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ARTICLE

Oleanolic Acid from *Fragaria ananassa* calyx Leads to Inhibition of α-MSH-induced Melanogenesis in B16-F10 Melanoma Cells

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Abstract Natural products with non-toxic and environmentally friendly properties are good sources for skin-whitening and brightening cosmetic agents. Strawberries (*Fragaria ananassa*), and their parts are used as cosmetic agents, because they contain high levels of bioactive substances. We isolated and identified compounds from *F. ananassa* calyx. Oleanolic acid has multiple biological activities, including anti-tumor, anti-angiogenic, anti-inflammatory, anti-oxidant, and pro-apoptotic effects. However, no study has investigated the influence of oleanolic acid on melanin synthesis in B16-F10 melanoma cells. In the present study, we investigated the effect of oleanolic acid on melanin biosynthesis in B16-F10 melanoma cells stimulated with α-melanocyte stimulating hormone (α-MSH). Oleanolic acid-mediated melanogenesis inhibition was studied by measuring intracellular and secreted melanin levels and by using Western blot and semi-quantitative reverse transcriptase-polymerase chain reaction analyses. Oleanolic acid suppressed melanin release and expression, resulting in a significant dose-dependent decrease in secreted and intracellular melanin levels and cellular tyrosinase activity. Furthermore, it inhibited the expression of melanogenesis-associated factors, including tyrosinase, tyrosinase-related proteins-1 and -2, and microphthalmia-associated transcription factor, in α-MSH-stimulated B16-F10 melanoma cells. The results of the present study can contribute to the development of cosmetic agents utilizing the skin whitening and brightening effect of oleanolic acid, which will likely have a wide range of applications in the cosmetic industry and/or clinical practice in the future.

Keywords anti-melanogenesis · B16-F10 melanoma cells · *Fragaria ananassa* · microphthalmia-associated transcription factor · oleanolic acid

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Introduction

Strawberries (*Fragaria ananassa*) are significant source of bioactive substances, because they contain high levels of folate, vitamin C, and phenolic constituents, which have antioxidant capabilities *in vitro* and *in vivo* (Giampieri et al., 2012). Flavonoid compounds have been identified in strawberries (Munoz et al., 2010), and the leaf and calyx of strawberries contain oleanolic and ursolic acids (Takada et al., 2010; Karlund et al., 2014).

Oleanolic acid is a naturally occurring, biologically active pentacyclic triterpenoid compound that has been isolated from more than 1,620 plant species including many food and medicinal plants (Niikawa et al., 1993; Cui et al., 2006). It has multiple biological activities, including anti-tumor, anti-angiogenic, anti-inflammatory, anti-oxidant, and pro-apoptotic effects (Kim et al., 2000; Andersson et al., 2003; Ovesna et al., 2004). One of the most important pharmacological properties attributed to oleanolic acid is its hepatoprotective effect. Oleanolic acid effectively protects the liver from acute chemically induced injury and also prevents fibrosis and cirrhosis caused by chronic liver diseases

(Liu, 1995; 2005). Furthermore, oleanolic acid had hypoglycemic and hypolipidemic effects in diabetic rats (Jang et al., 2009). To date, effects of oleanolic acid on melanogenesis have not been studied.

Melanin production is principally responsible for skin color, and melanin pigmentation is a major defense mechanism against UV radiation from the sun. Melanin biosynthesis proceeds through a complex series of enzymatic and chemical reactions in melanocytes (Hearing et al., 1982; Hearing and Jiménez, 1987; Prota, 1988; Kuzumaki et al., 1993). Melanocytes respond to different environmental stimuli and factors produced by adjacent cells in the skin (Tuerxuntayi et al., 2014). These include UV radiation, agouti signal protein, cytokines, and melanocyte-stimulating hormone (MSH) (Bellei et al., 2008). In particular, α-MSH is the most well characterized hormone involved in melanin production and released by skin melanocytes (Langan et al., 2010). Melanogenesis involves three melanocyte-specific enzymes: tyrosinase, tyrosinase-related protein-1 (TRP-1), and dopachrome tautomerase (DCT, TRP-2) (Ando et al., 2007). The expression of these tyrosinase family enzymes is tightly controlled by microphthalmia-associated transcription factor (MITF) (Tuerxuntayi et al., 2014). Tyrosinase catalyzes the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) as well as oxidation of DOPA to DOPA quinine (Plonka PM and Grabacka M, 2006). DCT converts dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Tuerxuntayi et al., 2014), and TRP-1 oxidizes DHICA to produce indole-5,6-quinone-carboxylic acid (del Marmol and Beermann, 1996). This tyrosinase reaction is involved in the abnormal accumulation of melanin pigments (hyperpigmentation) (Mishima, 1994; Virador et al., 2001). Hence, tyrosinase inhibitors, such as kojic acid and arbutin, have been established as important constituents of cosmetic products and depigmentation agents for hyperpigmentation treatment (Perez-Bernal et al., 2000; Parvez et al., 2006). Moreover, melanogenesis inhibitors, including plant extracts such as phenolic and linoleic acids, have been studied in detail (Shigeta et al., 2004; Mitani et al., 2013). However, effect of oleanolic acid on melanin synthesis in B16-F10 melanoma cells has not been studied.

