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# Inhibitory effect of glyceollin isolated from soybean against melanogenesis in B16 melanoma cells

*Young-Sang Lee[1,2], Hyun-Kyoung Kim[2], Kyung Ju Lee[2], Hye Won Jeon[2], Song Cui[2], You Mie Lee[1], Byung Jo Moon[1], Yong-Hoon Kim[2,*] & Young-Sup Lee[1,*]*

[1]School of Life Science and Biotechnology, College of Natural Sciences, Kyungpook National University, Daegu 702-701, [2]N&B Co., Ltd., Techno Building 406, Kyungpook National University, Daegu 702-832, Korea

**Natural products with non-toxic and environmentally friendly properties are good resources for skin-whitening cosmetic agents when compared to artificial synthetic chemicals. Here, we investigated the effect of glyceollin produced to induce disease resistance responses of soybean to specific races of an incompatible pathogen, *phytophthora sojae*, on melanogenesis and discussed their mechanisms in melanin biosynthesis. We found that glyceollin inhibits melanin synthesis and tyrosinase activity in B16 melanoma cells without cytotoxicity. To elucidate the mechanism of the effect of glyceollin on melanogenesis, we conducted western blot analysis for melanogenic enzymes such as tyrosinase, tyrosinase-related protein-1 (TRP-1), and TRP-2. Glyceollin inhibited tyrosinase and TRP-1 protein expression. Additionally, glyceollin effectively inhibited intracellular cAMP levels in B16 melanoma cells stimulated by α-melanocyte stimulating hormone (α-MSH). These results suggest that the whitening activity of glyceollin may be due to the inhibition of cAMP involved in the signal pathway of α-MSH in B16 melanoma cells. [BMB reports 2010; 43(7): 461-467]**

## INTRODUCTION

Melanin, which is the major pigment of skin, plays an essential role in protection against UV injury under normal physiological conditions (1-3). Melanin biosynthesis is regulated by melanogenic enzymes such as tyrosinase, tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2) (4). Tyrosinase is a key enzyme involved in melanin synthesis that can catalyze three different reactions, the hydroxylation of tyrosine to 3, 4-dihydroxyphenylalanine (DOPA), the oxidation of DOPA to DOPA quinone, and the oxidation of 5, 6-dihydroxyindole (DHI) to indole-quinone (5). In the absence of thiols, DOPA quinone changes to DOPA chrome and then to DHI or indole 5, 6-quinone 2-carboxylic acid (DHICA). In addition, there are two further steps in this melanogenic pathway, conversion of DOPA chrome to DHICA, which is catalyzed by TRP-2 (DOPA chrome tautomerase), and the oxidation of DHICA, which is catalyzed by TRP-1 (DHICA oxidase). Microphthalmia-associated transcription factor (MITF) strongly stimulates tyrosinase, TRP-1 and TRP-2, which indicates that it is an important regulator of melanogenesis (6-10).

cAMP increases the expression of MITF through activation of the cAMP-dependent protein kinase A (PKA), which in turn stimulates tyrosinase gene expression to allow melanin synthesis (11). Accordingly, up-regulation of the cAMP pathway by forskolin or α-MSH enhances tyrosinase gene expression to allow melanogenesis and the cAMP pathway is a key physiologic regulator of skin and hair pigmentation in mammals, including humans (12, 13).

Currently, numerous reported pharmacologic and cosmetic agents inhibit melanin biosynthesis targets. Many skin lightening products such as linoleic acid, hinokitol, kojic acid, naturally occurring hydroquinone and catechol have been reported to inhibit melanogenesis (14); however, these compounds exhibited side effects, toxicity and low clinical activity. These adverse effects have led to the search for compounds that lack side effects, such as natural molecules derived from plant extract based skin lightening products (15). Therefore, many plants have been investigated to determine their potential for use as cosmetic agents.

