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RESEARCH ARTICLE

Dual Inhibitions of Lemon Balm (*Melissa officinalis*) Ethanolic Extract on Melanogenesis in B16-F1 Murine Melanocytes: Inhibition of Tyrosinase Activity and Its Gene Expression

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Abstract The effects of wild type and UV-irradiated lemon balm (*Melissa officinalis*) ethanolic extracts (MOE and UMOE) on melanogenesis *in vitro* were examined. UMOE showed potent antioxidant activity and significantly inhibited the mushroom and melanocyte tyrosinase activity, and lowered cellular melanin content by 49% at 200 μg/mL in B16-F1 melanocytes. The key gene and protein expression of tyrosinase, tyrosinase-related protein (TRP)-1, and TRP-2 were reduced (-73% for TRP-1 protein at 200 μg/mL UMOE, $p<0.05$). MOE showed similar results to a slightly lesser degree. We found that myo-inositol, a major compound in lemon balm extracts, significantly reduced cellular melanin synthesis and its effect was greater than arbutin at 1 mM. These suggest that both MOE and UMOE have anti-melanogenic role by both direct inhibition of tyrosinase and down-regulation of gene expressions in melanogenesis. UV-irradiation slightly improved the anti-melanogenic activities. UMOE may be useful as natural anti-melanogenic biomaterials for functional foods and cosmetics.

Keywords: lemon balm (*Melissa officinalis*), hypopigmentation, tyrosinase, UV-irradiation

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Introduction

Melanin is synthesized in the epidermal melanocytes with UV radiation in skin and hair follicles and is a major factor to determine skin color and plays important roles in the prevention of sun-induced skin injury (1). Melanin in skin is a major defense mechanism against sunlight, however, the over-synthesis of melanin can cause various dermatological disorders, such as age spots, freckles, melasma, and lipid spots, which are serious concern especially among female populations (2).

Three enzymes called tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2) are considered as the key enzymes in the melanogenesis including the complicated network of gene expression and signalling pathways (3). Tyrosinase, the rate-limiting enzyme in melanin biosynthesis, catalyses the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and mediates the oxidation of DOPA to dopaquinone (4). TRP-2, a dopachrome tautomerase, mediates the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (5) while TRP-1 oxidises DHICA to a carboxylated indole-quinone that result in the formation of eumelanins (6). Consequently, direct inhibition of these enzymes or the reduced expressions of the genes encoding these enzymes, together with the inhibition of general melanogenic pathways, could be useful in skin hypopigmentation.

Lemon balm (*Melissa officinalis*), a member of Lamiaceae, has a documented medicinal history back to the 'Materia Medica' in approximately 50-80 BC. Its consumption had widely spread throughout Europe in the middle ages, and it is believed that lemon scented herb lemon balm has been in use as a pancultural medicinal treatment for more than

2 millennia (7). Recently, lemon balm is utilized more broadly as food additives, teas, cosmetics ingredients as well as traditional medicines. In addition, lemon balm has been favorite sources for aroma therapy, and biological activities including antioxidant (8), antibacterial, and anti-inflammatory effects have been recently investigated (9). Significant levels of myo-inositol and phenolic substances are contained in lemon balm (10-13), which may be responsible for the various biological activities.

Prevention and treatment of hyperpigmentation often need pharmaceutical therapy, however, functional foods containing hypopigmenting compounds isolated from medicinal plants could be utilized as cosmetic purposes. The secondary metabolism in plants produces wide range of chemicals from flavonoids, glucosinolates, to sugar derivatives such as myo-inositol. The production of secondary metabolites is not only species-specific, but depends on various environmental factors and UV radiation, which is one important factor that in many cases stimulates the production of secondary metabolites (14). The part of the UV daylight spectrum that is particularly variable, and therefore worth paying special attention to, is the UV-B band, 280-315 nm. Secondary metabolites have often been obtained in plants and cell cultures when UV-B (radiation of wavelength 280-315 nm) radiation has been applied (15,16).

In the study, wild type lemon balm plant was exposed to UV-B ray (7 W/m^2/s) for 20 days for modest increase of secondary metabolite concentrations. This method is commonly applied to increase the secondary metabolite synthesis in plant cells (17). Then, we investigated the hypopigmentation effects of wild type (MOE) and UV-irradiated lemon balm (UMOE). The composition analysis was performed using GC-MS, and DPPH radical scavenging assay was used to evaluate antioxidant activity. To examine the effects of MOE and UMOE on melanogenesis, tyrosinase activity, and melanin contents were measured, and the expressions of tyrosinase, TRP-1, and TRP-2 were analyzed in B16-F1 melanocytes.

