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Full Length Research Paper

Melanogenesis inhibition by a crude extract of *Magnolia officinalis*

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In the present study, we investigated the inhibitory effect of a crude extract from *Magnolia officinalis* (MOE) on melanogenesis in both mouse B16 melanoma cells and zebrafish. Our results showed that MOE inhibited melanogenesis in either α-melanocyte stimulating hormone (α-MSH)- or 3-isobutyl-1-methylxanthin (IBMX)-stimulated B16 cells in a dose-dependent manner with an IC_{50} value of 9.3 µg/ml. In addition, MOE also inhibited cellular tyrosinase activity with an IC_{50} value of 13.4 µg/ml while no inhibitory activity was found by MOE against cell-free tyrosinase activity. Moreover, western blotting and real time reverse-transcription polymerase chain reaction (qRT-PCR) analyses, respectively confirmed that MOE downregulated levels of tyrosinase protein but not that of its mRNA in α-MSH-stimulated B16 cells. These results demonstrated that MOE inhibits melanogenesis of B16 cells by a pre-translational regulation on tyrosinase gene expression. On the other hand, when using zebrafish as a depigmenting assay system, MOE could inhibit both melanogenesis and tyrosinase activity in the *in vivo* model. From the present study, MOE was proven to be a good candidate as a skin-whitening agent for treatment of skin hyperpigmentation.

Key words: *Magnolia officinalis*, melanogenesis, tyrosinase, melanin, inhibition.

INTRODUCTION

Skin pigmentation is produced by dermal melanocytes. Melanogenesis has been defined as the entire process leading to the formation of dark macromolecular pigments, that is, melanin, which are formed by a combination of enzymatically catalyzed and chemical reactions (Chang, 2009). Melanogenesis is initiated in melanosomes, the special organelles of melanocytes, with the first step of L-tyrosine oxidation to L-dopa (L-3,4-dihydroxyphenylalanine) and then to dopaquinone, which is catalyzed by tyrosinase. This is a rate-limiting step in melanin synthesis because the remainder of the reaction sequence can proceed spontaneously at a physiological pH value. Although, melanin mainly plays a photoprotective role, the accumulation of abnormal amounts of melanin in different parts of the skin, which results in pigmented patches of skin, might become an esthetic problem. Therefore, several studies have focused on the inhibition of tyrosinase activity and the prevention of abnormal pigmentation (Chang, 2007; Chang et al., 2007; Ding et al., 2009).

A previous study has reported that some cytokines and growth factors play importantly regulatory roles in melanogenesis (Imokawa, 2004). α-Melanocyte stimulating hormone (α-MSH) is the most well-studied hormone. This hormone binds to its receptor, melanocortin receptor 1 (MC1R), on the membrane of melanocytes and stimulates melanogenesis via the GPCR (G protein-coupled receptor)-cAMP-MITF (microphthalmia-associated transcription factor) pathway

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where the melanogenesis-related enzymes, including tyrosinase and tyrosinase-related proteins 1 and 2 (TRP1 and TRP2) are up-regulated. Hence, agents blocking the signal pathway would also exhibit depigmentation against melanocytes (Solano et al., 2006; Ando et al., 2007; Zhu and Gao, 2008; Chang and Chen, in press; Chang and Lin, 2010).

In the present study, we screened more than 200 crude extracts of traditional Chinese medicinal herbs to identify their applicability as skin-lightening agents. The *Magnolia officinalis* extract (MOE) was found to have strong inhibitory activity on melanogenesis in both mouse B16 melanoma cells and zebrafish. The inhibitory effect of MOE on melanogenesis was investigated in both B16 cells and zebrafish.

MATERIALS AND METHODS

Preparation of MOE

"Houpo", *M. officinalis* Rehd. et Wils.**,** was supplied from Kwong-Te Company, Kaohsiung, Taiwan and was identified by professor H. C. Lin of National Defense Medicine Center. The dried powder of the bark (20.0 Kg) was extracted with 95% (v/v) ethanol at room temperature overnight four times followed by filtration. The flow-through was concentrated in vacuo to yield dark-brown syrup (2.85 Kg).

