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Sargassum fusiforme Extract Induces Melanogenesis through the cAMP/PKA/CREB Signaling Pathway

Hayeon Kim, Seoungwoo Shin, Youngsu Jang, Eunae Cho, Deokhoon Park and Eunsun Jung *

BioSpectrum Life Science Institute, U-TOWER 18th FL, 767, Sinsu Ro, Suji Gu, Yongin 16827, Republic of Korea; bioyu@biospectrum.com (H.K.); biost@biospectrum.com (S.S.); biogc@biospectrum.com (Y.J.); biozr@biospectrum.com (E.C.); pdh@biospectrum.com (D.P.)

* Correspondence: bioso@biospectrum.com; Tel.: +82-31-698-3122; Fax: +82-31-698-3123

Abstract: The aim of this study was to investigate the effect of *Sargassum fusiforme* extract (SFE) on melanogenesis and its mechanism both in vitro and ex vivo. The melanogenic-inducing effect of SFE was evaluated using a melanin contents assay and a cellular tyrosinase activity assay. To investigate whether SFE could protect melanocytes against oxidative stress, hydrogen peroxidase was used. The molecular mechanism underlying the effect of SFE on melanogenesis was determined via Western blot analysis of tyrosinase, a microphthalmia-associated transcription factor (MITF), and a phosphorylated cAMP response element-binding protein (p-CREB) expression. The degree of pigmentation in a 3D skin model was determined by measuring the L* values. Contents of melanin in ex vivo human hair follicles were evaluated via Fontana–Masson staining. SFE significantly increased melanin contents and cellular tyrosinase activity in human epidermal melanocytes. SFE also increased the phosphorylation of CREB and the protein levels of tyrosinase and MITF. Moreover, SFE attenuated oxidative stress-induced cytotoxicity and depigmentation. Finally, the melanogenesis promoting effect of SFE was confirmed in both a 3D skin model and ex vivo human hair follicles. These findings suggest that SFE can induce melanogenesis via the cAMP/PKA/CREB signaling pathway in human epidermal melanocytes through its hyperpigmentation activity.

Keywords: *Sargassum fusiforme*; melanogenesis; pigmentation; hair treatment

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1. Introduction

Skin is the largest organ that can protect the body from the outer environment, keep the body hydrated, and play an essential role as an immunologic barrier [1,2]. The epidermis is the outermost layer of the skin containing melanin which determines skin color. Melanin plays a crucial role in photoprotection through absorbing UV and scavenging free radicals. Melanogenesis, a process of melanin formation in melanocytes, is a multistage process of tyrosine oxidation and polymerization melanin [3,4]. Melanogenesis involves the regulation of various signal molecules and transcription factors that influence the microphthalmia transcription factor (MITF) and its downstream proteins, including tyrosinase-related protein-1 (TRP-1), tyrosinase-related protein-2 (TRP-2), and tyrosinase [5].

Hyperpigmentation causes dark spots and freckles on some areas of the skin. On the contrary, a lack of melanin can cause skin diseases such as skin cancer and vitiligo [6]. The difference in the extent of skin photoprotection in accordance with the content and composition of melanin contributes to the incidence of melanoma. It is well reported that the prevalence of skin cancer is higher in light-skinned people than dark skin [7]. Hair greying is caused by a loss of melanin in hair follicles [8]. Therefore, it is important not only to reduce melanin pigment, but also to induce melanogenesis. Hair greying is the most representative hair aging symptom caused by a decrease in the number of follicular melanocytes or a decrease in melanin production [9]. In this study, *Sargassum fusiforme* was noted as a candidate that could increase melanin related to the cAMP/PKA/CREB signaling pathway.

The cAMP/PKA/CREB signaling pathway plays a crucial role in regulating melanogenesis, starting with activation of the G protein-coupled receptor, melanocortin 1 receptor (MC1R), via the melanocyte-stimulating hormone (MSH) [10]. This leads to activation of the G protein, which in turn activates adenylyl cyclase, an enzyme that converts ATP to cAMP. Increased cAMP levels activate protein kinase A (PKA), an enzyme that phosphorylates various target proteins, including the cAMP-response element binding protein (CREB), a transcription factor that regulates the expression of genes involved in melanogenesis [11]. Phosphorylated CREB binds to cAMP response elements (CREs) on specific DNA sequences and upregulates the expression of enzymes, such as tyrosinase, TRP-1, and TRP-2 involved in melanogenesis, which lead to an increase in melanin pigmentation and a darker skin color. This mechanism is responsible for the tanning response to UV radiation [12]. This has also been applied to hypopigmentation disorders such as vitiligo and grey hair.

Sargassum fusiforme is a species of brown seaweed that belongs to the genus *Sargassum*. It grows abundantly along the rocky coastline of Asian countries such as Korea, Japan, and China. Along with being used as a food source, seaweed is also a source of structurally diverse active compounds that have biomedical potential to develop functional substances for foods and cosmetics [13]. Among compounds isolated from *S. fusiforme*, polysaccharides have received considerable attention in the scientific community due to their potential health promotion effects [14]. Polysaccharides isolated from *S. fusiforme*, which include lamiginine, alginate, fucoidan, and dietary fiber, have been of particular interest due to their potential health promotion effects [15]. These polysaccharides have various biological activities and are the subject of much scientific research.

Although *Sargassum fusiforme* extract (SFE) is commonly used in cosmetic products due to its potential skin benefits such as moisturizing, anti-inflammatory, and antioxidant effects, its hyper-pigmenting effect has not been reported yet [16,17].

Thus, this study aimed to investigate the effect of *Sargassum fusiforme* extract (SFE) on melanin synthesis in human epidermal melanocytes. It was found that SFE could induce melanogenesis by increasing the expression of MITF gene via activation of the cAMP/PKA/CREB signaling pathway.