Materials and Methods

Preparation of MOE and UMOE Both wild-type and UV irradiated leaves of lemon balm (*Melissa officinalis*) were collected in April 2009 in Korea University Greenhouse, Seoul, Korea. For UV irradiation, UV-B (7 W/m^2/s) was exposed to the plant for 20 days during growing period (17). The collected leaves were washed in cold water, freeze-dried, crushed with grinder, and then extracted in 80%(v/v) ethanol at room temperature for 24 h. After filtering with Whatman #1 paper, the solution was freeze dried, then dried extracts were used for experiment.

GC-MS analysis The composition of MOE and UMOE was analyzed by GC-MS analysis. The sample was extracted with 1 mL of metabolite extraction solvent (MeOH:chloroform:dH_2O=2.5:1:1, v/v/v). The mixture was vortexed at −20ºC for 30 min and then centrifuged at 18,000×g for 4 min. The phase was seperated after adding water and subsequent centrifugation. The polar phase was transferred into another 1.5-mL Eppendorf tube and dried in a speed vacuum concentrator. The dried sample was subsequently derivatized by methoxymation and trimethylsilylation prior to analysis into GC-MS. For methoxymation, 50 μL of 20 mg/mL methoxyamine chloride in pyridine was added and placed in a shaking water bath at 30ºC for 90 min, 20×g. Eighty μL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide was added and placed in a water bath 37ºC for 30 min. The metabolites of MOE and UMOE were analyzed by GC-MS (5975C; Agilent Technologies, Wilmington, DE, USA). One μL of sample was injected in the split mode (split ratio 100) onto a DB-5MS capillary column (30 m×25 mm, 0.25 μm film thickness) with helium as a carrier gas. The oven temperature was 100ºC for 2 min and then 100-280ºC at a rate of 10ºC/min and held for 10 min. By using perfluorotributyl amine, the parameters of the mass spectrometer were optimized for the best resolutions at 69, 219, 502, and 614 *m/z*. The measurement of mass was conducted by using an EI positive ion source at 240ºC in the SCAN mode in the mass range of 50-550 *m/z*.

Determination of total avonoid concentration An aliquot of 0.5 mL was added to test tubes containing 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 M aqueous potassium acetate and 4.3 mL of 80% ethanol. After 40 min incubation at room temperature, the absorbance of the solution was determined at 415 nm for total flavonoid concentration. Quercetin was used to generate standard curve (18).

MTT assay B16-F1 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone Laboratories, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories) and 1% penicillin/streptomycin. The cells were subsequently seeded into 24-well culture plates. After they reached confluence, they were treated with MOE, UMOE or arbutin (positive control) for 72 h. Next, MTT reagent at 1 mg/mL in the culture media was added to each well, and then incubated for 2 h at 37ºC. The medium was removed and 100 μL of dimethyl sulfoxide was added to each well. The plate was gently rotated for 1 h to completely dissolve the precipitation. The absorbance was measured at 570 nm with a microplate reader (Bio-Rad, Hercules, CA, USA).

DPPH free radical scavenging activity The free radical scavenging capacity of MOE and UMOE were analyzed using DPPH, as previously described (19). Samples at various concentrations were added to a DPPH solution in ethanol and the mixtures were incubated at 37ºC for 30 min to measure the absorbance at 532 nm. The percentage inhibition of DPPH was calculated using the following equation: Radical scavenging activity (%) = $(A_{sample(517\ nm)}/A_{control(517\ nm)})\times100$, where $A_{sample(517\ nm)}$ is the absorbance of the sample and $A_{control(517\ nm)}$ is the absorbance of the control at 517 nm.

Mushroom tyrosinase assay DOPA oxidase activity of mushroom tyrosinase was determined based on the previously described method with minor modifications (20). Fifty μL of 0.03% tyrosine solution in distilled water, 75 μL of 0.1 M phosphate buffer (pH 6.8) with or without test sample were added to a 96-well microplate, and then 25 μL of mushroom tyrosinase (400 U/mL 0.1 M phosphate buffer) was mixed. After incubation at 37ºC for 20 min, the amount of DOPAchrome produced in the reaction mixture was determined at 475 nm. Inhibitory effects on the enzyme activity by test samples were represented as % of inhibition, $[1-(\text{sample } OD_{475}/\text{control } OD_{475})]\times100$.

Cellular tyrosinase assay Tyrosinase activity in B16-F1 cells was assayed as described previously with modifications (20,21). Cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed with 20 mM Tris-0.1% Triton X-100 (pH 7.5). Tyrosinase activity was analyzed spectrophotometrically by following the oxidation of DOPA to DOPAchrome at 475 nm. The reaction mixture containing 140 μL of freshly prepared substrate solution [0.1% L-DOPA in 0.1 M sodium phosphate (pH 6.0)] and 70 μL of enzyme solution was incubated at 37ºC. The absorbance of samples was measured during the first 2 h of the reactions. The increase of the absorbance was linear within this time range. Auto-oxidation of L-DOPA was adjusted. Enzyme activities were expressed as a percentage of the control cells.