Glyceollin is one of a group of phytoalexins produced in soybeans under stress conditions (16, 17). Glyceollin (mixture of glyceollin I, II, and III), which has been produced in high concentrations using several elicitors, exerts antimicrobial activity against several plant pathogens (18, 19). Existing reports previously identified glyceollin as anti-estrogenic agents that may be useful in the prevention or treatment of prostate, breast and ovarian carcinoma (20, 21). However, the effects of glyceollin on melanogenesis have not been evaluated to date. In the present study, we evaluated the inhibitory activity of glyceollin against melanin biosynthesis and its mechanism of action in B16 melanoma cells.

*Corresponding author. Yong-Hoon Kim, Tel: 82-53-954-2074; E-mail: fcchiral@yahoo.co.kr, Young-Sup Lee, Tel: 82-53-950-6353; Fax: 82-53-954-2094; E-mail: yselee@knu.ac.kr

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## RESULTS

### Effects of glyceollin on the cytotoxicity of B16 melanoma cells

Pure glyceollin (mixture of glyceollin I, II, and III) was isolated from elicited soybean using the procedure developed by Boue et al. (22). To determine if pure glyceollin has cytotoxic effects, we treated B16 melanoma cells with glyceollin at various concentrations. The cell viability was then determined by an MTT assay. As shown in Fig. 1, glyceollin does not exert a cytotoxic effect against B16 melanoma cells at concentrations ranging from 0.1-50 μM.

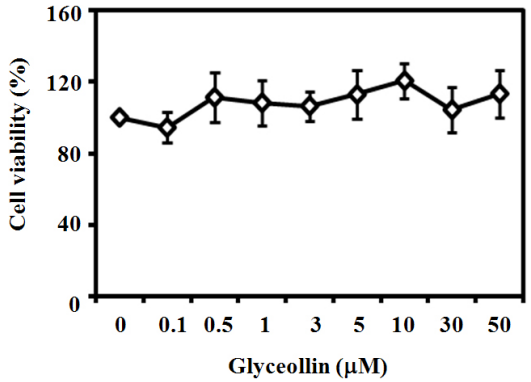


**Fig. 1.** Effect of glyceollin on cell viability in melanoma cells. Melanoma cells ($5 \times 10^3$) were cultured for 48 h with different doses (0.1, 0.5, 1, 3, 5, 10, 30, and 50 μM) of glyceollin. Cell viability was determined by an MTT assay. Values are the means ± SD of three independent experiments.

### Inhibitory effects of glyceollin against α-MSH enhanced melanogenesis and tyrosinase activity

Melanocytes can be stimulated by many effectors, including ultraviolet radiation (UV) and α-MSH. In this study, we used α-MSH (10 nM) to stimulate melanogenesis in B16 melanoma cells. As shown in Fig. 2A, the extracellular melanin contents of cells decreased significantly in the glyceollin range (0.1-10 μM). Moreover, intracellular melanin reduction by glyceollin was observed (Fig. 2B). These results indicate that glyceollin inhibits melanin synthesis in B16 melanoma cells.

Tyrosinase is a key enzyme involved in melanin biosynthesis. Therefore, the inhibition of tyrosinase is a major strategy for development of new whitening agents. The effects of glyceollin on the catalytic activities of tyrosinase are shown in Fig. 2C. The pure glyceollin has inhibitory effects against the oxidation activity of mushroom tyrosinase in a dose-dependent fashion. Furthermore, in the cell-based tyrosinase assay, inhibition of the tyrosinase activity by glyceollin was found to occur in a dose-dependent manner in B16 melanoma cells (Fig. 2D). It should be noted that the reduced melanin contents were attributed to the suppression of tyrosinase activity in a cell-free system and under cellular conditions.

