

Article

Sulforaphane, L-Menthol, and Dexpanthenol as a Novel Active Cosmetic Ingredient Composition for Relieving Hair Loss Symptoms

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Abstract: Sulforaphane increases the expression of the dihydrotestosterone (DHT)-degrading enzyme, 3 α -hydroxysteroid dehydrogenase (3 α -HSD) in the liver, which accelerates DHT degradation, thereby inhibiting hair loss in the animal model. In this study, we elucidated its underlying mechanism and demonstrated that sulforaphane has hair loss inhibitory functions in RAW264.7 macrophage cells and Hepa1c1c7 cells at the cellular and gene levels. The gene expression level of an isoform of 3 α -HSD, Akr1c2, increased in a dose-dependent manner when these cells were treated with sulforaphane, but there were no significant differences at the gene levels of Akr1c2 and Dhrr9 for the negative control mixture of biotin, dexpanthenol, and L-menthol. These studies indicated that sulforaphane is involved in regulating the gene expression of Akr1c2. To further determine whether this hair product has effects on alleviating hair loss symptoms, clinical trials were also conducted for 18 weeks. We performed a visual evaluation of the parietal and frontal lines of 23 patients before and after using the product, and then calculated the total number of hairs. This clinical study showed that the parietal lines and bangs visually improved and the number of hairs increased by 6.71% from before using the test product to 18 weeks after using the test product. Taken together, these cellular and clinical studies strongly suggest that sulforaphane may be an active ingredient that significantly alleviates hair loss symptoms.

Keywords: sulforaphane; hair loss; cosmetic ingredient; functional cosmetics



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1. Introduction

Androgen alopecia (AGA) is a common form of hair loss in men and women of different ages [1,2]. AGA is associated with high levels of dihydrotestosterone (DHT) released from testosterone by the enzymatic activity of 5 α -reductase (5 α -R). DHT binds to the androgenic receptor (AR) to form a hormone-receptor complex. The expressions of 5 α -R and AR are enhanced in patients with AGA [3]. In addition to its action on follicular androgen receptors, DHT stimulates growth factor TGF- β in the dermal papillary cells of cellular apoptosis, which cause hair follicles to die [4].

DHT is mainly degraded in the liver by hydroxysteroid dehydrogenases (HSDs), such as 3 α -hydroxysteroid dehydrogenase (3 α -HSD), 3 β -HSD, and 17 β -HSD [5–7]. Aldoketo reductases (ARKs), as one of two protein superfamilies within the 3 α -HSD, can bind to DHT [8] and degrade DHT by acting as a 3-ketosteroid reductase in the presence of dihydronicotinamide adenine dinucleotide phosphate (NADPH) [9]. Currently, 5 α -reductase inhibitors that suppress the conversion of testosterone into DHT are widely used to treat AGA [5,6]. However, the effect of the DHT-degrading enzymes on hair growth is less clear and has not been clinically tested.

Many active ingredients of synthetic origin are available for the treatment of baldness [7,10]. Only two drugs, finasteride and minoxidil, have been approved by the U.S.

Food and Drug Administration (FDA) for the treatment of AGA. Finasteride is primarily used as an oral drug for the treatment of androgen-related hair disorders due to its inhibitory activity on 5α -R [11,12], as shown in Figure 1.

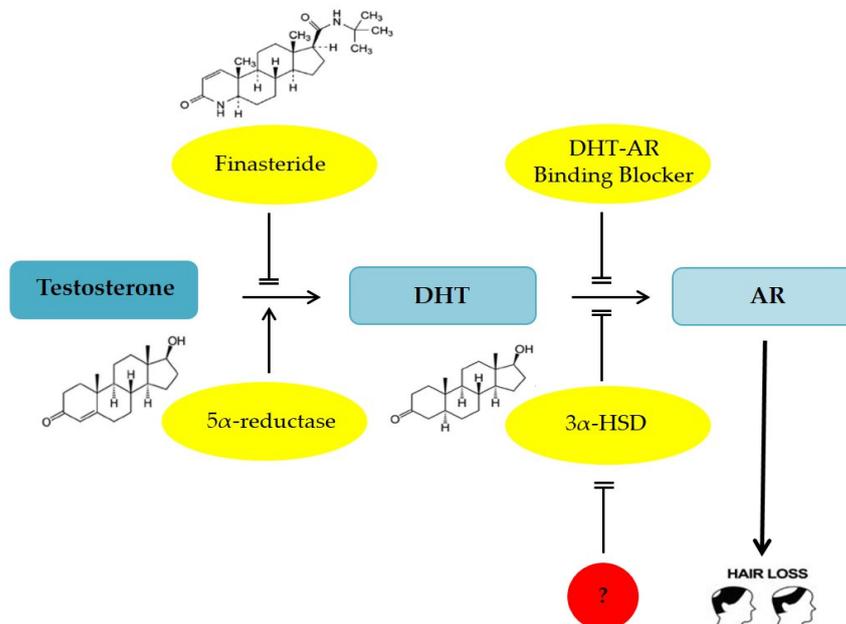


Figure 1. Schematic representation on the effect of the 5α -reductase, dihydrotestosterone (DHT), 3α -hydroxysteroid dehydrogenase (3α -HSD), finasteride, and DHT-AR (androgen receptor) blockers actions for hair loss.