Melanin content Cells were washed with PBS and lysed with 20 mM Tris-0.1% Triton X-100 (pH 7.5). After centrifugation, pellet was dissolved in 1 N NaOH for 1 h at 60ºC. The absorbance of samples was measured at 400 nm. Cellular melanin content was adjusted according to the protein concentration of the samples. The protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad).

Reverse transcription-PCR (RT-PCR) Total cellular RNA was isolated using TRIzol reagent (Invitrogen) as described previously (22). To synthesize cDNA, 2 μg of mRNA from cells was reverse transcribed with oligo dT and Superscript II (Invitrogen). The reaction was performed in a 20 μL final volume for 90 min at 42ºC and subsequently for 15 min at 70ºC. The resulting cDNA templates were subject to PCR amplification. The PCR was performed in a 20 μL final volume containing cDNA, 10× PCR buffer, 2.5 mM dNTPs, 10 mM forward, and reverse primers, DNA polymerase, and sterile water. The cDNA was used in RT-PCR with primers for tyrosinase, TRP-1 and TRP-2 as follows. Tyrosinase upstream 5'-GGC CAG CTT TCA GGC AGA GGT-3'; downstream 5'-TGG TGC TTC ATG GGC AAA ATC-3': TRP-1 upstream 5'-GCT GCA GGA GCC TTC TTT CTC-3'; downstream 5'-AAG ACG CTG CAC TGC TGG TCT-3': TRP-2 upstream 5'-GGA TGA CCG TGA GCA ATG GCC-3'; downstream 5'-CGG TTG TGA CCA ATG GGT GCC-3' (23). Specific primers for the β-actin: upstream 5'-TGC TGT CCC TGT ATG CCTCT-3'; downstream 5'-AGG TCT TTA CGG ATG TCA ACG-3' were added as a control for the same reverse transcriptase product. The PCR products (10 μL) were electrophoresed on 1.2% agarose gels in a constant 100 V field and stained with ethidium bromide before visualization using UV light. Quantitation of RT-PCR products was performed by measuring the intensity of each band using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA).

Immunblot analysis Immunoblotting was performed as previously described (24,25). The cells were lysed in RIPA buffer (10 mM Tris-HCl, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) at 4ºC. The lysate was clarified by centrifugation at 12,000×g for 5 min at 4ºC. The protein concentration was determined using a Bio-Rad protein kit (Bio-Rad) with bovine serum albumin (BSA, Sigma-Aldrich) as a standard. Protein samples were separated using 7.5% SDS-PAGE and were blotted onto a PROTRAN nitrocellulose membrane (PerkinElmer, Boston, MA, USA). Blocking buffer, PBS (pH 7.4) with 0.1% Tween 20 and 5% skimmed milk, was incubated for 1 h at room temperature. Primary antibodies were diluted in PBS containing 5% BSA as followings: anti-tyrosinase (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:500; anti-TRP-1 (Santa Cruz Biotechnology), 1:500; anti-TRP-2 (Santa Cruz Biotechnology), 1:500; anti-α-tubulin (Santa Cruz Biotechnology), 1:500. After incubation, membranes were washed several times with PBS-0.1% Tween 20, incubated with secondary antibody, anti-goat IgG conjugated with peroxidase (1:1,000 dilution, Santa Cruz Biotechnology). Immunoreactive bands were detected by ECL Western blotting detection reagents (GE Healthcare

Life Sciences, Uppsala, Sweden) and exposed to high performance chemiluminescence film (Fujifilm, Tokyo, Japan) for 1 to 2 min. The density of each band was quantified using Gel-Pro analysis Software (Media Cybernetics).

Statistical analysis All data were expressed as mean± standard error (SE). Treatments were compared using one-way analysis of variance (ANOVA) followed by Tukey test. Student's *t*-tests were performed to confirm the comparisons between groups.

Results and Discussion

Composition MOE and UMOE The composition analysis showed that UV irradiation mildly affected the level of secondary metabolites. The GC-MS analysis results of MOE and UMOE showed significant levels of myo-inositol also known as vitamin B8 (Table 1; 9.28 and 11.47% in MOE and UMOE, respectively). Total flavonoid concentrations of MOE and UMOE were 74.3 and 82.8 μg/mg(w/w), respectively, and the level in UMOE was 11% higher than the level in MOE (Table 2). UV radiation is a key factor to stimulate the production of secondary metabolites, which may have health-promoting functions, in plants. Secondary metabolites production by UV radiation depends on the overall developmental program of a plant. For example, UV-inducible accumulation of flavonoids is phased out 2 to 3 weeks after leaves become fully extended (17). We exposed the MO plants with UV-B light (7 $W/m^2/s$) for 20 days during growing period before harvesting the leaves for experiments to induce the synthesis of potential hypopigmenting chemicals. Approximately 7 $W/m^2/s$ of UV-B radiation levels have been used as moderate levels to enhance secondary metabolism of plants (26).