Chemicals and antibodies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), arbutin, Triton X-100, phenylmethylsulfonyl fluoride (PMSF), L-dopa, dimethyl sulfoxide (DMSO), trypsin/EDTA, synthetic melanin, 1-phenyl-2-thiourea (PTU), IBMX, and α-MSH were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-tyrosinase antibodies (#62914) and protease inhibitor cocktail were obtained from Abcam (Cambridge, MA, USA). Anti-□-actin antibodies (#3662) were purchased from Bio Vision Inc. (Irvine, CA, USA). All other chemicals were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan) and were of analytic reagent grade.

Cell cultures and drug treatments

Mouse B16 melanoma cells (4A5) were obtained from the Bioresources Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37℃ in a humidified, CO_2-controlled (5%) incubator. The cells were seeded at an appropriate cell density in a 24-well or a 6-well plate. After 1 d of incubation, the cells were treated with various concentrations of the drugs in the absence or presence of a stimulation agent (10 nM of α-MSH or 100 μM of IBMX) for another 8 h (qRT-PCR), 16 h (tyrosinase activity assay and western blot), 48 h (melanin determination). Thereafter, the cells were harvested and used for various assays.

Measurements of cell viability

MTT assay was performed to examine the viability of cells. After the cells were incubated with the samples for 48 h, the culture medium was removed and replaced with 1 mg/ml MTT solution dissolved in phosphate-buffered saline (PBS) and incubated for an additional 2 h. The MTT solution was then removed and DMSO was added, following which the absorbance of the dissolved formazan crystals was determined at 570 nm by a spectrophotometer.

Determination of melanin content

At the end of cell cultivation, the cells were harvested and washed twice with PBS. The pelleted cells were homogenized in lysis buffer containging 20 mM sodium phosphate (pH 6.8) and 1% Triton X-100 at 4℃ with 30 strokes in a Dounce homogenizer. After centrifugation at 15,000 × g for 15 min, the melanin pellets were dissolved in 1 N NaOH containing 20% DMSO for 1 h at 95℃. The absorbance at 490 nm was measured, and the melanin content was measured using the authentic standard of synthetic melanin. The protein content in the supernatant was determined using a Bradford assay with bovine serum albumin (BSA) as the protein standard. The specific melanin content was adjusted by the amount of protein in the same reaction.

Measurements of cellular tyrosinase activity

To determine the tyrosinase activity in the crude extract, a source of crude cellular tyrosinase was obtained by homogenizing drug-treated or untreated cells in 20 mM sodium phosphate (pH 6.8), 1% Triton X-100, and 1 mM PMSF at 4℃ with 30 repeated strokes in a Dounce homogenizer. Detergent was used to release the membrane-bound tyrosinase from the melanosomes. The lysates were centrifuged at 15,000 rpm for 15 min to obtain the supernatant as the source of crude cellular tyrosinase. The protein content in the supernatant was determined using a Bradford assay with BSA as the protein standard. Tyrosinase activity was then determined as follows: 1 ml of the reaction mixture contained 50 mM of phosphate buffer (pH 6.8), 2.5 mM of L-dopa, and 500 μg of the supernatant protein, and was incubated at 37℃ for 15 min, following which the dopachrome formation was monitored by measuring absorbance at a wavelength of 475 nm. One unit of tyrosinase activity is defined as the total enzyme that catalyzes the formation of 1 μmole of dopachrome in 1 min. The amount of dopachrome in the reaction was calculated using the Lambert-Beer Law while the molar extinction coefficient of dopachrome is 3600 $M^{-1} \cdot cm^{-1}$ (Neeley et al., 2009). The specific tyrosinase activity was normalized with protein content in the reaction.

Measurements of cell-free tyrosinase activity

The crude enzyme source of solubilized tyrosinase was prepared as described above, with the exception that untreated cells were used in this measurement. To determine the inhibitory effect of MOE toward cell-free tyrosinase activity, the reaction mixture contained 50 mM phosphate buffer (pH 6.8), 2.5 mM of L-dopa, the tested concentration of the drug, and 500 μg of the supernatant protein with a total volume of 1 ml of crude tyrosinase. After incubation at 37℃ for 15 min, dopachrome formation was monitored by measuring the absorbance at 475 nm. The relative tyrosinase activity was obtained by dividing the enzyme activity of the reaction mixture containing a drug by that without drugs.