In the present study, we isolated oleanolic acid from *F. ananassa* calyx and investigated the effect of oleanolic acid on melanin biosynthesis in B16-F10 melanoma cells stimulated with α-MSH. The oleanolic acid-mediated inhibition of melanogenesis was studied by measuring intracellular and secreted melanin levels as well as by Western blot and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analyses.

Materials and Methods

General. The SiO_2 and ODS resins used for column chromatography were Kiesel gel 60 (Merck, Germany). TLC analysis was carried out by using Kiesel gel 60 F_{254}, 10% H_2SO_4 solution, and Spectroline Model ENF-240 C/F (Spectronics Corporation, USA). The melting point was determined by using a Fisher-Johns apparatus (Fisher Scientific, USA) and was not corrected. Optical rotation was measured by using a JASCO P-1010 digital polarimeter (Japan). The IR spectrum was obtained by using a Perkin Elmer Spectrum One fourier transform infrared spectrometer (England). The electronic ionization mass spectrometry (EI/MS) data were recorded by using a JEOL JMS-700 mass spectrometer (Japan). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra ware recorded by using a Varian Unity Inova AS-400 FT-NMR spectrometer (USA).

Plant material. The dried calyxes of *F. ananassa* Duch (Rosaceae) were provided by GFC Co., LTD in June 2010. A voucher specimen (KHU20100608) was reserved at the Laboratory of Natural Products Chemistry, Kyung Hee University, Korea.

Extraction and isolation. The dried calyxes of *F. ananassa* (8.5 kg) were extracted with 80% aqueous MeOH (3×55 L) at room temperature for 12 h. The MeOH extract was suspended in H_2O (6.5 L) and then extracted with EtOAc (3×6.5 L), and the organic layer was concentrated to produce residue of the EtOAc fraction (FACE, 246 g). The EtOAc extract (FACE, 239 g) was subjected to SiO_2 column chromatography (c.c.; ϕ 14.5×14 cm) and eluted with $CHCl_3$-MeOH [18:1 (14 L)→13:1 (3 L)→10:1 (3 L)→7:1 (5 L)→5:1 (16 L)→2:1 (15 L)] and $CHCl_3$-MeOH-H_2O (6:4:1, 9 L). The eluting solutions were monitored by TLC to produce ten fractions (FACE-1 to FACE-10). Fraction FACE-1 [78 g, elution volume/total volume (V_e/V_t) =0–0.05 was subjected to SiO_2 c.c. (ϕ 11×15 cm) and eluted with *n*-hexane-EtOAc [7:2 (18 L)→2:1 (27 L)→1:3 (2 L)] and $CHCl_3$-MeOH [12:1 (8 L) 10:1 (4 L)], yielding 15 fractions (FACE-1-1 to FACE-1-15). Fraction FACE-1-7 (10.4 g, V_e/V_t =0.10–0.32) was subjected to SiO_2 c.c. (ϕ 8×14 cm) and eluted with *n*-hexane-EtOAc [4:1 (20 L)→3:1 (11 L)→5:2 (8 L)→2:1 (5 L)], yielding nine fractions (FACE-1-7-1 to FACE-1-7-9). Fraction FACE-1-7-6 (603 mg, V_e/V_t =0.20–0.22) was subjected to SiO_2 c.c. (ϕ 3.5×15 cm) and eluted with *n*-hexane-$CHCl_3$-MeOH (6:2:1, 2 L), yielding 13 fractions (FACE-1-7-6-1 to FACE-1-7-6-13). Fraction FACE-1-7-6-5 (47 mg, V_e/V_t =0.20–0.30) was subjected to ODS c.c. (ϕ 2×5 cm) and eluted with acetone-H_2O (7:2, 500 mL) to ultimately produce oleanolic acid [6.7 mg, V_e/V_t =0.35–0.40, TLC (ODS F_{254S}) R_f 0.46 in acetone-H_2O =5:1].

Oleanolic acid: white powder (C_5H_5N); m.p. 196–198°C; $[\alpha]_D^{25}$ +65° (*c* =0.10, $CHCl_3$); IR_ν (KBr, cm^{-1}) 3400, 1680, 1630; EI/MS *m/z* 456 $[M]^+$; ^{1}H-NMR (400 MHz, C_5D_5N, δ_H) 5.48 (1H, br. S, H-12), 3.44 (1H, dd, *J* =10.4, 5.6 Hz, H-3), 3.31 (1H, dd, *J* =15.0, 4.0 Hz, H-18), 1.70 (1H, t-like, *J* =8.8 Hz, H-9), 1.27 (3H, s, H-27), 1.22 (3H, s, H-23), 1.01 (6H, s, H-24, H-26), 0.89 (3H, s, H-25), 0.99 (3H, s, H-30), 0.93 (3H, s, H-29); ^{13}C-NMR (100 MHz, C_5D_5N, δ_C) 180.23 (C-28), 144.85 (C-13), 122.54 (C-12), 78.10 (C-3), 56.34 (C-5), 48.13 (C-9), 46.68 (C-19), 46.52 (C-17), 42.19 (C-18), 41.92 (C-14), 39.77 (C-8), 39.32 (C-4), 38.77 (C-1), 37.29 (C-10), 34.17 (C-21), 33.25 (C-22), 33.20 (C-7), 33.13 (C-29), 30.94 (C-20), 28.76 (C-23), 28.32 (C-15), 28.09 (C-2), 26.07 (C-27), 23.72 (x2, C-11, C-16), 23.67 (C-30), 18.80 (C-6), 17.33 (C-

26), 16.49 (C-24), 15.54 (C-25).