Antioxidant activity of MOE and UMOE In DPPH radical scavenging assay, MOE and UMOE showed antioxidant activities in a dose-dependent manner (Fig. 1). Skin damage is caused by multiple environmental factors and an oxidative stress induced by UV light represents a major environmental threat to the skin increasing its risk of photo-oxidative damage by UV-induced reactive oxygen species (ROS). Increased ROS load has been implicated in several pathological states including photo-aging and photo-carcinogenesis of the skin (27) and generated ROS assists melanogenesis (28,29). It has been reported that ROS-induced melanogenesis was reduced by the topical application and oral administration of such as antioxidants as vitamin C and E to the skin (30,31). Antioxidant can suppress the reactive ROS on melanogenesis because it can

Table 1. Composition of wild type (MOE) and UV-irradiated (UMOE) lemon balm ethanolic extracts

Class	Compound	Area (%)	
		MOE	UMOE
Amino acid	L-Valine	0.22	ND[1)]
	L-Norleucine	0.18	ND
	L-Isoleucine	0.19	ND
	Proline	1.41	ND
	Serine	0.26	0.12
	L-Threonine	0.10	ND
	Norvaline	0.11	ND
	L-Asparagine	0.28	ND
	L-Alanine	ND	0.16
Amine	Silanamine	0.33	ND
Acid	Butanedioic acid	0.56	0.59
	Propanoic acid	0.16	0.37
	Malic acid	0.27	ND
	Pentanedioic acid	2.29	0.42
	Risedronic acid	0.15	ND
	Tartaric acid	ND	0.46
	Succinic acid	0.25	ND
	Galactonic acid	0.14	0.19
	1-Cyclopropanecarboxylic acid	ND	0.20
	Hexadecanoic acid	0.09	ND
	D-Gluconic acid	ND	0.13
	2-Propenoic acid	2.12	2.75
	Benzoic acid	0.21	ND
	1-Cyclohexene-1-carboxylic acid	0.53	1.05
	Ethyl phosphoric acid	ND	0.12
	Butanoic acid	ND	0.27
Sugar	D-Ribose	0.13	0.60
	D-Ribofuraose	0.15	ND
	D-Fructose	18.34	8.62
	Talose	2.04	ND
	Sorbopyranose	ND	0.75
	D-Gluco-hexodialdose	ND	7.66
	D-Mannose	3.79	13.90
	D-Galactose	ND	14.42
	D-Glucose	31.59	10.79
	Maltose	ND	1.53
	α-D-Glucopyranoside	ND	7.30
	Galactose	3.85	ND
	Melibiose	0.40	0.32
Alcohol	1-Piperazineethanol	ND	0.84
Aldehyde	Butanal	0.28	ND
	Benzaldehyde	ND	4.64
Vitamin	Myo-inositol	9.28	11.47
Metal	Molybdenum	1.46	ND
Unidentified	Unidentified	17.98	4.02
	Total peak area (%)	99.15	93.66

[1)]Not detected

Table 2. Total flavonoids concentration of MOE and UMOE

	Total flavonoid concentration (μg/mg)[1)]
MOE	74.3±0.1[2)]
UMOE	82.8±0.0*

[1)]Measured using quercetin as a standard
[2)]Data are expressed as the mean±SE of 3 independent experiments; *p<0.05 compared with MOE

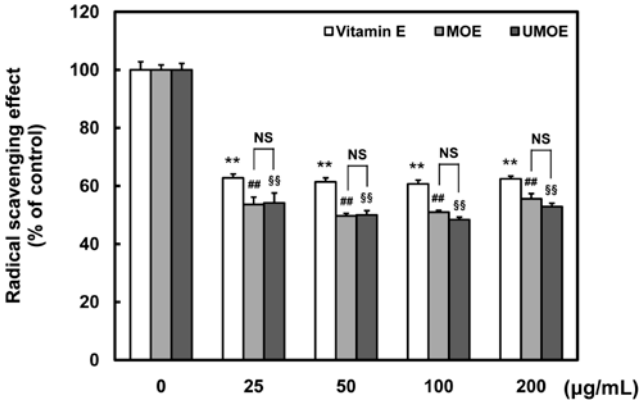

Fig. 1. Antioxidant activities of MOE and UMOE in DPPH radical scavenging activity assay. Values represent the mean±SE (n=3); *$^{,\#,\S}p$<0.05, **$^{,\#\#,\S\S}p$<0.001 vs. control of each group; NS, not significant

prevent ROS accumulation in the cell or tissue (32). Moreover, many studies have suggested new therapeutic strategies with topical or systemic administration of antioxidants in skin ageing and in UV-dependent cancer (33,34). Several animal studies have proven the efficacy of antioxidants such as vitamin E, vitamin C, and β-carotene in inhibiting skin reaction caused by UV irradiation or formation of DNA oxidation products and immunosuppression. In this study, both MOE and UMOE effectively removed free radicals compared with positive control (vitamin E), which may inhibit ROS-induced melanogenesis in skin tissue.

