drugs, particularly for those with hydroxyl groups [42,43]. CDs are also known to enhance drug stability by reducing oxidation [44]. Therefore, the complexation of CDs may not be advantageous for in vitro models involving extracts rich in polyphenols with hydroxyl groups. Additionally, it has been reported that complex antioxidant abilities may not be detectable using certain in vitro test systems, such as DPPH, FRAP, and CUPRAC, as these methods measure distinct properties of the complex [44,45].

Nonetheless, a study carried out by a group of researchers, as indicated by Escobar-Avello et al., yielded results that demonstrated minimal distinctions between the effects of pure extracts and encapsulated extracts [46]. For instance, when investigating encapsulated β-CD, it was revealed that their antioxidant capability measured at 5300 μmol TE/g [46] was quite comparable to the reading of 4612 μmol TE/g obtained from the pure extract [47]. This observation underscores that the encapsulation process utilizing β-CD seemed to have a negligible impact on enhancing the antioxidant potential of the compound.

On the contrary, numerous authors propose the notion that the antioxidative potential of phenolic compounds experiences enhancement through their encapsulation within β-CD [48]. This encapsulation process within cyclodextrins is believed to bolster the antioxidant capacity of phenolic compounds, according to several scholarly perspectives.

Furthermore, the variation in results across different antioxidant tests reflects the diversity of mechanisms by which the compounds present in the extracts can scavenge free radicals and other reactive species. The DPPH, ABTS, CUPRAC, FRAP, PBD, and MCA tests utilize distinct mechanisms to assess antioxidant activity, which can explain why certain active substances exhibit superior performance in a specific test but not in others. To gain more insights into the relationship between bioactive compounds and antioxidant abilities, we designed circle and Pearson's correlation heat maps. Based on the circle heat map, the extracts/complex were divided into different groups. Polar extracts, namely ethanol, 70% ethanol and water, were divided into the same group. Others (ethyl acetate, DCM, and n-hexane) were placed in a different group. In addition, the β-CD complexes were very close to each other (Figure 5A). Based on Pearson's correlation values, the total phenolic content was highly correlated with radical scavenging, reducing and metal chelating abilities ($R > 0.8$).

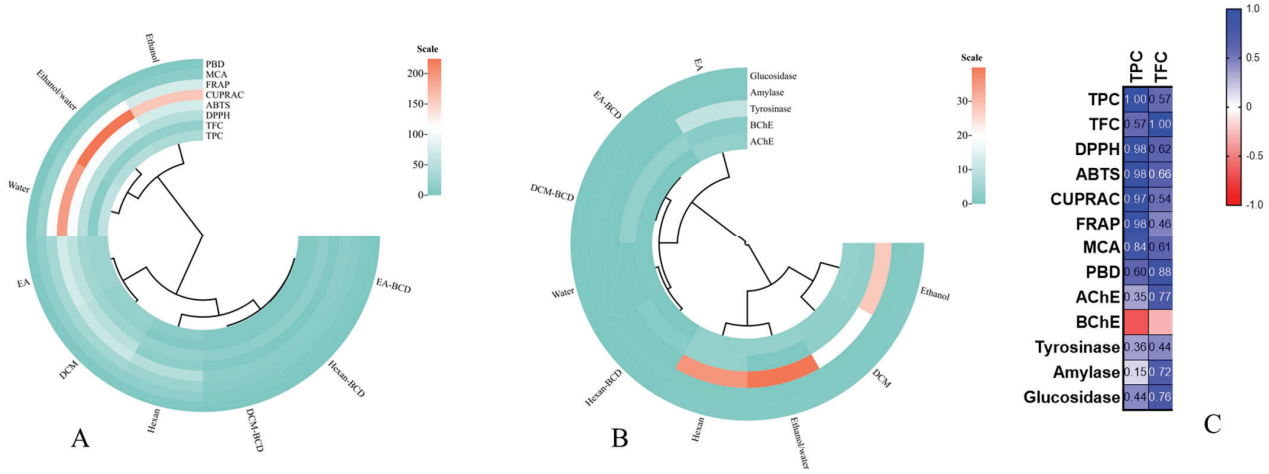


**Figure 5.** Circle heat maps for the results from antioxidant (**A**) and enzyme inhibitory assays (**B**) and Pearson correlation values between total bioactive compounds and biological activities (**C**) (TPC: Total phenolic content; TFC: Total flavonoid content; ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; DPPH: 1,1-diphenyl-2-picrylhydrazyl; CUPRAC: Cupric reducing antioxidant capacity; FRAP: Ferric reducing antioxidant power; AChE: acetylcholinesterase; BChE: butyryl-cholinesterase; MCA: Metal chelating assay; PBD: Phosphomolybdenum).

