Hindawi
Journal of Food Biochemistry
Volume 2024, Article ID 6668222, 12 pages
https://doi.org/10.1155/2024/6668222

Research Article

The Antimelanogenic Activity of the Extract of Heukharang Lettuce (*Lactuca sativa* L.) Leaf

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Received 19 November 2023; Revised 16 January 2024; Accepted 19 January 2024; Published 2 February 2024

Academic Editor: Ignazio Restivo

Heukharang (*Lactuca sativa* L.), the artificially developed lettuce cultivar for enhancement of lactucin content, exhibits antioxidant activities and sleep-promoting effects. However, potential of Heukharang as a raw material for the skin-whitening agent has not been investigated yet. This study evaluated the effects of Heukharang extract (HHE) on α-melanocyte-stimulating hormone (α-MSH)-induced melanogenesis *in vitro* and *in vivo* models. Our findings revealed that HHE (25–100 μg/mL) effectively inhibited α-MSH-induced melanin synthesis in both 2D and 3D cell culture environments without compromising the viability of the B16F10 murine melanoma cell line. Furthermore, HHE suppressed intracellular tyrosinase activity, along with both gene and protein expression of tyrosinase (TYR) and tyrosinase-related protein (TRP)-1 and TRP-2. HHE downregulated the mRNA level of microphthalmia-associated transcription factor (*MITF*) and the phosphorylation of cAMP response element-binding (CREB) protein. The antimelanogenic activity of HHE (50 and 100 μg/mL) was also confirmed using a zebrafish embryo model, highlighting its efficacy in inhibiting α-MSH-induced melanogenesis through the downregulating CREB/MITF/TYR signaling pathways. Moreover, our study demonstrated that a corresponding amount of lactucin (5–20 μM) to the HHE inhibited and regulated melanogenesis. These results collectively suggest that HHE, enriched with lactucin, holds promise as a potential skin-whitening agent for nutricosmetic industries.

1. Introduction

The skin, constituting 16% of the total body weight, stands as the largest organ in the human body. Its vitality and aesthetics play a significant role in enhancing the overall quality of human life. Uniform skin color is one of the critical factors in determining skin health, but hyperpigmentation which is darkened patches or spots on the skin related to melanin synthesis can cause discomfort; thus, several researchers have tried to find out novel substances or agents inhibiting pigmentation of skin cells [1, 2]. The color of the skin is determined by the synthesis and distribution of melanin, a pigment produced through the process of melanogenesis. This intricate biological mechanism involves epigenetic regulation, where melanin is synthesized within melanocytes and subsequently transported to keratinocytes. The orchestration of multiple genes and signaling pathways is integral to the melanogenesis process [3]. Thus, hyperpigmentation can be alleviated by controlling or regulating the process of melanin synthesis and transport in various ways, and an omnidirectional molecular investigation is required to determine whether specific materials are effective for skin whitening or not [4, 5].

Tyrosinase (TYR) is a crucial enzyme for melanin synthesis, initializing melanogenesis by catalyzing the hydroxylation of tyrosine and the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) to o-dopaquinone. This o-dopaquinone serves as a primary substrate for the subsequent synthesis of melanin [2]. The tyrosinase family genes, including TYR, tyrosinase-related protein 1 (TRP-1), and 2 (TRP-2) [6], are regulated by microphthalmia-associated transcription factor (MITF) [7, 8], which plays a key regulatory role in melanin synthesis. Especially in an eumelanogenesis pathway, both 2-carboxy-2,3-dihydroindole-5,6-quinone (L-DOPA chrome) and TRP-1 were involved, generating eumelanin [9, 10]. In addition, α-melanocyte-stimulating hormone (α-MSH) is produced by keratinocytes in response to UV exposure [11]. α-MSH plays a role in elevating intracellular cyclic adenosine monophosphate (cAMP) levels and activating protein kinase A (PKA) [12, 13]. A cAMP response element-binding (CREB) protein can be activated by the cAMP/PKA signaling pathway that can phosphorylate CREB at serine^{133} (p-CREB), finally promoting melanogenesis (PKA/CREB pathway) [14, 15]. Upon activation, the CREB protein can directly bind to the MITF promoter region, thereby stimulating MITF transcription [16]. MITF, in turn, governs the expression of numerous pigmentation genes, playing a crucial role in promoting melanocyte differentiation [17]. Thus, the interaction between these series of molecular biological and biochemical factors was collectively referred to as cAMP/PKA/CREB/MITF cascade [14].

On the other hand, lettuce (*Lactuca sativa* L.) enjoys widespread cultivation and global popularity, with significant consumption in regions such as China, the United States, and Western Europe. Its nutrition composition varied depending on the lettuce type, but all of them contain an abundance of moisture (~95%) [18]. Lettuce exhibits a diverse range of varieties distinguished by colors, sizes, and shapes. Within each type, further subdivisions into subtypes are possible, characterized by shared morphological and genetic similarities. However, the extensive genetic and morphological diversity of lettuce has led to the absence of a standardized classification system for this versatile plant. Instead, Mou [19] proposed a classification system comprising six primary lettuce types, categorized based on distinct characteristics such as had formation, leaf shape, size, stem type, and texture as follows: butterhead lettuce, crisphead lettuce, Latin lettuce, leaf or cutting lettuce, romaine or cos lettuce, and stem or stalk lettuce. A cultivar is a variety selected for desirable traits for cultivation, and several researchers have tried to select a pure line for their own purposes: higher yield, appearances [20], heat resistance [21], etc.

In Korea, Jang et al. [22] have collected and selected specific cultivars from native seeds based on their traits and productivity from 2011 to 2015 to develop a new Korean lettuce variety which has excellent functional component contents and good texture. They successfully developed a specific and novel type of lettuce named Heukharang, which was characterized by its more pungent, bitter taste, higher hardness, black-red leaf color, even distribution of anthocyanin color throughout the leaves, and substantially higher level of lactucin ($C_{15}H_{16}O_5$, MW = 276.28 g/mol) exceeding those found in the typical lettuce, red skirt, by more than 100 times. Due to the improvement in lactucin content, several researchers have been interested in the potential use of Heukharang for developing food and/or health functional food products. Consequently, investigations into its physiological and/or biochemical effects have been conducted, encompassing areas such as antioxidant activities [22] and sleep-promoting effects [23]. Lactucin, the target compound where the Korean researchers artificially promoted its content in Heukharang, is a sesquiterpene lactone, and it has attracted attention owing to its multiple biological activities, including antimalarial [24], antihyperalgestic and anti-abiogenic [25], antiandrostenone metabolism [26], and anticancer [27]. However, the effect of Heukharang and/or lactucin on skin cells has not been reported. This study examined whether Heukharang extract (HHE) and its bioactive compound lactucin would affect melanogenesis and explored the potential use of HHE as a skin-whitening agent in the nutricosmetic industry. The expression of TYR, TRP-1, TRP-2, and cAMP/PKA/CREB/MITF cascade was also investigated to elucidate how the HHE treatment on skin cells contributes to regulating melanogenesis, and the antimelanogenic effect of the HHE treatment was confirmed *in vivo* using a zebrafish embryo model.

