



Effects of phenolics from *Oplismenus undulatifolius* in α -MSH-stimulated B16F10 melanoma cells

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Abstract In this study, the efficacy of melanoma cell B16F10 was investigated using the Korean native plant *Oplismenus undulatifolius* (OU). First, the cell viability of the extract was more than 90% when treated with 15 μ g/mL of phenolics from OU. The results showed that melanin biosynthesis and cellular tyrosinase synthesis were inhibited by treatment with α -melanocyte-stimulating hormone-stimulated mouse melanoma cell B16F10 at a concentration of 15 μ g/mL of phenolics for cell-line efficacy. The expression of tyrosinase, tyrosinase-related protein (TRP)-1, TRP-2, and microphthalmia transcription factor (MITF) protein was confirmed by western blot to investigate the effect of phenolics from OU on melanin biosynthesis. When treated with phenolics from OU 15 μ g/mL, tyrosinase, TRP-1, TRP-2, and MITF decreased the protein expression level. In particular, tyrosinase, TRP-1, and MITF inhibited the production amount to a level similar to that of the non-treated normal group, indicating that the effect was excellent. Therefore, phenolics from OU acts as an inhibitor of tyrosinase, TRP-1, TRP-2, and its transcription factor MITF, and participates in melanin biosynthesis mechanism. These results suggested the potential for development as a material.

Keywords B16F10 mouse melanoma cells, Extract · α -Melanocyte-stimulating hormone-stimulation · *Oplismenus undulatifolius*

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Introduction

In 1992, according to the United Nations Convention on Biological Diversity, the exclusive right of each country's plant resources was recognized. As a result, competition for industrialization is intensifying, for example, by searching for and obtaining plant genetic resources among countries [1]. In accordance with such international changes and trends, the importance of national support and active development and utilization of forest resource plants is emphasized.

Among the vast array of physiologically active compounds present in plants, plant-produced anti-oxidative polyphenolic compounds are known to protect the plants from various hazardous conditions, such as cell-damaging ultraviolet irradiation, cellular oxidative stress, and pest attacks. Notably, some of these polyphenolic compounds (e.g., flavonoids and acidic phenolic compounds) are known to have diverse beneficial physiological activities in humans, including anti-oxidative, anti-allergic, anti-diabetic, anticancer, anti-inflammatory, skin whitening, and anti-wrinkle effects [2,3]. Owing to its antioxidant potential with low toxicity, several articles have assessed its use as an active extract of cosmetic ingredients. Therefore, the development of health-related functional foods and food ingredients has considerable scope in terms of efficient utilization of natural resources, the development of new food resources, and the development of new materials.

Oplismenus undulatifolius (OU) is a perennial herbaceous plant of Gramineae that is commonly found in the forests and is widely distributed in the Far East, mainly in Central Asia in the Korean and Mediterranean coasts, northern India [4]. OU is currently used as livestock feed, but it is rarely known as a medicinal resource, and all of the natural substances contained in this plant are isoarborinol, cylindrin, and triedelin, all of which are triterpenoid components [5].

Recently, many types of researches and developments have been made in the field of functional foods and functional cosmetics for superior functional materials derived from medicinal plant

resources that have been proven to be effective [6,7]. Also, significant research is underway in terms of the efficient use of natural resources and contribution to the development of new materials [8].

Melanin is a pigment that forms between the lower layer of the epidermis and the dermis. It is a beneficial substance that is produced to protect cells against external stimuli such as ultraviolet rays [9]. However, excessive or abnormal melanin production in the body may cause skin pigmentation, freckles, and skin irritation as well as skin cancer [10]. Tyrosinase is the most important enzyme in the production of melanin in the skin. It converts tyrosine in the melanosome of melanocyte into DOPA and DOPA quinone during melanin biosynthesis. DOPA chrome is metabolized by indole-5,6-quinone carboxylic acid and indole-5,6-quinone by enzymatic action and autoxidation. Finally, melanin is synthesized [11]. In this process, the expression of cellular tyrosinase, microphthalmia transcription factor (MITF), tyrosinase-related protein (TRP)-1, and TRP-2 protein, which are involved in the production of melanin pigment by molecular biology, increases in cells. When excessive melanin is produced, the skin tone becomes dark and dark circles, spots and freckles appear on the surface of the skin.