Inhibitory effects of MOE and UMOE on mushroom tyrosinase activity MOE and UMOE inhibited mushroom tyrosinase activity in a dose-dependent manner, demonstrating UMOE showed stronger inhibitory effects on tyrosinase activity than those of MOE (Fig. 2). MOE and UMOE, at 25 to 1,000 μg/mL, reduced DOPA oxidase activity by 3 to 32 and 4 to 36%, respectively.

Effects of MOE and UMOE on melanin synthesis and cellular tyrosinase activity in the B16-F1 melanocytes Cellular melanin content in B16-F1 melanocytes with various doses of MOE and UMOE was quantified using arbutin as a positive control. Treatment concentrations were selected based on MTT assay. Both MOE and UMOE did not show the cytotoxicity upto 200 μg/mL

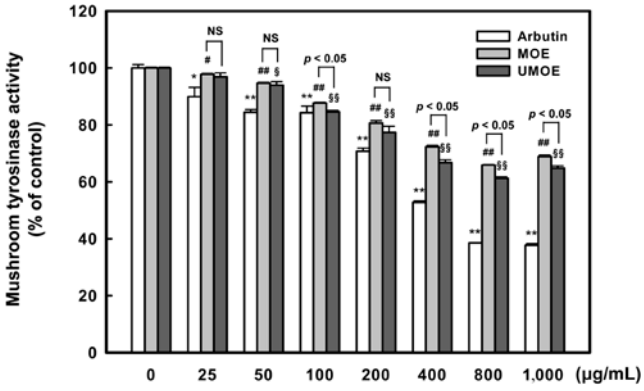

Fig. 2. Inhibitory effects of MOE, UMOE, and the positive control, arbutin on the activity of mushroom tyrosinase. Activity was determined spectrophotometrically using tyrosinase as the substrate. Values represent the mean±SE (n=3); *$^{,\#,\S}p$< 0.05, **$^{,\#\#,\S\S}p$<0.001 vs. control of each group

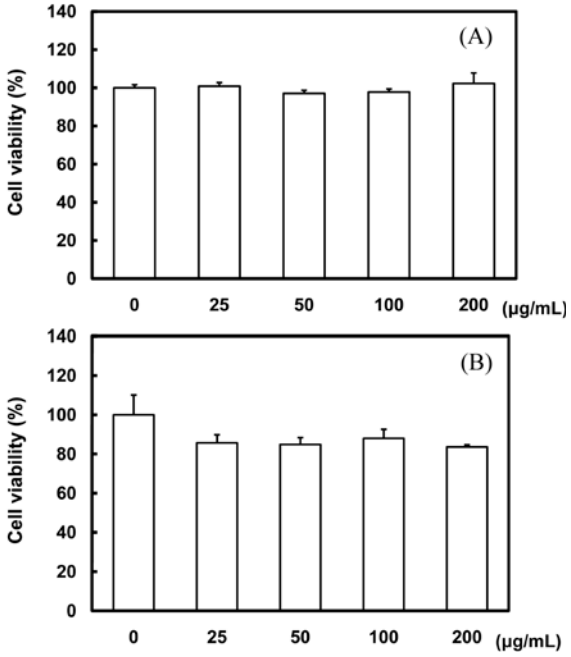

Fig. 3. Cytotoxicity of MOE and UMOE. B16-F1 cells were treated with MOE (A) and UMOE (B) for 72 h, and the cell viability was determined with MTT assay. Values represent the mean±SE (n=3).

(Fig. 3), thus the concentrations were used for further study. Arbutin at 125 μg/mL is a known non-toxic and effective concentration with anti-melanogenic activity (35). The cellular tyrosinase activity was decreased in MOE- and UMOE-treated cells (Fig. 4A). MOE treatments reduced cellular tyrosinase by 20 (100 μg/mL) and 27% (200 μg/mL), respectively. Melanin production was decreased by MOE and UMOE by 31 and 49%, respectively, at 200 μg/mL (Fig. 4B). The effect of UMOE was slightly higher than those of MOE, and similar to the activity of arbutin at the same concentrations (w/v). These results suggested that

