Department of Chemistry and Cosmetics[1], Jeju National University, Jeju; Department of Chemistry BT-Convergent Pharmaceutical Engineering[2], Sunmoon University, Chungnam, Republic of Korea

8-Methoxycoumarin enhances melanogenesis *via* the MAPKase signaling pathway

You Chul Chung[1,#], Seung-Young Kim[2,#], Chang-Gu Hyun[1,*]

Received May 8, 2019, accepted June 7, 2019

**Corresponding author: C-G. Hyun, Department of Chemistry and Cosmetics, Jeju National University, Jeju 63243, Republic of Korea*
cghyun@jejunu.ac.kr
[#]These authors contributed equally to this work

Pharmazie 74: 529-535 (2019) *doi: 10.1691/ph.2019.9553*

8-Methoxycoumarin (8-methoxy-chromen-2-one), isolated from *R. graveolens* L., is able to alleviate arthritis by inhibition of proinflammatory cytokines. However, its effects on melanogenesis have largely remained unreported. The present study examined the effects of 8-methoxycoumarin on melanogenesis in B16F10 murine cells, together with its effect on the mechanism of melanin synthesis. The cells were treated with different concentrations of 8-methoxycoumarin; α-MSH was used as the positive control. We found 8-methoxycoumarin to significantly increase the melanin content of the cells without exerting any cytotoxicity. In addition, it significantly upregulated the expression of tyrosinase and tyrosinase-related protein-1 and 2 *via* inducing the expression of microphthalmia-associated transcription factor. Furthermore, we demonstrated the involvement of mitogen-activated protein kinase (MAPK) pathway-mediated phosphorylation of p38 and c-Jun N-terminal kinase (JNK), and inhibition of phosphorylation of extracellular signal-regulated kinase (ERK) to be responsible for enhanced melanin production. Use of SB203580 (p38 inhibitor) and SP600125 (p-JNK inhibitor) corroborated these findings. Additionally, we investigated the effects of 8-methoxycoumarin on protein kinase B (AKT) phosphorylation and protein kinase A (PKA) signaling pathway (using H89, a PKA inhibitor). These results suggested that 8-methoxycoumarin increases melanogenesis *via* the MAPK signaling pathway. Based on these findings, we conclude that 8-methoxycoumarin could serve as a potential compound for treating hypopigmentation disorders. It could also serve as a promising chemical for hair depigmentation treatment in the cosmetic industry.

1. Introduction

Melanin is the major pigment produced by specialized organelles, called melanosomes present in the melanocytes (Bonaventure et al. 2013; Briganti et al. 2003). Melanocytes are dendritic cells derived from the neuroectoderm and are found in the basal layer of the skin epidermis; these produce vesicles called melanosomes and deliver them to the surrounding keratinocytes (Sturm et al. 2001; Tadokoro et al. 2005; Schadendorf et al. 2015; Gibbs et al. 2000; Cichorek et al. 2013). Melanin has many types, such as the brownish black eumelanin and reddish yellow pheomelanin, which are responsible for determining the skin and hair color (Thody et al. 1991; Lamoreux et al. 2001; Hearing et al. 2005). Another important function of melanin in the epidermis is to prevent the skin from damage from harmful UV radiation by scavenging free radicals or dispersing the incoming UV light (Anderson et al. 1981; Brenner et al. 2008). Consequently, a defective melanin synthesis or melanogenesis leads to hypopigmentation and increases the risk of skin cancer and various pigmentary disorders.

Melanin biosynthesis is a complex process that occurs inside melanocytes and involves tyrosinase and other melanogenic enzymes, such as tyrosinase-related protein (TRP)-1 and 2. Tyrosinase serves as the rate-limiting enzyme in a two-step synthesis of melanin: It catalyzes the hydroxylation of l-tyrosine to 3,4-dihydroxyphenyl-l-alanine (l-DOPA; diphenol) and oxidation of l-DOPA to dopaquinone (Hearing et al. 1987). The expression of tyrosinase is regulated by microphthalmia-associated transcription factor (MITF) that binds to the M-box of the tyrosinase promoter to activate its expression and of other melanogenic enzymes (Bentley et al. 1994; Vachtenheim et al. 2010).

Recent studies have implicated mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 in melanogenesis through regulation of MITF expression (Ahn et al. 2008; Widlude et al. 2003; Saha et al. 2006). An increased phosphorylation of p38 and JNK increases the expression of MITF and melanogenic enzymes, leading to melanogenesis (Kang et al. 2015; Han et al. 2016). In addition, a reduced phosphorylation of ERK increases the transcription of tyrosinase, which activates melanogenesis (Wu et al. 2011). Thus, regulation of the MAPK signaling pathway is considered a strategic target for the regulation of melanogenesis. Furthermore, c-AMP-dependent protein kinase A (PKA) has been reported to activate MITF transcription via phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) (Chan et al. 2014; Kim et al. 2013). Other reports suggest inhibition of the phosphatidylinositol-3-kinase PI3K/AKT signaling pathway to upregulate melanogenensis by MITF degradation (Kim et al. 2015; Chung et al. 2018).

Recently, many researchers have focused on melanogenesis stimulators as potential options to treat hypopigmentation of skin and hair (Niu et al. 2017). For instance, several compounds, especially methylated compounds isolated from natural sources, have been reported to induce melanogenesis in B16F10 cells (Niu et al. 2017; Alesiani et al. 2009; Yang et al. 2006; Yoon et al. 2015; Chung et al. 2017; Yoon et al. 2007).