Western blot analysis

The cells were washed 3 times in ice-cold PBS, and lysed in cold lysis buffer (20 mM sodium phosphate (pH 6.8), 1% Triton X-100,

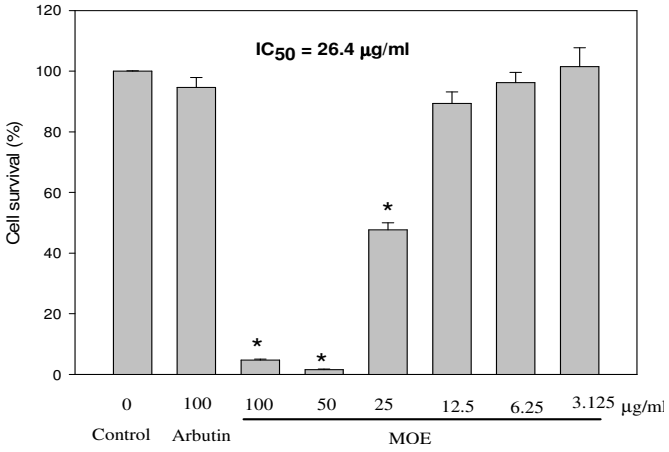

Figure 1. Effect of MOE on B16 cell viability. The cells were seeded in 24-well plates for 1 d and then treated with various dosages of MOE (100–3.125 μg/ml) for 2 d. The cell viability was then examined by the MTT assay.

1 mM PMSF, 1 mM EDTA) containing protease inhibitors cocktail (Abcam, Cambridge, UK). An aliquot of the lysate was used to determine the protein content with a Bradford assay using BSA as the standard. The proteins (100 μg) were separated using 10% SDS-polyacrylamide gel electrophoresis and blotted onto polyvinyl difluoride (PVDF) membranes (MP Biomedicals Co., Irvine, CA, USA). The membranes were blocked with 5% non-fat skim milk in TBS-T buffer. Tyrosinase and β-actin (as an internal control) were detected using a rabbit polyclonal antibodies and mouse monoclonal anti-β-actin antibodies, respectively. The membranes were further incubated with horseradish peroxidase-conjugated secondary antibody. All bound antibodies were then detected using an Amersham ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The signal intensity of each band was quantified with a densitometer system GS-700 (Bio-Rad, CA, USA) equipped with an integrator, and normalized with that of the internal control.

Quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR were performed on the ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, U.S.A.) using Fast SYBR® Green Master Mix (Applied Biosystems). Total RNA was extracted using an RNeasy® mini Kit (Qiagen, CA, USA) according to the manufacturer's instructions. The quality of the total RNA sample was evaluated by determining the OD_{260}/OD_{280} ratio. To prepare a cDNA pool from each RNA sample, total RNA (2 μg) was reverse transcribed at 42℃ for 90 min in the presence of oligo(dT) primers (MD Bio. Co., Taipei, Taiwan) and reverse transcriptase (Roche Molecular Biochemicals, Mannheim, Germany). The oligonucleotides primers for mouse tyrosinase (forward, 5′-GGCCAGCTTTCAGGCAGAGGT-3′; reverse, 5′-TGGTGCTTCATGGGCAAAATC-3′) and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (forward, 5′-ACCACAGTCCATGCCATCAC-3′; reverse, 5′-TCCACCACCCTGTTGCTGTA-3′) were used. After the initial incubation of 2 min at 50℃, the cDNA was denatured at 95℃ for 10 min followed by 40 cycles of PCR (95℃, 15 s, 60℃, 60 s). All results were obtained from at least three independent experiments. The mRNA level of tyrosinase was normalized using GAPDH as an internal control.

Determinations of depigmenting activity in zebrafish

The assays of evaluation of depigmenting activity in zebrafish were according to a publish by Choi et al. (2007). Adult zebrafishes were obtained from a commercial dealer and kept in acrylic tanks with a 14/10 h light/dark cycle at 28℃. Synchronized embryos were obtained from natural spawning that was induced at the morning by turning on the light.

Test compounds were dissolved in 0.1% DMSO, and then added to the embryo medium from 9 to 48 hpf (hour post ferterization). The developed zebrafish larval were photographed with a digital camera under a stereomicroscope and sonicated in cold lysis buffer (20 mM sodium phosphate (pH 6.8), 1% Triton X-100, 1 mM PMSF, 1 mM EDTA) containing protease inhibitors cocktail. An aliquot of the lysate was used to determine the protein content with a Bradford assay using BSA as the standard. The lysate was clarified by centrifuging at 10,000 x *g* for 10 min. The melanin precipitation in each tube was photographed with a digital camera and then resuspended with 0.2 ml of 1 N NaOH/20% DMSO at 95℃ for 1 h. The absorbance at 490 nm was measured, and the melanin content was measured using the authentic standard of synthetic melanin. The specific melanin content was adjusted by the amount of protein in the same reaction.