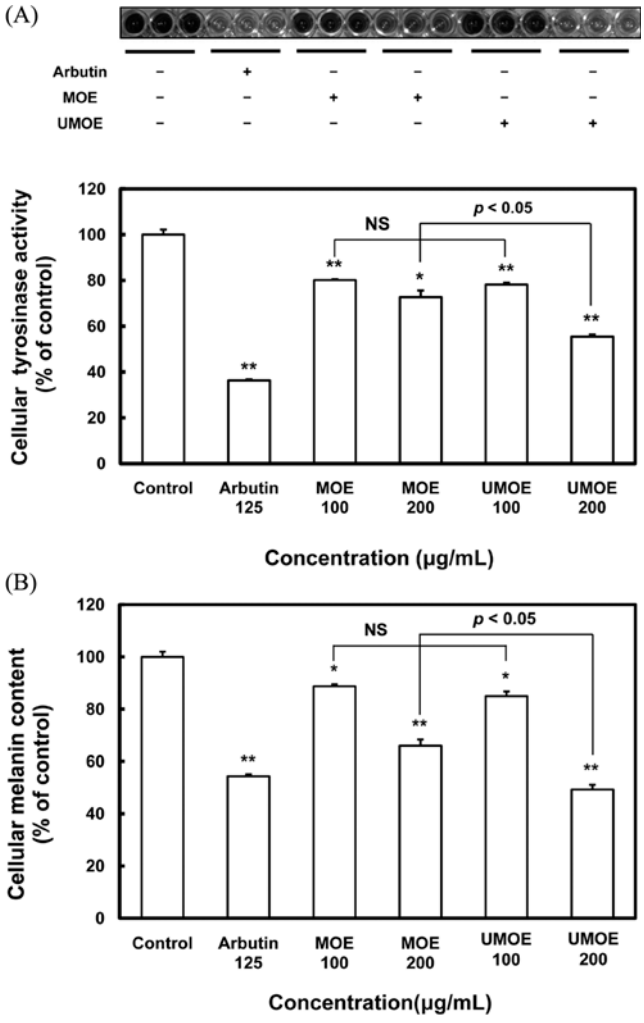

Fig. 4. Cellular tyrosinase activity and melanin contents. A, cellular tyrosinase activity. B16-F1 cells were incubated with arbutin, MOE and UMOE for 72 h and the activity were measured from cell lysates; B, melanin contents. Melanin contents were determined in B16-F1 cells treated with arbutin, MOE or UMOE for 72 h. Values represent mean±SE (n=3); *p<0.05, **p<0.001 vs. control; NS, not significant

the hypopigmentation effects of MOE and UMOE were, at least in part, due to the inhibition of enzyme activity. MOE and UMOE reduced the melanin contents of the B16-F1 melanocyte dose dependently (Fig. 4B). The melanin contents of MOE- and UMOE (200 μg/mL)-treated cells were reduced by 34 and 49%, respectively, thus UMOE was more effective than MOE.

Effects of MOE and UMOE on the gene expression of tyrosinase, TRP-1, and TRP-2 As tyrosinase, TRP-1, TRP-2 are major genes in melanogenesis, we determined their mRNA expression levels after MOE and UMOE treatments. MOE and UMOE reduced tyrosinase, TRP-1 and TRP-2 transcription levels in B16-F1 melanocyte (Fig. 5). The Fig. 5A shows the amplified gene products by RT-PCR. The 200 μg/mL of UMOE significantly inhibited tyrosinase mRNA expression level by 43% (Fig. 5B). However, the mRNA expressions of TRP-1 and TRP-2 did not show significant changes in both MOE and UMOE (Fig. 5C, 5D).

Effects of MOE and UMOE on the protein expression levels of tyrosinase, TRP-1 and TRP-2 The protein levels of tyrosinase, TRP-1 and TRP-2 were assessed by immunoblotting (Fig. 6A). The UMOE reduced the tyrosinase protein levels by 67 and 56% at 100 and 200 μg/mL, respectively (Fig. 6B). Significant reductions were also observed for TRP-1 in all concentrations of both the extracts (Fig. 6C; -77% at 100 μg/mL MOE, -73% at 200 μg/mL UMOE). TRP-2 expression levels were also decreased by 39 and 76% at 100 μg/mL of MOE and UMOE, respectively. MOE and UMOE showed stronger effects on protein expressions than mRNA levels. Although the mRNA levels of the melanogenic genes are not changed significantly, an increased rate of proteolytic degradation can attribute to the reduction of protein levels (36). Thus, MOE and UMOE might reduce the expressions of melanogenic genes through regulating post-translational stage.

Myo-inositol is an active anti-melanogenic compound in MOE and UMOE GC-MS analysis showed that myo-inositol was the main secondary metabolite in both MOE and UMOE, and its content was increased by 24% in UMOE compared with MOE. Myo-inositol is one of the essential polyols in eukaryotes, and can be synthesized from D-glucose-6-phosphate via the formation of D-myo-inositol-3-monophosphate or produced from degradation of phytic acid. In cytosol, D-myo-inositol (1,4,5)-triphosphate (IP_3), a second messenger molecule in signal transduction and lipid signaling in eukaryotic cell, is synthesized by phosphoinositide 3-kinase (PI 3-kinase) for stimulation of the PKC signaling pathway. Extensive studies showed that myo-inositol is a chemopreventive agent against cancers of the breast, colon, liver, leukemia, prostate, and skin by potentially interfering downstream signaling pathway of PI 3-kinase (37,38). Additionally, it has been suggested that increase in immunity and antioxidant properties of myo-inositol is responsible for anti-cancer activity (39).