2. Materials and Methods

2.1. Sargassum Fusiforme Extract

The aerial part of *Sargassum fusiforme* was obtained from the Bomyeongsanghoe Co. (Jeju, Republic of Korea). They were thoroughly washed to completely remove impurities, dried at 35 °C to 40 °C, and extracted with water at 100 °C for 2 h and filtered. After cooling the filtrate, 2% of calcium chloride compared to the raw material was added, and a precipitate was formed via the reaction between calcium chloride and alginate, and the precipitate was removed via filtering. The extract obtained after removing the alginate was precipitated by adding 3 folds of ethanol. After precipitation, only the supernatant was collected by passing through a 5 μm filter and concentrated in a vacuum concentrator (Rotavapor® R-215, Bu-chi, Flawil, Switzerland). Then, it was freeze-dried (TEFIC TF-10D, Tefic Biotech CO, Xi'an, China) to prepare *Sargassum fusiforme* extract (SFE) [18].

2.2. Cell Culture

Normal Human Epidermal Melanocytes, neonatal, and moderately pigmented donors (HeMn-MP) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). These cells were cultured in a M254 medium supplemented with a human melanocyte growth solution (HMGS) at 37 °C in 5% CO_2. All experiments were performed using the cells in passages 4–9.

2.3. Chemical Reagents and Antibodies

Tyrosine, 3,4-dihydroxyphenilalanine (L-DOPA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies against MITF, TRP-1, tyrosinase, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA,

USA). Antibodies against CREB, p-CREB, H89 dihydrochloride, and the protein kinase A (PKA) inhibitor were obtained from Cell Signaling Technology (Beverly, MA, USA). Culture medium M254, the human melanocyte growth solution (HMGS), and the hydrogen peroxide solution were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.4. Cell Viability Measurement and Melanin Contents Assay

HeMn-MP were initially seeded into 6-well plates at a density of 1.5×10^5 cells/well and incubated at 37 °C with 5% CO_2 overnight. The next day, the cells were treated with a medium containing various concentrations of SFE for 5 days. Prior to cell collection, cell viability was measured using a WST-1 solution (DoGenBio, Seoul, Republic of Korea) after 4 h of incubation. Afterward, the supernatant was transferred to a new 96-well plate for absorbance measurement at 450 nm using a spectrophotometer (BioTek, Winooski, VT, USA).

The remaining cells were used for determining melanin content. The cells were washed with PBS and lysed with 1 N of NaOH for 1 h at 60 °C. Following this, the lysate was centrifuged, and the supernatant was collected to determine the amount of melanin. The absorbance of supernatant was measured at 450 nm.

2.5. Oxidative Stress Induction with Hydrogen Peroxide

HeMn-MP were seeded in the same way as described in the melanin contents assay section. After seeding, they were exposed to 10 to 150 μg/mL of SFE for 6 h before H_2O_2 treatment. After 200 μM of H_2O_2 was added to each well, the cells were incubated with H_2O_2 for 5 days. Cell viability and melanin contents were measured using the same method as described in the previous section.

2.6. Western Blot Analysis

The expression levels of melanogenesis-related proteins in HeMn-MP were detected using Western blot analysis. HeMn-MP cells at a density of 2×10^5 cells per dish were cultured in 6 cm dishes. After adding various concentrations of SFE to the culture medium, the cells were incubated for 5 days. The cells were lysed with a cell lysis buffer (CytoBuster™; Merck, Darmstadt, Germany) and the protein concentration was determined using the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of proteins (30 μg/lane) were loaded and separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by an electrotransfer to polyvinylidene difluoride (PVDF) membranes. Blots were blocked with 5% skim milk for 30 min at room temperature and probed with primary antibodies (MITF, tyrosinase, CREB, p-CREB, TRP-1, or β-actin) overnight on ice. These membranes were then incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibody at room temperature for one hour. Subsequently, these membranes were washed with Tris-buffered saline with 0.1% Tween 20 Detergent (TBS-T) for 10 min in three times, and the bands were visualized using an enhanced chemiluminescence system (PicoEPD Western reagent; LPIS-Biotech, Daejeon, Republic of Korea) with an LAS 500 image analyzer (GE Healthcare Life Sciences, Seongnam-Si, Republic of Korea). The expression of each protein was analyzed using the ImageJ program (National Institutes of Health, Bethesda, MD, USA) and visualized as a graph. Results were confirmed by three independent experiments.

2.7. Tyrosinase Zymography

HeMn-MP cells were incubated with SFE for 3 days and washed three times with Dulbecco's phosphate-buffered saline (DPBS). The cells were subsequently lysed with a cell lysis buffer (CytoBuster™; Merck, Darmstadt, Germany), and the protein content was measured using a BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of proteins (20 μg) were mixed with a 4× sample buffer and resolved via 4–12% SDS-PAGE. After electrophoresis, the tyrosinase activity of lysates was determined by an additional process. SDS gel was incubated with a rinse buffer (0.1 M of sodium phosphate

buffer, pH 6.8) for 20 min at room temperature. Then, the gel was rinsed and incubated with a rinse buffer containing 5 mM of L-DOPA, a substrate in melanin synthesis, at 37 °C in the dark for 15 min.

2.8. Measurement of Cellular Tyrosinase Activity

For cellular tyrosinase activity, HeMn-MP cells were seeded into 24-well plates at a density of 5×10^4 cells/well and stabilized in a culture medium overnight. The next day, the cells were treated with a medium containing various concentrations of SFE for 72 h. The medium was removed and replaced with 1% Triton X-100 containing 0.1 M of a phosphate buffer (pH 6.5) and 0.1 mM of phenyl methane sulfonyl fluoride (PMSF). The mixture was frozen at −80 °C, thawed at room temperature, and then centrifuged at 13,000 rpm for 30 min at 4 °C. Reaction mixtures consisting of 50 mM of the phosphate buffer (pH 6.5), 100 μg of the supernatant protein and 2 mg/mL of L-DOPA were assayed on a 96-well plate at 37 °C for 30 min and the absorbance was measured at 490 nm using a spectrophotometer (BioTek, Winooski, VT, USA).