For the tyrosinase activity assay, 250 μg of total protein in the lysate was added into a reaction mixture containing 50 mM phosphate buffer (pH 6.8) and 2.5 mM of L-dopa. After incubation at 37℃ for 60 min, dopachrome formation was monitored by measuring the absorbance at 475 nm. The specific tyrosinase activity was normalized with protein content in the reaction.

Statistical analysis

All the data in the present study were obtained as averages of experiments that were performed in triplicate and are expressed as means ± S.D. Statistical analysis was performed by the Student's *t* test. A value of $p < 0.05$ was considered to be statically significant.

RESULTS AND DISCUSSION

Effect of MOE on cell viability

Safety is the prime criteria for a skin-whitening agent. Hence, in the front of investigating the effect of MOE on melanogenesis of mouse B16 melanoma cells, the concentration range of the extract that is nontoxic to the cells should be determined. Cell viability of drug-treated cells was determined by the MTT method. Arbutin was used as the control in the present study. As illustrated in Figure 1, 12.5 μg/ml MOE does not exert significant cytotoxicity to mouse B16 melanoma cells, and the cytotoxic IC_{50} value of MOE toward B16 cells was determined to be 26.4 μg/ml. In order to ignore the cytotoxic effect of MOE, we used MOE concentration of up to 12.5 μg/ml in the depigmenting experiments of the present study.

Effect of MOE on melanogenesis and tyrosinase activity in B16 cells

To study the anti-melanogenic effect of MOE in B16 cells, the melanin content of the cells in each treatment was