Little is known about the mechanisms of other synthetic substances that are used to decrease hair loss. In addition to its frequent side effects, the length of treatment is often uncertain. Some synthetic substances should be taken for at least 6 months to decrease hair loss and 12 months to restore hair growth [7,10]. For these reasons, there is a need for alternative treatments, including cosmetic treatments, supplements, and the use of herbal extracts [8,13,14]. Recently, attention has been given toward the development of new alternative treatments that promote the relief of hair loss symptoms, which has focused on the discovery of new and safer remedies, which were often provided by natural functional products.

For new alternative pharmacological treatments, plant preparations have many advantages over synthetic substances, considering their higher compliance, fewer side effects, wider activity spectrum, and lower cost of treatment. Due to the diverse composition of plant extracts, plants often possess more than one biochemical action that is effective for treating hair loss [15]. Many researchers have searched continually for new medicinal plants with active ingredients that could present a viable natural alternative to already established hair loss treatments using synthetic substances.

Sulforaphane, which is an isothiocyanate isolated from broccoli, is a potential modulator of DHT degradation and possesses anti-oxidative effects through the activation of the nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) pathway [9,16]. It has been reported that sulforaphane induces DHT-degradation enzymes, such as aldo-keto reductase 1c1 (AKR1C1) [17] and aldo-keto reductase 1c2 (AKR1C2) [18] in human cultured cells. It was previously reported that sulforaphane increased the mRNA and protein levels of two forms of 3α -hydroxysteroid dehydrogenase (3α -HSD), AKR1C1 and dehydrogenase/reductase (SDR family) member 9 (DHRS 9) in the liver of the mice and in cultured murine hepatocyte Hepa1c1c7 cells. [19,20].

This study aims to provide insight into the possibility of using sulforaphane as a potent and functional hair cosmetic ingredient as an alternative pharmacological treatment.

The potential effect of sulforaphane is its ability to promote the relief of hair loss symptoms by the expression of two isozymes of 3 α -HSD, AKR1C2 and DHRS9, which are the murine homologous of human 3 α -HSD isozymes that are encoded by *Akr1c2* [21] and *Dhrs9* [22]. To the best of our knowledge, sulforaphane has not been reported as a functional cosmetic material that induces DHT-degrading enzymes to promote the relief of hair loss symptoms in humans.

To further understand the underlying mechanism, our laboratory recently tested whether sulforaphane could enhance the mRNA and protein levels of *Akr1c2* and *Dhrs9* in murine Hepa1c1c7 cells. In these experiments, we employed RAW 264.7 macrophage cells and Hepa1c7c7c cells for the cytotoxicity and genetic identification of *Akr1c2* and *Dhrs9* at the RNA and protein levels, respectively. As negative controls, four common ingredients were also employed, including dexpanthenol, biotin, l-menthol, and zinc pyrithione, which are known as functional cosmetic materials that help relieve hair loss symptoms, according to the Ministry of Food and Drug Safety Food and Drug Administration (MFDS) in Korea. Subsequently, based on a clinical application test for hair loss, we evaluated whether potential functional cosmetics containing sulforaphane, l-menthol, and dexpanthenol as active ingredients promoted the relief of hair loss symptoms.

2. Materials and Methods

2.1. Chemicals and Reagents

Sulforaphane, biotin, dexpanthenol, L-menthol, zinc pyrithione, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Murine RAW264.7 macrophage cells and murine Hepa1c1c7 cells were purchased from the Korea Cell Line Bank (Seoul, Korea). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the cell proliferation assay kit (Quanti-Max™) were purchased from Biomax (Seoul, Korea).

2.2. Cell Culture

RAW264.7 macrophage cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, Grand Island, USA) and 1% penicillin/streptomycin (P/S) (100 U/mL, 100 mg/mL, respectively). In the logarithmic growth phase, RAW264.7 macrophage cells were maintained in DMEM at 37 °C. The Hepa1c7c7 cells were also cultivated in DMEM supplemented with 10% FBS and 1% P/S (100 U/mL and 100 mg/mL, respectively). The cells were maintained in a minimum essential medium eagle-alpha modifier (α -MEM) under incubation at 37 °C and 5% CO₂.

2.3. Cell Viability

The MTT assay was used to determine the cytotoxicity of biotin, dexpanthenol, L-menthol, zinc pyrithione, and sulforaphane, which are substances that relieve hair loss, and to test the cytotoxicity of sulforaphane, which is expected to be effective in relieving hair loss symptoms. RAW264.7 macrophage cells and Hepa1c1c7 cells were seeded into 96-well plates at a concentration of 5×10^4 cells per well and were treated with different concentrations (0 μ g/mL to 50 μ g/mL) of biotin, dexpanthenol, l-menthol, and zinc pyrithione for 24 h. Subsequently, an MTT solution of 5 mg/mL (Quanti-Max™, Biomax, Korea) was added to each well and then maintained in an incubator under the conditions of 37 °C and 5% CO₂ for 4 h. Finally, a UV MAX kinetic microplate reader (Synergy™ microplate reader, BioTek, Winwooski, USA) was used to measure the absorbance at 450 nm. The experiment was independently repeated three times under the same experimental conditions.