Cell culture. Murine B16-F10 melanoma cells were obtained from ATCC (USA), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere containing 5% CO_2. The cells were harvested by incubation in phosphate-buffered saline containing 1 mM EDTA and 0.25% trypsin for 5 min at 37°C and were used for subsequent bioassays.

Cell viability. B16-F10 cell were plated at a density of 5×10^3 cells per well. After 24 h, oleanolic acid was added at concentrations ranging from 1.56 to 50 μM, and the cells were incubated at 30°C for 40 h. Treated cells were examined and compared to control cells by using the EZ-Cytox Cell Viability assay. The cells were incubated with DMEM medium (90 μL/well) and EZ-Cytox reagent (10 μL/well) at 37°C for 2 h. Cell viability was determined by measuring the absorbance at 450 nm using a microplate reader (Synergy HT, Bio-Tek instruments, USA).

Determination of melanin content in B16-F10 cells. Melanin content was measured as described by Bellei et al. (2008), with minor modifications. Briefly, the cells were cultured in DMEM containing 10% FBS without phenol red in the presence or absence of test compounds for 72 h. The medium was collected and 100 μL aliquots of medium were placed in 96-well plates, and the optical density of each culture well at 450 nm was measured by using a microplate reader. After concentration by centrifugation, the cell pellets were dissolved in 1 mL of 1 N NaOH at 80°C for 60 min and centrifuged again for 30 min at 17,000×*g*. The optical densities of the supernatants at 450 nm were measured by using a microplate reader.

Measurement of cellular tyrosinase activity. Tyrosinase activity was determined as described by del Marmol and Beermann, (1996), with L-DOPA as the substrate. B16-F10 melanoma cells cultured in the presence or absence of test compounds for 72 h were collected, lysed in lysis buffer (NaCl 150 mM, Tris 50 mM, Igpal 1%, protein inhibitor cocktail; Roche, complete Mini, DTT 1 μM), and centrifuged at 17,000×*g* for 30 min. After determining the protein concentration, duplicate 50 μL cell lysate samples (containing equal amounts of protein) were incubated with 50 μL of 10 mM L-DOPA at 37°C for 1 h. The absorbance at 450 nm was measured.

Western blot analysis. Melanoma cells (1.0×10^5 cells) were seeded in 60-mm dishes and cultured for 24 h. Subsequently, the cells were incubated with or without oleanolic acid and arbutin in the presence of α-MSH (50 μM) for 72 h. After incubation, the cells were lysed in lysis buffer containing a proteinase inhibitor (Roche, USA). The protein concentrations of cell lysates were determined by using the Protein Assay kit (Thermo Scientific, USA). Equal amounts of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were probed with primary antibody diluted in Tris-buffered saline containing 0.1% Tween 20 (TBST). The membranes were washed three times with TBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were detected by using the Clarity Western ECL Substrate (Bio-Rad Laboratories, USA) and analyzed by using a Chemi-Doc XRS (Bio-Rad), according to the manufacturer's instructions.

Semi-quantitative RT-PCR. Melanoma cells (1.0×10^5 cells) were seeded in 60-mm dishes and cultured for 24 h. Then, the cells were incubated with the test compound and 50 μM α-MSH for 24 h. Total RNA was extracted from the cells by using the RNeasy Mini kit (Qiagen, USA) according to the manufacturer's instructions. The concentration and purity of the RNA were determined by measuring the absorbances at 260 and 280 nm and determining the ratio. cDNA synthesis, and PCR was performed by using the ReverTra Ace qPCR RT kit (Toyobo, Japan) according to the manufacturer's instructions. The PCR conditions were 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min for 25 cycles for tyrosinase, 30 cycles for Trp-1, Trp-2, and MITF, and 25 cycles for β-actin. PCR products were separated on 1.5% agarose gels and visualized by using ethidium bromide staining. The primer pairs were: tyrosinase, 5'-CAGATCTCTGATGGCCAT-3' and 5'-GGATGACATAGACTGAGC-3'; TRP-1, 5'-CTTTCTCCCTTCCTTACTGG-3' and 5'-TGGCTTCATTCTTGGTGCTT-3'; TRP-2, 5'-TGAGAAGAAACAAAGTAGGCAGAA-3' and 5'-CAACCCCAAGAGCAAGACGAAAGC-3'; MITF, 5'-CCCGTCTCTGGAAACTTGAT-3' and 5'-ACCTGCTGCTCAGAGTACAG-3'; and β-actin, 5'-GGGAAATCGTGCGTGACAT-3' and 5'-CAGGAGGAGCAATGATCTC-3'. β-actin was used as an internal control for normalization.