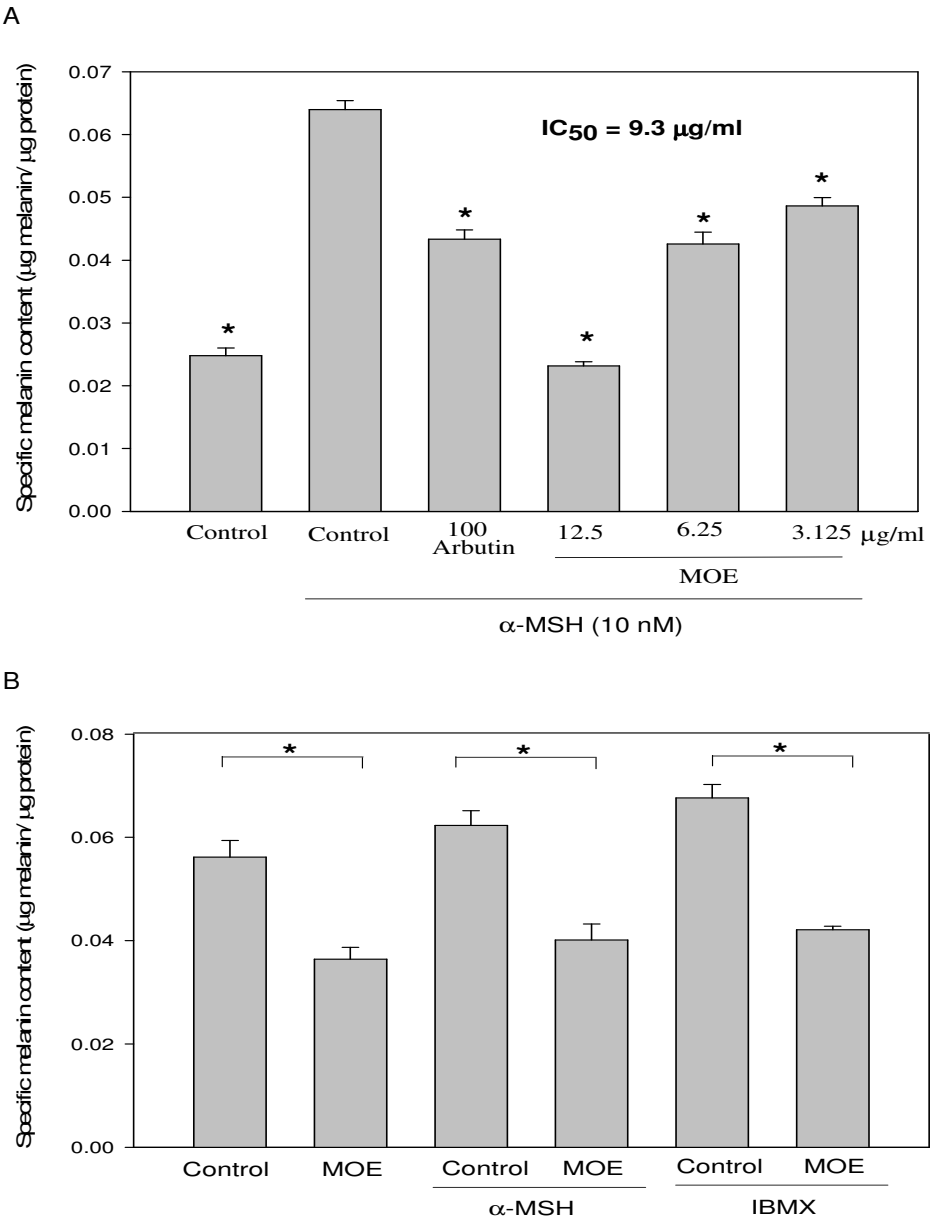

Figure 2. Effect of MOE on melanogenesis in B16 cells. The cells were cultivated for 1 d and then stimulated with 10 nM of α-MSH (A and B) or 100 μM of IBMX (B) for 2 d in the absence or presence of 6.25 μg/ml MOE. The melanin and protein contents of cells were determined using spectrometry, as described in the Experimental section, and the specific melanin content of each reaction was calculated by dividing the melanin content by the protein content in the same reaction.

determined. The results are shown in Figure 2. The melanin content of B16 cells increased considerably after stimulation by α-MSH. Thereafter, MOE treatment resulted in significant decrease in the melanin content of α-MSH-stimulated B16 cells, and 12.5 μg/ml MOE-treated cells contained similar level of the melanin as that in non-α-MSH-stimulated cells (Figure 2A). In addition, the melanogenic inhibition of MOE showed a dose-dependent manner with an IC_{50} value of 9.3 μg/ml. When the inhibitory potency of MOE and arbutin were compared, MOE exhibited more than 8-fold stronger activity than that of arbutin. In addition to α-MSH, we used another stimulating agent IBMX, which is an intracellular cAMP elevation agent, to stimulate B16 cells and found that MOE also inhibited melanogenesis in the IBMX-stimulated cells (Figure 2B).

Because tyrosinase plays an important role in melanogenesis, we determined the effect of MOE on