2.9. Reconstituted Three-Dimensional Human Skin Model

To investigate the effect of SFE on melanogenesis in a 3D skin model, a commercially available reconstituted human skin model (KeraSkin-M, BioSolution, Seoul, Republic of Korea) was used. The skin model contained both human epidermal keratinocytes and melanocytes. The skin model was cultured with SFE for 13 days in a maintenance medium (BioSolution Science, Seoul, Republic of Korea). To measure the degree of melanin synthesis, L* values were measured with a CR-300 chromameter (Minolta, Tokyo, Japan). Following this, the skin model was treated with 1 N of NaOH and incubated at 70 °C for 4 h to dissolve melanin. The amount of dissolved melanin was measured using a spectrophotometer (BioTek, Winooski, VT, USA) at 450 nm. All image data were analyzed with Image-Pro Plus (Media Cybernetics, Rockville, MD, USA) and expressed with statistically significant L* values.

2.10. Ex Vivo Human Hair Follicles

Pigmented hair follicles were obtained from a female donor. On a scalp plasty for a 67-year-old woman, 56 entire hair follicles (from the bulb to the infundibulum) were isolated via microdissection. Hairs exhibiting a low pigmentation in the hair shaft and medium pigmentation in the bulb were selected for this study. Hair follicles were placed in 48-well plates and incubated at 37 °C in a 5% CO_2 incubator. They were then treated with 50 or 100 μg/mL of SFE for 9 days in William's medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with L-glutamine, insulin–transferrin–selenium G, hydrocortisone, penicillin and streptomycin antibiotics, and antifungal amphotericin B (300 μL per well).

After incubation, hair follicles were fixed in a buffered formalin solution for 30 min. After that, the samples were dehydrated, embedded in paraffin, sectioned (5 μm thick serial sections) using a microtome, and mounted on histological glass slides. Microscopical observations were performed using a Leica DMLB, an Olympus BX43, or a BX63 microscope. Images were captured with a DP72 or DP74 Olympus camera with CellˆD or CellSens Dimension (version 1.6) software (Olympus Life Science, Tokyo, Japan).

To assess the cell viability of the hair bulb, Masson's trichrome staining was performed on formalin-fixed paraffin-embedded (FFPE) skin sections and observed microscopically. Melanin was visualized after silver impregnation according to the Fontana–Masson staining method using FFPE skin sections. Staining was assessed via microscopical observation and image analysis. Melanin contents in hair follicles were quantitatively measured as the melanin surface normalized to the hair follicle area.

2.11. Statistical Analysis

All experiments were conducted independently three times, and the data are presented as mean ± standard deviations. To assess the statistical significance of the results, unpaired

two-tailed Student's *t*-test was used to compare the treated cells with the control (untreated) cells. The significance level was set at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, indicating a significant difference.

3. Results

3.1. SFE Induces Melanogenesis in Human Epidermal Melanocytes

To assess the potential cytotoxicity of SFE on human epidermal melanocytes (Figure 1A), cell viability was evaluated.

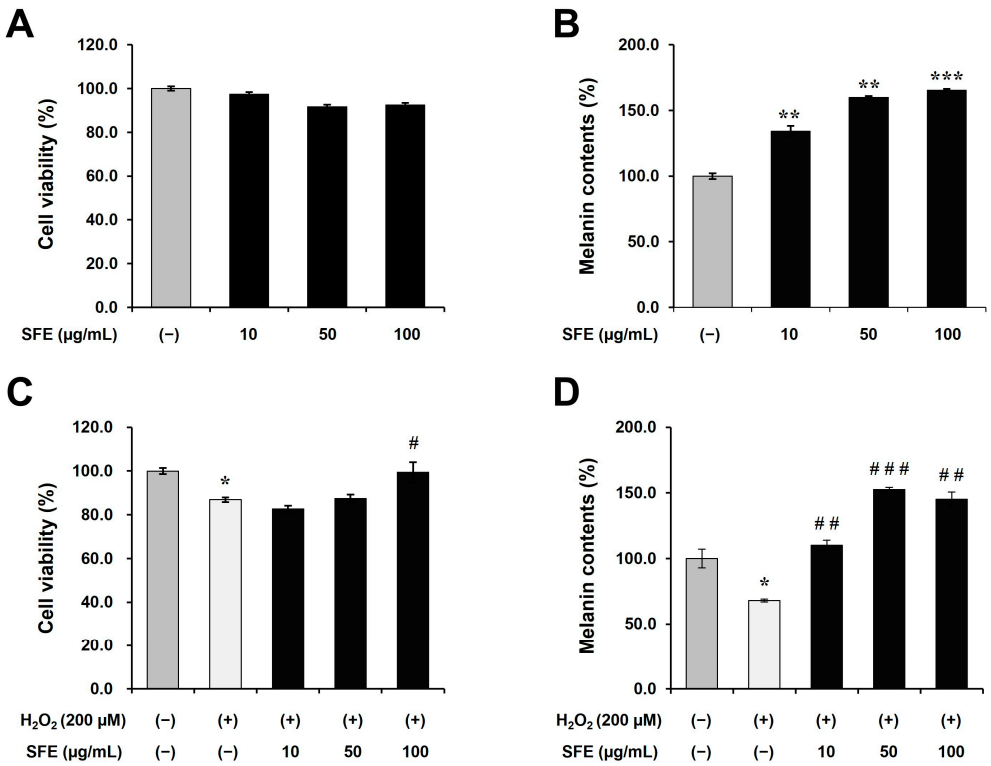

Figure 1. SFE induces melanogenesis in human epidermal melanocytes. (**A**) Cell viability and (**B**) melanin contents of Human Epidermal Melanocytes (moderately pigmented) treated with SFE. Melanocytes were cultured in the presence of SFE for 5 days and cell viability was determined via a WST-1 assay. Melanin contents were determined under the same condition as described in the main text. All results are expressed as percentages of untreated control. After cells were treated with both H_2O_2 and SFE, (**C**) cell viability and (**D**) melanin contents were measured in the same way. Values are expressed as mean ± SD (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. untreated control, # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. H_2O_2-treated control).