Ruta graveolens L., an herb commonly known as rue, has been used in traditional medicine for treating diseases such as rheumatism and dermatitis, and eye ailments (Gentile et al. 2015). Recently, a study demonstrated the inhibitory activity

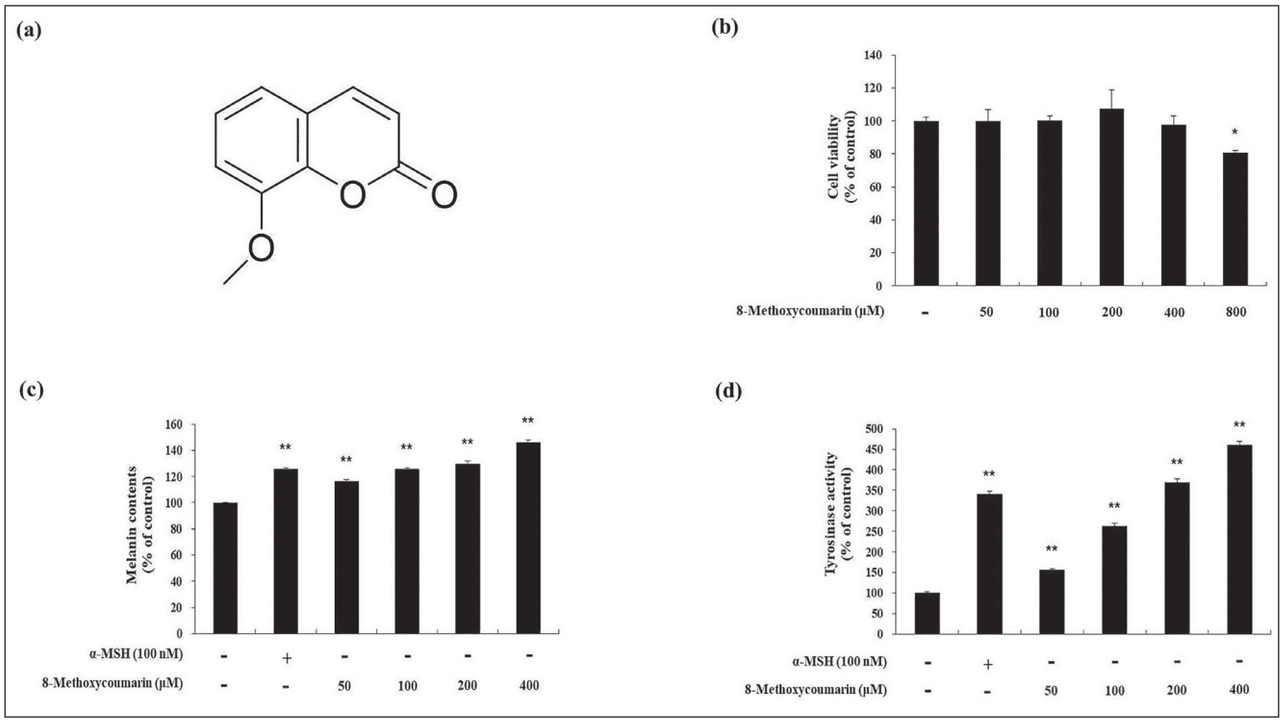

Fig. 1: (a) Structure of 8-methoxycoumarin. (b) The cell viability test that B16F10 cells were treated with 8-methoxycoumarin (50, 100, 200, 400, and 800 μM) for 48 h. (c) The melanin content, and (d) tyrosinase activity test that B16F10 cells were treated with 8-methoxycoumarin (50, 100, 200, and 400 μM) for 72 h. α-MSH (100 nM) was used as the positive control. Cell viability, melanin content, and tyrosinase activity test are expressed as percentages compared to the respective values obtained for the control. The data are presented as mean ± standard deviation (SD) of at least three independent experiments. * indicates $p < 0.001$, ** indicates $p < 0.001$.

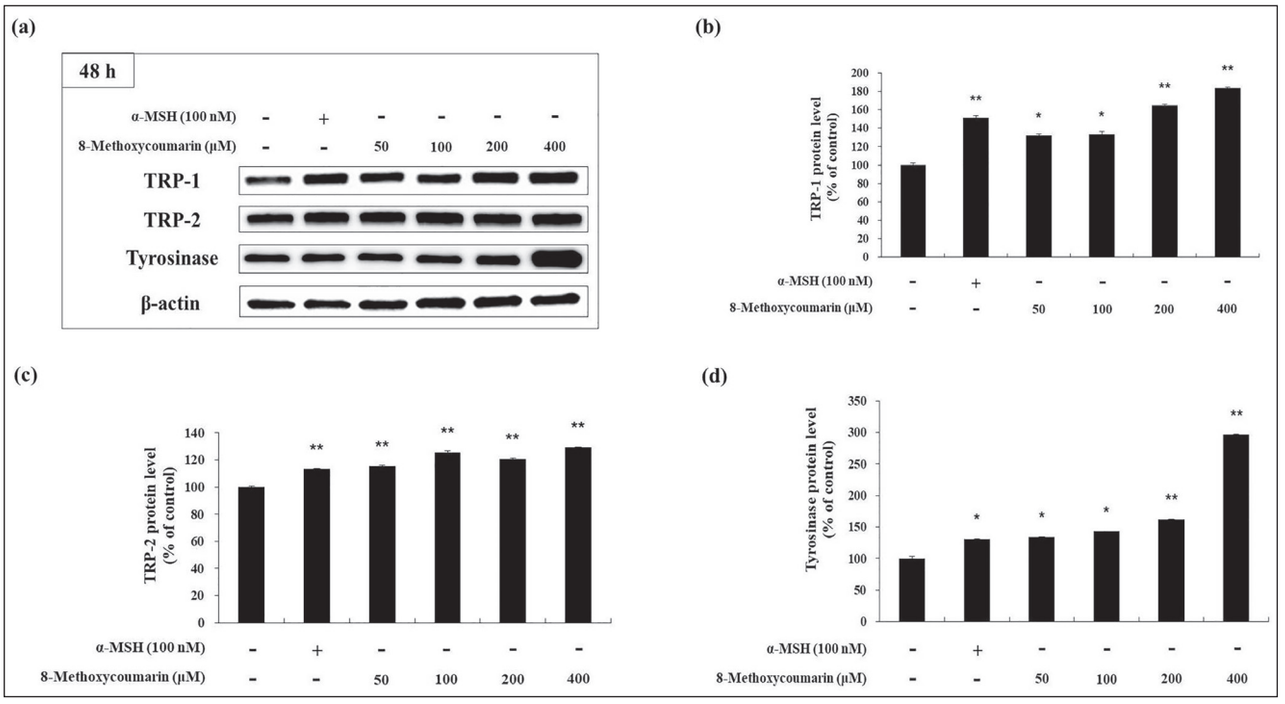

Fig. 2: Effect of 8-methoxycoumarin on TRP-1, TRP-2, and tyrosinase expression in B16F10 cells. Cells were treated with various concentrations of 8-methoxycoumarin (50, 100, 200, and 400 μM) for 48 h. Protein levels were examined by western blotting. (a) Result of western blotting, and protein levels of (b) TRP-1, (c) TRP-2, and (d) tyrosinase. Results are expressed as a percentage of the control. The data are presented as mean ± SD of at least three independent experiments. * indicates $p < 0.01$, whereas ** indicates $p < 0.001$. SD: standard deviation, TRP: tyrosinase-related protein.

of 8-methoxycoumarin (8-methoxy-chromen-2-one), isolated from *R. graveolens* L. against proinflammatory cytokines (Sahu et al. 2015). However, its effect on melanogenesis has not been elucidated. Thus, the present study investigated the melanogenic effect of 8-methoxycoumarin on B16F10 cells and the underlying molecular mechanisms.