The observed antioxidant ability of the extracts can be attributed to the presence of certain compounds. Chlorogenic and other caffeoylquinic acids, which are common in the Asteraceae family, are main players in the antioxidant mechanisms of chlorogenic acid due to their hydroxyl groups [49]. Rutin, which was detected in all extracts, also plays an essential role in the antioxidant properties of the extracts due to its 3′ OH group [50]. In addition, 4-hydroxybenzoic and 3-hydroxybenzoic acids, which were detected in all samples, are significant antioxidants with hydroxyl groups [51]. Sesquiterpenes are also important groups in the chemical composition of the tested samples and can contribute to the observed antioxidant properties. For example, partenolide has been reported as a protective shield against oxidative stress in moderate dosages [52]. Therefore, the antioxidant ability of the extracts can be attributed to the presence of these compounds.

In summary, these results shed light on the complexity of the antioxidant activity of *I. sarana* extracts and their complex with β-CD. They underscore the importance of understanding the interactions between active compounds, solvents, and complex forms to optimize the antioxidant properties of the extracts. This study provides crucial insights to guide future research aimed at exploring underlying mechanisms and harnessing these extracts for applications beneficial to human health.

### *3.5. Enzyme Inhibition Effects*

#### 3.5.1. α-Amylase and α-Glucosidase

Diabetes, also referred to as diabetes mellitus, stands as one of the most widespread epidemic diseases globally [53]. It is characterized by the inhibition of enzyme activities responsible for the conversion of dietary starch into glucose, namely α-amylase and α-glucosidase [54]. Nature has consistently proven to be a generous reservoir of valuable compounds known for their potential health benefits. Among these, plants emerge as a substantial source of secondary metabolites, notably phenolic and flavonoid compounds, with recognized inhibitory properties against α-amylase and α-glucosidase activities [55].

The investigation into the inhibitory activity of *I. sarana* and its complex with β-CD on the enzymes amylase and glucosidase has unveiled intriguing insights into their potential as regulators of carbohydrate digestion. The results reveal significant variations in inhibition values among the different extracts and the complex. Extract obtained from ethyl acetate exhibited the highest inhibition values for amylase, averaging at 0.49 mmol

ACAE/g dry extract. This suggests that these extracts might contain compounds capable of modulating amylase activity, which is involved in breaking down starch into simple sugars. The n-hexane and DCM extracts also displayed notable inhibitory activity against amylase, with values of 0.47 and 0.44 ACAE/g dry extract, respectively. Conversely, water extract exhibited lower inhibition values, indicating less pronounced amylase inhibitory activity (Table 3).