2.4. Isolation of Total RNA and Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated with an RNeasy Mini Kit (RNeasy Mini kit, Qiagen, Germantown, MD, USA). RNA was reverse-transcribed with an oligo (dT) primer using the SuperScript First-strand Synthesis System (Intron Power cDNA Synthesis Kit, Intron, Ko-

rea). RT-PCR was performed using recombinant Taq DNA polymerase according to the manufacturer's instructions (2xPCR master mix solution, Intron, Korea). The GAPDH mRNA level was used as a control. The following primers were used: Dhhrs9: Forward 5'-GGAAACTTAGCAGCCAGAAC-3' and Reverse 5'-AACACGCCAAGAACCACCAG-3'; and Akr1c2: Forward 5'-AATGGCCCTGAAACCAGGAG-3' and Reverse 5'-GACCACAATCCCACGCTGTA-3'; GAPDH: Forward 5'-ACCACAGTCCATGCCATCAC-3' and Reverse 5'-CACCACCCTGTTGCTGTAGCC-3'. The conditions for PCR were 30 cycles of 94 °C denaturation for 30 s, 60 °C annealing for 30 s, and 72 °C extension for 30 s. The PCR products were analyzed by electrophoresis on 2% agarose gels stained with RedSafe staining solution (Intron, Korea).

2.5. Western Blotting

Cell extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE), transferred to a polyvinylidene fluoride (PVDF) membrane and immunoblotted with anti-rabbit DHRS9 polyclonal (MBS768606, MyBioSource, San Diego, CA, USA), anti-rabbit polyclonal AKR1C2 (PA5-36572, Invitrogen, Waltham, MA, USA), or anti- β -Actin rabbit polyclonal antibodies (Ab8227, Abcam, Cambridge, MA, USA), followed by incubation with secondary anti-goat antibody (Ab6721, Abcam, Cambridge, MA, USA). Reactive bands were detected by chemiluminescence using a western lightning reagent (Clarity™ Western ECL Substrate, Bio-Rad, Hercules, CA, USA).

2.6. Prototype Preparation of a Functional Cosmetic Formulation to Relieve Hair Loss

A prototype formulation was prepared for the clinical application test to promote the relief of hair loss symptoms. Besides the active ingredients, including dexpanthenol, biotin, l-menthol, and sulfuraphane, which are functional cosmetic materials that relieve hair loss, according to the MFDS in Korea. Most the other ingredients listed in the formula ingredients statement were purchased from Sigma-Aldrich (St. Louis, MO, USA). A prototype formulation was manufactured at a small scale at a pilot plant in Interkos, Inc. (Inchon, Korea).

2.7. Clinical Application Test for Hair Loss

2.7.1. Purpose of the Trial

This test will be used to evaluate the capability of the prototype formulation to relieve hair loss symptoms after 18 weeks of use. The trial target formulation was tentatively designated as The Fact/BHCG (Biodegradable Hair Control Gel).

2.7.2. Description for the Trial Target

Men and women from 18 to 54 years old were diagnosed by a basic and specific (BASP) classification. The basic type was grouped from male and female with an androgenic alopecia that was classified autonomously as M1 or higher, C1 or higher, or U1 or higher. The specific type was also grouped from male and female, with androgenic alopecia classified autonomously as V1 or higher, or F1 or higher. Men and women were diagnosed as 2 or 2A or higher by Norwood-Hamilton classification, and as more than 1 by Ludwig classification.

2.7.3. Evaluation Methods

All measurements and evaluations were carried out in a space without air movement or direct sunlight. Based on the skin stability of the subjects under constant temperature and humidity conditions (22 ± 2 °C and $50 \pm 5\%$), the evaluation was performed before and after use of the test product implemented in the trial.

2.7.4. Visual Evaluation for the Crown of the Head and Forehead Line

In this test, a high-resolution digital camera (DSLR, Canon Inc., Tokyo, Japan) was used to take pictures of the crown of the head at a 90-degree angle and the forehead line at

a 45-degree angle under the same conditions. After 6, 12, and 18 weeks of treatments, the test product was implemented for the trial, and the structure and location with relief of hair loss were observed from the crown of the head and forehead line [23].

2.7.5. Quantitative Evaluation Using Phototricogram

In this test, a folliscope (LeadM, Seoul, Korea) was used to evaluate hair loss and growth rates by magnifying areas of scalp or hair before and after 6, 12, and 18 weeks treatments of the test product that was implemented for the trial. After cutting the hair on areas with hair loss, a small dot tattoo with a diameter of 1 mm was applied to evaluate the center of the tattoo each time using a phototricogram. It was evaluated statistically whether the test product group showed a trend of an increase in hair growth after using the product from 6 weeks to 18 weeks.