2. Investigations and results

2.1. Effect of 8-methoxycoumarin on cell viability of B16F10 cells

To determine whether 8-methoxycoumarin has a cytotoxic effect on B16F10 melanoma cells, we investigated cell viability using

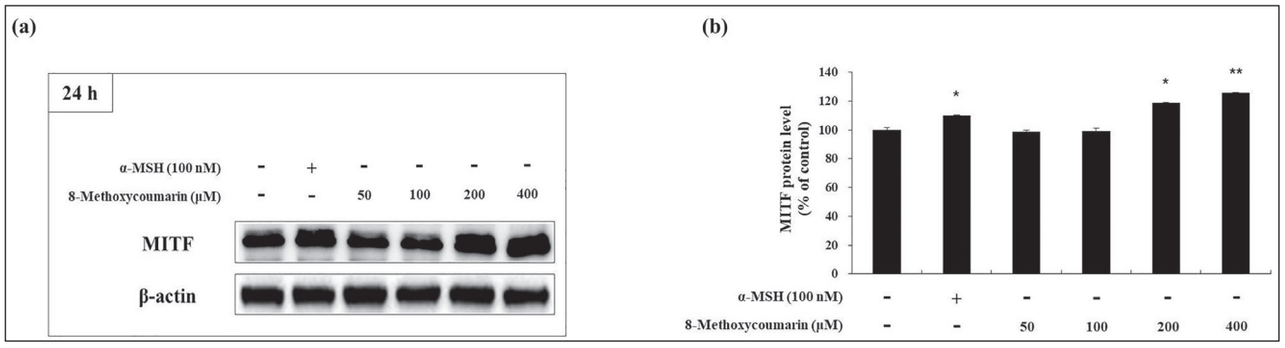

Fig. 3: Effect of 8-methoxycoumarin on MITF expression in B16F10 cells. Cells were treated with various concentrations of pratol (50, 100, 200, and 400 μM) for 24 h. (a) Result of western blotting, and (b) protein levels MITF were examined by western blotting. Results are expressed as a percentage of the control. The data are presented as mean ± SD of at least three independent experiments. * indicates $p < 0.01$, whereas ** indicates $p < 0.001$. MITF: microphthalmia-associated transcription factor, SD: standard deviation.

the MTT assay. Briefly, the cells were treated with various concentrations of 8-methoxycoumarin (50, 100, 200, 400, and 800 μM) for 48 h. No significant differences on cell viability were observed with concentrations ranging from 50 to 400 μM. However, the cells treated with 800 μM MTT showed a 20% decrease in viability, indicating cytotoxicity (Fig. 1B). Therefore, we used 50, 100, 200, and 400 μM concentrations of 8-methoxycoumarin for further investigations.

2.2. Effect of 8-methoxycoumarin on melanin production

To verify the effects of 8-methoxycoumarin on melanin synthesis, we examined melanin content in cells treated with various concentrations of 8-methoxycoumarin for 72 h. We observed a dose-dependent increase in the melanin content upon treatment with 8-methoxycoumarin. In particular, 8-methoxycoumarin at 400 μM enhanced the intracellular melanin content by 46% as compared with the untreated group, which was 18% higher than that observed for the positive control (Fig. 1C).

2.3. Effect of 8-methoxycoumarin on intracellular tyrosinase activity

Tyrosinase is the key enzyme for inducing melanogenesis in B16F10 cells. To determine the effect of 8-methoxycoumarin on melanogenesis, we examined the intracellular tyrosinase activity in 8-methoxycoumarin-treated B16F10 cells. As shown in Fig. 1D, all concentrations of 8-methoxycoumarin increased the tyrosinase activity as compared with the control group. In particular, 400 μM of 8-methoxycoumarin increased the tyrosinase activity by 360% as compared with the untreated group, which was 120% higher than that observed for the positive control.

2.4. Effect of 8-methoxycoumarin on expression of melanogenic enzymes and MITF in B16F10 cells

The expression of melanogenic enzymes, such as TRP-1, TRP-2, and tyrosinase, is related to melanogenesis in B16F10 cells (Bentley et al. 1994; Vachtenheim et al. 2010). On the other hand, MITF