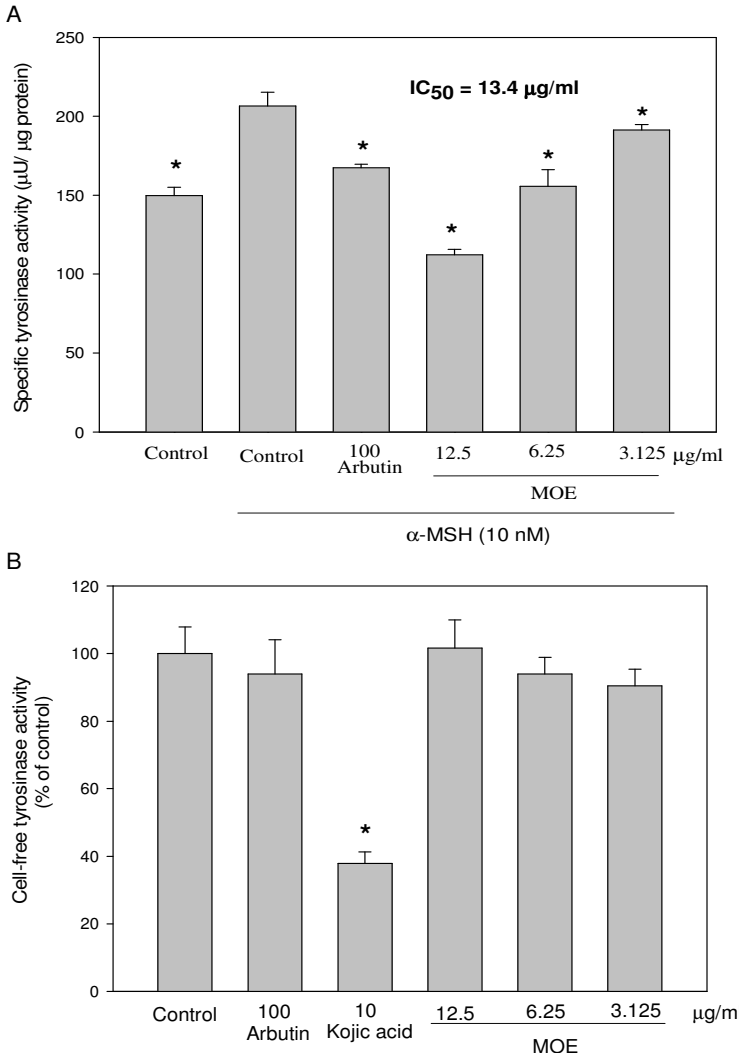

Figure 3. Effect of MOE on tyrosinase activity of B16 cells. (A) Cellular tyrosinase activity. The cells were cultivated for 1 d and stimulated with 10 nM of α–MSH in the absence or presence of various dosages of MOE or arbutin for 2 d. Both the protein content and cellular tyrosinase activity in the cells were determined using spectrometry. Specific tyrosinase activity was calculated by dividing the amount of the tyrosinase activity by the protein content, as described in the Experimental section. (B) Cell-free tyrosinase activity. B16 cells were cultured for 3 d and harvested and lysed to obtain crude protein extract containing murine tyrosinase. The tyrosinase activity of each reaction was determined by a spectrometric method as described in the experimental section, and the relative tyrosinase activity was calculated by dividing the tyrosinase activity of each reaction by that of the control.

tyrosinase activity. After MOE treatments, B16 cells were lysed to obtain cellular tyrosinase. We measured the enzyme activity by using L-dopa as an enzyme substrate. The result is shown in Figure 3. MOE treatments would inhibit cellular tyrosinase activity with a dose-dependent manner and an IC_{50} value of 13.4 μg/ml (Figure 3A). However, the deduced cellular tyrosinase activity was not achieved by a direct inhibition of tyrosinase activity by MOE judged by the result from the inhibitory assay of cell-free tyrosinase activity (Figure 3B).