2.7.6. Statistical Analysis

To verify the statistical significance before and after the test product was used, a statistical analysis was performed using Embedded on SPSS Statistics 26 (IBM, Armonk, NY, USA), and the significance was confirmed when the significance probability was $p < 0.05$ in the 95% confidence interval. The resulting value, which was calculated through device evaluation, was expressed by obtaining the mean and standard deviation as a continuous variable, and the resulting value of the questionnaire evaluation was expressed as a frequency and percentage as categorical variables.

The normality of the data was verified using the SPSS Shapiro–Wilk test. In the case of repeating measured data with a measurement point of three or more times, if normality was satisfied, the test was performed using repeated measures analysis of variance, which is a parametric method, followed by Bonferroni correction, and the normality was not satisfied [24]. If not, it was tested with Friedman test, which is a nonparametric method, followed by Bonferroni correction, and post-tested with the Wilcoxon signed rank test [25].

3. Results

3.1. Cytotoxicity of Raw264.7 Cells with Biotin, Dexpanthenol, L-Menthol, and Zinc Pyrithione Using Raw264.7 Cells

Previous results on the cytotoxicity of sulfuraphane treatment showed cell viability of 100%, 100%, and 99% at concentrations of 1, 5, and 10 μM , respectively [26]. To determine whether biotin, dexpanthenol, L-menthol, and zinc pyrithione have effects on cell viability, Raw264.7 cells were treated with biotin, dexpanthenol, and L-menthol at various concentrations (1, 2.5, 5, 10, and 20 μM) for 24 h. Biotin, dexpanthenol, L-menthol, and zinc pyrithione showed no effects on macrophage cells with viability of 92–132%. However, zinc pyrithione showed 72–94% of cell viability. In conclusion, there was no significant difference in the cell viability between the control group and the experimental groups under all conditions with biotin, dexpanthenol, and L-menthol. Each material was dissolved in DMSO solvent. The experimental group treated with the same volume of DMSO was used as 100% control (Figure 2). That is why the control appears all the same at 100%. Raw264.7 cells were used to determine whether the 1~25 μM concentration affects cell viability. In the same experimental setting, 1~25 μM Biotin, Dexpanthenol, and L-menthol did not affect cell viability, but 2.5 μM Zinc-pyrithione affected the cell viability (Figure 2). Therefore, these results suggested that biotin, dexpanthenol, and L-menthol have no cytotoxicity under these experimental conditions (Figure 2).

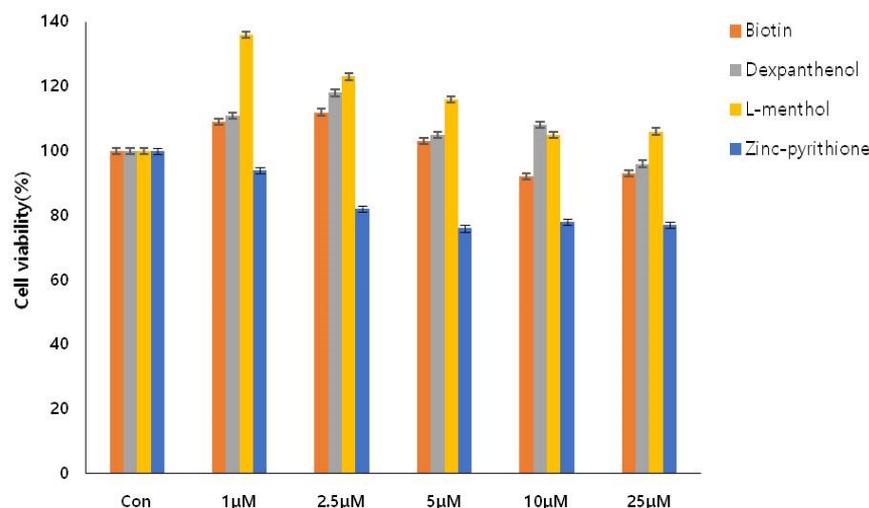


Figure 2. Effects of biotin, dexpanthenol, l-menthol, and zinc pyrithione on cell viability using Raw264.7 cells. The cells were treated with various concentrations (1–25 μ M) of biotin, dexpanthenol, l-menthol, and zinc pyrithione for 24 h, respectively. Cell survival was measured by MTT assay.

3.2. Cytotoxicity of Hepa1c1c7 Cells with Sulforaphane, Biotin, Dexpanthenol, L-Menthol, and Zinc Pyrithione

To determine whether sulforaphane, biotin, dexpanthenol, L-menthol, and zinc pyrithione may affect cell viability, Hepa1c1c7 cells were treated with sulforaphane, biotin, dexpanthenol, and L-menthol at various concentrations (1, 2.5, 5, 10, and 20 μ M) for 24 h. Sulforaphane, biotin, dexpanthenol, L-menthol, and zinc pyrithione showed no effects on Hepa1c1c7 cell viability with 93–110%. However, zinc pyrithione showed 70–94% cell viability. We showed that treatment of the product from low concentration (1 μ M) to high concentration (25 μ M) did not show any significant effects on cell viability (Figure 3). In this experimental setting, the standard was treated with the same amount of DMSO dissolved in the product. This suggested that this product does not affect cell viability at various concentrations (1–25 μ M). In conclusion, there was no significant difference in the cell viability between the control group and the experimental groups under all conditions with biotin, dexpanthenol, and L-menthol. Therefore, these results suggested that biotin, dexpanthenol, and L-menthol have no cytotoxicity in these experimental conditions. However, the survival rate of cells treated with zinc pyrithione was significantly low, even at a low concentration of zinc pyrithione; therefore, it was confirmed that zinc pyrithione is not suitable for use in the experiment, as it was confirmed that it is toxic to cells (Figure 3).