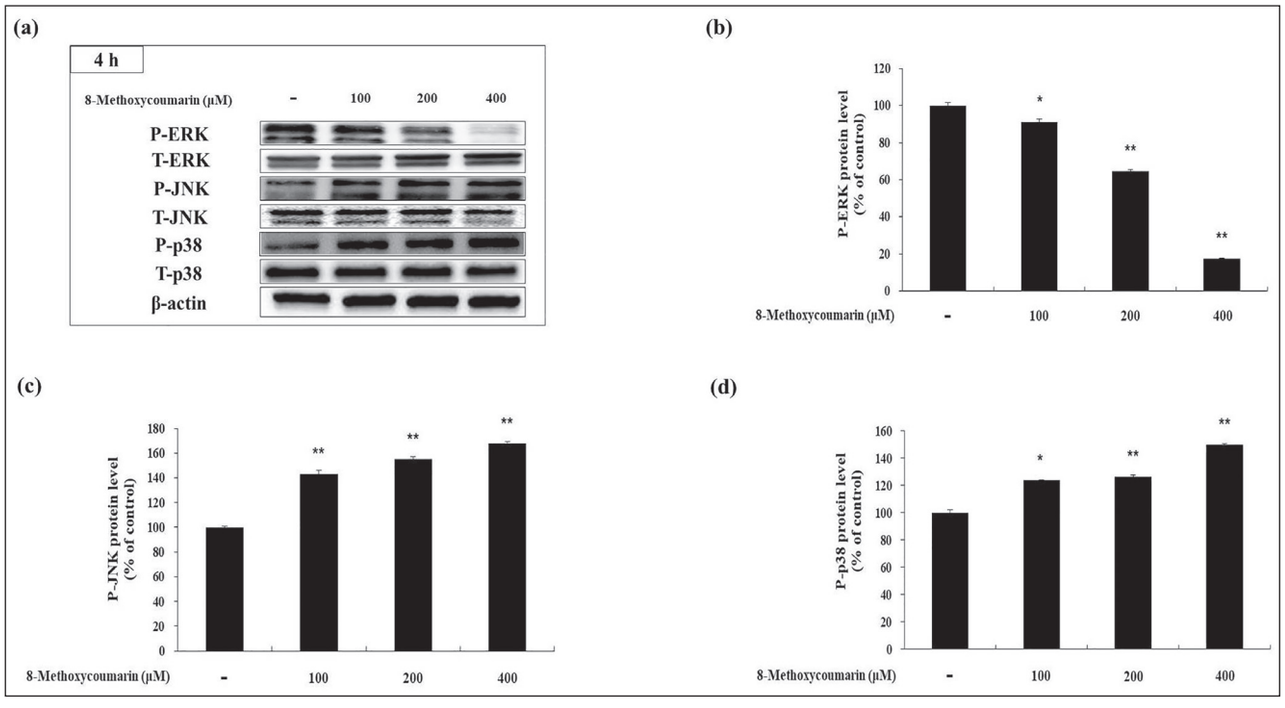

Fig. 4: Effects of 8-methoxycoumarin on phosphorylation of p-ERK, p-p38, and p-JNK. The B16F10 cells were treated with 8-methoxycoumarin at the indicated concentrations for 4 h. (a) Result of western blotting and protein levels of (b) p-ERK, (c) p-JNK, and (d) p-p38. The data are presented as mean ± SD of at least three independent experiments. * indicates $p < 0.01$, whereas ** indicates $p < 0.001$. ERK, extracellular signal-regulated kinase, JNK: c-Jun N-terminal kinase, P: Phosphorylated, S: standard deviation, T: Total.

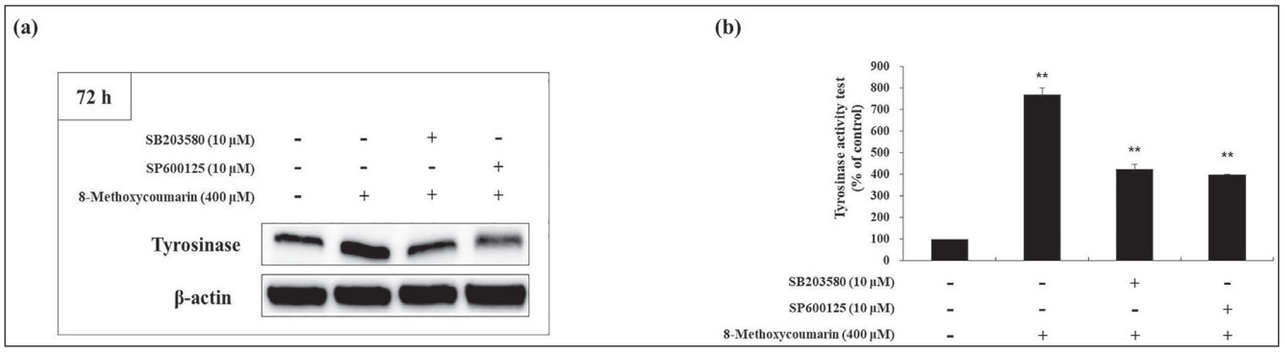

Fig. 5: Effect of MAPK inhibitors on 8-methoxycoumarin-induced tyrosinase activation in B16F10 cells. To determine the involvement of MAPK enzymes in melanogenesis, (a) western blotting and (b) a cellular tyrosinase activity were conducted using the following MAPK inhibitors: SP600125 (JNK inhibitor) and SB203580 (p38 inhibitor). The data are presented as mean ± SD of at least three independent experiments. ** indicates $p < 0.001$. JNK: c-Jun N-terminal kinase, MAPK: mitogen-activated protein kinase, SD: standard deviation.

is an important transcriptional regulator that binds to the M-box in the promoters of melanogenic enzymes, thereby inducing their expression. To determine the effect of 8-methoxycoumarin on the expression of MITF and melanogenic enzymes, western blotting was performed in B16F10 cells. As shown in Fig. 2, the expression of TRP-1 and tyrosinase was significantly increased in 8-methoxycoumarin-treated B16F10 cells. To understand the relationship between melanogenic enzymes and MITF expression, we treated B16F10 cells with various concentrations of 8-methoxycoumarin. Figure 3 depicts western blot results, showing a dose-dependent increase in MITF expression. Therefore, these results suggested that 8-methoxycoumarin enhances expression of melanogenic enzymes by increasing MITF expression.

2.5. Effect of 8-methoxycoumarin on MAPK phosphorylation in B16F10 cells

Recent studies have reported MAPK signaling pathways to be related to melanin production (Ahn et al. 2008; Widlude et al. 2003; Saha et al. 2006). To determine the effect of 8-methoxycoumarin on MAPK phosphorylation, western blot analysis was performed in B16F10 cells treated with various concentrations of 8-methoxycoumarin. As shown in Fig. 4, 8-methoxycoumarin dramatically inhibited phosphorylation of ERK while increased phosphorylation of JNK and p38 in a dose-dependent manner. To determine whether an increase in JNK and p38 phosphorylation upregulates tyrosinase production, we treated the cells with