2. Materials and Methods

2.1. Reagents and Antibodies. Heukharang lettuce freeze-dried powder was purchased from HJ Biotech (Yeonggwang, Republic of Korea). The MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was obtained from Promega (Medison, WI, USA). Arbutin, α-melanocyte-stimulating hormone (α-MSH), dimethylsulfoxide (DMSO), lactucin, L-DOPA, and phenazine methosulfate (PMS) were obtained from Sigma (St. Louis, MO, USA). Tyrosinase (TYR) and TYR-related protein (TRP)-2 antibodies were acquired from Abcam (Cambridge, UK). TRP-1 and CREB protein 1 (CREB-1) antibodies were obtained from Santa Cruz (Dallas, TX, USA). The vinculin antibody was bought from Invitrogen (Waltham, MA, USA), and the phosphorylated CREB (p-CREB) antibody was acquired from Cell Signaling (Danvers, MA, USA).

2.2. Sample Preparation. To prepare Heukharang extract (HHE), the dried powder of Heukharang (30 g) was heated in distilled water (900 mL) and was put in a water bath (70°C, 60 rpm, 3 h). The extract solution was obtained by vacuum filtration using filter paper (Whatman, Buckinghamshire,

UK). After freeze-drying, a Heukharang water extract powder was obtained (yield of extraction = 36.53%). A stock solution of HHE (100 mg/mL) and lactucin (50 mM) was dissolved in dimethylsulfoxide (DMSO).

2.3. Cell Culture. The B16F10 cell line from murine melanoma was obtained from the Korean Cell Line Bank (Seoul, Republic of Korea). B16F10 cells were seeded and grown in Dulbecco's modified Eagle medium (DMEM) (HyClone, Logan, UT, USA) with 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco, Waltham, MA, USA) at 37°C (5% CO_2).

2.4. Cell Viability Assay. The MTS assay was employed to assess the cell viability of B16F10 cells. The B16F10 cells (1×10^4) were cultured in a 96-well plate for 1 day, followed by treatment with HHE (12.5–400 μg/mL), arbutin (25–400 μg/mL), or lactucin (5–40 μM) for 72 h. After the replacement of the fresh medium, 20 μL MTS (MTS: PMS = 20 : 1) solution was put into individual wells. The 96-well plate containing the treated cells was incubated at 37°C with 5% CO_2. After 30 min of the incubation period, the absorbance at 490 nm was measured using a Cytation 1 microplate reader (BioTek, Winooski, VT, USA) at the Biopolymer Research Center for Advanced Material (BRCAM).

2.5. Melanin Content Assay. B16F10 cells were seeded at a density of 2.4×10^5 cells per 60 mm dish) and treated with HHE (25–100 μg/mL) for 60 min prior to α-MSH treatment. The cells were treated with 100 nM of α-MSH and incubated at 37°C for 72 h. A three-dimensional melanoma cell culture system was conducted by the forced-floating method [28, 29] with slight modifications. 1×10^4 cells were cultured in an ultra-low attachment (ULA) 96-well round plate (SPL, Seongnam, Republic of Korea) at 37°C. Next day, the cells were cotreated by 100 nM α-MSH, HHE (25–100 μg/mL) or lactucin (5–20 μM). A ULA plate was incubated for 3 days at 37°C. In both 2D and 3D cell cultures, 100 μL of the cultured medium was put on each well, and melanin contents were assayed at 490 nm absorbance.

2.6. Intracellular Tyrosinase Activity Assay. B16F10 cells were seeded at a density of 2.4×10^5 cells per 60 mm dish and incubated at 37°C overnight. After pretreatment with HHE or lactucin for 1 h, the cells were treated with 100 nM α-MSH (37°C for 72 h). Subsequently, the conditioned medium was discarded, and the cells were washed with PBS twice. Cell lysis was conducted using cell lysis buffer (Cell signaling Technology, Danvers, MA, USA), followed by centrifugation (15,000g, 10 min). The supernatant was mixed with 10 mM L-DOPA and reacted (37°C for 2 h). Quantitative protein concentrations were performed using the Pierce BCA protein assay kit (Thermo Fisher, Waltham, MA, USA). The absorbance of dopachrome was measured at 490 nm.

2.7. L-DOPA Staining Assay. The L-DOPA staining assay was conducted following the procedure outlined in prior studies [30, 31], with certain modifications. The B16F10 cells were harvested with lysis buffer, and protein extracts were mixed with 10 mM Tris-HCl buffer (pH 7.0) including 1% sodium dodecyl sulfate (SDS), without β-mercaptoethanol or heating. 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Following electrophoresis, the gel was rinsed twice with 0.1 M sodium phosphate monobasic buffers (pH 6.8) containing 10 mM L-DOPA for 30 min each at room temperature (RT), followed by incubation in the dark at 37°C for 1 h. TYR activity was confirmed with dark color bands on the gels.

2.8. Western Blotting. Protein extracts obtained from 2.7 were used for western blotting. Protein samples were subjected to 5 min heat treatment (95°C), followed by centrifugation (15,000g for 2 min at 4°C), and subsequently separated by 8% SDS-PAGE. The gels were transferred onto polyvinylidene difluoride membranes, and these membranes were blocked with Blotting-Grade Blocker (Bio-Rad, Hercules, CA, USA) for 1 h at RT. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies (diluted 1 : 1,000) in 1× Tris-Buffered Saline with 0.1% Tween® 20 detergent (TBST). Following incubation with horseradish peroxidase-conjugated secondary antibodies (diluted 1 : 5,000) for 40 min at RT, the bands were detected using a chemiluminescence reader (LuminoGraph III Lite; ATTO, Tokyo, Japan) at the Biopolymer Research Center for Advanced Material (BRCAM, Seoul, Republic of Korea). Relative band intensity was calculated by the ImageJ software program (National Institutes of Health, Bethesda, MD, USA).