After treatment with 10, 50, and 100 μg/mL of SFE for 5 days, the cytotoxicity of SFE was evaluated using a WST-1 solution. Cell viabilities were 97.5%, 91.6%, and 92.5% after treatment with 10, 50, and 100 μg/mL of SFE, respectively. Thus, these concentrations of SFE were used for observing its effect on melanogenesis.

To confirm the effect of SFE on melanin synthesis, we measured melanin contents in human epidermal melanocytes using non-cytotoxic concentrations of SFE. HEMn-MP were exposed to SFE for 5 days in the same way as described above (Figure 1B). Melanin contents were increased by about 134.3%, 159.8% and 165.4% after treatment with 10, 50, and 100 μg/mL of SFE, respectively, compared to the control group. These results indicate that SFE can increase melanin production without affecting cell viability.

3.2. SFE Attenuates Both Cytotoxicity and Depigmentation in H_2O_2-Treated Human Epidermal Melanocytes

To determine the appropriate dose of hydrogen peroxide (H_2O_2), various concentrations of H_2O_2 were applied to human epidermal melanocytes. Cell viability was then evaluated via a WST-1 assay (the data for other concentrations of H_2O_2 except 200 μM were not shown because the viability at the selected H_2O_2 concentration was measured again in the pre-treatment experiment set using SFE). Based on the study results, 200 μM of H_2O_2 was chosen as a concentration with the appropriate cytotoxicity for subsequent experiments. The experiment was conducted as described below to explore the potential of SFE in mitigating H_2O_2-induced cytotoxicity and depigmentation.

After pretreating HEMn-MP cells with different concentrations of SFE for 4 h, the cells were then exposed to 200 μM of H_2O_2 for 5 days. As a result, H_2O_2 decreased the viability of HeMn-MP cells by 13.1%. However, pretreatment with SFE at 100 μg/mL attenuated the cytotoxicity of H_2O_2 and restored the cell viability up to 99.4% compared to the control group (Figure 1C). Melanogenesis of the cells after treatment with 200 μM of H_2O_2 was also significantly suppressed by about 32% compared to that of the control cells (Figure 1D). The cells treated with 200 μM of H_2O_2 showed a lower level of pigmentation than the control cells when the same cell number was used for comparison. However, SFE pretreatment greatly improved the melanin content in the same number of cells. Melanin contents were increased by about 110%, 152.5%, and 145.2% after treatment with 10, 50, and 100 μg/mL of SFE, respectively, compared to that in the control group. These results indicate that SFE can prevent oxidative stress-induced cytotoxicity and restore decreased melanin production caused by oxidative stress.

3.3. SFE Increases Expression Levels of Melanogenesis-Related Proteins in Human Epidermal Melanocytes

Since it was confirmed that SFE could increase melanin production, we investigated the effect of SFE on the expression levels of melanogenesis-related proteins. HEMn-MP cells were treated with 10, 50, and 100 μg/mL of SFE and incubated for 5 days. The protein levels of tyrosinase, MITF, TRP-1, CREB, and phospho-CREB were analyzed via Western blot as shown in Figure 2A.

The relative value of each image analysis via β-actin is shown in a graph (Figure 2B). The results showed that compared to the untreated control, SFE treatment increased the expression levels of tyrosinase and TRP-1 in a dose-dependent manner. In addition, the expression levels of MITF and p-CREB protein were increased in the SFE treatment group compared to those in the untreated control. Taken together, these results indicate that SFE can induce melanogenesis by regulating the expression of tyrosinase-related genes as well as MITF.

3.4. Effects of SFE on Cellular Tyrosinase Activity

Tyrosinase is a key enzyme that plays a crucial role in the process of melanogenesis. It catalyzes the conversion of amino acid tyrosine into an intermediate compound dopaquinone, which is then converted into melanin pigments. Using zymography, the effect of SFE on cellular tyrosinase was assessed, and the findings showed that tyrosinase activity increased in a dose-dependent manner upon treatment with SFE (Figure 2C). At a concentration of 100 μg/mL of SFE, the tyrosinase activity increased by up to 2-fold.

Additionally, the cellular tyrosinase levels were measured to confirm the effect of SFE on enzyme activity. As shown in Figure 2D, relative cellular tyrosinase activity was increased about 29.6%, 25.7%, and 35.4% after treatment with 10, 50, and 100 μg/mL of SFE, respectively. These results clearly demonstrated that tyrosinase activity was induced by SFE. It was noteworthy that SFE significantly increased tyrosinase activity even at 10 μg/mL, the lowest concentration used in this study.