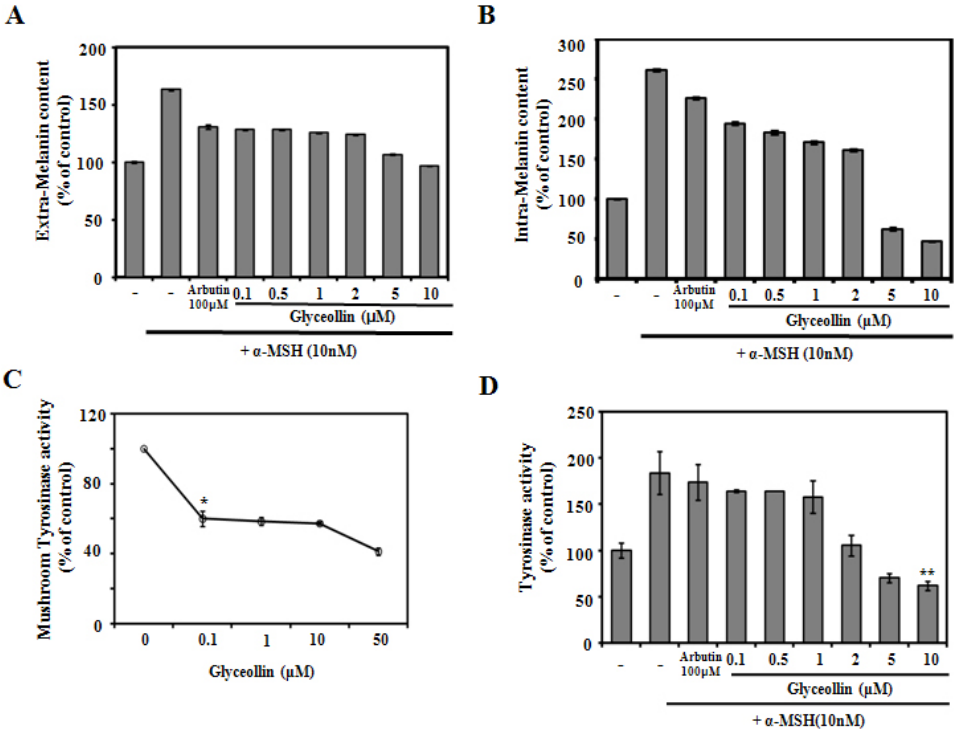


**Fig. 2.** Effect of glyceollin on melanin synthesis and tyrosinase activity in melanoma cells. (A, B) After the melanoma cells were incubated with the indicated dose of glyceollin for 40 h the extra- and intra-melanin content was determined. (C) Cell-free tyrosinase activity was determined using mushroom tyrosinase. (D) The effect on cellular tyrosinase activity was tested at the indicated doses of glyceollin for 40 h. Data are expressed as a percent (%) of the control. Values shown are the means ± SD of three independent experiments. Significantly different from the control group (*$P < 0.05$, **$P < 0.01$).

### Effects of glyceollin on protein and mRNA expression of melanogenic related genes

To characterize the expression of tyrosinase and melanogenic related proteins, Western blot analysis was conducted using specific antibodies. As shown in Fig. 3A, B16 melanoma cells treated with glyceollin showed significantly decreased TRP-1, MITF and tyrosinase protein expression levels when compared with arbutin treated cells as a positive control; however, TRP-2 protein expression was not changed. In addition, an RT-PCR assay of B16 melanoma cells treated with glyceollin and α-MSH was conducted to determine if glyceollin could also suppress the mRNA level of the TRP-1, TRP-2, MITF and tyrosinase genes. As shown in Fig. 3B, the tyrosinase level was significantly decreased by glyceollin when compared with the control. However, we did not observe a change in the TRP-1, TRP-2 and MITF gene expression levels. These results suggested that glyceollin may affect melanin synthesis through tyrosinase.