2.9. Real-Time Quantitative Polymerase Chain Reaction (qPCR). B16F10 cells were cultured at a density of 2.4×10^5 cells per 60 mm dish and incubated. The cells were treated with HHE (25–100 μg/mL) for 1 h, followed by the treatment of 100 nM α-MSH for 4.5 h. Total RNA was prepared using an EcoPURE Total RNA kit (Mn, Woodbury, USA). Quantitative purified RNA concentration was performed by using a NanoDrop One^c Microvolume UV-Vis spectrophotometer (Thermo Fisher). Complementary DNA was synthesized using amfiRivert cDNA synthesis platinum master mix (GenDEPOT, TX, USA) in a 20 μL volume, comprising 800 ng of total RNA. cDNA was amplified in a CFX 96 Touch Real-Time PCR Detection system (Bio-Rad) using specific primers and AccuPower® 2X GreenStar™ qPCR Master Mix (Bioneer, Daejeon, Republic of Korea). The following primer sequences were used as follows: 5′-ATC AGC AAC TCC TGT CCA GC-3′ (forward), 5′-TGC TTC AGA CTC TGT GGG GA-3′ (reverse) for MITF; 5′-TCT TCA CC ATG CTT TTG TGG-3′ (forward), 5′-ATA GGT GCA TTG GCT TCT GG-3′ (reverse) for TYR; 5′-TGG TCT GTG AAT CCT TGG AA-3′ (forward), 5′-CAT TTC CAG CTG GGT TTC TC-3′ (reverse) for TRP-1; 5′-CGT GCT GAA CAA GGA ATG C-3′ (forward), 5′-CGA AGG ATA TAA GGG CCA CTC-3′ (reverse) for

TRP-2; and 5′-CAT CAC TGC CAC CCA GAA GAC TG-3′ (forward), 5′-ATG CCA GTG AGC TTC CCG TTC AG-3′ (reverse) for GAPDH.

2.10. Maintenance of Zebrafish. Adult zebrafish were housed in a 3-liter acrylic tank for a period of 24 h. The maintenance system was operated on a 14/10 h light/dark cycle, and the tank temperature was maintained at 28.5°C. Zebrafish were fed three times a day. Embryos were collected from natural spawning within 30 min by turning on the light, which induced sunrise. HHE (50 and 100 μg/mL) was treated zebrafish embryos at 8 h after fertilization. Then, the effects of the HHE treatment on the melanogenesis of zebrafish were observed under the stereomicroscope.

2.11. Measurement of Melanin Contents in Zebrafish Embryos. Protein from zebrafish embryos was extracted using zebrafish embryos using PRO-PREP™ protein extraction solution (Intron, Sungnam, Republic of Korea), followed by centrifugation for collecting the pellet. After exposing to 500 μL of 1 N NaOH (60°C for 30 min), the melanin contents in the suspension were quantified at 490 nm using a microplate reader (BioTeK, Santa Clara, CA, USA).

2.12. High-Performance Liquid Chromatography (HPLC) Analysis. Contents of lactuin in HHE were quantified using the HPLC system (Agilent Technologies, Santa Clara, CA, USA). Sample separation was used by Agilent Eclipse XDB-C18 (4.6 mm ID × 250 mm, 5 μm) at 0.8 mL/min of the flow rate. The mobile phase was applied by solvent A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile), and the gradient program is summarized in Table S1.

2.13. HPLC-High-Resolution-MS/MS Analysis. To identify lactucin in the HHE, LC-MS/MS analysis was performed by a method described in a previous study with some modifications [32]. For the HPLC analysis, an ACQUITY UPLC BEH C18 column (130 Å, 1.7 μm, 2.1 mm × 50 mm) (Waters, Milford, MA, US) was used at 45°C, and the column was equilibrated for 1 min. The mobile phase was composed of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) with 0.3 mL/min of the flow rate. Sample volume was used 10 μL to an injector, and the detection wavelength was 210 nm. HHE (200 mg/mL) and lactucin (1 μg/mL) were used for this experiment. An elution gradient program was as follows: 0–1 min, 2% B; 1–15 min, 20% B; 15–15.5 min, 90% B; 15.5–17.5 min, 90% B; and 17.5–18 min, 2% B. Mass spectrometry analysis was conducted by using an Orbitrap Exploris 120 mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK) with a heated electrospray ionization (H-ESI) interface (Thermo Fisher Scientific). The mass spectrometric conditions were performed as follows: spray voltage, 2500 V in negative ion mode; collision gas (nitrogen); sheath gas, 50 arbitrary unit; auxiliary gas, 10 arbitrary unit; sweep gas, 1 arbitrary unit; ion transfer tube temperature, 325°C; and vaporizer temperature, 350°C. The full-MS scan mode was detected at 120,000 resolution at m/z 100–1500 and RF Lens at 70%. Precursor ions were fragmented by higher energy collisional dissociation with a normalized collision energy of 15, 30, and 60% detected at 15,000 resolution. All data were analyzed using Freestyle software (Thermo Fisher Scientific).

2.14. Statistical Analysis. All experimental data were presented as the mean ± standard deviation. Statistical analysis was conducted using Student's *t*-test with Microsoft Excel 2019 (Redmond, WA, USA). $p < 0.05$ was accepted to show a significant difference.

3. Results

3.1. HHE Shows No Cytotoxicity and Suppresses Melanogenesis. Before testing the effect of HHE on melanogenesis, a cell viability assay was conducted to assess the potential effect of HHE on B16F10 cells. HHE demonstrated no significant cytotoxicity up to a concentration of 400 μg/mL (Figure 1(a)), compared to that of 400 μg/mL of arbutin (positive control) that showed significant cytotoxicity (Figure S1(d)). When we measured extracellular melanin contents of the HHE (25–100 μg/mL)-treated cells, induction of melanogenesis by α-MSH (100 nM) was significantly decreased in both 2D (Figure 1(b)) and 3D melanoma cell culture systems (Figure 1(c)). In a specific range of concentration (25–100 μg/mL), a dose dependency of the effect of HHE was clearly observed in a 2D culture ($p < 0.05$), and all HHE treatment groups reduced melanin contents to the similar level of the control group in a 3D culture. The plateau of the effect was also observed in a higher concentration of HHE-treated groups (100–400 μg/mL) in a 2D culture (Figure S1(e)).