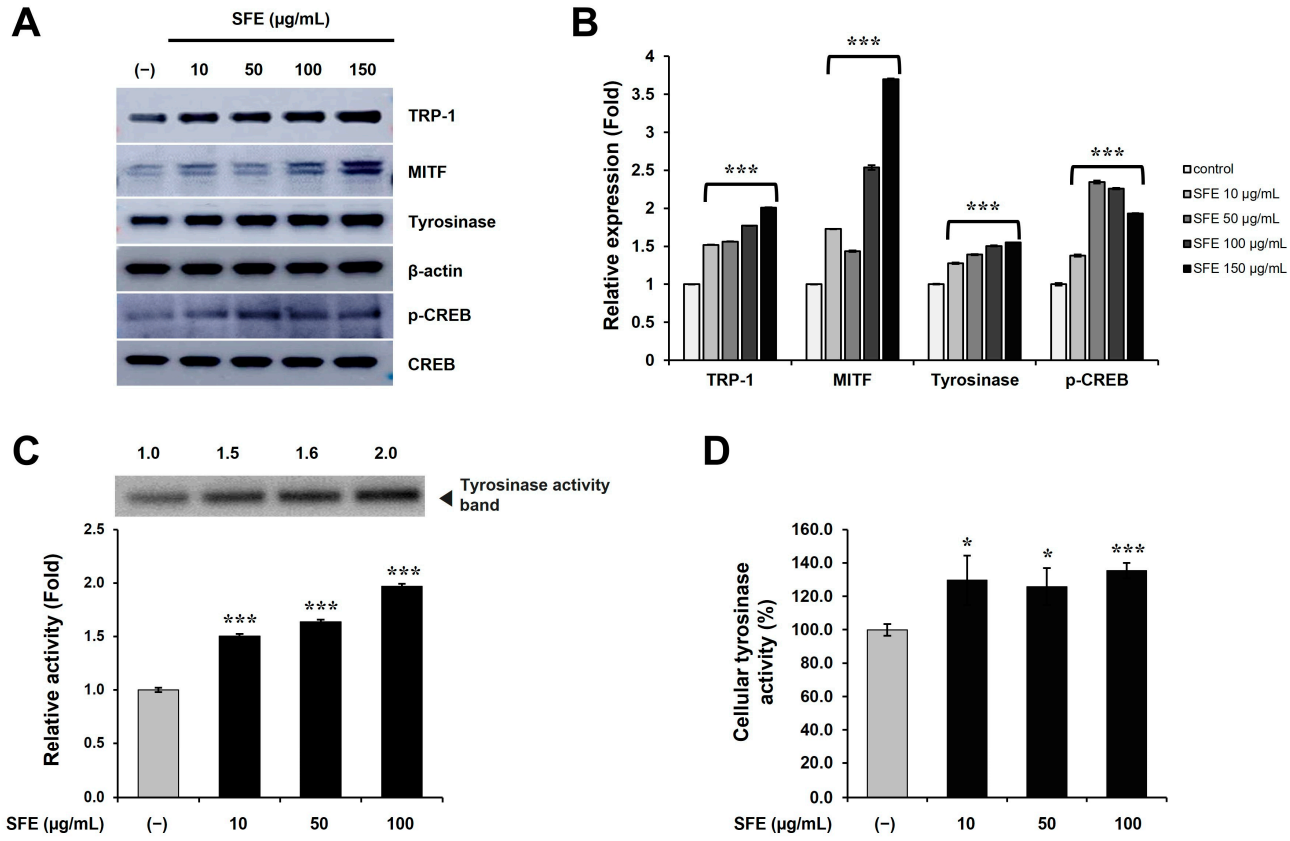

Figure 2. Effects of SFE on melanogenesis-related protein expression and cellular tyrosinase activity in human epidermal melanocytes. Human Epidermal Melanocytes (moderately pigmented) were incubated with SFE (10, 50, 100, and 150 μg/mL). (**A**) Expression levels of proteins including TRP-1, MITF, Tyrosinase, CREB, and p-CREB were determined via Western blot assay. (**B**) Expression levels are presented in ratios compared with β-actin as an internal control. CREB, cAMP-response element-binding protein; MITF, microphthalmia-associated transcription factor; p-CREB, Phosphorylated CREB; TYR, tyrosinase. (**C**) Tyrosinase zymography analysis and (**D**) cellular tyrosinase activity. Values are expressed as mean ± SD (* $p < 0.05$ and *** $p < 0.001$ vs. untreated control).

3.5. SFE Promotes Melanogenesis by Regulating cAMP/PKA/CREB Pathway

In this study, we found that SFE promoted melanin production through the upregulation of MITF, resulting in increased expression of tyrosinase, TRP1, and the phosphorylation of CREB. It is well known that the cAMP signaling pathway contributes to melanin production [10,11]. To determine whether cAMP was involved in the melanogenesis activity by SFE, H89 was used as a specific inhibitor of protein kinase A (PKA). As shown in Figure 3A, melanin content of the control group was reduced by H89 pre-treatment.

The increase in melanin by SFE at a concentration of 100 μg/mL or 150 μg/mL was reduced to a level similar to that of the control group.

In addition, the effect of SFE treatment with H89 on cell tyrosinase was confirmed via zymography (Figure 3B). The cells were pretreated with H89 for 1 h. They were then treated with SFE for 3 days. The activity of cellular tyrosinase was confirmed on bis-tris gel via zymography. With the same tendency as melanin content, tyrosinase activity was decreased by about 0.4-fold after treatment with H89 compared to the control group. However, treatment with 100 μg/mL and 150 μg/mL of SFE increased the tyrosinase activity by 1.2-fold and 1.3-fold, respectively. When PKA was inhibited by H89 treatment, tyrosinase activity decreased by 0.5-fold and 0.4-fold, respectively.