We examined the effect of myo-inositol on melanogenesis. Interestingly, myo-inositol dose-dependently inhibited mushroom tyrosinase activity (Fig. 7A). The inhibitory effects were found especially in 10 and 20 mM concentration range. The reduction rate was 12.6 and 19.4% at 10 and 20 mM myo-inositol, respectively (p<0.01). The cellular melanin content was also significantly decreased by 26, 36, and 47% at 0.1, 0.5, 1 mM myo-inositol, respectively (Fig. 7B). The effects were greater than those of arbutin. In the

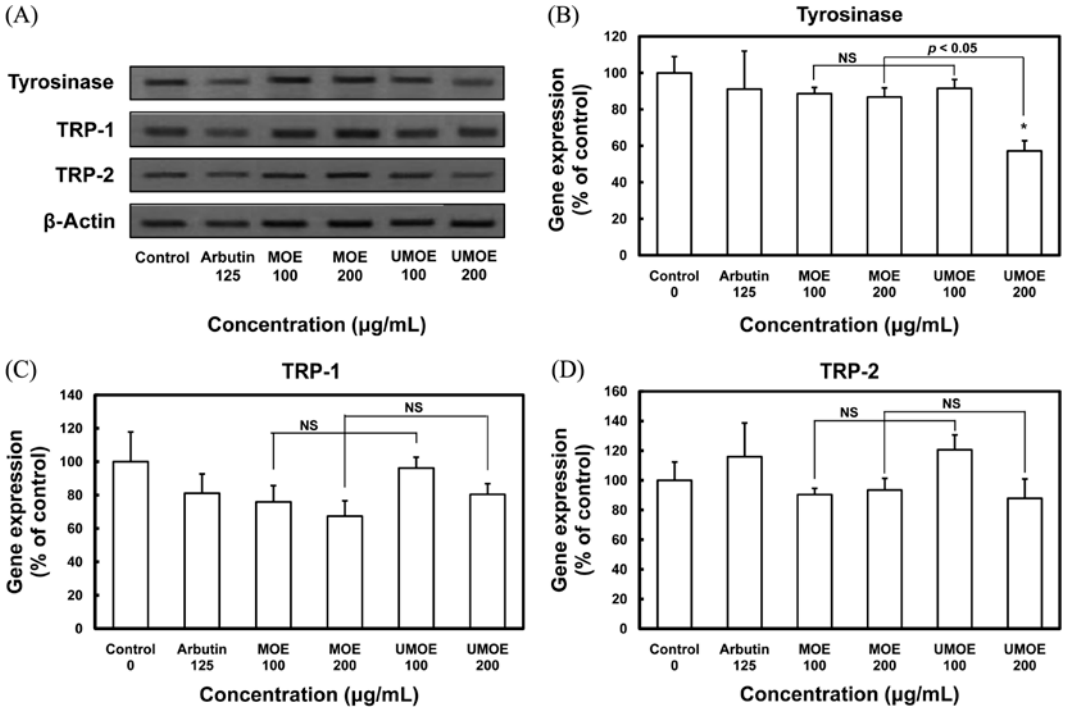

Fig. 5. Effect of MOE and UMOE on the mRNA expression of melanogenesis-related genes. B16-F1 cells were treated with MOE and UMOE for 72 h, and total RNA was extracted. PCR products (A) and mRNA expression of tyrosinase (B), TRP-1 (C), and TRP-2 (D) were analyzed with RT-PCR. Sizes of amplified gene products were 528 bp for β-actin, 477 bp for tyrosinase, 286 bp for TRP-1, and 1,044 bp for TRP-2. Values represent the mean±SE (n=3); *p<0.05 vs. control; NS, not significant