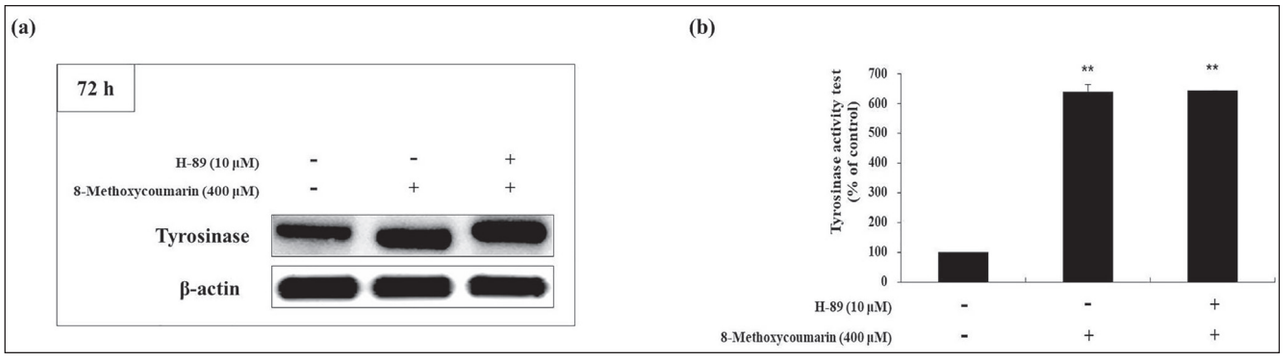

Fig. 6: Involvement of cAMP pathway in melanogenesis. The PKA inhibitor (H-89) was subjected to (a) western blotting assay and (b) a cellular tyrosinase activity test. The data are presented as mean ± SD of at least three independent experiments. ** indicates $p < 0.001$. cAMP: cyclic adenosine monophosphate, PKA: protein kinase A.

specific inhibitors of JNK and p38 pathways, namely SP600125 and SB203580. As shown in Fig. 5, western blot and tyrosinase activity test results indicated a reduced tyrosinase expression after treatment with inhibitors as compared with 8-methoxycoumarin treatment alone.

2.6. Effect of 8-methoxycoumarin on PKA signaling pathway

Activation of PKA causes phosphorylation of CREB, which, in turn, induces MITF expression, resulting in melanogenesis *via* increased expression of melanogenic enzymes, such as TRP-1, TRP-2, and tyrosinase (Chan et al. 2014; Kim et al. 2013). To understand the involvement of PKA signaling pathway in 8-methoxycoumarin-regulated melanogenesis, B16F10 cells were treated with H-89 (PKA inhibitor) in the presence or absence of 8-methoxycoumarin. As shown in Fig. 6, western blot and tyrosinase activity test results showed that the combination of 8-methoxycoumarin and H-89 did not result in a significant change in PKA activity as compared to 8-methoxycoumarin treatment alone. These results indicated 8-methoxycoumarin was not related to PKA signaling pathway in enhancing tyrosinase expression in B16F10 cells.

2.7. Effect of 8-methoxycoumarin on AKT signaling pathway

Recent studies have reported the role of PI3K/AKT signaling pathways in regulating melanogenesis (Kim et al. 2015; Chung et al. 2018). To determine the effect of 8-methoxycoumarin on AKT phosphorylation in B16F10 cells, western blot analysis was performed after treatment of cells with varying concentrations of 8-methoxycoumarin. The result showed a dose-dependent increase in the expression of AKT and its phosphorylation (Fig. 7). To further understand the involvement of AKT signaling pathway in 8-methoxycoumarin-regulated melanogenesis, B16F10 cells were treated with LY294002 (AKT inhibitor). As shown in Fig. 8, treatment with 8-methoxycoumarin increased tyrosinase expression as compared with untreated cells. In addition, treatment with

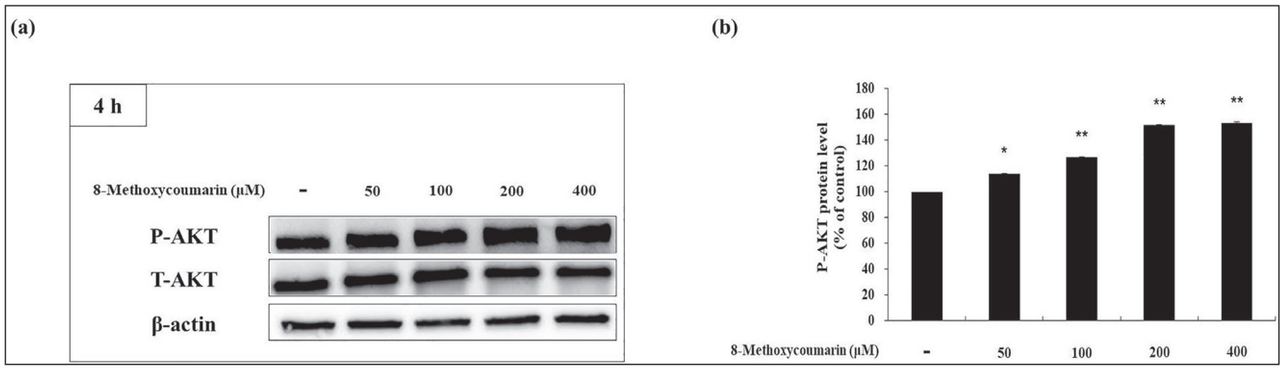

Fig. 7: Effects of 8-methoxycoumarin on AKT phosphorylation. The B16F10 cells were treated with various concentrations of 8-methoxycoumarin (50, 100, 200, and 400 μM) for 4 h. (a) Protein expression levels were investigated by western blotting. (b) Results are expressed as a percentage of the control. The data are presented as mean ± SD of at least three independent experiments. * indicates $p < 0.01$, whereas ** indicates $p < 0.001$. P: Phosphorylated, SD: standard deviation, T: Total.

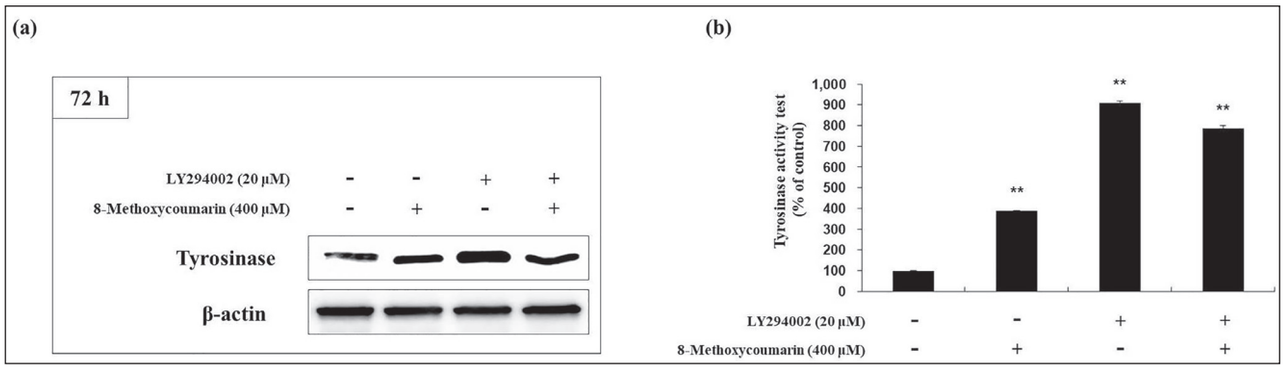

Fig. 8: Effect of AKT inhibitors on 8-methoxycoumarin-induced tyrosinase activation in B16F10 cells. To determine the involvement of AKT in melanogenesis, (a) western blotting assay and (b) a cellular tyrosinase activity were conducted using LY294002 as the AKT inhibitor. The data are presented as mean ± SD of at least three independent experiments. ** indicates $p < 0.001$. SD: standard deviation.