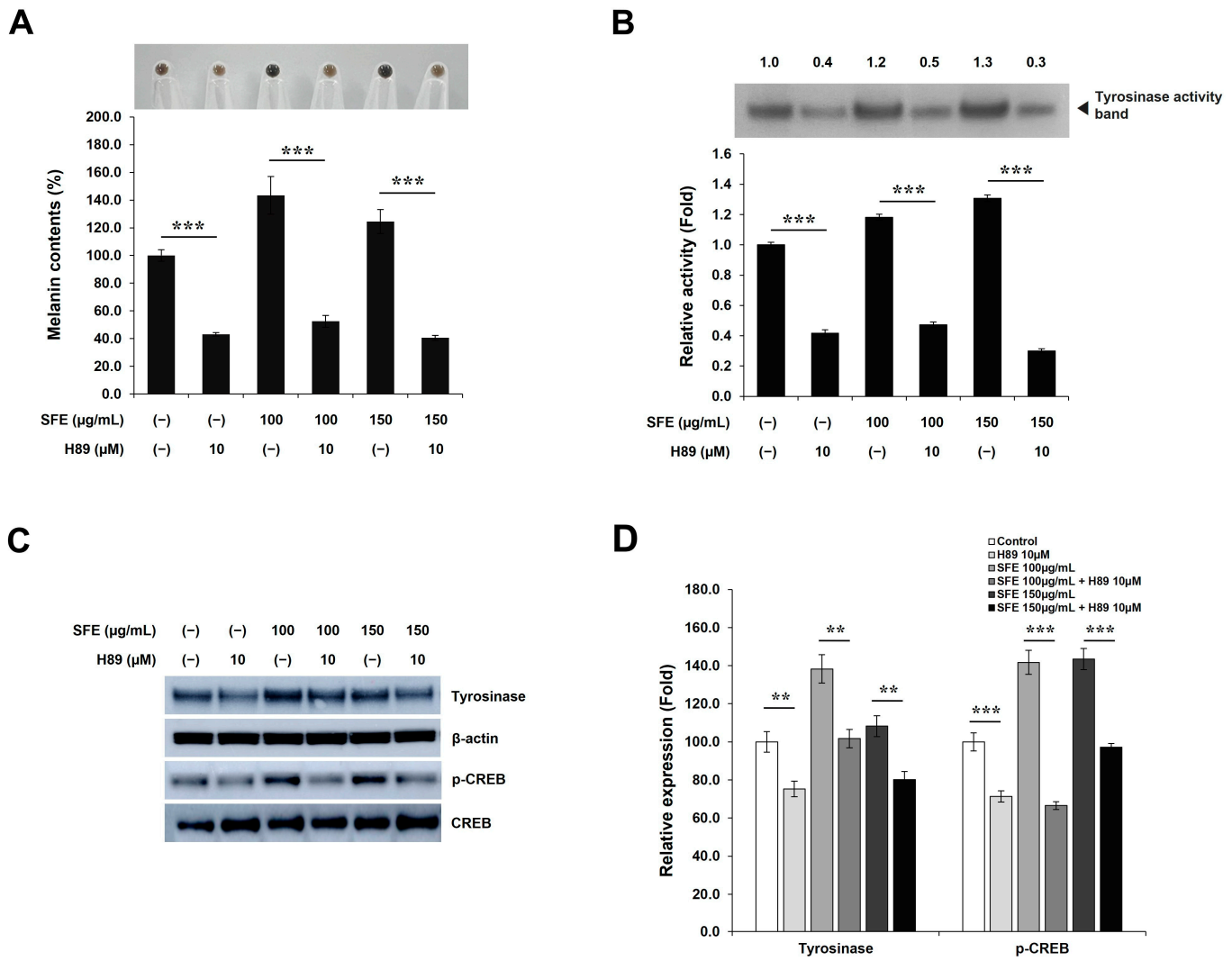

Figure 3. Promoting effects of SFE on melanogenesis by regulating cAMP/PKA/CREB signaling pathway in human epidermal melanocytes. (**A**) Human Epidermal Melanocytes (moderately pigmented) were incubated with or without 10 μM of H89 for 1 h. HeMn-MP cells were incubated with a medium containing 100 or 150 μg/mL of SFE for 5 days and melanin contents was determined. (**B**) Tyrosinase zymography analysis was performed under H89 pretreated condition. (**C**) Expression levels of proteins including Tyrosinase, CREB, and p-CREB were determined via Western blot assay. (**D**) Expression levels are presented in ratio compared with β-actin as an internal control. All results are expressed as percentages of H89 untreated control. Values are expressed as mean ± SD (** $p < 0.01$ and *** $p < 0.001$ vs. untreated control).

When SFE and H89 were used for treatment, the effect on the expression of melanogenesis-related proteins, especially tyrosinase and the phosphorylation of CREB, was investigated via Western blot (Figure 3C). The relative value of each image analysis normalized by β-actin is shown in a graph (Figure 3D). As a result, it was confirmed that tyrosinase and the phosphorylation of CREB were inhibited by H89 not only in the control group, but also in the SFE-treated group. Tyrosinase protein expression was increased significantly by 100 μg/mL of SFE but decreased by H89. The same tendency was observed when the cells were treated with SFE at a concentration of 150 μg/mL (i.e., tyrosinase was increased by SFE but suppressed by H89, although the effect was not as much as that in the group treated with SFE at 100 μg/mL). The phosphorylation of CREB was increased similarly between the group treated with SFE at 100 μg/mL and the group treated with SFE at 150 μg/mL. In both groups, the phosphorylation of CREB was decreased by inhibition

of PKA following treatment of H89. Consistent with these findings, we found that SFE promoted melanogenesis via the cAMP/PKA/CREB pathway.

3.6. Hyperpigmentation Effect of SFE on a Reconstituted Human Skin Model

To confirm the effect of SFE on melanogenesis in an equivalent condition of human skin, a reconstituted three-dimensional human skin, KeraSkin-M™ (BioSolution, Seoul, Republic of Korea) containing normal epidermal keratinocytes and melanocytes, was incubated with SFE (10, 50, 100, and 150 μg/mL) for 14 days. As shown in Figure 4A, when 3D skin treated with SFE was observed through a microscope, it was confirmed that more melanocytes were distributed in the SFE-treated group compared to those in the untreated control.