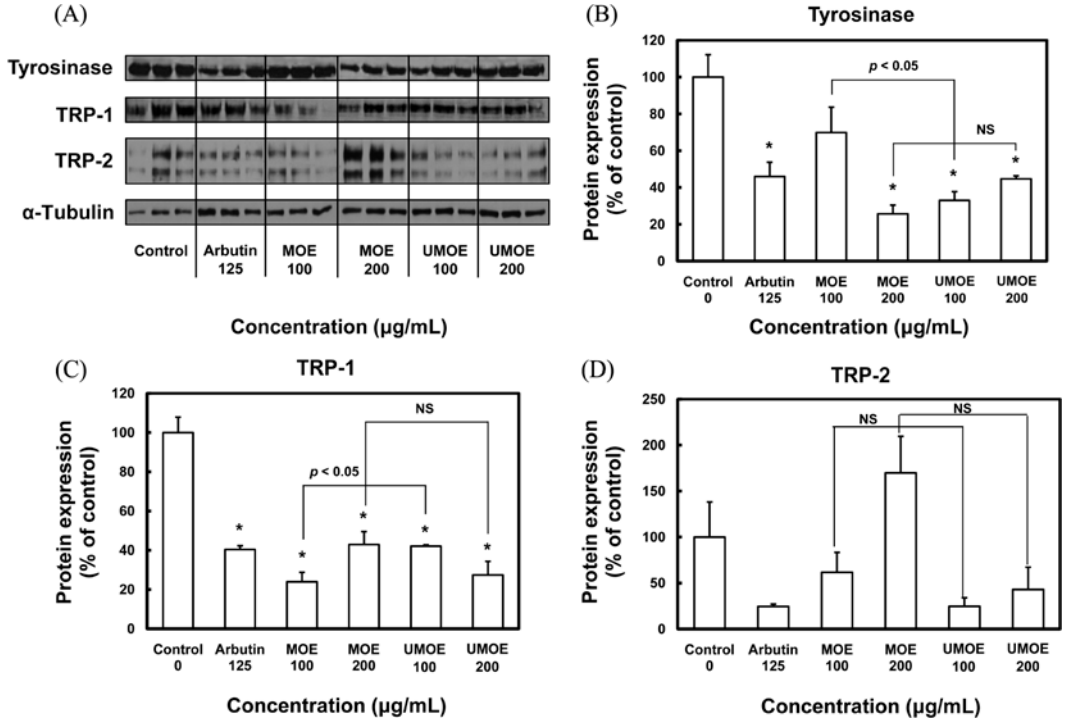

Fig. 6. Effect of MOE and UMOE on the protein expression of melanogenesis-related genes. B16-F1 cells were incubated with MOE and UMOE for 72 h. The protein was isolated from the cells and analyzed for the expression of specific proteins by immunoblotting. A, representative immunoblot; B, C, D, the protein levels of tyrosinase (B), TRP-1 (C), TRP-2 (D). Size of specific protein was 55, 75, 80, and 59 KDa for α-tubulin, tyrosinase, TRP-1, and TRP-2, respectively. Values represent the mean±SE (n=3); *p<0.05 vs. control

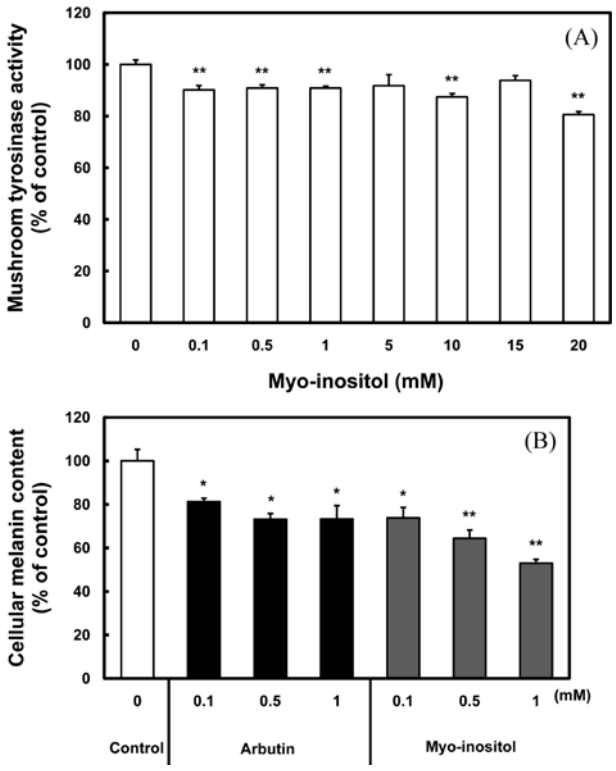

Fig. 7. Effects of myo-inositol on mushroom tyrosinase activity and cellular melanin contents. A, mushroom tyrosinase activity; B, cellular melanin contents were determined with B16-F1 cells treated with myo-insitol for 72 h. Values represent the mean±SE (n=3); *p<0.05, **p<0.01 vs. control; NS, not significant

present study, we found that myo-inositol reduced cellular melanin synthesis. It is possible that myo-inositol may inhibit protein kinase C (PKC)-dependent melanogenesis in skin tissues and further studies are needed to verify the mechanism of myo-inositol on melanogenesis.

This study suggests that MOE and UMOE may decrease melanogenesis with inhibiting tyrosinase activity and reducing the expression of major melanogenic genes. Both MOE and UMOE significantly reduced the protein levels of tyrosinase and related enzymes (TRP-1 and TRP-2). However, we found that UMOE was slightly more effective than MOE on melanogenesis. Lemon balm may be useful as skin-whitening functional foods or functional cosmetics. This effect may be achieved at least partly by myo-inositol. However, further *in vivo* study is needed to verify safety and enhancement of skin color by elevated skin-whitening index, L*-value, using one type of tristimulus reflectance instrument, which is used for measuring the color of skin directly *in vivo*, for the development of skin-whitening functional products (40).

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