Statistical analysis. All statistical analyses were performed by using R-program. All experimental groups were analyzed by using Student's t-test for each experimental period. p values <0.05 were considered statistically significant. Data are expressed as the mean ± standard deviation (n =6).

Results

Oleanolic acid (Fig. 1) showed absorbance bands in the IR spectrum due to the hydroxyl group (3,400 cm^{-1}), the carboxyl group (1,680 cm^{-1}), and the double bond (1,630 cm^{-1}). The molecular weight was determined to be 456 based on the molecular ion peak $[M]^+$ at m/z 456 in the EI/MS spectrum. In the ^{1}H-NMR (400 MHz, C_5D_5N) spectrum, an olefin methine proton at δ 5.48 (1H, br. s), an oxygenated methine proton at δ 3.44 (1H, dd, J=10.4, 5.6 Hz), and a methine proton at δ 3.31 (1H, dd, J= 15.0, 4.0 Hz) were observed. Additionally, in the high magnetic field, seven singlet methyl proton signals at δ 1.27 (3H, s), 1.22 (3H, s), 1.01 (6H, s), 0.99 (3H, s), 0.93 (3H, s), and 0.89 (3H, s) were observed, indicating that the isolated compound could be an oleanane-type triterpenoid. The ^{13}C-NMR (100 MHz, C_5D_5N) spectrum showed 30 carbon signals, including one carboxyl carbon at δ 180.23 (C-28), one oxygenated methine carbon at δ 78.10 (C-3), one olefin quaternary carbon at δ 144.85 (C-13), one

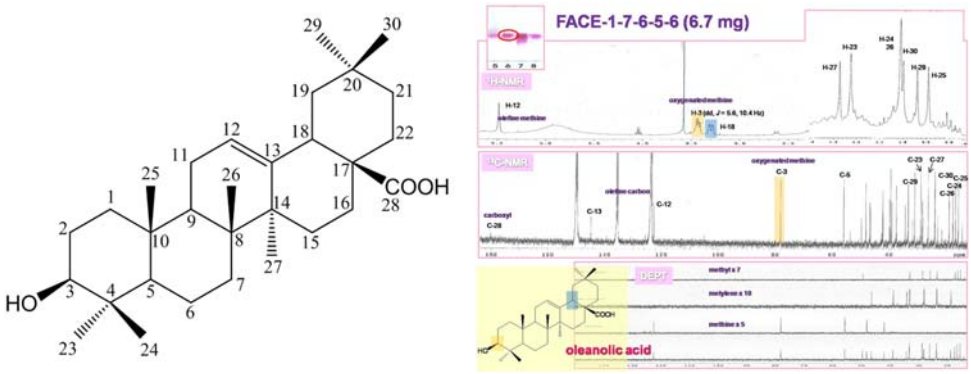

Fig. 1 Chemical structure of oleanolic acid and NMR data. Oleanolic acid was isolated and identified from *Fragaria ananassa* calyx.