B16F10 mouse melanoma cell inhibited cellular tyrosinase inhibitory activity and inhibited the expression of proteins involved in melanin production such as MITF, TRP-1, and TRP-2. Based on the experimental results, the efficacy of the effect was confirmed.

Materials and Methods

Preparation of phenolic samples

Lee et al. [12] reported that the biological activities of OU for functional cosmetic ingredients or other physiological supplements were expressed by phenolics in OU. Thus, the preparation of phenolics from OU has carried out the following step.

OU, which is a native herbaceous plant on the hillside, Korea, was sampled and dried at 50 °C in a dry oven and pulverized to 40 mesh. The sample was vacuum packed and stored at 4 °C for cold storage. Phenolic extraction from the sample was performed by adding 500 mL of 70% ethanol to 10 g of dried OU powder and stirring at room temperature for 24 h at 120 rpm. The phenolic solution was filtered through Whatman No. 1 filter paper. After removing all of the ethanol by using a rotary vacuum evaporator (Eyela NE, Tokyo, Japan), it was lyophilized and used as a sample.

Cell culture

In this study used mouse melanoma cell B16F10, was purchased from American type culture collection (ATCC; Manassas, VA, USA). Cell culture was performed with 10% fetal bovine serum (FBS, HyClone Laboratories, Inc, Logan, Utah, USA) and 1%

penicillin/streptomycin (100 U/mL, HyClone Laboratories, Inc.) in Dulbecco's Modified Eagle's Medium (DMEM, HyClone Laboratories, Inc.) was added and the cells were subcultured in a 5% CO₂ incubator at 37 °C and seeded at the appropriate numbers into wells of the cell culture plate for further experiments.

MTT assay

To determine the quality of the various extracts the viability of cells following treatment with extracts was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. B16F10 cells were seeded on 48 well plates at 5×10^3 cells/well using the method of Carmichael et al. [13] for measuring the cell viability of the samples. And incubated in a 5% CO₂ incubator at 37 °C for 24 h to stabilize. After the culture medium was removed, the phenolics were treated with concentrations of 1, 5, 10, 15, and 20 µg/mL and cultured for 24 h. Then, 5 mg/mL MTT (Sigma-Aldrich Co., Louis, MO, USA) reagent was added and reacted for 4 h. After completion of the incubation, all of the culture medium was removed, 500 µL of dimethyl sulfoxide was added to each well, reacted at room temperature for 10 min, and absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) reader (SPECTRO star Nano, BMG LABTECH, Ortenberg, Germany). Cell viability was expressed as the absorbance reduction rate of the sample solution addition group and the no addition group.

Cell stimulus and phenolics treatment

To stimulate B16F10 cells, 100 nM α -melanocyte stimulating hormone (MSH) was added to each dish except for the non-treated group after seeding to 5×10^5 cells/well in a 100 nm cell culture dish. After 1 h, the phenolics were treated with concentrations (1, 5, 10, 15, and 20 µg/mL) and cultured for 24 h.

Inhibitory activity of cellular tyrosinase

All of the culture broth was removed from B16F10 cells after stimulation and phenolics treatment. After washing twice with cold PBS, 250 µL of lysis buffer per well was added to dissolve the cells. The lysis buffer was a mixture of mammalian protein extraction reagent (M-PER) (Thermo Fisher Scientific, Waltham, MA, USA) and protease inhibitor (Thermo Fisher Scientific). The mixture was centrifuged at 13,000 rpm for 15 min at 4 °C to remove protein components. Then, 40 µL of the supernatant was added to each well of a 96 well plate and mixed with 160 µL of 10 mM 3,4-Dihydroxy-L-phenylalanine (L-DOPA) substrate dissolved in 0.1 M sodium phosphate buffer (pH 6.8). After incubation at 37 °C for 1 h, the amount of DOPA chrome produced was measured at 490 nm with an ELISA reader (SPECTRO star Nano, BMG LABTECH) to confirm cellular tyrosinase inhibitory activity.