3.3. Identification of Ak1c21 and Dhhrs9 at the RNA and Protein Levels Using Hepa1c1c7 Cells with Sulforaphane and a Mixture of Biotin, Dexpanthenol, and L-Menthol

Previous studies have shown that several genes are involved in the hair loss process [26]. To understand the molecular mechanism of the hair loss process regulated by sulforaphane, dexpanthenol, and l-methol, we determined whether the cellular expression levels of these genes that are involved in hair loss process could be regulated by sulforaphane, biotin, dexpanthenol, and L-memthol. First, Hepa1c1c7 cells were treated with 2.5, 5, 10, and 20 μ M sulforaphane to determine the genes of Ak1c21 and Dhhrs9 at the RNA and protein levels. When treated with only sulforaphane, the gene level of Ak1c21 was increased in a dose-dependent manner. On the other hand, in the case of Dhhrs9, there was no significant difference in the RNA level. Depending on the concentration, the protein level increased as the concentration of the test product increased (Figure 4a).

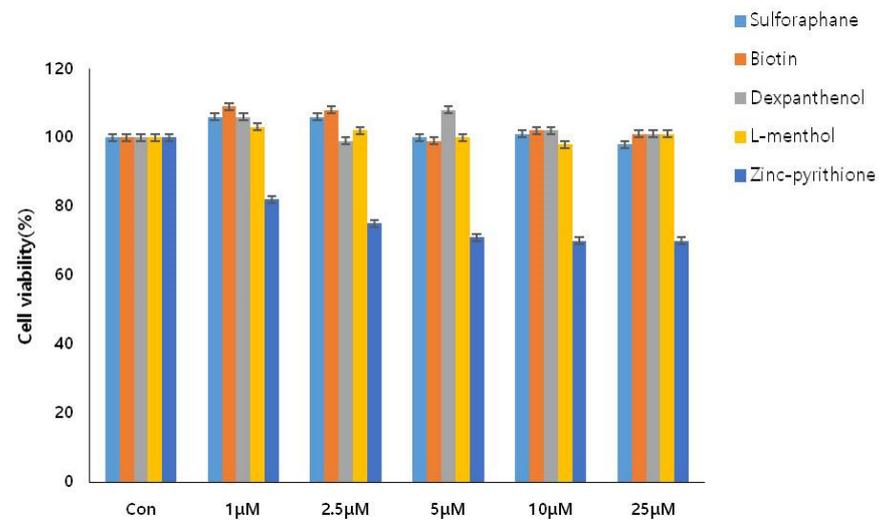


Figure 3. Effects of sulforaphane, biotin, dexpanthenol, l-menthol, and zinc pyrithione on cell viability using Hepa1c1c7 cells. The cells were treated with various concentrations (1–25 μ M) of sulforaphane, biotin, dexpanthenol, l-menthol, and zinc pyrithione for 24 h, respectively. Cell survival was measured by MTT assay.