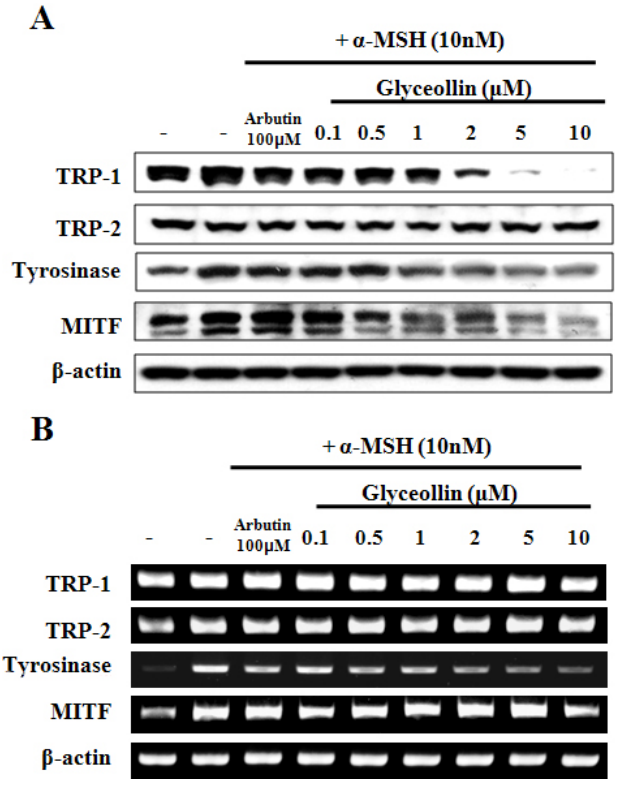


**Fig. 3.** Expression of protein and mRNA in arbutin- and glyceollin-treated melanoma cells. (A) Cells were treated with test compounds for 40 h. Cells were harvested, and the lysates (30 μg protein) were separated using 8% SDS-PAGE, followed by electrophoresis and immunoblotting with antibodies to TRP-1, TRP-2, tyrosinase and MITF. β-actin was used as an internal control. (B) Total RNA was collected from melanoma cells treated with drugs for 24 h. TRP-1, TRP-2, Tyrosinase and MITF expression were investigated using RT-PCR. The findings were normalized against the expression of β-actin. Values shown are the means ± SD of three independent experiments.

### Glyceollin reduced melanogenesis through cAMP signaling

Because glyceollin decreased melanin synthesis and tyrosinase expression, we evaluated glyceollin to determine if it influenced the expression of cAMP, which induces the expression of MITF, a master transcriptional regulator for melanogenic enzymes and tyrosinase family proteins. To accomplish this, we evaluated glyceollin to determine if it reduces cAMP production in B16 melanoma cells. As shown in Fig. 4A, we found that cAMP production decreased upon treatment with glyceollin in a dose dependent manner.

Since we established that glyceollin reduced the cAMP production in B16 cells, we investigated whether it is involved in the down-regulation of MITF and tyrosinase expression. To accomplish this, we conducted western blot using forskolin, which is a cAMP-dependent protein kinase A activator. As shown in Fig. 4B, the presence of glyceollin led to a significant decrease in the expression of MITF and tyrosinase. Consistent with these findings, the tyrosinase activity induced by forskolin was suppressed by glyceollin (Fig. 4C), indicating the involvement of cAMP signaling in glyceollin-inhibited melanogenesis. Collectively, our data indicate that glyceollin-reduced melanogenesis is correlated with cAMP activation.

## DISCUSSION

Soybean plants (*Glycine* max L. Merr.) produce phytoalexin in response to a number of stimuli such as freezing, exposure to UV light, and microorganisms. Phytoalexin are low molecular weight antimicrobial agents that are synthesized by plants and accumulate after exposure to microorganisms (18).

Glyceollin is a phytoalexin found in soybeans that occurs in a series of at least three isomers. This compound often accumulates at the site of infection by pathogens such as *Phytophthora sojae*, *Meloidogyne incognita* and *Pseudomonas syringae* pv. *glycinea*. This group of compounds has a broad interaction between soybeans and microorganisms and exhibits a range of toxicity against nematodes, fungi, insects and bacterial disease (19). Glyceollin has been evaluated with respect to its anti-estrogenic activity that occurs through the inhibition of estrogen receptor α and β when compared with well known phyto-estrogenic photochemicals such as genistein (20). It has also recently been recognized that glyceollin can suppress human breast and ovarian carcinoma tumorigenesis and may modulate potential estrogenic properties in the breast through anti-estrogenic effects (21). However, the biological activity of glyceollin has been poorly researched to date. To the best of our knowledge, the results of the present study provide the first evidence that glyceollin has a potent inhibitory effect against melanogenesis.