the inhibitor significantly enhanced the expression of tyrosinase as compared with 8-methoxycoumarin treatment alone. However, a combination of the inhibitor and 8-methoxycoumarin resulted in a decreased tyrosinase expression as compared with inhibitor treatment alone. These results suggested 8-methoxycoumarin-induced increase in melanin to be independent of the AKT signaling pathway.

3. Discussion

Melanogenesis, the production of melanin pigments, is an important process that is responsible for the specific color of skin and hair. Several compounds have been isolated from natural sources that are able to stimulate melanogenesis in B16F10 cells (Niu et al. 2017). For example, coumarins that are widely distributed in many plants, such as *Ammi majus* L., *Psoralea corylifolia* L., *Ficus carica* L., and *R. graveolens* L., and isolated from their seeds, leaves, and fruits, are known to regulate the biosynthesis of melanin (Späth et al. 1936; Ekiert et al. 2000; Innocenti et al. 1982). In addition, various coumarin derivatives, especially methoxy-coumarins, have been reported to increase melanogenesis (Alesiani et al. 2009; Yang et al. 2006). The current study evaluated the effects of 8-methoxycoumarin on melanogenesis in B16F10 murine melanoma cells with respect to melanin content and tyrosinase activity. Further, to understand the mechanism of melanogenesis in B16F10 cells, we studied the expression of tyrosinase gene family (TRP-1, TRP-2, and tyrosinase), as well as their regulators, such as MITF, and signaling pathways. The results of our study show that 8-methoxycoumarin induces melanogenesis in B16F10 melanoma cells. Western blot analysis confirmed the phosphorylation of AKT and MAPK, regulators of MITF expression. Increased MITF expression was shown to be responsible for enhanced tyrosinase expression observed in 8-methoxycoumarin-treated cells.

The MTT assay showed 8-methoxycoumarin to exert cytotoxicity at concentrations above 800 μM; however, it did not affect cell viability at concentrations ranging from 50 to 400 μM. In addition, 8-methoxycoumarin treatment resulted in a dose-dependent increase in tyrosinase activity and melanin content (Fig. 1).

Recent studies have reported the tyrosinase gene family to be positively regulated by MITF (Hearing et al. 1987; Jiménez-Cervantes et al. 1994; Tsukamoto et al. 1992), resulting in activation of the tyrosinase gene family and stimulation of melanogenesis in B16F10 cells. This is evident by the increased expression of MITF as well as TRP-1 and tyrosinase in 8-methoxycoumarin-treated cells in a dose-dependent manner (Fig. 2 and 3). Thus, 8-methoxycoumarin increases the expression of MITF, which results in increased TRP-1 and tyrosinase expression and finally increased melanin synthesis.

Recently, phosphorylation of various signaling pathway proteins, such as MAPK, p38, and JNK, has also been linked to enhanced melanogenesis by increasing MITF expression. In addition, previous studies have identified phosphorylated ERK to induce MITF degradation and thus melanogenesis (Ahn et al. 2008; Widlude et al. 2003; Saha et al. 2006; Kang et al. 2015; Han et al. 2016; Wu et al. 2011). The western blot analysis in the present study demonstrated 8-methoxycoumarin to hyperphosphorylate p38 and JNK and decrease ERK phosphorylation. We further checked tyrosinase expression using SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor) to study the relation of this pathway to melanogenesis. The reduced expression of tyrosinase in 8-methoxycoumarin-treated B16F10 cells post inhibitor treatment indicated the role of MAPK signaling pathway in 8-methoxycoumarin-induced melanogenesis in B16F10 cells (Figs. 4 and 5). Results from the present study show the ability of 8-methoxycoumarin to induce melanogenesis in B16F10 murine melanoma cells without exerting cytotoxicity. In addition, 8-methoxycoumarin decreased expression of p-ERK and

increased that of p-p38 and p-JNK in the MAPK pathways, with no involvement of AKT and PKA signaling pathways. To conclude, 8-methoxycoumarin may serve as a potential compound for treating hypopigmentation disorders and for development of skin-tanning and anti-gray hair products.

4. Experimental

4.1. Chemicals and reagents

8-Methoxycoumarin, dimethyl sulfoxide (DMSO), α-MSH, NaOH, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), radioimmunoprecipitation assay (RIPA) buffer, L-DOPA, and H-89 (PKA inhibitor) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and trypsin-ethylenediaminetetraacetic acid were purchased from Thermo Fisher Scientific (Waltham; MA, USA). LY294002 and antibodies against tyrosinase, TRP-1, TRP-2, and MITF were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against p-p38, p38, p-JNK, JNK, p-ERK, ERK, p-AKT, AKT, and β-actin were procured from Cell Signaling Technology (Danvers, MA, USA). SP600125 (JNK inhibitor) and SB203580 (p38 inhibitor) were purchased from Cayman Chemical (Ann Arbor, MI, USA) and Calbiochem (San Diego, CA, USA), respectively. Enhanced chemiluminescence (ECL) kit and 2× Laemmli sample buffer were obtained from Biosesang (Seongnam, Gyeonggi-do, Korea) and Bio-Rad (Hercules, CA, USA), respectively.

4.2. Cell culture

Murine melanoma cells, B16F10, were purchased from the Korean Cell Line Bank (Seoul, Korea). These were cultured in DMEM supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10 % FBS. The cells were incubated in a humidified atmosphere containing 5 % CO_2 at 37 °C and were sub-cultured every 4 days.