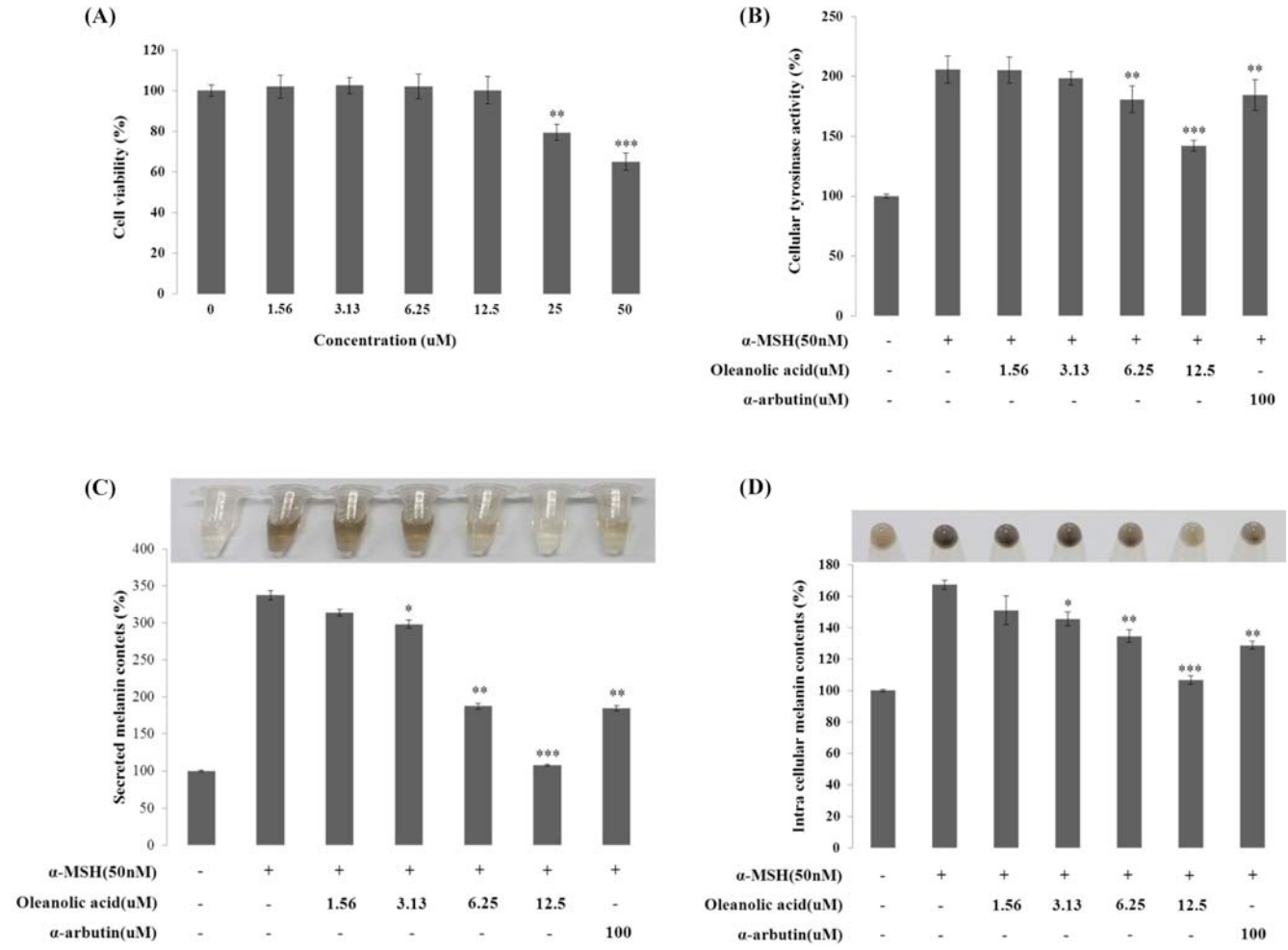

Fig. 2 Inhibitory effect of oleanolic acid on melanogenesis in B16-F10 melanoma cells. (A) Cell viability was determined by using the EZ-Cytox method, which showed that oleanolic acid was cytotoxic at concentrations >25 μM. (B) Cellular tyrosinase activity decreased in a dose-dependent manner following oleanolic acid treatment. (C) Secreted and (D) intracellular melanin levels decreased in a dose-dependent manner in oleanolic acid-treated B16-F10 melanoma cells. Data are expresses as the mean ± SD of three independent experiments. *p* values indicate significant differences as compared to control cells, which were treated with α-MSH only (**p* <0.5, ***p* <0.05, ****p* <0.005).