Inhibitory activity of melanin production

The culture medium was removed from the B16F10 cells that had

been subjected to stimulation and phenolics treatment by the method of Hosoi et al. [14] and washed twice with cold PBS. Then, a mixture of M-PER and protease inhibitor was used as lysis buffer and 250 μ L was added to dissolve the cells, and the supernatant was removed by centrifugation at 13,000 rpm for 15 min at 4 °C, and the pellets of the separated cells were collected and dried. To the isolated pellet, 200 μ L of 1 N NaOH was added and reacted at 70 °C for 1 h. Melanin content was measured at 405 nm using an ELISA reader (SPECTRO star Nano, BMG LABTECH) to confirm melanin biosynthesis inhibitory activity.

Western blot analysis

Western blot analysis was performed using Tsareva et al. [15]. After the culture medium was removed, stimulated and phenolics-treated B16F10 cells were washed twice with cold PBS, and the cells were lysed by adding 250 μ L of lysis buffer (M-PER and protease inhibitor mixture) per well. After centrifugation at 13,000 rpm for 15 min at 4 °C, the supernatant was separated and proteins were quantitated using the BCA assay kit (Thermo Fisher Scientific). Then, 20 μ L of protein was separated by electrophoresis using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred to a PVDF membrane at 60 V for 2 h and 30 min to inhibit the nonspecific binding of the antibody. The resulting protein was transferred to 5% bovine serum albumin dissolved in 1 \times Tris-Buffered Saline Tween (TBST)-and the background was removed. After washing with 1 \times TBST every 10 min three times, the primary antibodies were diluted in proportions with tyrosinase (T311, 1:500, Thermo Fisher Scientific), MITF (N2C1, 1:500, GeneTex, Irvine, CA, USA), TRP-1 (G-9, 1:100, Santa Cruz Biotechnology, Dallas, TX, USA), TRP-2 (E-10, 1:100, Santa Cruz Biotechnology) and GAPDH (GA1R, 1:1,000, Thermo Fisher Scientific), respectively, and reacted overnight at 4 °C. The membranes were washed three times with 1 \times TBST for 10 min, the cells were incubated with goat anti-mouse IgG1-HRP (sc-2060, 1:500, Santa Cruz Biotechnology) for tyrosinase, TRP-1, TRP-2, and GAPDH as secondary antibodies were used. MITF was reacted with mouse anti-rabbit IgG-HRP (sc-2357, 1:500, Santa Cruz Biotechnology) at room temperature for 1 h 30 min. After washing with 1 \times TBST for 10 min three times, it was reacted with Super Signal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) solution. And it was transferred to film. The band was developed using a C 300 image analyzer (Azure Biosystems, Dublin, CA, USA). And density was quantitated.

Statistical analysis

All tests were performed in triplicate (n=3). Statistical analysis was conducted using one-way ANOVA in IBM SPSS Statistics 25 (Statistical Package for Social Science, Chicago, IL, USA). Duncan's multiple range tests were performed to analyze differences. A *p*-value of less than **p*<0.05, ***p*<0.01 was considered to indicate a significant difference.

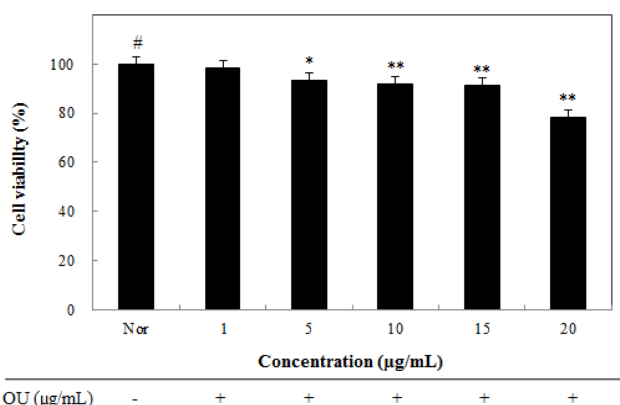


Fig. 1 Cell viability in B16F10 melanoma cells by phenolics from OU. 5×10^3 cells were seeded in serum-free medium for 24 h, the cells were treated from normal or different concentration (1, 5, 10, 15, and 20 μ g/mL) of phenolics for 24 h. Nor: not treated ethanol extracts. Data represent means \pm SD of three independent experiments (n=3). The values are mean \pm SD of three independent experiments. #*p*<0.05 compared with the Nor group, **p*<0.05, ***p*<0.01 compared with the Nor group