3.2. HHE Reduces Intracellular TYR Activity. We applied L-DOPA, a substrate for the TYR reaction, to test the effect of HHE treatment on the intracellular TYR activity. The pretreatment of HHE significantly suppressed intracellular TYR activity (Figure 2(a)). The HHE pretreatment induced a decreased level of TYR activity, and intracellular melanogenesis induced by α-MSH was also significantly reduced as TYR activity decreases (Figure 2(b)). A dose dependency of the HHE treatment was clearly observed in both results.

3.3. HHE Inhibits and Regulates the Expression of Melanogenic Enzymes. To validate the downregulatory effect of the HHE treatment on melanogenic enzymes, protein expressions of TYR, TRP-1, and TRP-2 in the HHE-treated B16F10 cells were compared by western blotting analysis (Figure 3(a)). Figure 3(b) shows that the HHE treatment significantly decreased gene expression of the key enzymes associated with melanogenesis (TYR, TRP-1, and TRP-2). To check the effect of the HHE treatment on the upstream regulatory action of the cAMP/PKA/CREB/MITF cascade, changes in mRNA expression of *MITF* were confirmed (Figure 3(c)). We found that the HHE downregulated *MITF* expression. In addition, the

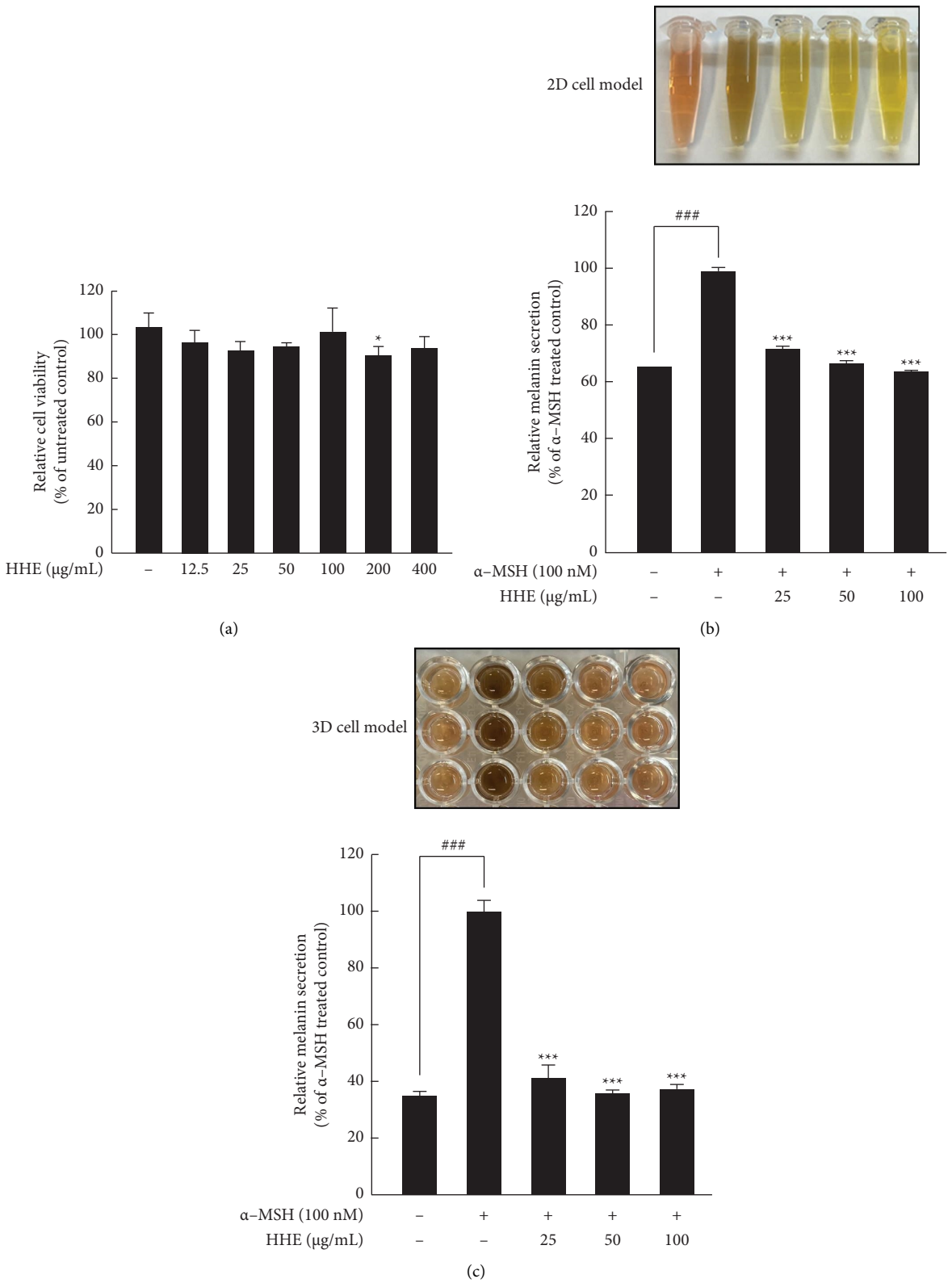


Figure 1: Effects of Heukharang (HHE) on cytotoxicity and melanin synthesis in B16F10 cells. (a) Cell viability of the HHE-treated B16F10 cells ($^{*}p < 0.05$ in comparison with the untreated group). (b) Extracellular melanin contents of the 2D cell culture system. (c) Extracellular melanin contents of the 3D cell culture system ($^{###}p < 0.001$ versus the untreated group; $^{***}p < 0.001$ in comparison with the α-MSH-treated group).