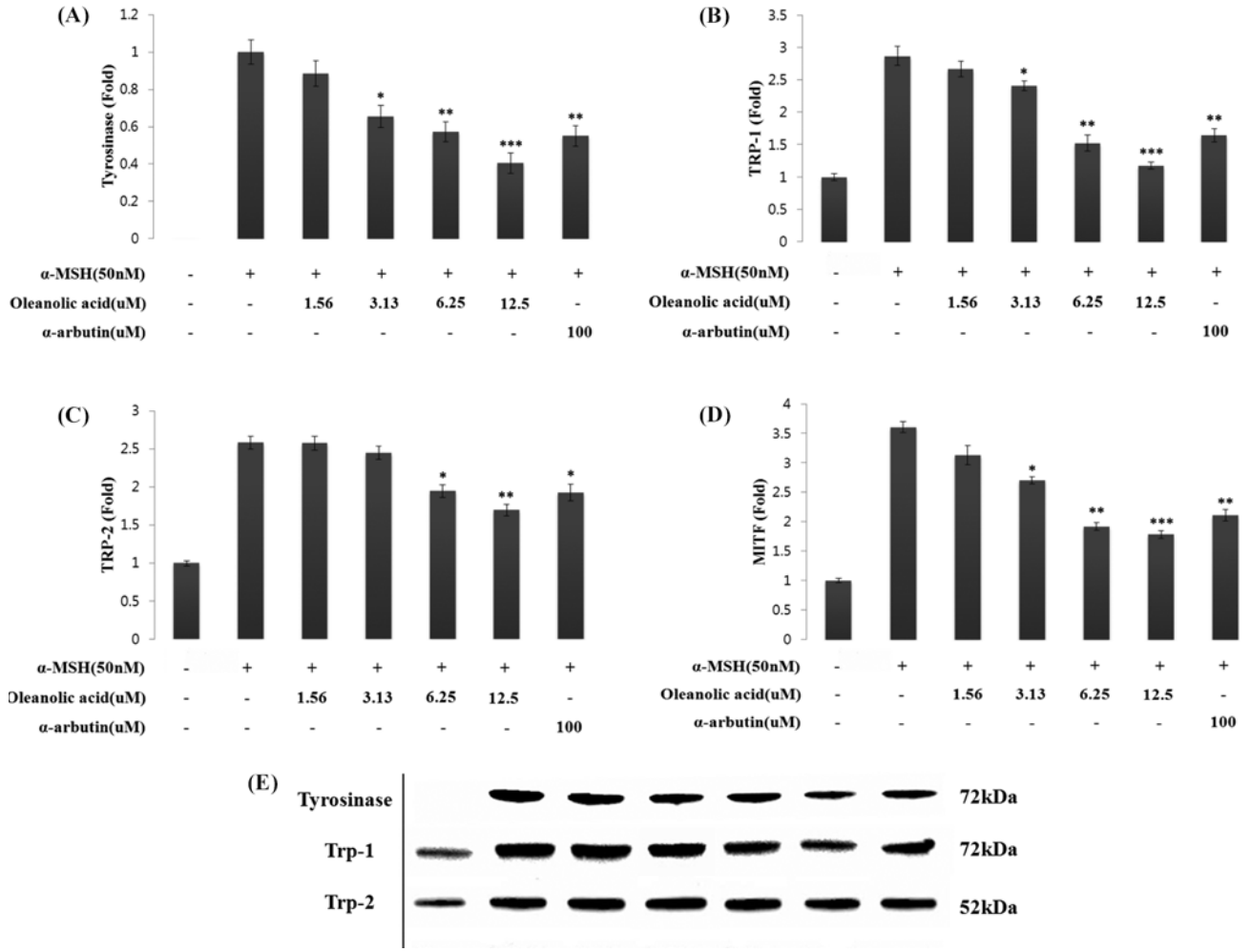

Fig. 3 Effect of oleanolic acid on tyrosinase, TRP-1, TRP-2, and MITF protein expressions levels in B16-F10 melanoma cells. Intensities of tyrosinase (A), TRP-1 (B), TRP-2 (C), and MITF (D) protein bands detected by using Western blot (E). The levels of melanogenesis-associated proteins decreased in a dose-dependent manner after treatment with oleanolic acid as indicated by Western blot analysis. Note that the band intensity in the control treatment (addition of α-MSH only) was the highest, and oleanolic acid treatment (12.5 μM) resulted in a decrease in band intensity. Data are expressed as the mean ± SD of three independent experiments. *p* values indicate significant differences as compared to control cells, which were treated with α-MSH only (**p* <0.5, ***p* <0.05, ****p* <0.005).

olefin methine carbon at δ 122.54 (C-12), seven methyl carbons at δ 33.13 (C-29), 28.76 (C-23), 26.07 (C-27), 23.67 (C-30), 17.33 (C-26), 16.49 (C-24), and 15.54 (C-25), ten methylene carbons at δ 46.68 (C-19), 38.77 (C-1), 34.17 (C-21), 33.25 (C-22), 33.20(C-7), 28.32 (C-15), 28.09 (C-2), 23.72 (C-11, 16), and 18.80 (C-6), three methine carbons at δ 56.34 (C-5), 48.13 (C-9), and 42.19 (C-18), and six quaternary carbons at δ 46.52 (C-17), 41.92 (C-14), 39.77 (C-8), 39.32 (C-4), 37.29 (C-10), and 30.94 (C-20), the multiplicity of which was determined by performing distortionless enhancement by polarization transfer experiments. The chemical shift of a carboxyl group at δ 180.23 (C-28) and two sp^2 hybridized carbons at δ 122.54 (C-12) and δ 144.85 (C-13) indicated that the oleanane-type triterpenoid was olean-12-en-28-oic acid. Thus, the compound was identified as the oleanane-type pentacyclic-triterpenoid 3β-hydroxy-olean-12-en-28-oic acid (oleanolic acid) through comparison of the spectroscopic data with those in the literature (Baek and Lee, 2010).

To identify the biological activity of oleanolic acid, B16-F10 melanoma cells were treated with the extract. The effect of oleanolic acid isolated from a strawberry calyx extract on the viability of B16-F10 melanoma cells was examined by using the EZ-Cytox reagent. The cells were treated with different concentrations (1.56, 3.13, 6.25, 12.5, 25, and 50 μM) of oleanolic acid. The results showed that oleanolic acid was not toxic at concentrations up to 12.5 μM as compared with the untreated control group (0 μM). However, the number of viable cells was significantly reduced with increasing concentrations of oleanolic acid (Fig. 2A).

Next, melanin levels and tyrosinase activity were measured. Cellular tyrosinase activity was examined to investigate the mechanisms responsible for the decreased pigmentation induced