Results and Discussion

Cell viability

The results of the MTT assay to evaluate the cytotoxicity of B16F10 melanoma cells of phenolics from OU were shown in Fig. 1. The cell viability of the untreated group was normalized to 100%. Phenolics of OU were treated at concentrations of 1, 5, 10, 15, and 20 μ g/mL. At the concentrations of 1, 5, 10, and 15 μ g/mL, cell viability was about 90.0% or higher. However, the cell viability was 78.5% at the concentration of 20 μ g/mL. This means that it is toxic at a concentration of 20 μ g/mL. Based on the above results, the experiment was conducted at a maximum concentration of 15 μ g/mL in the subsequent experiments. This is because the cell survival rate was more than 90.0% at the concentration of 15 μ g/mL.

Inhibition of melanin production by phenolics from OU in B16F10 melanoma cells

Melanin is a pigment component that forms between the lower layer of the epidermis and the dermis. If it is produced excessively on the skin due to external stimuli, skin pigmentation, freckles, skin inflammation, and skin cancer can occur [9,10]. The inhibitory effect of phenolics of OU on melanin biosynthesis against B16F10 melanoma cell stimulated with α -MSH was confirmed in Fig. 2. Controls were only stimulated with α -MSH. The amount of melanin produced by the control was 100%. And the treatment of phenolics from OU at 10, and 15 μ g/mL showed 87.7%, and 81.8% production, respectively. And the amount of production was decreased in a concentration-dependent manner.

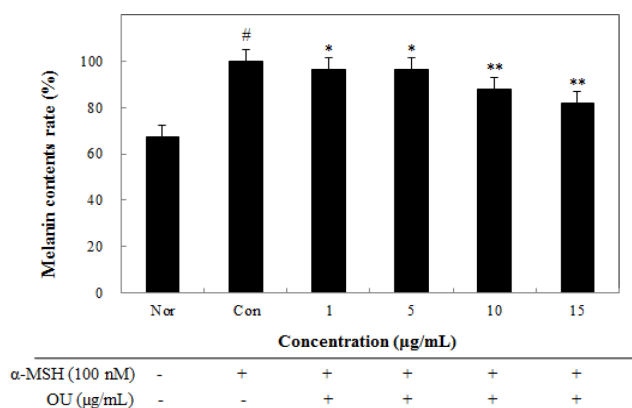


Fig. 2 Inhibitory effect of melanin contents by phenolics from OU treatment in B16F10 melanoma cells. 5×10^5 cells were seeded and stimulated to α -MSH 100 nM for 1 h, the cells were treated from control or different concentration (1, 5, 10, and 15 μ g/mL) of phenolics for 24 h. Nor: α -MSH not induced group, Con: α -MSH induced group not treated phenolics. The values are mean \pm SD of three independent experiments. [#] $p < 0.05$ compared with the Con group, ^{*} $p < 0.05$, ^{**} $p < 0.01$ compared with the Con group

Inhibition of intracellular tyrosinase activity by phenolics from OU in B16F10 melanoma cells

In Fig. 3 shows the results of the measurement of cellular tyrosinase activity in B16F10 melanoma cells stimulated with α -MSH to confirm tyrosinase inhibitory activity, a key enzyme involved in melanin biosynthesis. The activity level of cellular tyrosinase was 89.1, 82.9, and 73.3%, respectively, when phenolics of OU was treated at 5, 10, and 15 μ g/mL, respectively. Especially at the concentration of 15 μ g/mL, cellular tyrosinase activity was similar to that of normal without stimulation. At this time, the amount of normal was 72.0%. Therefore, it was confirmed that phenolics of OU markedly inhibited cellular tyrosinase enzyme activity. These results suggest that phenolics of OU inhibit the synthesis of intracellular tyrosinase in melanocyte and inhibit melanin biosynthesis. As a result, it was confirmed to have efficacy.