**Table 3.** Enzyme inhibitory effects of the tested extracts *.

| Extracts | AChE (mg GALAE/g Dry Extract/Complex) | BChE (mg GALAE/g Dry Extract/Complex) | Tyrosinase (mg KAE/g Dry Extract/Complex) | Amylase (mmol ACAE/g Dry Extract/Complex) | Glucosidase (mmol ACAE/g Dry Extract/Complex) |
|---|---|---|---|---|---|
| n-Hexane | $2.99 \pm 0.06^{c}$ | $3.37 \pm 0.31^{a}$ | $35.48 \pm 2.19^{b}$ | $0.47 \pm 0.01^{a,b}$ | $0.89 \pm 0.01^{b}$ |
| Ethyl acetate | $3.17 \pm 0.08^{b,c}$ | $1.43 \pm 0.22^{c}$ | $10.32 \pm 1.38^{e}$ | $0.49 \pm 0.02^{a}$ | $0.80 \pm 0.01^{c}$ |
| DCM | $2.69 \pm 0.06^{d}$ | $2.37 \pm 0.38^{b}$ | $19.57 \pm 1.08^{d}$ | $0.45 \pm 0.01^{b,c}$ | $0.90 \pm 0.03^{a,b}$ |
| Ethanol | $3.42 \pm 0.12^{a}$ | $2.82 \pm 0.18^{a,b}$ | $27.87 \pm 0.57^{c}$ | $0.44 \pm 0.01^{c}$ | $0.88 \pm 0.01^{b}$ |
| 70% Ethanol | $3.18 \pm 0.05^{b}$ | $0.07 \pm 0.01^{d}$ | $39.88 \pm 0.46^{a}$ | $0.33 \pm 0.01^{d}$ | $0.93 \pm 0.01^{a}$ |
| Water | $0.12 \pm 0.06^{e}$ | na | na | $0.05 \pm 0.01^{f}$ | $0.21 \pm 0.01^{d}$ |
| n-Hexane/β-CD | na | $1.27 \pm 0.23^{c}$ | na | $0.10 \pm 0.01^{e}$ | na |
| Ethyl acetate/β-CD | na | $2.97 \pm 0.06^{a}$ | na | $0.07 \pm 0.01^{e,f}$ | na |
| DCM/β-CD | $0.16 \pm 0.03^{e}$ | $2.95 \pm 0.09^{a,b}$ | na | $0.09 \pm 0.01^{e}$ | na |

* Values are reported as mean ± SD of three parallel measurements. GALAE: Galantamine equivalent; KAE: Kojic acid equivalent; ACAE: Acarbose equivalent; na: not active. Different letters ("a" indicates the strongest ability) in the same column indicate significant differences in the tested extracts and complexes ($p < 0.05$).

Regarding glucosidase, 70% ethanol extract showcased the highest inhibitory activity, with average values of 0.93 mmol ACAE/g dry extract. This might suggest that this extract contain compounds capable of modulating the digestion and absorption of simple carbohydrates. DCM extract also exhibited high inhibition values for glucosidase, with values of 0.90 mmol ACAE/g. Conversely, the water extract displayed weaker inhibitory activity, indicating a limited impact on glucosidase.

The inhibitory activity observed in the extracts of the complex with β-CD might suggest a potential modulation of digestive enzymes through complex formation, although further in-depth research is needed to understand the underlying mechanisms.

The results from the evaluation of the inhibitory activity of *I. sarana* and its complex with β-CD on the enzymes amylase and glucosidase provide significant insights into their potential in regulating carbohydrate digestion, with implications for glycemic management and metabolic health.

The observed variations in inhibition values among the different extracts and the complex underscore the importance of selecting the appropriate extraction solvent for the final composition of the extracts and their inhibitory activity [36,56]. Seventy percent ethanol extract exhibited the highest inhibition values for both amylase and glucosidase, suggesting that these extracts might contain specific bioactive compounds capable of modulating the activity of these digestive enzymes [57–59]. The ethanol extract also displayed significant inhibitory activity, confirming the positive impact of ethanol as an extraction solvent.

The observation of lower inhibitory activities in water extract suggests that the bioactive compounds responsible for the inhibitory activity might be more soluble in organic solvents. This raises questions about the specific compounds present in the organic extracts that are responsible for this inhibitory activity.

The formation of the complex with β-CD exhibited some inhibitory activity, although the results are less conclusive due to the presence of "na" values for certain extracts. However, this could suggest that complex formation may potentially influence the interactions between the extracted compounds and the target enzymes, thereby modulating their activity. This phenomenon can be elucidated by considering the involvement of hydroxyl groups in phenolic and flavonoid compounds in the formation of β-CD complexes. The depletion of free hydroxyl groups in these compounds may lead to a reduction in enzyme inhibition.

3.5.2. Cholinesterase Inhibitory Activity

Alzheimer's Disease (AD) is a globally recognized neurodegenerative condition that has sparked widespread concern, currently affecting over 35 million individuals across the world [60]. Within the realm of potential therapeutics for this condition, the inhibition of cholinesterase enzymes emerges as a pivotal target. Specifically, the AChE and BChE enzymes play essential roles in terminating cholinergic neurotransmission by facilitating the hydrolysis of acetylcholine. As disease progresses, acetylcholine levels decrease, and using AChE and BChE inhibitors has been shown to effectively relieve AD symptoms [61]. Notably, BChE enzyme levels exhibit an upward trend as the disease advances and in correlation with an individual's age. Consequently, the exploration of selective AChE and BChE inhibitors holds great promise and has garnered significant attention from researchers. Additionally, it's noteworthy that plant extract inhibitors are known to possess potent and undesirable side effects [62,63], highlighting one of the primary objectives of this investigation.