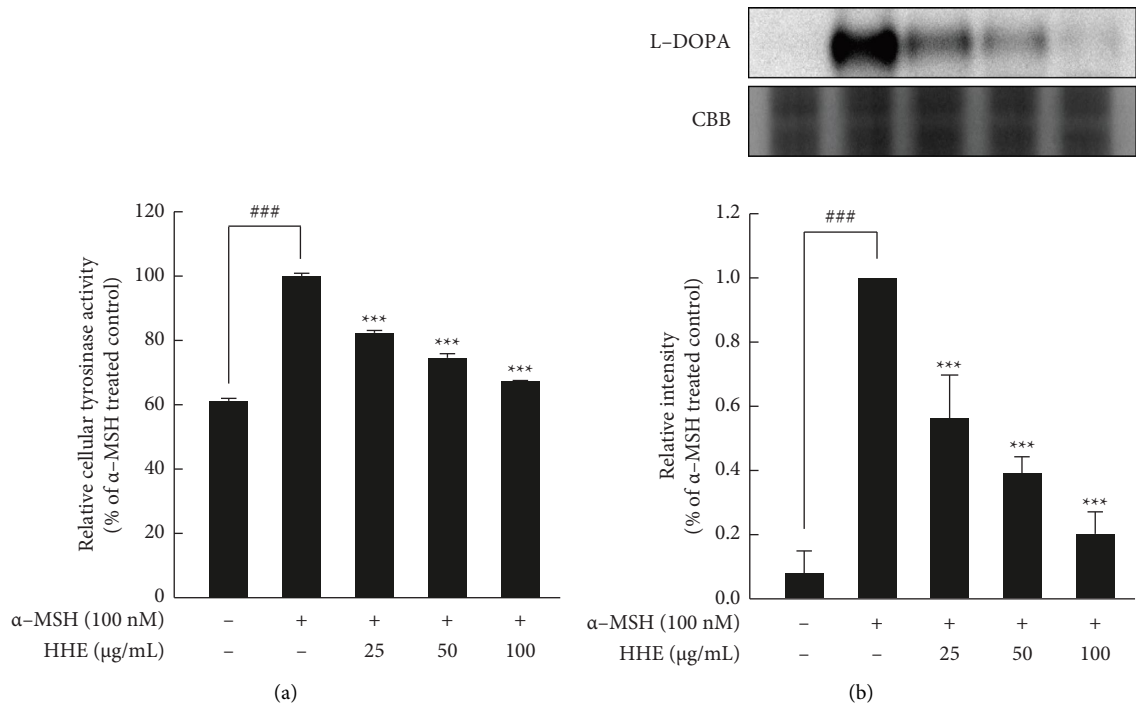
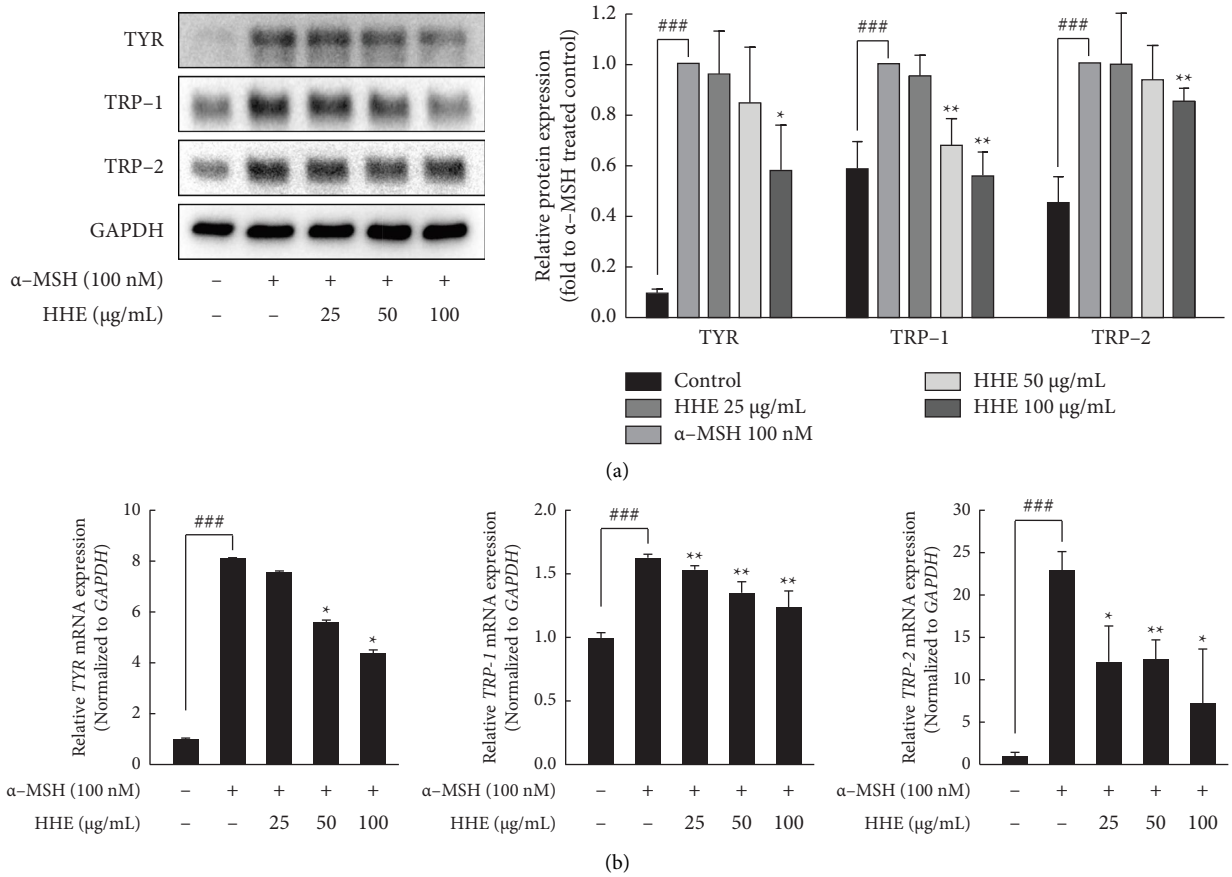


Figure 2: Effects of Heukharang (HHE) on intracellular tyrosinase (TYR) enzyme activity in B16F10 cells. (a) Intracellular TYR activity of the HHE-treated B16F10 cells. (b) L-DOPA staining results and intracellular melanin contents of the HHE-treated B16F10 cells ($^{###}p < 0.001$ in comparison with the untreated group; $^{***}p < 0.001$ in comparison with the α-MSH-treated group).