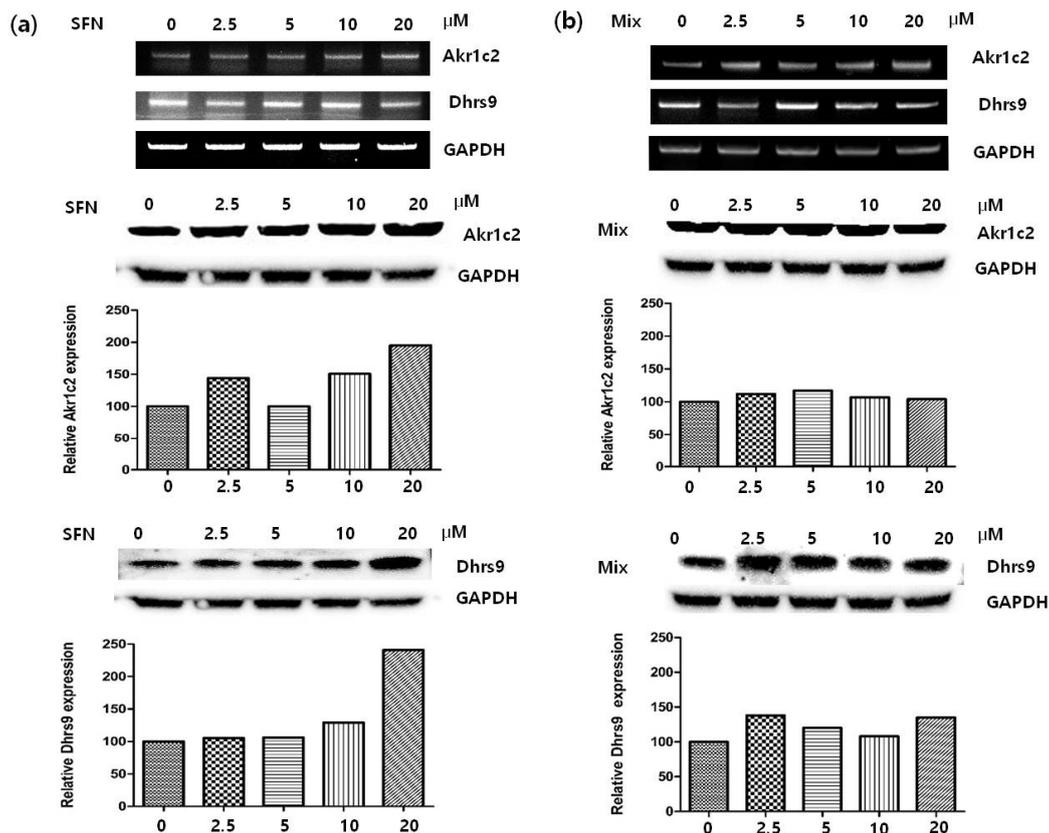


Figure 4. The RNA and protein expression levels of Akrlc2 and Dhhrs9. The levels were detected by RT-PCR and Western blot by treatment with (a) sulforaphane and (b) a mixture of biotin, dexpanthenol, and l-menthol in dose-dependent manner. Hepa1c1c7 cells were treated with various concentrations of (2.5–20 μ M) with sulforaphane and a mixture of biotin, dexpanthenol, and L-menthol.

Hepa1c1c7 cells were treated with a mixture of biotin, dexpanthenol, and l-methol, which are known as hair loss notices, at the same concentrations of 2.5, 5, 10, and 20 μ M.

It was confirmed that the levels of the Ak1c21 and Dhrr9 genes in the RNA and protein levels were not significantly different, even when the concentration was increased. These results showed that sulfuraphane might be a candidate compound that controls the hair loss mechanism and has an effect on hair loss, but a mixture of biotin, dexpanthenol, and l-menthol of other hair loss notification substances appeared to be unrelated to the hair loss mechanism. This suggests that sulfuraphane has a better effect on inhibiting the hair loss process (Figure 4b).

3.4. Preparation of a Prototype Formulation to Reduce Hair Loss

The prepared prototype formulation was proposed to include sulfuraphane, dexpanthenol, and l-menthol as the main active ingredients for testing potential effects for the relief of hair loss symptoms and other various functional ingredients that are conventionally used in the field of cosmetics production. Herein a summary stating the formula ingredients for our clinical application test for hair loss is shown in Table 1.

Table 1. Summary of formula ingredients that were proposed for the clinical application test.

Key Functional Agents	Major Ingredients
Hair loss conditioners	Sulfuraphane, Dexpanthenol, L-Menthol
Skin conditioners	Trideceth-10, Sodium Hyaluronate
Thickeners	Carbomer
Surfactants	PEG-60, Hydrogenated Castor Oil
Moisturizers	Glycerin

3.5. Clinical Application Test for Hair Loss

Before using the test product and after 6, 12, and 18 weeks of using the test product, the visual evaluation score of the parietal region increased from 0.00 to 0.87. The visual evaluation scores of the bangs line are shown as the increase from 0.00 to 0.70. When we compared the difference between before using the test product and after 18 weeks of using the test product, the visual evaluation scores of the parietal and forehead regions increased significantly (Table 2). This result suggests that this test product has specificity in acting on both parietal and forehead regions.

Table 2. Visual evaluation for the crown of the head and forehead line.

Point of View	Parietal, Evaluation Score ⁽¹⁾	<i>p</i> -Value ⁽²⁾	Bangs, Evaluation Score ⁽¹⁾	<i>p</i> -Value ⁽²⁾
Before using the test product (D0)	0.00 ± 0.00	-	0.00 ± 0.00	-
After 6 weeks of use (D42)	0.61 ± 0.50	<0.001	0.09 ± 0.29	-
After 12 weeks of use (D84)	0.74 ± 0.54	<0.001	0.30 ± 0.47	0.008
After 18 weeks of use (D236)	0.87 ± 0.69	<0.001	0.70 ± 0.56	<0.001

⁽¹⁾ N = 23 (No.01–23), mean ± standard deviation. ⁽²⁾ *p*-value: Significant Friedman test, post-hoc Wilcoxon signed-ranks test with Bonferroni correction (*p* < 0.001, comparison to initial value (D0)).

Five representative images obtained from using a high-resolution digital camera are shown in Figures S1 and S2. In these Figures S1 and S2 pictures, we can notice the difference in the trend of hair growth beyond general hair loss among untreated group (a), 6 weeks treated group (b), 12 weeks treated group (c), and 18 weeks treated group (d). This result also supports our suggestions that this test product has specificity in acting on both parietal and forehead regions.