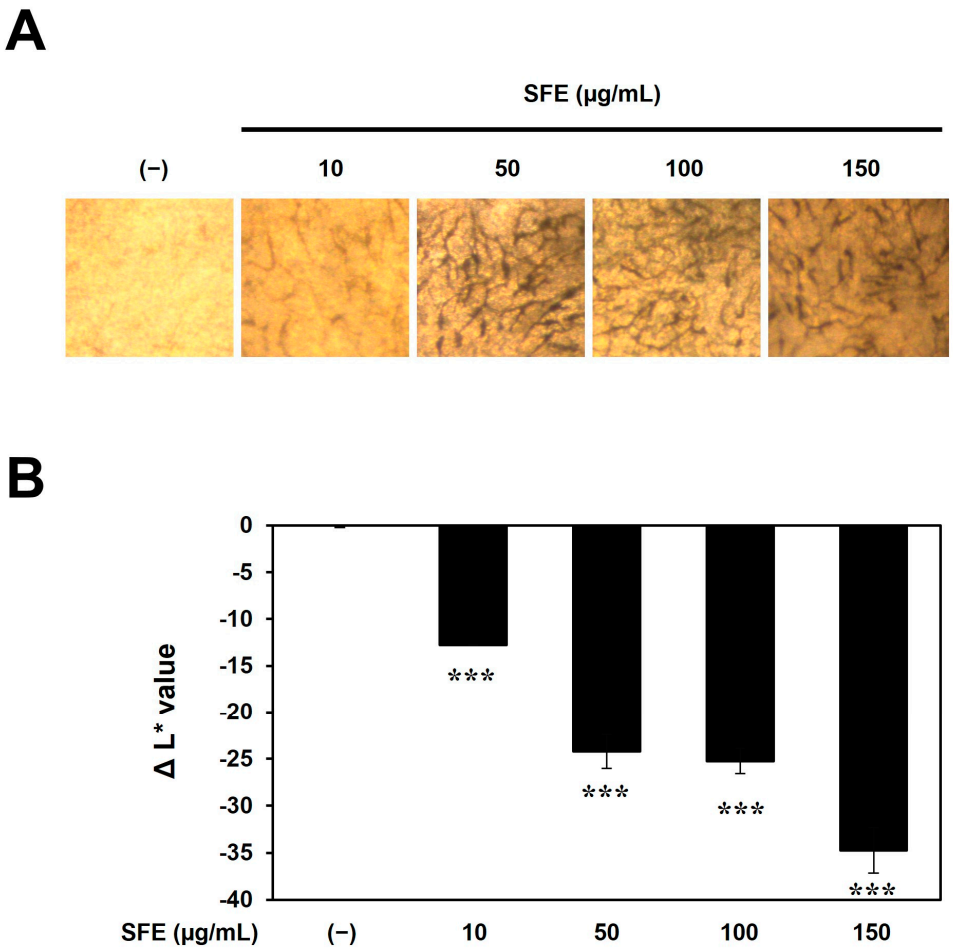

Figure 4. Hyperpigmentation effect on reconstituted human skin model treated with SFE. Reconstituted human skin model composed of human epidermal keratinocytes and melanocytes were treated with SFE (10, 50, 100, and 150 μg/mL) every two or three days for 13 days. (**A**) All pictures of skin were analyzed with Image-Pro Plus (Media Cybernetics, Rockville, MD, USA), and (**B**) L* values of cell pigmentation were measured using a chromameter. Values are expressed as mean ± SD (*** $p < 0.001$ vs. untreated control).

As a result of measuring the L* value (a color value used in color management systems to represent the lightness of a color), it was confirmed that the L* value decreased after treatment with SFE (Figure 4B). Through this, the same hyperpigmentation efficacy of SFE was confirmed not only under in vitro cell conditions, but also in a three-dimensional reconstituted human skin model, which closely mimics human skin.

3.7. Hyperpigmentation Effect of SFE on Ex Vivo Human Hair Follicles

We further confirmed whether SFE affected pigmentation of the hair through ex vivo studies.

After ex vivo hair follicles were treated with 50 or 100 μg/mL of SFE for 9 days, melanin content was evaluated using Fontana–Masson staining, and the amount of melanin in the hair bulb and shaft area was observed microscopically (Figure 5A). After treatment with 50 or 100 μg/mL of SFE, the melanin content was significantly increased compared with that in the untreated control (Figure 5B).