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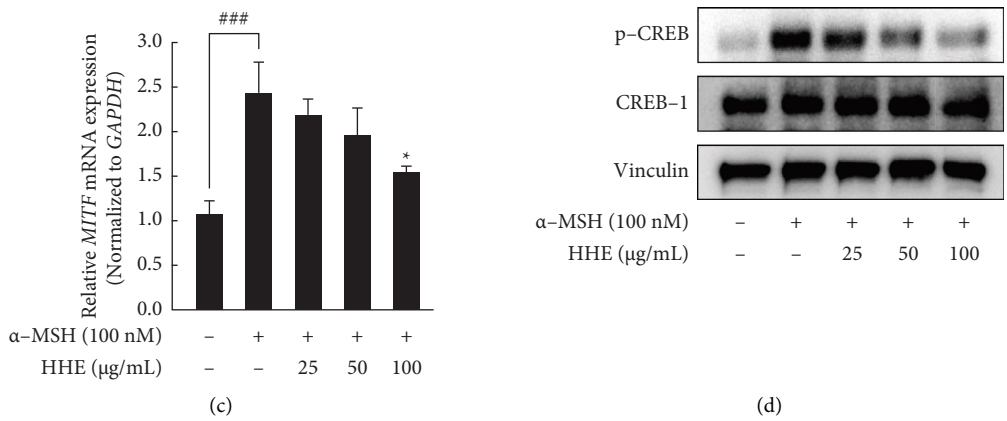


Figure 3: Effects of Heukharang (HHE) on the expression of melanogenic enzymes in B16F10 cells. (a) Western blotting expression of tyrosinase (TYR), tyrosinase-related protein 1 (TRP-1), and 2 (TRP-2) in the HHE-treated B16F10 cells. (b) Relative mRNA expression of *TYR*, *TRP-1*, and *TRP-2* in the HHE-treated B16F10 cells. (c) Relative mRNA expression of microphthalmia-associated transcription factor (*MITF*) in the HHE-treated B16F10 cells. (d) Western blotting analysis of phosphorylated CREB (p-CREB) and cAMP response element-binding protein 1 (CREB-1) in the HHE-treated B16F10 cells ($^{###}p < 0.001$ in comparison with the untreated group; $^{*}p < 0.05$ and $^{**}p < 0.01$ in comparison with the α-MSH-treated group).

phosphorylation level of CREB was confirmed (Figure 3(d)). Expression of phosphorylated CREB was decreased by HHE treatment, dose-dependently.

3.4. HHE Inhibits Melanogenesis in Zebrafish. Figure 4(a) shows that the HHE treatment inhibited the body pigmentation of zebrafish with more than 90% of the survival and hatching rate (Figures S1(b) and S1(c), respectively). In addition, HHE treatment significantly reduced melanin contents in an embryo of zebrafish in a dose-dependent manner (Figure 4(b)).

3.5. Lactucin Is an Active Constituent of the HHE on Melanogenesis. HHE used in this study contained 0.23 mg of lactucin/g measured by HPLC analysis (Figures S2(a) and S2(b)), and the lactucin in the HHE was identified by LC-MS/MS analysis (Figures S2(b)–(d)). Figures 5(a) and 5(b) show that lactucin prepared based on the measured amount (5–20 μM) also significantly decreased melanin secretion induced by α-MSH with a slight cytotoxicity. We confirmed that intracellular melanogenesis of B16F10 cells was also significantly reduced by lactucin treatment with both L-DOPA staining assay (Figure 5(c)) and western blotting expressions of TYR, TRP-1, and TRP-2 (Figure 5(d)).

4. Discussion

Heukharang which is well known for a sleep-promoting effect reported higher antioxidant activity compared with red skirt lettuce [23, 33]. Plant extracts with antioxidant activity could have potential of inhibitory effects on melanogenesis [34]. Heukharang extracts used in this study showed antioxidant activities including 8.31 ± 0.61 mg gallic acid equivalent/g of phenolic contents, 18.81 ± 1.67 mg quercetin equivalent/g of flavonoid contents, and increasing DPPH radical scavenging activity, dose-dependently (Figure S1(a)).

This study represents the first elucidation of the potential use of HHE as an antimelanogenic agent in the nutricosmetic industry. The *in situ* test was performed by exposing the *in vitro* cell culture model to the HHE treatment and then α-MSH, inducer of melanogenesis [12, 13] and/or substrate (L-DOPA) of key melanogenic enzyme action [35]. The skin-whitening effect of the HHE at an *in vivo* level was also confirmed using one of the animal alternative test methods, the zebrafish model.

Indeed, pretreatment of the HHE only for 1 h greatly inhibited α-MSH-induced melanogenesis without affecting cell viability of the B16F10 cells, more than 90% of the cells remained viable even after 72 h of exposure to HHE at concentrations up to 400 μg/mL. This study employed the melanocyte aggregates as an alternative to the artificial skin model, bridging the experimental gap between 2D cell culture systems and animal models [29]. Notably, pretreatment with HHE (25–100 μg/mL) effectively inhibited α-MSH (100 nM)-induced melanin synthesis in both the 2D and 3D culture systems, as illustrated in Figures 1(b) and 1(c). In comparison of the experimental results reported in the previous study [36], which treated 50 μg/mL of arbutin, a well-known melanogenesis inhibitor in cosmetics and medicines, on 100 nM of α-MSH-stimulated B16F10 cells, the HHE applied in this study indicated a remarkable reduction effect on melanin synthesis (Figure S1(e)).

When intracellular responses of the cells to the HHE pretreatment were observed, increased conversion L-DOPA to L-DOPA chrome induced by α-MSH in eumelanogenesis was successfully suppressed. The HHE decreased TYR