The total number of hairs using a phototricogram increased from 28.48 to 30.39 and 28.48 to 29.52 before and after 18 weeks and 12 weeks of use of the test product, respectively (N = 23). The test product group showed a 6.71% and 3.65% increase in the total number of hairs after using the product, respectively (Table 3). This suggests that changes in the

number of hairs increased statistically in a significant manner after 18 weeks and 12 weeks of use (Table 3).

Table 3. Evaluation of the total number of hairs using a phototricogram.

Point of View	Total Hair Count (N/cm ²) ⁽¹⁾	<i>p</i> -Value ⁽²⁾
Before using the test product (D0)	28.48 ± 7.18	-
After 6 weeks of use (D42)	29.57 ± 6.95	0.008
After 12 weeks of use (D84)	29.52 ± 7.07	0.004
After 18 weeks of use (D236)	30.39 ± 6.95	0.001

⁽¹⁾ N = 23(No.01–23), Mean ± Standard deviation. ⁽²⁾ *p*-value: Significant Friedman test, post-hoc Wilcoxon signed-ranks test with Bonferroni correction ($p < 0.01$, comparison to initial value (D0)).

Herein, representative images obtained from using phototricogram are shown in Figure 5. In these Figure 5 pictures, we can notice the difference in the trend of hair growth beyond general hair loss among the untreated group (A), 6 weeks treated group (B), 12 weeks treated group (C), and 18 weeks treated group (D). When we compare the pictures of (A) and (D), there is a significant difference in the amount of hair growth (Figure 5). These results clearly suggest that this test product is may be an effective agent in enhancing the total number of hairs.

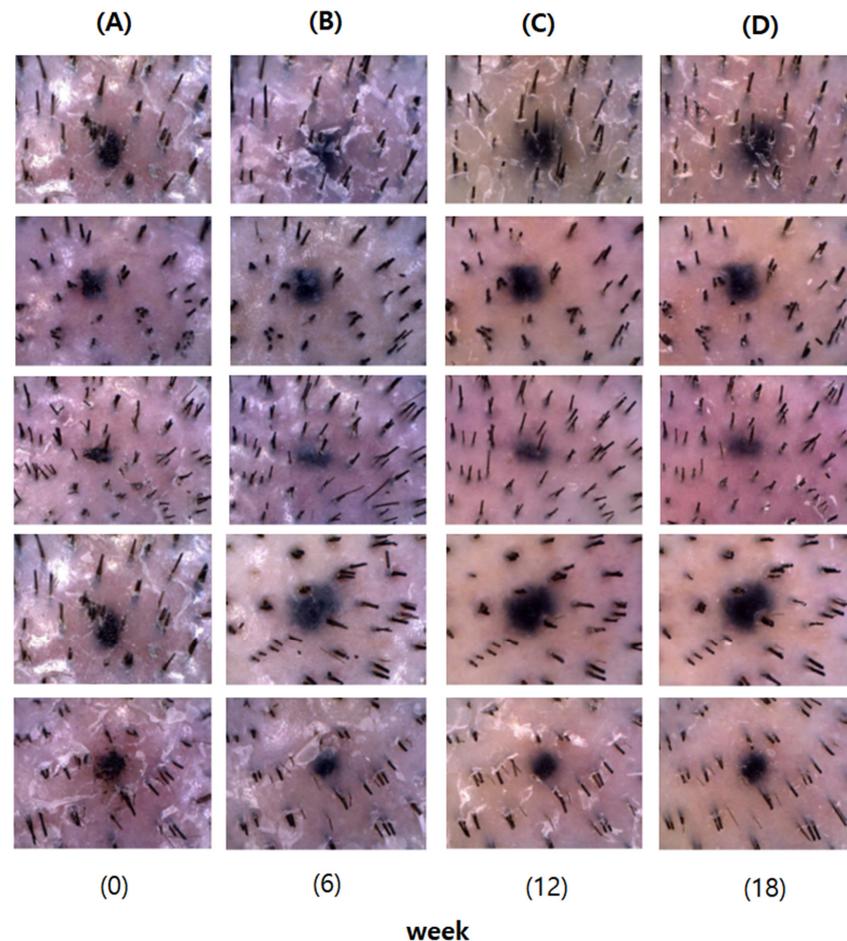


Figure 5. Five representative images obtained from using a phototricogram after 6, 12, and 18 weeks of use compared to before using the test products. Images are from (A) before using the products or after (B) 6 weeks, (C) 12 weeks, and (D) 18 weeks of using the test products (No. 9, 10, 11, 12, and 21).