4.3. Measurement of cell viability

The B16F10 cells (1.5 × 10^{1}4 cells/well) were pre-incubated into 24–well plates for 24 h; the cells was treated with various concentrations of 8-methoxycoumarin (50, 100, 200, 400, and 800 μM) for 48 h. The MTT solution was added to the wells for 3 h, after which the solution was removed and the insoluble formazan crystals were dissolved in 1 mL DMSO for 1 h. The solution in each well of the 24-well plate was transferred to a 96-well plate, and absorbance of the wells was measured at 540 nm using a microplate reader (Tecan; Männedorf, Switzerland).

4.4. Measurement of intracellular melanin content

The B16F10 cells were treated in a 6-well plate with various concentrations of 8-methoxycoumarin (50, 100, 200, and 400 μM) and α-MSH (100 nM) for 72 h. Here, α-MSH served as the positive control. After removal of DMEM, cells were twice washed with cold phosphate-buffered saline (PBS) and dissolved in 1 N NaOH for 1 h at 80 °C. Next, 200 μL of aliquots of the media were placed into 96-well plates, and absorbance was measured at 405 nm using a microplate reader. All experiments were performed in triplicate.

4.5. Measurement of intracellular tyrosinase activity

The B16F10 melanoma cells (1.5 × 10^5 cells/well were seeded into 100-mm dishes and treated with various concentrations of 8-methoxycoumarin (50, 100, 200, and 400 μM) and α-MSH (100 nM) for 72 h. After DMEM was removed, cell pellets were collected into micro-tubes and lysed with RIPA buffer containing 1 % protease inhibitor cocktail. Lysates were clarified by centrifugation at 15,000 rpm for 30 min. The amount of protein in each lysate was quantified using the bicinchoninic acid (BCA) kit. Next, 20 μL of each lysate was mixed with 80 μL of L-DOPA (2 mg/mL) in a 96-well plate. After incubation at 37 °C for 2 h, the absorbance was measured at 490 nm using a microplate reader.

4.6. Western blot analysis

The B16F10 cells were seeded into 100-mm dishes and then incubated with α-MSH (100 nM) and various concentrations of 8-methoxycoumarin (50, 100, 200, and 400 μM). After incubation, the cells were washed thrice with cold PBS and treated with trypsin/EDTA to collect cell pellets into 1.5 mL micro-tubes. The cells were then lysed for 1 h in RIPA buffer containing 1% protease inhibitor cocktail. After centrifugation at 15,000 rpm for 15 min at -4 °C, the supernatant was collected into a new micro-tube. The amount of protein in each cell lysate was quantified using the BCA kit. After adjusting the protein levels, the western blot samples were denatured at 100°C for 5 min, following which 20 μL of each sample was loaded onto 10% sodium dodecyl sulfate (SDS) polyacrylamide gels for 1 h at 150 V. The gels were transferred to polyvinylidene difluoride membranes for 7 min. Membranes were blocked using TBST containing 5 % skim milk solution for 1 h. These were then washed six times every 10 min with Tris-buffered saline (TBS; 20 mM Tris base, 137 mM NaCl, pH 7.6) containing 0.4% Tween 20 (TBST) for 1 h. Next, the membranes were incubated for 24 h at 4 °C with specific primary antibodies (1:1,000). The membranes were washed with TBST every 10 min for 1 h to wash off any unbound primary antibody; each membrane was then incubated with rabbit anti-mouse secondary antibody (IgG, 1:3,000) for 1 h, and then washed with TBST every 10 min for 1 h. The antibody-bound protein bands were detected using the ECL kit.

4.7. Statistical analysis

Student's *t*-test was used for statistical analysis and $p < 0.01$ (*) or $p < 0.001$ (**) was considered statistically significant. All data are expressed as means ± SD of at least three independent experiments.

Acknowledgements: This research was financially supported by the Ministry of Trade, Industry, and Energy (MOTIE), Korea, under the "Regional Specialized Industry Development Program"(reference number P0006063) supervised by the Korea Institute for Advancement of Technology (KIAT).

Conflicts of interest: None declared.

References

Ahn JH, Jin SH, Kang HY (2008) LPS induces melanogenesis through p38 MAPK activation in human melanocytes. Arch Dermatol Res 300: 325–329.

Alesiani D, Cicconi R, Mattei M, Bei R, Canini A (2009) Inhibition of Mek 1/2 kinase activity and stimulation of melanogenesis by 5,7-dimethoxycoumarin treatment of melanoma cells. Int J Oncol 34: 1727-1735.

Anderson RR, Parrish JA (1981) The optics of human skin. J Invest Dermatol 77: 13-19.

Bentley NJ, Eisen T, Goding CR (1994) Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. Mol Cell Biol 14: 7996-8006.

Bonaventure J, Domingues MJ, Larue L (2013) Cellular and molecular mechanisms controlling the migration of melanocytes and melanoma cells. Pigment Cell Melanoma Res 26: 316-325.

Brenner M, Hearing VJ (2008) The protective role of melanin against UV damage in human skin. Photochem Photobiol 84: 539-549.

Briganti S, Camera E, Picardo M (2003) Chemical and instrumental approaches to treat hyperpigmentation. Pigment Cell Res 16: 101-110.

Chan CF, Huang CC, Lee MY, Lin YS (2014) Fermented broth in tyrosinase- and melanogenesis inhibition. Molecules 19: 13122-13135.

Chung YC, Kim S, Kim JH, Lee GS, Lee JN, Lee NH, Hyun CG (2017) Pratol, an O-methylated flavone, induces melanogenesis in B16F10 melanoma cells via p-p38 and p-JNK upregulation. Molecules 22: E1704.

Chung YC, Ko JH, Kang HK, Kim S, Kang CI, Lee JN, Park SM, Hyun CG (2018) Antimelanogenic effects of *Polygonum tinctorium* flower extract from traditional jeju fermentation via upregulation of extracellular signal-regulated kinase and protein kinase B activation. Int J Mol Sci 19: 2895.

Cichorek M, Wachulska M, Stasiewicz A, Tymińska A (2013) Skin melanocytes: biology and development. Postępy Dermatol Alergol 30: 30-41.

Ekiert H, Gomółka E (2000) Coumarin compounds in *Ammi majus* L. callus cultures. Pharmazie 55: 684-687.