Effect of MOE on tyrosinase protein and its mRNA levels in B16 cells

To study the inhibitory effects of MOE on melanogenesis

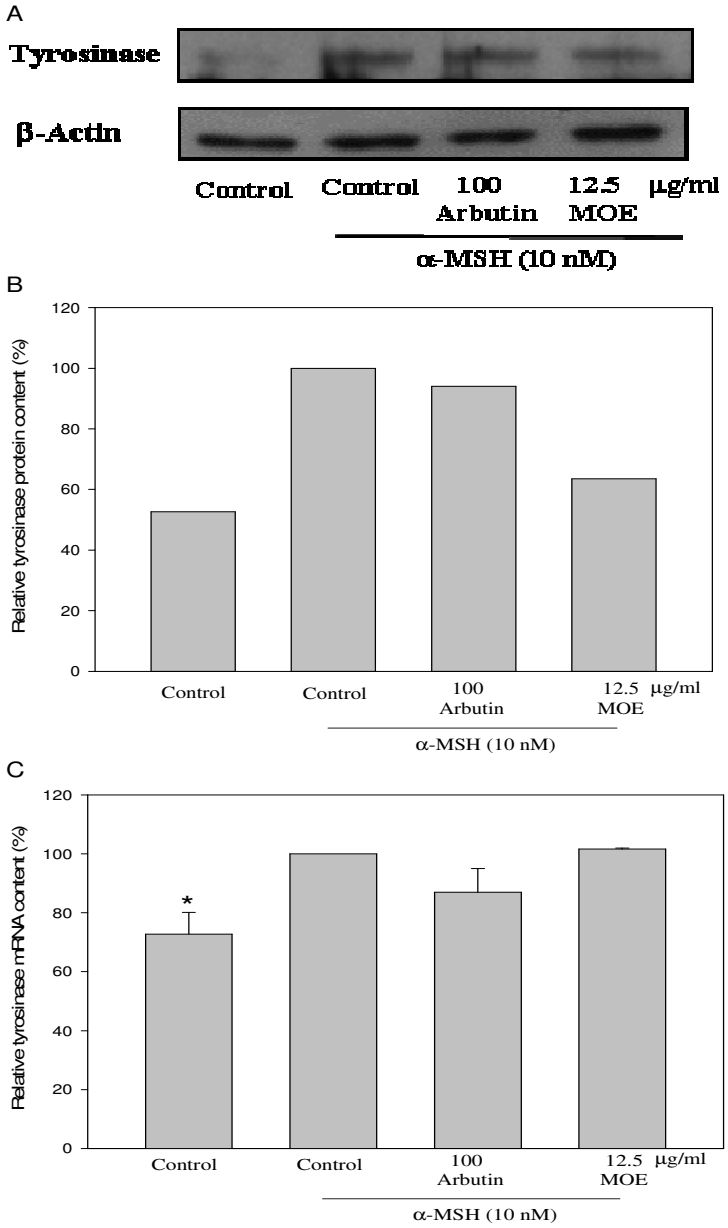

Figure 4. Effect of MOE on amounts of tyrosinase protein and its mRNA in B16 cells. Cells were inoculated in 24-well plates for 1 d and then stimulated by 10 nM α-MSH with or without the test drug. The cells were harvested and the tyrosinase protein and mRNA were analyzed as described in the experimental section. (A) Western blot analysis of tyrosinase and β-actin. (B) The band intensity of tyrosinase from result of western blot was normalized by that of β-actin, and the normalized band intensity in the α-MSH-stimulated control was recalculated to be 100. (C) qRT-PCR analysis of tyrosinase and GAPDH.

more detail, we conducted western blotting and qRT-PCR to verify levels of tyrosinase protein and its mRNA in the MOE-treated cells. The result is shown in Figure 4. After stimulation by α-MSH, both tyrosinase protein (Figure 4A and B) and its mRNA (Figure 4C) were significantly increased. However, the increased level of tyrosinase protein was significantly reduced by the treatment of 12.5 μg/ml MOE (Figure 4A and B). In contrast, the stimulated tyrosinase mRNA in the α-MSH-stimulated cells was not reduced by the MOE treatment (Figure 4C). These results demonstrated that MOE inhibits melanogenesis of B16 cells by a pre-translational regulation on tyrosinase gene