4. Discussion

In this present study, we determined the hair loss-relieving efficacy of a mixture of sulforaphane, dexpanthenol, and l-menthol. To measure its efficacy, we performed a clinical application test using the prototype formulation. The results after 18 weeks of using the prototype formulation revealed a significant difference in the hair loss-relieving efficacy when compared to that before using the product. To further confirm this effect at the molecular and cellular level, we then tested the expression levels of Ak1c21 and Dh9 in the *in vitro* cell culture experiment. Hepa1c1c7 cells were treated with sulforaphane or a mixture of biotin, dexpanthenol, and l-menthol. This study showed that sulforaphane alone achieved a hair loss-relieving effect in our experimental cell culture conditions. Although the mixture of biotin, dexpanthenol, and l-menthol did not show any difference under a time- or dose-dependent manner in our *in vitro* experiments, we speculated the possibility that these mixtures might have a hair loss-relieving effect in the clinical trial test to relieve hair loss, because the mechanisms of action of these mixtures are still not fully understood yet. Actually, the MFDS, a cosmetics regulatory authority in Korea, approved dexpanthenol, biotin, l-menthol, and zinc pyrithione as functional cosmetic materials that can help to relieve hair loss symptoms. We excluded biotin and zinc pyrithione from our *in vitro* cell culture experiments since biotin and zinc pyrithione are widely used in cosmetic products and show cytotoxicity at certain temperature ranges. Though the use of biotin or zinc pyrithione as a hair growth supplement is prevalent, research demonstrating the efficacy of biotin [27] or zinc pyrithione is limited [28].

As one of the main ingredients promoting the relief of hair loss symptoms in our experiments, topical dexpanthenol has emerged as a frequently used formulation in the field of dermatology and skin care. Various studies have confirmed that dexpanthenol offers moisturizing [29] and skin barrier enhancing potential [30]. Other human studies also demonstrated an increased concentration of pantothenic acid in the hair and hair roots after topical administration [31]. Although discovered decades ago, the action mechanisms of dexpanthenol have not been fully clarified. Recently, various trends in the applications of new technologies focused on dexpanthenol's mode of action at the molecular level.

Regarding the effect of l-menthol on the relief of hair loss in previous *in vivo* experiments, the transcriptome analysis of skin from the dorsal side of a mouse treated with l-menthol compared to that of the controls revealed significant changes in keratin, keratin-associated protein, forkhead box, sonic hedgehog, fibroblast growth factor 10, desmoglein 4, deoxyribonuclease-like 2, and cadherin 3, which are known to play roles in promoting hair growth [32].

Our finding that sulforaphane induces Ak1c21 is consistent with previous studies [33]. It was also shown that sulforaphane not only induced Ak1c21 expression in cultured murine Hepa1c1c7 hepatocytes but also alleviated hair loss symptoms in a clinical application test for 23 people with AGA. Sulforaphane treatment induced the expression of another isoform of 3 α -HSD, Dh9, in an *in vitro* cell culture experiment. Dh9 has several NE-F2 binding sites in the promoter region that bind to Nrf2, which are known to be induced by sulforaphane [18,20]. Thus, it is highly likely that sulforaphane might enhance the degradation of DHT, not only via the induction of degrading enzymes 3 α -HSD, but also by functional activation of these enzymes. Further studies remain to test this possibility soon.

The synergistic effect of sulforaphane, dexpanthenol, and l-menthol in promoting the relief of hair loss also remains to be further investigated. Notably, our synergistic effects would be drawn from the potential effects of dexpanthenol and l-menthol at the molecular levels, even though the action mechanisms of the two substances have not been fully elucidated.

Our present results suggest that sulforaphane may enhance efficacy to relieve hair loss with the two substances described above, possibly synergistically. Although the mechanism of action of sulforaphane, dexpanthenol, and l-menthol is not fully understood, we believe that these potential bioenhancers may be applied to develop functional cosmetics applications for the relief of hair loss. Actually, based on the clinical application results

obtained from this test formulation, we expect that we may be able to launch new excellent functional cosmetics in the global market.

5. Conclusions

We demonstrated that sulfuraphane has the potential to become a highly effective functional hair cosmetic to relieve hair loss with AGA. We expect that sulfuraphane will emerge as an alternative pharmacological treatment for AGA. As a functional cosmetic material, sulfuraphane induces DHT-degrading enzymes to promote the relief of hair loss symptoms in humans. We could further understand that sulfuraphane enhances the mRNA and protein levels of Akr1c2 and Dhhr9 in the cell cultures of RAW 264.7 macrophages without cytotoxicity. We also achieved genetic identification of Ak1c21 and Dhhr9 at the RNA and protein levels of Hepa1c7c7c cells. In the end, we determined that potential functional cosmetics containing sulfuraphane, l-menthol, and dexpanthenol as active ingredients promote the relief of hair loss symptoms, based on a clinical application test for hair loss.

Supplementary Materials: Available online at <https://www.mdpi.com/article/10.3390/cosmetics8030063/s1> & s2, Figure S1: Five representative images for forehead line obtained from using a high-resolution digital camera after 6, 12, and 18 weeks of use compared to before using the test products, Figure S2: Five representative images for crown of the head obtained from using a high-resolution digital camera after 6, 12, and 18 weeks of use compared to before using the test products.

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Institutional Review Board Statement: The clinical application tests for the studies were led by Human Skin Clinical Trial Center located in Seoul, Korea. All tests were conducted according to the clinical trial plan, the good clinical practice (GCP) guideline, the related regulations of Ministry of Food and Drug Safety (MFDS), and the standard operating procedure (SOP) of the clinical trial center. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Human Skin Clinical Trial Center (Protocol Code: HM-P20-0159 and Date of Approval: 18 January 2021).

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

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

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