Conformation of inhibition of tyrosinase, MITF, TRP-1, and TRP-2 protein expression using western blot analysis with phenolics of OU

Melanin in the skin is synthesized by enzymatic reactions such as tyrosinase, TRP-1, and TRP-2 [16]. MITF, a transcription factor involved in the expression of tyrosinase and TRP-1, is involved in melanin biosynthesis by promoting the expression of tyrosinase enzyme, which has the greatest effect on melanin biosynthesis [17]. In this study, the expression level of tyrosinase, TRP-1, TRP-2, and MITF was confirmed to examine the effect of phenolics of OU on melanin biosynthesis. The results are shown in Fig. 4. When the amount of tyrosinase protein produced by the α -MSH-stimulated B16F10 melanoma cell was 100%, the expression of tyrosinase protein decreased to 42.5% at the concentration of 15 μ g/mL of phenolics from OU. This value was similar to that of

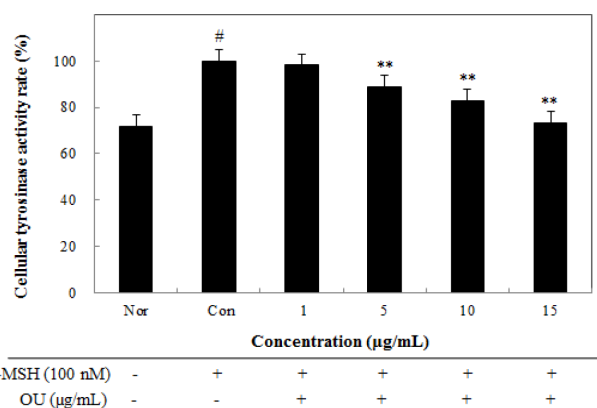


Fig. 3 Inhibitory effect of cellular tyrosinase activity by phenolics from OU treatment in B16F10 melanoma cells. 5×10^5 cells were seeded and stimulated to α -MSH 100 nM for 1 h, the cells were treated from control or different concentration (1, 5, 10, and 15 μ g/mL) of phenolics for 24 h. Nor: α -MSH not induced group, Con: α -MSH induced group not treated phenolics. The values are mean \pm SD of three independent experiments. [#] $p < 0.05$ compared with the Con group, ^{*} $p < 0.05$, ^{**} $p < 0.01$ compared with the Con group

normal without stimulation. The amount of normal was 55.3% (Fig. 4A). In the case of TRP-1, the expression level of 40.7% when treated with 15 μ g/mL of phenolics from OU, compared with control. This is better than normal (Fig. 4B). In the case of TRP-2, the expression level of 66.5% when phenolics of OU was treated at a concentration of 15 μ g/mL compared to control. This value inhibited 34.5% protein expression compared to control (Fig. 4C). In the case of MITF, the transcription factor, the expression level of MITF protein was 49.1% at the treatment with phenolics of OU 15 μ g/mL compared with control. It was confirmed that this level is similar to that of 50.9% protein expressed by normal (Fig. 4D). These results indicate that phenolics of OU inhibits melanin pigment biosynthesis by significantly inhibiting tyrosinase, TRP-1, TRP-2, and protein expression of their transcription factor MITF. Therefore, it was expected that phenolics of OU could be developed as a functional material.