The evaluation of the inhibitory activity of *I. sarana* and its complex with β-CD on the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) has provided valuable insights into their potential as neuroprotective agents. The results reveal significant variations in inhibition values among the different extracts and the complex. Extracts obtained from ethanol, 70% ethanol, and ethyl acetate exhibited the highest values for AChE inhibition, with values of 3.42, 3.18, and 3.1 mg GALAE/g dry extract, respectively. For BChE inhibition, the ethanol-based extracts also displayed higher values, with 2.82 and 1.43 mg GALAE/g dry extract for the ethanol and ethyl acetate extracts, respectively. Conversely, the water extract showed low AChE inhibition activity (0.12 mg GALAE/g dry extract), while the complex with β-CD exhibited significant BChE inhibition activity (2.95 mg GALAE/g dry complex) (Table 3).

The observation of varying inhibition values among the different extracts underscores the significance of solvent choices in the final composition of the extracts. Extracts obtained from ethanol, 70% ethanol, and ethyl acetate demonstrated significant AChE inhibition activity, indicating the presence of compounds capable of modulating this enzyme closely linked to cholinergic neurotransmission [64–66]. These extracts exhibited higher inhibitory activity against AChE, potentially attributed to the presence of specific bioactive compounds that possess particular affinities for this enzyme.

The inhibition values for BChE also exhibited variations, with the ethanol extract showing noteworthy activity. Inhibition of BChE is associated with positive effects on cognitive function and the prevention of neurodegenerative diseases [67–69]. It is intriguing to note that the complex with β-CD displayed significant BChE inhibition activity, suggesting that complex formation might influence interactions between the extracted compounds and the target enzymes.

3.5.3. Tyrosinase Inhibitory Activity

Tyrosinase, a copper-containing enzyme, plays a pivotal role in catalysing melanin synthesis through oxidation reactions, leading to undesired darkening resulting from the enzymatic oxidation of phenols [70]. This concern arises from its significant involvement not only in mammalian melanogenesis but also in enzymatic browning in fruits and fungi [71]. Consequently, the development of tyrosinase inhibitors holds broad applications, extending to medicinal [72] and cosmetic products [73], particularly in addressing hyperpigmentation issues. In light of this, downregulating or inhibiting tyrosinase activity has become a common approach, both in dealing with pigment disorders and as a whitening agent for aesthetic purposes serving as one of the focal points of this present study.

The evaluation of the inhibitory activity of *I. sarana* and its complex with β-CD on the enzyme tyrosinase has provided intriguing insights into their potential for melanogenesis regulation. The results unveiled significant differences in inhibition values among the various extracts and the complex. Extracts obtained from ethanol and 70% ethanol exhibited the highest tyrosinase inhibition values, with 27.87 mg KAE/g dry extract and 39.88 mg

KAE/g dry extract, respectively (Table 3). The ethanol extract displayed remarkable inhibitory activity, suggesting the presence of compounds capable of modulating melanin production and potentially applicable in cosmetic or dermatological applications. The weakest tyrosinase inhibitory effect was found in the ethyl acetate extract with 10.32 mg KAE/g dry extract. Other extracts displayed varying inhibition values, emphasizing the impact of solvent choice on the enzyme tyrosinase activity.

The observation of variable inhibition values among the different extracts and the complex underscores the impact of solvent choice on the chemical composition of the extracts and, consequently, their capacity to modulate tyrosinase enzyme activity. The highest inhibitory activity was observed in the ethanol and 70% ethanol extracts, indicating that these solvents favored the extraction of potentially bioactive compounds capable of regulating melanin production [74–76]. This observation aligns with ethanol's known properties to solubilize phenolic and flavonoid compounds, which are often associated with tyrosinase inhibitory activities.