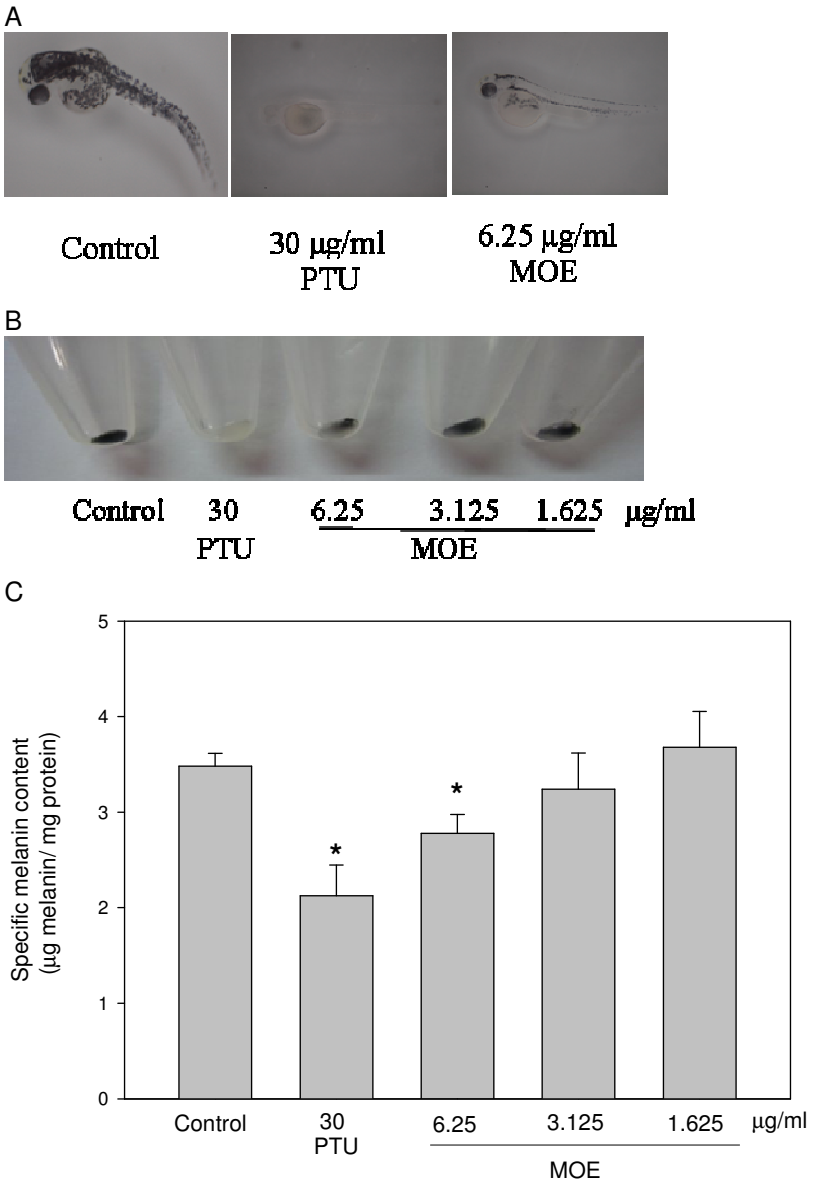

Figure 5. Effects of MOE on melanogenesis in zebrafish. 100 synchronized embryos were treated with drugs from 9 to 48 hpf and the effects on the pigmentation of zebrafish were observed under a stereomicroscope equipped with a digital camera (A). The embryos were then collected and sonicated. After centrifugation, melanin pigment was photographed (B) and determined (C) by a photometric method as described in the experimental section.

expression.

Effect of MOE on melanogenesis and tyrosinase activity in zebrafish

Zebrafish has been established as a new *in vivo* model for evaluating the depigmenting activity of melanogenic regulatory compounds (Choi et al., 2007). To determine the effects of melanogenic inhibition by MOE in advance, we verified the anti-melanogenic activity of MOE by the *in vivo* model. 100 embryos were included for each test condition and PTU was used as a positive standard. The results were showed in Figures 5 and 6. PTU (30 μg/ml) exhibited excellent depigmenting activity against zebrafish, while 6.25 μg/ml MOE also showed comparable depigmenting activity in the assay system (Figure 5A). In addition, MOE performed a dose-dependent effect on inhibition of both melanogenesis and tyrosinase activity (Figures 5B, C and 6).

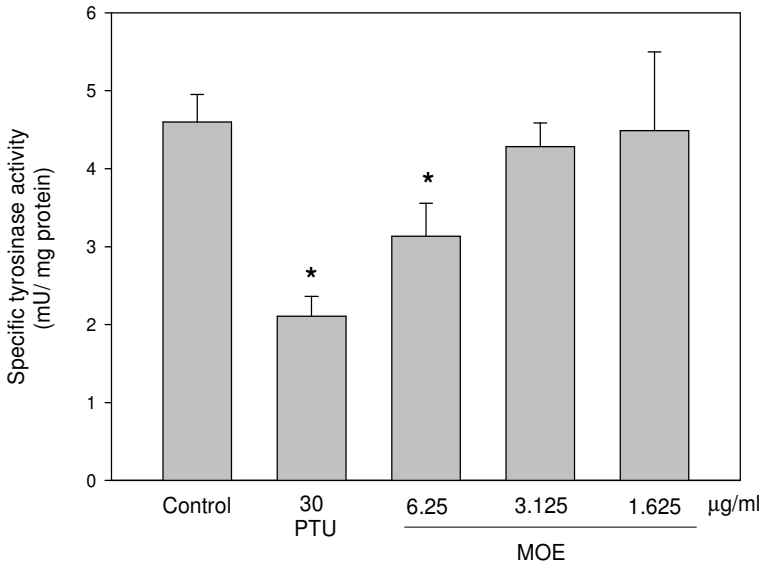

Figure 6. Effects of MOE on tyrosinase activity of zebrafish. 250 µg of total protein from lysates of 100 zebrafish larval as the treatment in Figure 5 was incubated with 2.5 mM of L-dopa, and then quantified using a photometric method as described in the experimental section.

In conclusion, our results clearly demonstrated that MOE is an effectively melanogenesis inhibitor, which functions by a pre-translational regulation on tyrosinase gene expression. In addition, MOE also exhibited depigmenting activity in an *in vivo* evaluation system. These results indicated that MOE may be useful in the treatment of skin hyperpigmenting.

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