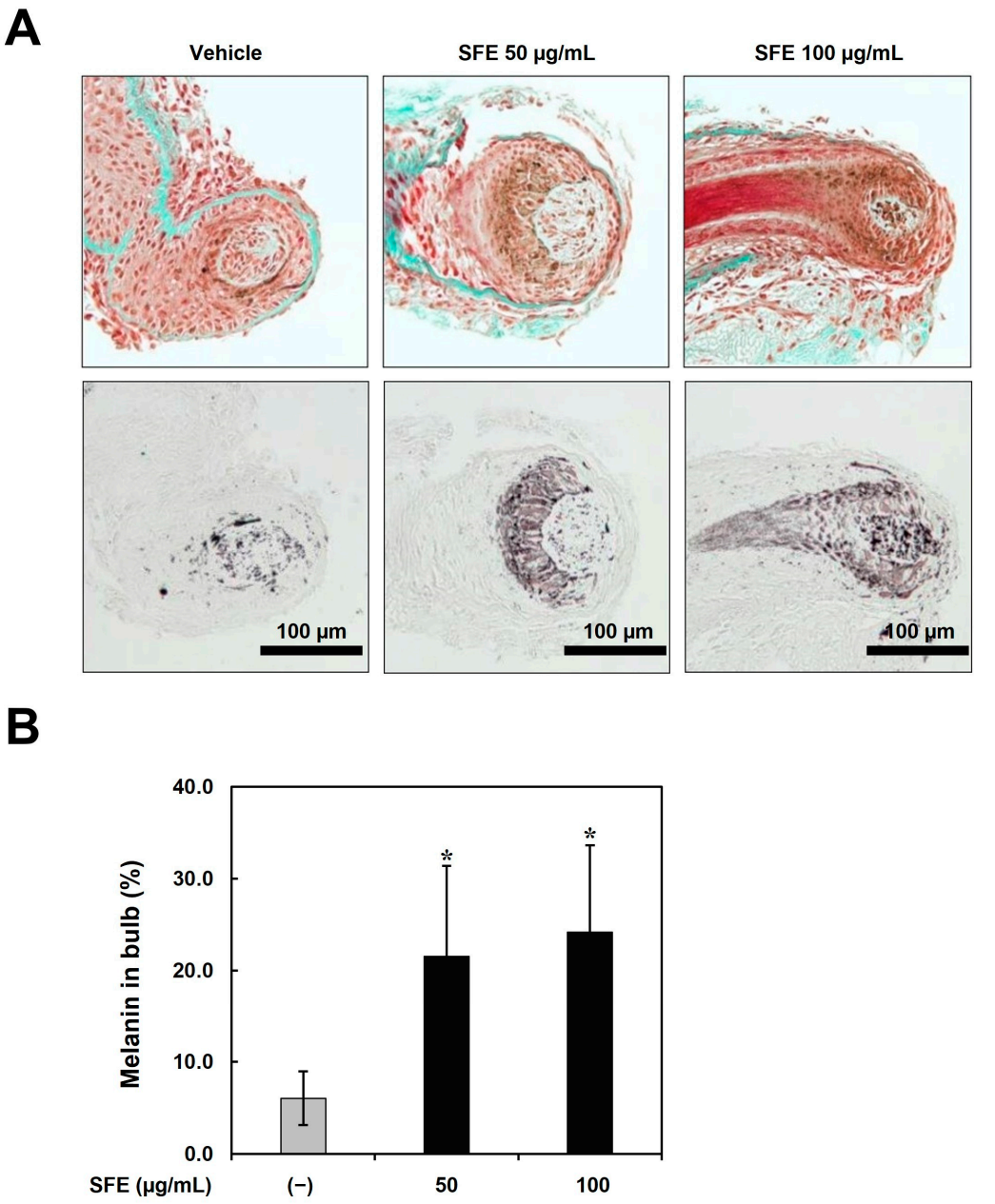

Figure 5. Ex vivo hyperpigmentation effect of SFE on human hair follicles. Human hair follicles were treated 50 or 100 μg/mL of SFE and incubated for 9 days. (**A**) Pictures of hair follicle model and (**B**) analyzed graph are shown. The hair follicle model was stained according to the Fontana–Masson staining method on formalin-fixed paraffin-embedded skin sections. The melanin content in the bulb was measured using the ImageJ program (National Institutes of Health, Bethesda, MD, USA). Values are expressed as mean ± SD (* $p < 0.05$ vs. untreated control).

4. Discussion

Sargassum is a genus of brown seaweeds sourced from a wide and diverse range, holding promise in various domains, including both food and alternative medical applications [19]. It is renowned not only for its exceptional nutritional value but also for being a rich repository of various natural antioxidant compounds such as polyphenols, carotenoids, meroterpenoids, and plant sterols. *Sargassum* possesses qualities that position it as a valuable resource with significant potential for the development of commercially viable products across a range of fields, including food, biofuel, agriculture, therapeutic, and cosmetic products [20].

Brown algae within the *Sargassum* genus have been shown to exhibit diverse physiological benefits, including antioxidative effects [21]. However, there has been no evidence of their ability to enhance melanogenesis. Additionally, these large *Sargassum* seaweeds can be cultivated through aquaculture and can also be produced in various forms, including powders, tablets, or capsules, all of which are now widely involved in various biological applications, which may be an advantage of *Sargassum* over other natural materials [22].

Previous studies have demonstrated that cAMP is involved in the regulation of melanogenesis, the process by which skin cells produce the pigment melanin [12]. The elevated levels of cAMP can activate protein kinase A (PKA), leading to the phosphorylation and activation of enzymes involved in melanin synthesis and ultimately resulting in increased melanogenesis. This process helps darken the skin in response to sun exposure, providing protection against ultraviolet radiation.

A recent study about the regulation of melanin synthesis has determined the melanogenic and antioxidant activities of Argan leaves extract. Phenol profiling was also performed via UPC-ESI-HRMS analysis to identify phenol compounds present in the extract [23]. Similarly, a study on *Vernonia anthelmintica* (L.) Willd, a traditional Chinese herbal medicine, has revealed its potential in promoting pigmentation for the treatment of skin depigmentation disorders by reducing the accumulation of ROS and inflammatory response [24,25].

Regarding oxidative stress, previous research conducted by Wood (2009) examined the effects of H_2O_2-induced oxidative damage on the human hair follicle, including the hair shaft, which is a critical factor in age-related hair greying [26]. Numerous studies have investigated the protective mechanisms of melanocytes against oxidative stress via the Nrf2 and MITF signaling pathways [27,28].

The present study aimed to investigate the effects of *Sargassum fusiforme* extract (SFE) on melanin content and tyrosinase activity without causing cytotoxicity in moderately pigmented human epidermal melanocytes. (Figure 1). This finding was also confirmed under oxidative stress conditions. Oxidative stress and melanogenesis have a complex relationship. Oxidative stress refers to an imbalance between production of reactive oxygen species and the cell's ability to detoxify them, leading to cellular damage. Many studies have investigated inhibitory effects of reactive oxygen species (ROS) on melanin production [29]. Reactive oxygen species (ROS) have been shown to inhibit melanin production by damaging melanocytes, which is a proposed mechanism for vitiligo and hair greying. Therefore, we investigated the potential of SFE to counteract this effect. As shown in Figure 1, the reduction in melanin content and cell viability were restored via SFE treatment. Additionally, we found that SFE increased melanogenesis by promoting the phosphorylation of CREB via the cAMP/PKA/CREB signaling pathway and upregulating MITF in human epidermal melanocytes (Figure 2). These results suggest that *Sargassum fusiforme* extract may have a promoting effect on pigmentation.

Additionally, we conducted HPLC profiling for several compounds by performing LC-MS/MS to find out which components of *Sargassum fusiforme* were responsible for this effect. However, we could not identify the candidates that caused pigmentation. In the results of the total equivalent analysis and total polyphenol analysis, the total sugar content was 58.8%. Contents of total polyphenols were analyzed to be 5.04 mg of GAE/g. However, based on a previous study, sugar is not expected to cause pigmentation. We

also found that SFE had a high iron content. Iron is known to catalyze decomposition of hydrogen peroxide to hydroxyl radical and hydroxyl ions with melanin through the Fenton reaction [30]. When melanin is exposed to the ferrous iron in the Fenton system, the rapid formation of melanin is observed [31]. Those results indicate that iron can contribute to enhancing melanogenesis via the Fenton reaction. Moreover, previous studies have already reported that iron components can improve hair greying [32]. Thus, it can be expected that melanin production is increased in iron-rich extracts. Additional research is needed on whether the ingredient that induces the pigmentation efficacy of SFE is iron.

5. Conclusions

Sargassum fusiforme extract (SFE) increased melanin contents and tyrosinase in human epidermal melanocytes via the cAMP/PKA/CREB signaling pathway. In addition, SFE pretreatment protected human epidermal melanocytes against H_2O_2-induced oxidative stress. Moreover, SFE increased melanin contents in a 3D skin model and an ex vivo hair follicle model. These results suggest that SFE has a hyperpigmentation activity in human epidermal melanocytes. Thus, it can be applied as a potential melanogenesis inducer to improve hair follicular depigmentation and vitiligo by stimulating melanin synthesis.

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