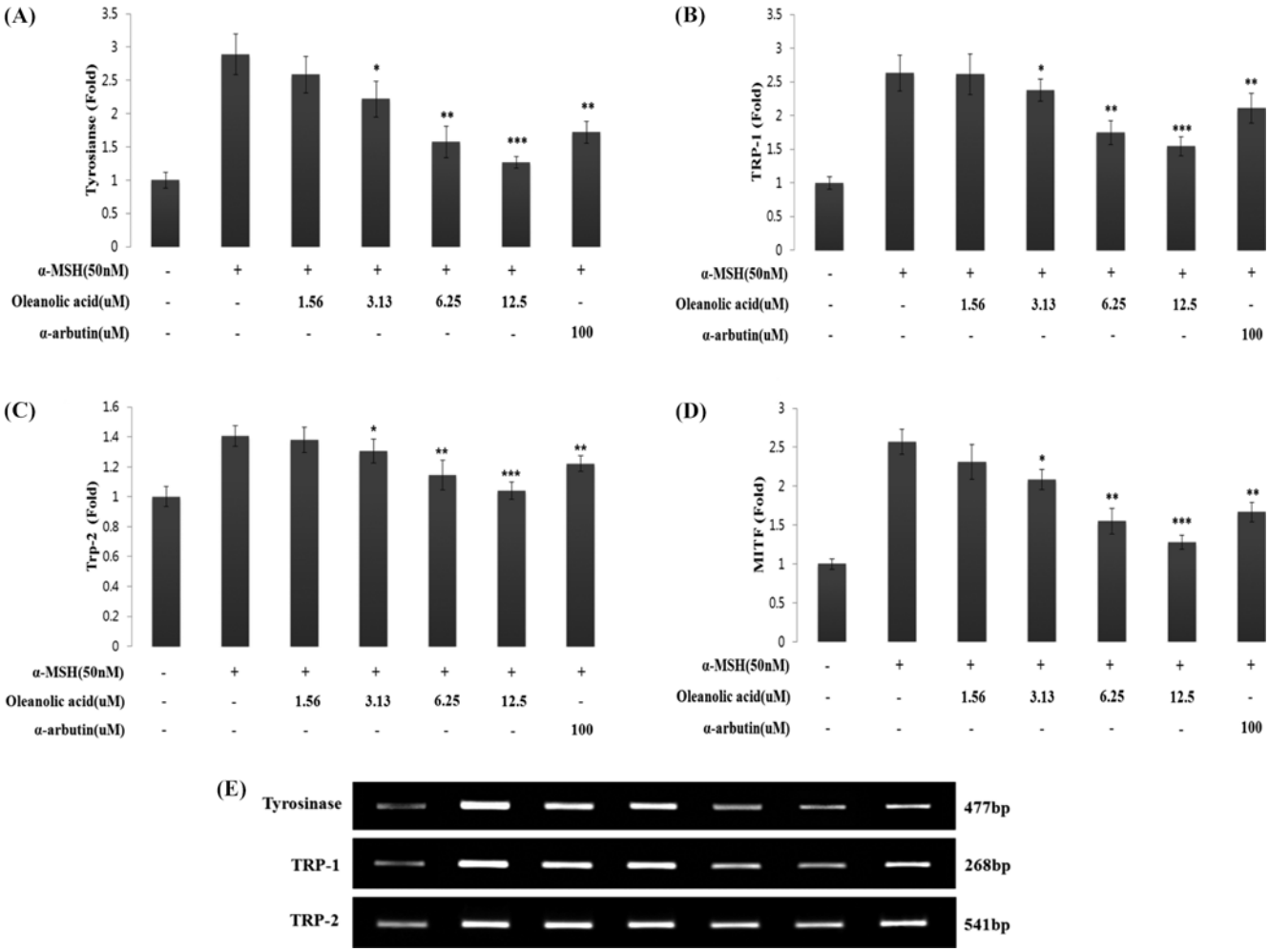

Fig. 4 Effect of oleanolic acid on tyrosinase, TRP-1, TRP-2, and MITF mRNA levels in B16-F10 melanoma cells. Intensities of tyrosinase (A), TRP-1 (B), TRP-2 (C), and MITF (D) cDNA bands determined by semi-quantitative RT-PCR (E). Quantitative PCR analysis results show that the expression of melanogenesis-associated genes decreased significantly following oleanolic acid treatment. Note that treatment with 12.5 μM oleanolic acid resulted in decreased intensity as compared to that in control samples (addition of α-MSH only). Data are expressed as the mean ± SD of three independent experiments. *p* values indicate significant differences as compared to control cells, which were treated with α-MSH only (**p* <0.5, ***p* <0.05, ****p* <0.005)

by oleanolic acid. Oleanolic acid treatment resulted in a significant and dose-dependent decrease in cellular tyrosinase activity (Fig. 2B). The effects of oleanolic acid on secreted and intracellular melanin levels were examined at concentrations of 1.56, 3.13, 6.25, and 12.5 μM and compared with those after treatment with 100 μM α-arbutin. α-MSH-stimulated cells were treated with oleanolic acid and α-arbutin for 3 days and the amounts of intracellular and secreted melanin were measured. Oleanolic acid decreased the amount of secreted and intracellular melanin. The oleanolic acid treatment resulted in a significant decrease in melanin secretion by α-MSH-stimulated B16-F10 melanoma cells, and cells treated with 12.5 μM oleanolic acid secreted at similar levels of melanin as those in unstimulated cells (Fig. 2C). Moreover, the oleanolic acid treatment resulted in a dose-dependent decrease in intracellular melanin levels in α-MSH-stimulated B16-F10 melanoma cells. Cells treated with 12.5 μM oleanolic acid contained similar levels of melanin as those in unstimulated cells (Fig. 2D). Comparison of the inhibitory effects of oleanolic acid and α-arbutin on melanin production revealed that oleanolic acid had greater activity than á-arbutin (Fig. 2C and D).