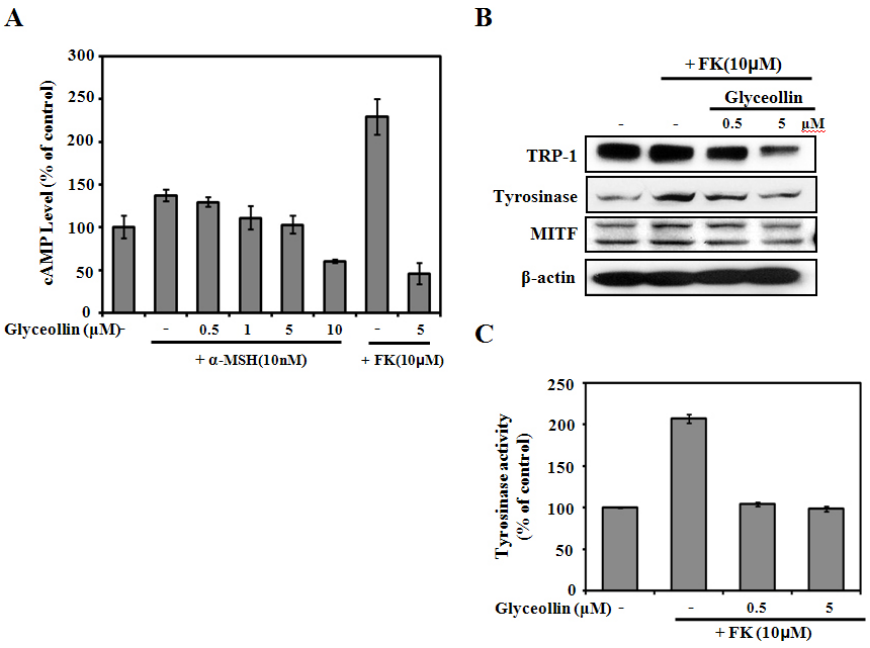


**Fig. 4.** Effect of glyceollin on α-MSH/forskolin-induced cAMP levels in melanoma cells. (A) Melanoma cells pretreated with glyceollin were stimulated with 10 nM α-MSH or 10 μM forskolin (FK) for 40 h. The total amount of cellular cAMP was assayed using enzyme the immunoassay protocol. (B, C) Cells treated with test compounds were induced with 10 μM forskolin (FK) for 40 h. Whole cell lysates were subjected to Western blot analysis using antibodies to TRP-1, TRP-2, tyrosinase and MITF. β-actin was used as an internal control. To test the effects of tyrosinase, tyrosinase activity was measured as described in the Materials and Methods. Values shown are the means ± SD of three independent experiments.

The first step in this study was determination of the potential cytotoxicity of the glyceollin based on its effects on cell viability. As shown in Fig. 1, no significant cytotoxic effect was observed in response to any of the concentrations tested. Next, the inhibitory action of glyceollin against melanogenesis was evaluated. As shown in Fig. 2, glyceollin had an inhibitory effect on melanin synthesis and tyrosinase activity at 0.1-10 μM. To determine the relative efficiency of glyceollin against other melanogenesis inhibitors, the effect of glyceollin was compared with arbutin, which is used as a popular skin-lightening agent. Interestingly, glyceollin (IC50 < 2.8 M) was shown to have a more powerful effect than arbutin (IC50 < 1.25 mM).