Gentile MT, Ciniglia C, Reccia MG, Volpicelli F, Gatti M, Thellung S, Florio T, Melone MA, Colucci-D'Amato L (2015) *Ruta graveolens* L. induces death of glioblastoma cells and neural progenitors, but not of neurons, via ERK 1/2 and AKT activation. PLoS One 10: e0118864.

Gibbs S, Murli S, de Boer G, Mulder AAT, Mommaas AM, Ponec M (2000) Melanosome capping of keratinocytes in pigmented reconstructed epidermis-effect of ultraviolet radiation and 3-isobutyl-1-methyl-xanthine on melanogenesis. Pigment Cell Res 13: 458-466.

Han JS, Sung JH, Lee SK (2016) Antimelanogenesis activity of hydrolyzed ginseng extract (GINST) via inhibition of JNK mitogen-activated protein kinase in B16F10 Cells. J Food Sci 81: H2085-H2092.

Hearing VJ (2005) Biogenesis of pigment granules: a sensitive way to regulate melanocyte function. J Dermatol Sci 37: 3-14.

Hearing VJ, Jimenez M (1987) Mammalian tyrosinase—the critical regulatory control point in melanocyte pigmentation. Int J Biochem 19: 1141-1147.

Innocenti G, Bettero A, Caporale G (1982) Determination of the coumarinic constituents of *Ficus carica* leaves by HPLC. Farmaco Sci 37: 475-485.

Jiménez-Cervantes C, Solano F, Kobayashi T, Urabe K, Hearing VJ, Lozano JA, Garcia-Borrón JC (1994) A new enzymatic function in the melanogenic pathway. The 5,6-dihydroxyindole-2-carboxylic acid oxidase activity of tyrosinase-related protein-1 (TRP1). J Biol Chem 269: 17993-18000.

Kang SJ, Choi BR, Lee EK, Kim SH, Yi H Y, Park HR, Song CH, Lee YJ, Ku SK (2015) Inhibitory effect of dried pomegranate concentration powder on melanogenesis in B16F10 melanoma cells; involvement of p38 and PKA signaling pathways. Int J Mol Sci 16: 24219-24242.

Kim A, Yim NH, Im M, Jung YP, Liang C, Cho WK, Ma JY (2013) Ssanghwa-tang, an oriental herbal cocktail, exerts anti-melanogenic activity by suppression of the p38 MAPK and PKA signaling pathways in B16F10 cells. BMC Complement Altern Med 13: 214.

Kim HJ, Kim IS, Dong Y, Lee IS, Kim JS, Kim JS, Woo JT, Cha BY (2015) Melanogenesis-inducing effect to cirsimaritin through increases in microphthalmia-associated transcription factor and tyrosinase expression. Int J Mol Sci 16: 8772-8788.

Lamoreux ML, Wakamatsu K, Ito S (2001) Interaction of major coat color gene functions in mice as studied by chemical analysis of eumelanin and pheomelanin. Pigment Cell Res 14: 23-31.

Niu C, Aisa Ha (2017) Upregulation of melanogenesis and tyrosinase activity: potential agents for vitiligo. Molecules 22: E1303.

Saha B, Singh SK, Sarkar C, Bera R, Ratha J, Tobin DJ, Bhadra R (2006) Activation of the Mitf promoter by lipid-stimulated activation of p38-stress signaling to CREB. Pigment Cell Res 19: 595-605.

Sahu D, Raghav SK, Gautam H, Das HR (2015) A novel coumarin derivative, 8-methoxy chromen-2-one alleviates collagen induced arthritis by down regulating nitric oxide, NFκB and proinflammatory cytokines. Int Immunopharmacol 29: 891-900.

Schadendorf D, Fisher DE, Garbe C, Gershenwald JE, Grob JJ, Halpern A, Herlyn M, Marchetti MA, McArthur G, Ribas A, Roesch A, Hauschild A (2015) Melanoma. Nat Rev Dis Primers 1: 1-20.

Späth E, Manjunath BL, Pailer M, Jois HS (1936) Synthese und Konstitution des Psoralens. Eur J Inorg Chem 69: 1087-1090.

Sturm RA, Teasdale RD, Box NF (2001) Human pigmentation genes: identification, structure and consequences of polymorphic variation. Gene 277: 49-62.

Tadokoro T, Yamaguchi Y, Batzer J, Coelho SG, Zmudzka BZ, Miller SA, Wolber R, Beer JZ, Hearing VJ (2005) Mechanisms of skin tanning in different racial/ethnic groups in response to ultraviolet radiation. J Investig Dermatol 124: 1326-1332.

Tsukamoto K, Jackson IJ, Urabe K, Montague PM, Hearing VJ (1992) A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase. EMBO J 11: 519-526.

Thody AJ, Higgins EM, Wakamatsu K, Ito S, Burchill SA, Marks JM (1991) Pheomelanin as well as eumelanin is present in human epidermis. J Invest Dermatol 97: 340-344.

Vachtenheim J, Borovansky J (2010) "Transcription physiology" of pigment formation in melanocytes: central role of MITF. Exp Dermatol 19: 617-627.

Widlude HR. Fisher DE (2003) Microphthalmia-associated transcription factor: a critical regulator of pigment cell development and survival. Oncoqene 22: 3035-3041.

Wu LC, Lin YY, Yang SY, Weng YT, Tsai YT (2011) Antimelanogenic effect of c-phycocyanin through modulation of tyrosinase expression by upregulation of ERK and downregulation of p38 MAPK signaling pathways. J Biomed Sci 18: 74.

Yang JY, Koo JH, Song YG, Kwon KB, Lee JH, Sohn HS, Park BH, Jhee EC, Park JW (2006) Stimulation of melanogenesis by scoparone in B16 melanoma cells. Acta Pharm Sin 27: 1467-1473.

Yoon HS, Lee NH, Hyun CG, Shin DB (2015) Differential effects of methoxylated *p*-coumaric acids on melanoma in B16F10 cells. Prev Nutr Food Sci 20: 73-77.

Yoon HS, Lee SR, Ko HC, Choi SY, Park JG, Kim JK, Kim SJ (2007) Involvement of extracellular signal-regulated kinase in nobiletin-induced melanogenesis in murine B16/F10 melanoma cells. Biosci Biotechnol Biochem 71: 1781-1784.