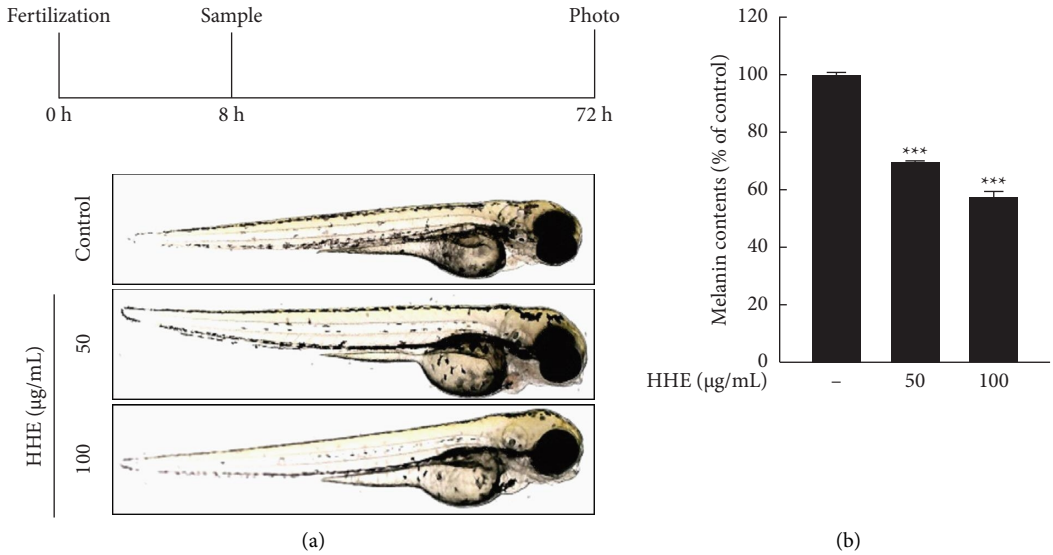
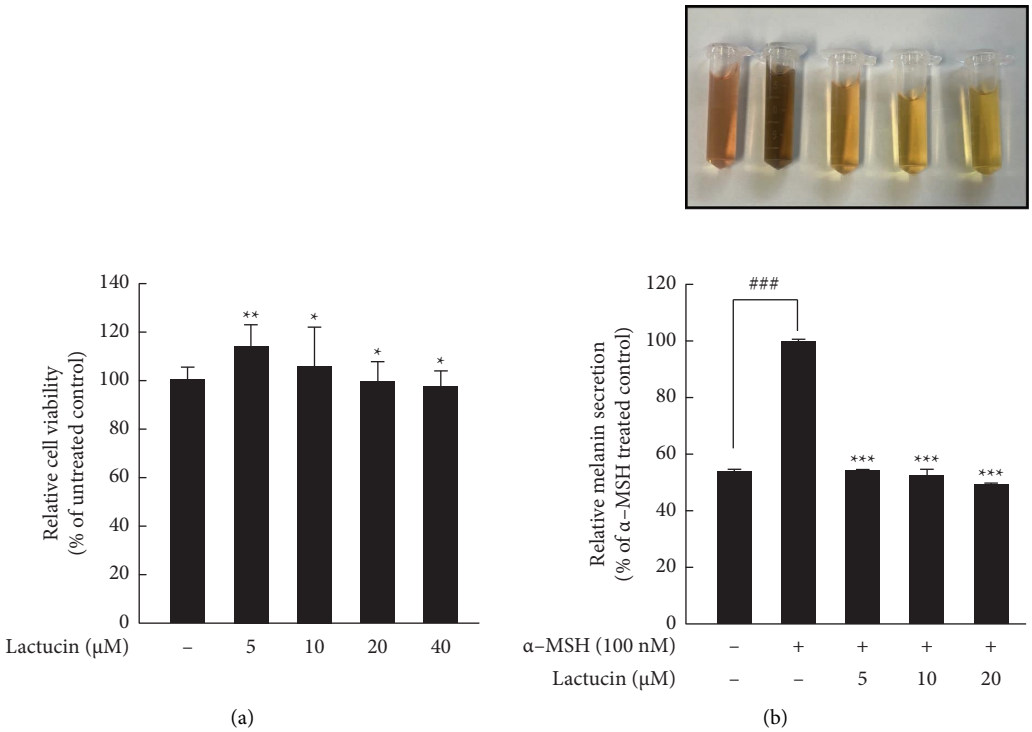


Figure 4: Effects of Heukharang (HHE) on melanogenesis in zebrafish. (a) Stereomicroscopic image of the HHE-treated zebrafish. (b) Melanin retention of the HHE-treated zebrafish embryos ($^{***}p < 0.001$ in comparison with the untreated group).



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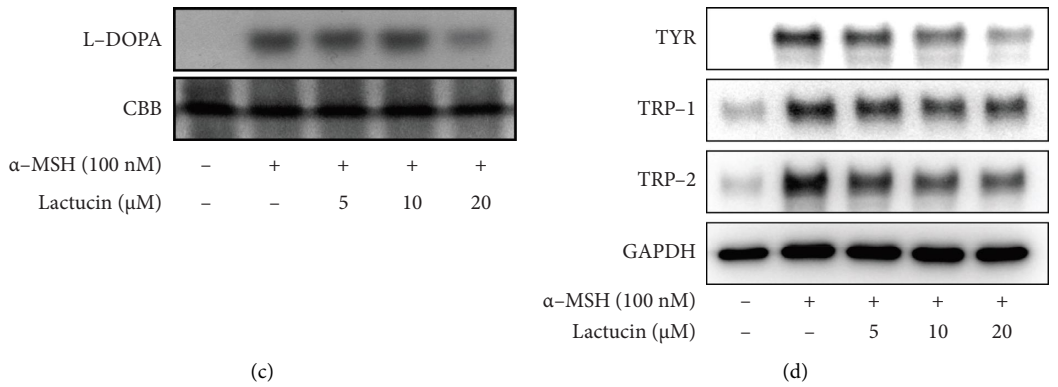

Figure 5: Effects of lactucin on melanogenesis in B16F10 cells. (a) Cell viability of lactucin-treated B16F10 cells. (b) Extracellular melanin contents of lactucin-treated B16F10 cells ($^{*}p < 0.05$ and $^{**}p < 0.01$ in comparison with the untreated group). (c) L-DOPA staining results of lactucin-treated B16F10 cells ($^{\#\#\#}p < 0.001$ versus untreated group; $^{***}p < 0.001$ in comparison with the α-MSH-treated group). (d) Western blotting analysis of tyrosinase (TYR), tyrosinase-related protein 1 (TRP-1), and 2 (TRP-2) in B16F10 cells treated with lactucin.