To explore the mechanism of oleanolic acid-mediated inhibition of melanin biosynthesis, we investigated the effect of oleanolic acid on the expression of melanogenesis-associated proteins. Tyrosinase, TRP-1, TRP-2, and MITF protein levels in melanoma cells were determined by using Western blot analysis. Melanogenesis-associated protein expression levels were lowest in B16-F10 melanoma cells treated with 12.5 μM oleanolic acid (Fig. 3A, B, C, and D). There were statistically significant differences in protein expression levels between oleanolic acid-treated cells and

untreated controls. Furthermore, treatment with oleanolic acid at high concentrations reduced protein expression levels to a greater extent than treatment with 100 μM α-arbutin (Fig. 3A, B, C, and D). Protein expression levels decreased significantly in a dose-dependent manner (Fig. 3E).

Next, we determined the expression of melanogenesis-associated genes. The cells were treated with 1.56, 3.13, 6.25, and 12.5 μM of oleanolic acid for 24 h, and total cellular RNA was extracted. The mRNA was amplified by using RT-PCR with specific primers for tyrosinase, TRP-1, TRP-2, and MITF. Treatment of B16-F10 melanoma cells with 12.5 μM oleanolic acid decreased the expression of all tested genes to the greatest extent as compared to the other tested concentrations (Fig. 4A, B, C, and D). There were statistically significant differences in transcript levels between oleanolic acid-treated and control cells. In addition, the inhibitory effect of oleanolic acid (12.5 μM) on the expression of genes involved in melanin synthesis was greater than that of 100 μM α-arbutin (Fig. 4A, B, C, and D). The mRNA expression of the four tested melanogenesis-associated genes decreased significantly in a dose-dependent manner (Fig. 4E).

Discussion

In the present study, we isolated and identified oleanolic acid from the EtOAc fraction from *F. ananassa* calyx. The effects of oleanolic acid on melanogenesis in α-MSH-stimulated B16-F10 melanoma cells were investigated. We demonstrated that oleanolic acid could suppress melanin release and expression. Oleanolic acid treatment resulted in a significant and dose-dependent decrease in secreted and intracellular melanin levels and cellular tyrosinase activity in α-MSH-stimulated melanoma cells. In particular, the levels of melanin in cells treated with 12.5 μM oleanolic acid were similar to those of unstimulated cells. The results showed that oleanolic acid was equally or more effective than α-arbutin in inhibiting cellular tyrosinase activity and decreasing melanin production in α-MSH-stimulated B16-F10 melanoma cells, although oleanolic acid was more cytotoxic than α-arbutin.

We observed that treatment with oleanolic acid at concentrations >25 μM induced cytotoxicity. This finding was consistent with a previous study using A375 melanoma cells (Cijo George et al., 2014). Because oleanolic acid was cytotoxic at high doses, B16-F10 melanoma cells were treated with lower concentrations, and the IC_{50} value for inhibition of melanin production (including secreted and intracellular melanin) was not determined.

To the best of our knowledge, this is the first report showing that oleanolic acid isolated from *F. ananassa* calyx specifically inhibited the expression of melanogenesis-associated factors, including tyrosinase, TRP-1, TRP-2, and MITF, in α-MSH-stimulated B16-F10 melanoma cells. Notably, this is the first study to show that oleanolic acid can inhibit melanin biosynthesis. The expression of tyrosinase, TRP-1, TRP-2, and MITF mRNA and protein decreased in a dose-dependent manner in α-MSH-induced B16-F10 melanoma cell treated with oleanolic acid. In addition, the results showed that the effect of oleanolic acid was comparable to that of α-arbutin. Notably, we confirmed that not only the levels of three melanogenic enzymes, but also MITF expression, decreased after oleanolic acid treatment during α-MSH-induced melanogenesis. Hence, these results indicated that the suppressive activity of oleanolic acid on melanin synthesis maybe due to the downregulation of MITF signaling pathways. α-MSH can increase melanin synthesis by binding melanocortin 1 receptor (MC1R) (Scott et al., 2002), which is a seven-transmembrane, G-protein-coupled receptor. The α-MSH-bound MC1R activates adenylyl cyclase, thereby inducing cyclic AMP (cAMP) production (Mountjoy et al., 1992; Gillbro and Olsson 2011), which leads to phosphorylation of the cAMP responsive-element-binding protein (CREB) transcription factor. CREB transcriptionally activates different genes, including MITF, which regulates the expressions of Trp-1 and Trp-2 (Levy et al., 2006). Therefore, it is maybe possible that oleanolic acid exerts its effect through inhibition of CREB, protein kinase A, or cAMP.

In conclusion, the findings of this study showed that the inhibitory effect of oleanolic acid on melanin production was similar to that of α-arbutin, and that oleanolic acid specifically inhibited melanogenesis-associated factors at the levels of transcription and protein expression. The results of this study can contribute to the development of cosmetic agents utilizing the skin whitening and brightening effects of oleanolic acid. Although the compound has low cytotoxicity, it will likely have a wide range of applications in the cosmetic industry and/or clinical practice in the future.

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