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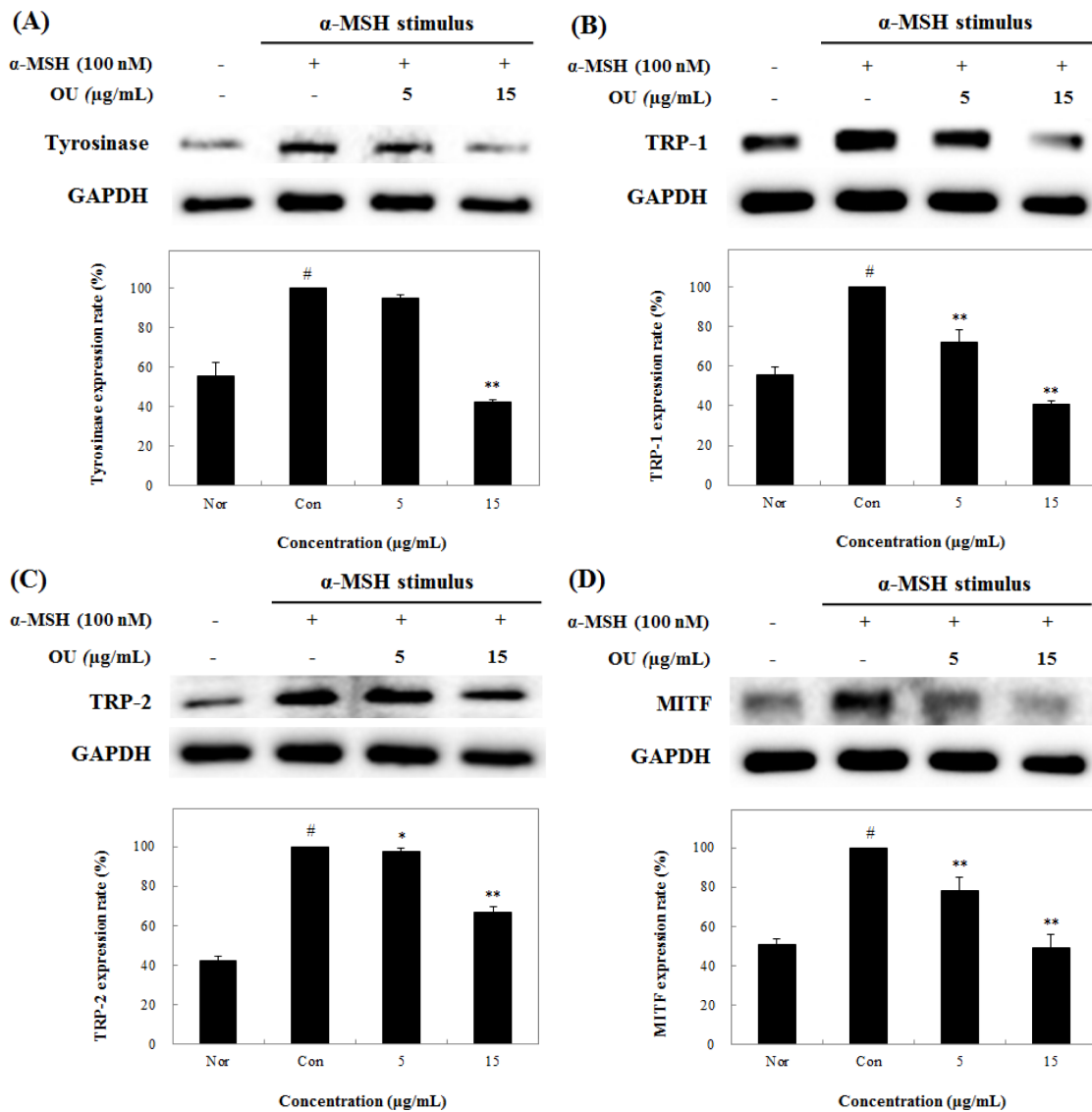


Fig. 4 Protein expression rate of tyrosinase (A), TRP-1 (B), TRP-2 (C), and MITF (D) by phenolics from OU in B16F10 melanoma cells. Lysates were prepared from control or different concentration (5 and 10 μ g/mL) of phenolics. Nor: α -MSH not induced group, Con: α -MSH induced group not treated phenolics. The values are mean \pm SD of three independent experiments. # p < 0.05 compared with the Con group, * p < 0.05, ** p < 0.01 compared with the Con group

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