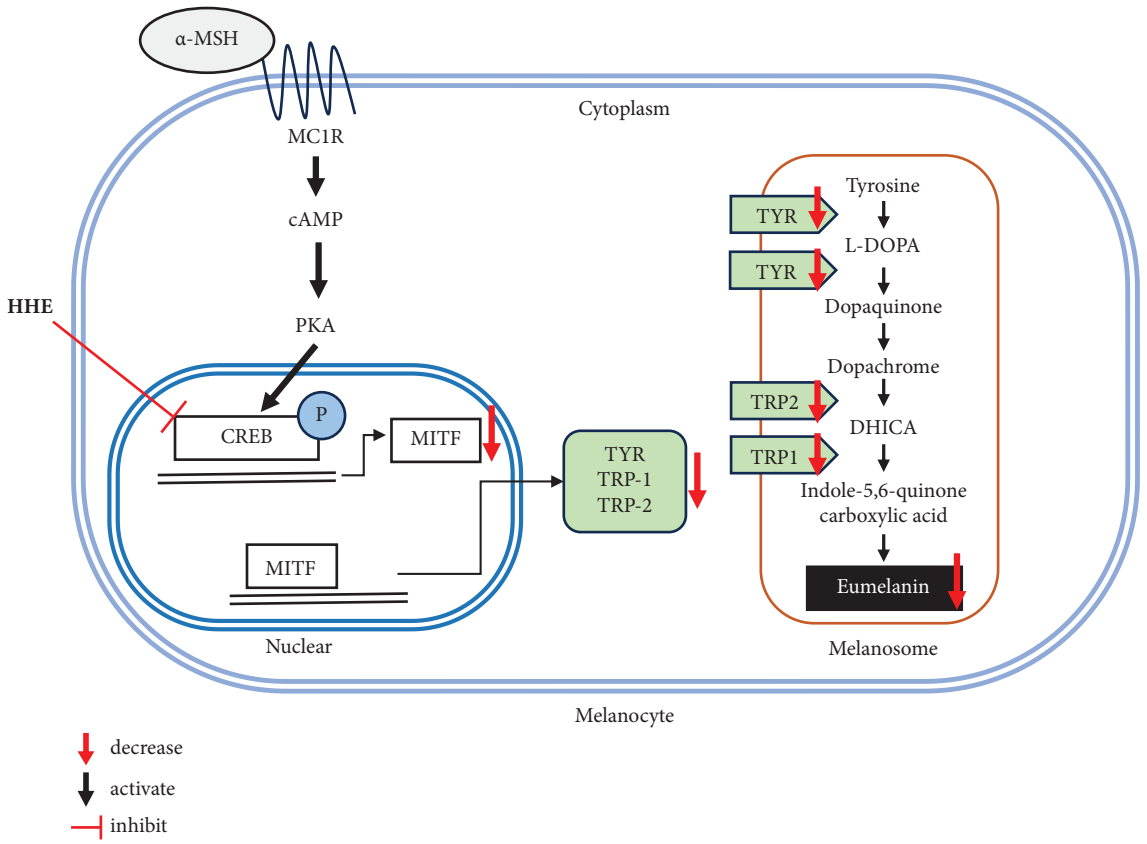

Figure 6: Schematic diagram of α-MSH-induced melanogenesis. HHE downregulates the CREB/MITF signaling pathway, leading to subsequent reduction in the expression of melanogenic proteins (TYR, TRP-1, and TRP-2) and melanin synthesis.

enzyme activity (Figure 2(a)) and also significantly reduced the production of dopachrome from L-DOPA, also called tyrosinase zymography. Protein expression of TYR is also significantly suppressed by the HHE treatment (Figure 3). TYR activity is crucial to melanogenesis [13]; thus, inhibition of enzymatic activity and/or expression of TYR by the HHE treatment exert a significant influence on the overall pathway of melanogenesis. In addition, the pretreatment of HHE exhibited a dose-dependent impact on the relative expressions of TRP-1 and TRP-2. Notably, our result showed that changes in TRP-1 expression were more noticeable than in TRP-2 (Figure 3(a)). In an eumelanogenesis pathway, TRP-1 oxidizes 5,6-dihydroxyindole-2-carboxylic acid (DHICA) to a carboxylated indolequinone, a precursor that is ultimately transformed into eumelanin. On the other hand, TRP-2 catalyzes the rearrangement of

dopachrome to DHICA [37]. It is assumed that pretreatment of HHE inhibits melanin synthesis by suppressing TYR itself and preventing reorganization L-DOPA chrome and oxidizing DHICA to an eumelanin precursor.

The process of melanin synthesis is very complex and involves a number of molecular biological factors [13, 14]. This study considered expressions of p-CREB, CREB-1, and MITF to examine the effect of the HHE on the B16F10 cells in a cAMP/PKA/CREB/MITF cascade [14]. The results of this study show that the HHE treatment downregulates p-CREB and mRNA of *MITF*. The role of MITF as a master regulator in melanocyte development has been extensively studied and established [8, 38]. α-MSH induces the production of cAMP, and the increase in cAMP levels triggers the phosphorylation of the CREB transcription factor, subsequently facilitating MITF activation. This cascade of events positively regulates the transcription TYR, TRP-1, and TRP-2 [39–42]. Thus, this study shows that treatment of the HHE negatively regulates melanogenesis by suppressing p-CREB and MITF, which are transcription factors of key melanogenic enzymes TYR, TRP-1, and TRP-2.

Zebrafish is a valuable model system to evaluate antimelanogenesis activity because it has similar genetic structures and organs to human beings [43]. To confirm the HHE treatment successfully control melanin synthesis without safety issue at an *in vivo* level, this study treated zebrafish with HHE (50 and 100 μg/mL). Melanogenesis in zebrafish is successfully controlled, and more than 90% of zebrafish survived and hatched. Thus, reviewing HHE as a skin-whitening agent in the nutricosmetic industry is reasonable.

5. Conclusions

This is the first report elucidating antimelanogenic activity of Heukharang and/or lactucin *in vitro* and *in vivo*. We suggest the mode of action of the HHE treatment on eumelanogenesis (Figure 6) based on scientific data demonstrating that pretreatment HHE on the skin inhibits melanin synthesis by regulating the CREB/MITF signaling pathway. In addition, lactucin, a well-known bioactive constituent in Heukharang lettuce, is one of the major active compounds in the HHE on antimelanogenesis. Therefore, HHE could be quite useful as a skin-whitening agent, preventing the abnormal accumulation of melanin in the cosmetic industry's melanogenesis process.

Data Availability

The string data that support the findings of this study are included within the article and the supplementary information. Also, the integer data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

H. Ko and Y. J. Shon contributed equally to this work.

Acknowledgments

This research was funded by the Ministry of Science and ICT and by the National Research Foundation of Korea (NRF) grant funded by the Korea Government (MSIT), grant no. 2020R1C1C100467011, by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science & ICT (NRF-2022M3A9I5082349). This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (2022R1A6A1A03055869).

Supplementary Materials

Table S1: mobile phase conditions of HPLC. Figure S1: antioxidant activity of the HHE and effect of HHE treatment on survival and hatching rate of zebrafish. (a) DPPH radical scavenging activity of the HHE. (b) Survival rate (%) of the HHE-treated zebrafish. (c) Hatching rate (%) of the HHE-treated zebrafish. (d) Relative cell viability when treated with arbutin (25–400 μg/mL). (e) Comparison of relative melanin secretion of HHE (100–400 μg/mL) and arbutin (100 μg/mL). Figure S2: chromatogram of lactucin in HHE using HPLC and LC-MS/MS analysis (a) HPLC chromatogram of the HHE. (b) HPLC chromatogram of lactucin. (c) Total ion chromatogram (TIC) of HHE and lactucin. (d) Full-scan MS1 spectrum of HHE and lactucin at 10.45 min (red line of (c)). (e) Fragment ion spectrum (MS/MS) of the precursor ion of m/z 275.09 in the MS1 spectrum. (*Supplementary Materials*)

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