The absence of inhibitory activity in the water extract raises questions about the active compounds responsible for this activity. This could be attributed to differences in solubility of bioactive compounds in water compared to organic solvents [77]. However, it is possible that the active compounds might be present in too low quantities in the water extract to generate observable inhibitory activity. The influence of the complex with β-CD on tyrosinase inhibitory activity was not extensively explored in this study, but it could be interesting for future research to investigate how complex formation might influence the regulation of melanin production.

Similar to antioxidant assays, we designed a circle heat map based on the enzyme inhibition results and the results are given in Figure 5B. In contrast to antioxidant ability, the extracts/complex based on the enzyme inhibitory were differently classified. This fact can be explained by different effects (synergetic or antagonistic) of chemicals on the enzymes and complex structures of active sites of each enzymes. As further insights, we examined the relationship between total bioactive compounds and enzyme inhibitory properties. In Figure 5C, we did not observe any correlation between total phenolic content and enzyme inhibitory properties ($R < 0.5$). However, AChE, amylase and glucosidase were moderately correlated with the total flavonoid content ($R > 0.7$).

When considering the collective findings, the observed inhibitory effect on enzymes in the tested samples can be attributed to the presence of specific compounds. For instance, chlorogenic acid has been recognized as a crucial inhibitor of both AChE and BChE, and its OH group, substituting quinic acid, can interact with the active sites of these enzymes, as detailed by Oboh et al. [78]. Likewise, the OH group participates in the formation of hydrogen bonds within the active site of amylase, as suggested by Zheng et al. [79]. Furthermore, compounds such as rutin [80], hyperoside [81], and hydroxybenzoic acid [82] are also known as significant inhibitors. Notably, the glycosylation and the number of hydroxyl groups within the flavonoid rings of their structure can impact their enzyme inhibitory effects, as discussed by Li et al. [83]. Additionally, partenolide has demonstrated the ability to enhance acetylcholine levels by inhibiting AChE activity in diabetic rats, as reported by Khare et al. [84].

## 4. Conclusions

The study findings showed that water and 70% ethanol extracts had the highest phenolic content, while ethyl acetate extracts contained notable flavonoids. This observation was further substantiated through the utilization of ESI MS/MS analysis. Moreover, the extracts of ethyl acetate, n-hexane, and DCM were effectively enclosed within an inclusion complex of β-CD, a fact that was verified by means of FTIR-ATR and SEM examinations.

It is noteworthy that the water-based extract and 70% ethanol extracts exhibited the highest level of antioxidant activity among all tested extract. This indicates a significant potential for these extracts to serve as potent sources of antioxidants, thereby holding promise for various applications in the realm of health and wellness.

Similarly, ethanol and n-hexane extracts displayed higher inhibition of the AChE and BChE, respectively. Moreover, the 70% ethanol extract exhibited elevated inhibition of the tyrosinase and α-glucosidase enzymes, and ethyl acetate demonstrated pronounced inhibition of the amylase enzyme. Notably, the inclusion complex displayed relatively less or no antioxidant efficacy and enzyme inhibitory potential compared to the unencapsulated extracts. This variation in inhibition values observed across the distinct extracts and the inclusion complex underscores the significant influence of solvent selection on the chemical composition of the extracts. Consequently, this choice of solvent directly affects their potential to effectively modulate enzyme activity. These findings emphasize the critical significance of comprehending the interplay between active compounds, solvents, and complex formations, as a means to enhance and optimize the antioxidant properties inherent in these extracts. The outcomes of this study offer invaluable insights for future investigations aimed at comprehending the fundamental mechanisms at play, thereby unlocking the potential of these extracts for applications that can distinctly contribute to enhancing human health and well-being.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox12101842/s1, Table S1: Specialized metabolites in *Inula sarana* extracts assayed by UHPLC-HRMS; Table S2: Specialized metabolites in *Inula sarana* extracts assayed by UHPLC-HRMS in positive ion mode; Table S3: The equations, range and R2 values of standard compounds in the biological activity assays; Figure S1: Extracted ion chromatograms of hydroxybenzoic and hydroxycinnamic acids and their derivatives; Figure S2: Extracted ion chromatograms of acylquinic acids; Figure S3: Extracted ion chromatograms of caffeoylhexaric acids; Figure S4. Extracted ion chromatograms of flavonoid glycosides; Figure S5: Extracted ion chromatograms of flavonoid aglycons. References [22,85] cited in the supplementary materials.

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