To better understand the molecular mechanisms involved in glyceollin-reduced melanogenesis, we evaluated the protein levels of tyrosinase, TRP-1, TRP-2 and MITF. As shown in Fig. 3A, glyceollin reduced the protein levels of tyrosinase, TRP-1 and MITF, but not TRP-2. Indeed, glyceollin significantly inhibited the TRP-1 protein expression in melanoma cells. Next, we evaluated the mRNA levels of tyrosinase, TRP-1, TRP-2 and MITF in glyceollin treated cells. An RT-PCR assay revealed that glyceollin decreased the tyrosinase mRNA level in melanoma cells while TRP-1, TRP-2 and MITF were unchanged. These findings suggest that glyceollin inhibited both the mRNA level and protein level of tyrosinase. However, glyceollin had no influence on the mRNA level of TRP-1 and MITF, even though glyceollin inhibited their protein levels. We assumed that this was due to post-translational events, such as protein degradation, modification, maturation or direct inhibition of the signal transduction pathway. It has been reported that normal human melanocytes respond to α-MSH by increasing the protein levels of TRP-1 and TRP-2 without inducing any noticeable effect on their mRNA (23). In addition, TGF-β1 has been shown to inhibit melanin synthesis through post-translation events leading to decreased protein levels of tyrosinase and TRP-1 (24), which is similar to the results of the present study. However, further investigation is needed to confirm this assumption.

Next, we examined the signaling pathway involved in glyceollin inhibited melanogenesis while focusing on the role of cAMP. It has been reported that α-MSH caused tyrosinase activation and melanogenesis mediated via cAMP activation in melanoma cells. We measured the intracellular cAMP levels in melanoma cells stimulated by α-MSH and forskolin. α-MSH, a peptide acting on the MC1-R of melanocytes, and forskolin, an adenyl cyclase activator, both increased the cellular melanin content in B16 cells (25-27). Glyceollin led to remarkable inhibition of cAMP production in melanoma cells stimulated by α-MSH or forskolin (Fig. 4A). We confirmed that glyceollin had a significantly inhibitory effect on forskolin induced tyrosinase activity and protein expression in melanoma (Fig. 4B, C). Furthermore, glyceollin blocked α-MSH enhanced tyrosinase activity, as well as protein and mRNA expression (Fig. 3). Therefore, glyceollin suppressed α-MSH-induced melanogenesis at the transcriptional level of tyrosinase, suggesting it influences the cAMP-dependent pathway.

Taken together, the results of this study revealed that glyceollin inhibited melanin production via decreased tyrosinase activity and consequent mRNA and protein levels of tyrosinase. We also found that glyceollin reduced MITF activation as well as intracellular cAMP activation, which stimulates MITF. Therefore, utilization of the depigmenting effect of gly-

ceollin might be useful as a cosmetic whitening agent.

## MATERIALS AND METHODS

### Cell culture

B16 melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin and 100 μg/ml streptomycin at 37°C under a humidified atmosphere containing 5% $CO_2$.

### Cell viability assay

B16 melanoma cells were plated at a density of $5 \times 10^3$ per well (96-well plates). After 24 h of culture, test drugs were added, and the cultures were incubated at 37°C for an additional 40 h. The treated cells were stained with MTT reagent [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] for 4 h, after which the precipitated formazan was dissolved by DMSO and the optical density was measured at 540 nm using a spectrophotometer.

### Measurement of melanin content

B16 melanoma cells were individually treated with the test preparations for 40 h. Cells were incubated at 60°C for 1 h in 1 ml of 1 N NaOH and then vortexed to solubilize the melanin, after which the samples were centrifuged for 10 min at 14,000 rpm. Spectrophotometric analysis of the melanin content was then conducted at 415 nm. Next, the melanin content was determined based on the absorbance/μg of protein. The protein concentration of the cells was determined using a BCA protein assay kit.

### Assay of tyrosinase activity in a cell culture free system

Test samples were dissolved in 10% DMSO. Briefly, 250 μl of the aqueous solution of mushroom tyrosinase (135 U/ml), 250 μl of the test sample solution and 250 μl of a 0.03% L-DOPA solution with 50 mM phosphate buffer (pH 6.5) were added to a test tube. The assay mixture was then incubated at 25°C for 90 min in the dark. Following incubation, the amount of dopachrome produced in the reaction mixture was determined based on the absorbance at 415 nm using a spectrophotometer.

### Assay of cellular tyrosinase activity

B16 melanoma cells were treated with the individual test drugs for 40 h, after which the cells were washed with potassium phosphate-buffered saline (PBS) and collected with lysis buffer. Next, the cells were then ruptured by freezing and thawing and the lysate was clarified by centrifugation at 14,000 × rpm for 20 min. The protein content was then determined using a BCA Protein Assay Kit (Thermo). After quantifying the protein levels, the concentrations were adjusted to contain the same amount of protein (20 μg). Each well of the 96-well plate contained the lysate and 10% 2 mg/ml L-DOPA in phosphate solution. Following incubation at 37°C for 1 h, the absorbance was measured at 415 nm using a spectrophotometer.

### Western blot analysis

Proteins were separated by 8% reducing SDS-PAGE and then immunoblotted onto nitrocellulose membranes in 20% methanol, 25 mM Tris, and 192 mM glycine. Membranes were then blocked with 5% non-fat dry milk and incubated with primary antibody overnight. Subsequently, membranes were washed in Tween-Tris buffer saline (TTBS), incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit (1 : 4,000) antibodies for 4 h, rewashed, and finally developed using an enhanced ECL system (KPL Inc., Gaithersburg, MD). The membranes were then reprobed with β-actin antibody as a control for protein loading.

### Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from melanoma cells using an easy-BLUE total RNA extraction kit (iNtRON, Biotechnology, Sungnam, Korea) according to the manufacturer's protocols and then quantified by measuring the absorbance at 260 nm. The RNA was then reverse-transcribed using 2.5 μM oligo-dT primers, 1 mM dNTPs, and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI), after which the resulting cDNA was amplified with Super-Therm DNA polymerase (SR Product, Kent, United Kingdom). The oligonucleotide primers used for the PCR were as follows: tyrosinase (5'-GGCCAGCTTTCAGGCAGAGGT-3' and 5'-TGGT GCATGGGCAAAATC-3'), TRP-1 (5'-GCTGCAGGAGCCTTCT TTCTC-3' and 5'-AAGACGCTGCACTGCTGGTCT), TRP-2 (5'-GGATGACCGTGAGCAATGGCC-3' and 5'-CGGTTGTGACC AATGGGTGGTGCC-3'), MITF (5'-CAGAGGCACCAGGTAAA GCA-3' and 5'-GGATCCATCAAGCCCAAAAT-3'), β-actin (5'-TTCTTCGTTGCCGGTCCACA-3' and 5'-GGTCTCCGGAGTCC ATCA CA-3'. The reaction was cycled 25 times for 30 s at 94 °C, 30 s at 55°C, and 30 s at 72°C. β-actin primers were used to standardize the amount of RNA in each sample. PCR products were resolved on 1.5% agarose gels and visualized by ethidium bromide staining.

### cAMP measurement assay

The cAMP concentration was measured using a cAMP immunoassay kit (Cayman, Ann Arbor, MI, USA). Briefly, B16 melanoma cells ($3 \times 10^5$) were lysed in 0.1 M HCl to inhibit the phosphodiesterase activity. The supernatants were then collected, neutralized, and diluted. After neutralization and dilution, a fixed amount of cAMP conjugate was added to compete with cAMP in the cell lysate for sites on rabbit polyclonal antibody immobilized on a 96 well plate. After washing to remove excess conjugated and unbound cAMP, substrate solution was added to the wells to determine the activity of the bound enzyme. The color development was then stopped, after which the absorbance was read at 415 nm. The intensity of

the color was inversely proportional to the concentration of cAMP in the cell lysate.

### Statistical analysis

The data for the melanin synthesis, cytotoxicity, and tyrosinase activity assay were statistically evaluated using an ANOVA-test followed by Dunnett's test. The data are given as the means ± SD. A P value of $<0.05$ was considered significant.

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