Journal of Applied Pharmaceutical Science Vol. 4 (01), pp. 166-169, January, 2014
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2014.40126
ISSN 2231-3354

Short Communication

Melanogenesis Inhibitory Activity in the Extracts of *Oreocnide fruticosa* (Gaudich.) Hand.-Mazz. Branches

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ARTICLE INFO

Article history:
Received on: 17/11/2013
Revised on: 19/12/2013
Accepted on: 26/01/2014
Available online: 30/01/2014

Key words:
Oreocnide fruticosa (Gaudich.) Hand.-Mazz. melanin, anti-melanogenesis, tyrosinase.

ABSTRACT

Development of anti-melanogenic agents from natural sources has long been the research project of our laboratory. In this study, ethyl acetate extracts of *Oreocnide fruticosa* (Gaudich.) Hand.-Mazz. branches (OBE) were examined for their melanin synthesis inhibitory activities using B16 melanoma cells. Our results indicated that OBE down-regulates melanin production in a dose-dependent pattern. To clarify the target of OBE action in melanogenesis, we performed Western blotting for the key melanogenic enzymes; tyrosinase, tyrosinase related protein-1 (TRP-1) and TRP-2. The results showed that OBE efficiently inhibited tyrosinase, TRP-1 and TRP-2 in a dose-dependent manner. Therefore, OBE is a good candidate for the further study including identification of the effective chemical constituents as well as their working mechanism for the application of whitening agent in the human skin.

INTRODUCTION

The pigment melanin is synthesized from the melanocytes localized in the epidemis of human skin. Upon exposure of the skin to external stimuli such as UV radiation, melanogenesis is enhanced to protect skin from harmful risk. However, the overproduction and accumulation of melanin in the skin could lead to pigmentary disorders, such as melasma, freckles and solar lentigo. Accordingly, the regulation of melanin production is an important strategy in the treatment of abnormal skin pigmentation for cosmetic and medicinal purposes (Cho *et al*., 2008; Gillbro and Olsson, 2011; Jung *et al*., 2011).

In the process of melanogenesis, tyrosinase is the key enzyme catalyzing the first two steps of melanin production: the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) and successive oxidation of the L-DOPA to L-DOPA quinone. The resulting L-DOPA quinone could be enzymatically transformed to 5,6-dihydroxyindole-2-carboxylic acid by tyrosinase-related protein-2 (TRP-2). There are two tyrosinase-related proteins, TRP-1 and TRP-2, which are structurally sharing 40~45% identity with tyrosinase. TRP-1 and TRP-2 existing within the melanosomes are useful markers for differentiation. The role of TRP-1 and TRP-2 is not totally clarified, however, they have been demonstrated to increase tyrosinase stability. (Ito and Wakamatsu, 2003; Lin and Fisher, 2007; Gillbro and Olsson, 2011). A number of anti-melanogenic agents have been reported from both natural and synthetic sources, but only a few of them are used as skin-whitening agents primarily due to various safety concerns. Therefore, search of medicinal plants possessing high efficacy with low toxicity has been conducted for cosmetic and medicinal purposes (Roh *et al*., 2004; Cho *et al*., 2011). *Oreocnide fruticosa* (Gaudich.) Hand.-Mazz. (Korean local name: biyang namu) is a shrub endemic to biyang-do in Jeju Island, Korea (Lee et *al*., 2001). As this tree growing up to 2 m height inhabits a specific area in the Island, this species is classified as protected plant in Korea. There is no report on the biological properties either on the chemical constituents on the extracts for this plant.

MATERIALS AND METHODS

Plant material

The branches of *Oreocnide fruticosa* were collected in June of year 2011 from Halla Botanical Garden in Jeju, the island located at the southernmost part of Korea.

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Extraction

The air-dried branches of *O. fruticosa* were extracted three times with 70% aq. ethanol using a mechanical stirrer at room temperature for 24 hr. The resulting ethanol solutions were combined and filtered. The filtrate was concentrated using a rotary evaporator at a temperature 37℃. Extract was suspended in water and successively partitioned to give *n*-hexane, ethyl acetate (EtOAc), *n*-butanol and water fractions.

Cell culture

B16F10 murine melanoma cell was purchased from the ATCC. These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin streptomycin at 37℃ in a humidified 95% air/5% CO_2 atmosphere.

Determination of cellular tyrosinase activity/Melanin contents

B16F10 murine melanoma cells were seeded into 6 well cell culture plates at a density of 5.0×10^4 cells/well. A day later, the cells were stimulated with α-MSH (50 nM) and treated with extracts. The resulting cells were incubated at 37℃ 5% CO_2 condition for two days. The cells were washed with 1X phosphate buffered saline (PBS) and then collected using 1X trypsin-ethylenediaminetetraacetic acid (EDTA), after which they were lysed with 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1% triton-X 100 in 67 mM sodium phosphate buffer (pH 6.8). The samples were sonicated and centrifuged at 15,000 rpm for 20 min at 4℃. Supernatant (80 μL) was placed in a 96 well plate and mixed with 25 mM L-DOPA (40 μL) and 67 mM sodium phosphate buffer (120 μL, pH 6.8). After reacting at room temperature for at least 1 hr, the absorbance was measured at 475 nm. To dissolve the melanin, 1 N NaOH was added to the pellets and subsequently incubated at 70℃ for 4 h. Absorbance of total protein was measured using the supernatant mixture and Bradford reagent.

Cell viability assay

The cell viability was determined by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] cell viability assay. Briefly, cells were seeded into 24 well cell culture plates. After 24 hr, the cells were stimulated with α-MSH (50 nM) and treated with extracts, and incubated for 48 h at 37℃ under 5% CO_2 condition. MTT reagent (2 mg/mL in phosphate buffered saline) was added to each well in a 1/10 volume of medium. Cells were incubated at 37℃ under 5% CO_2 condition for 3 hr. Finally, the media was removed, and the formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm. Cell viability was evaluated as the relative absorbance of the control group.

Western blot measurement of TYR, TRP-1 and TRP-2 in B16F10 melanoma cell

B16F10 murine melanoma cells stimulated with α-MSH (50 nM) were treated with each extract (*n*-hexane, ethyl acetate, *n*-butanol and water fractions), and were collected and lysed in 1X RIPA buffer. Protein (30 μg) per well were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently blotted onto nitrocellulose membranes. And then the nitrocellulose membranes were blocked with 5% dried milk in Tris buffered saline containing 0.1% Tween 20. Next, the blots were incubated with primary antibodies at a dilution of 1:1000 and then further incubated with horseradish peroxidase-conjugated secondary antibody.

The bound antibodies were then detected using an enhanced chemiluminescence kit (Western blot detection system, iNtRON BIOTECHNOLOGY)). Tyrosinase, TRP-1, TRP-2 and β-actin were purchased from Santa Cruz Biotech.

Statistical analysis

All data were obtained in triplicate and are represented as means ± standard error (SE). Significant differences between treatments were determined by the Student's *t* test in one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Melanin pigment plays a crucial role in protecting the skin against the damage caused by harmful ultraviolet light. However, overproduction and accumulation of dark melanin could create serious skin problems such as freckles, age pigment and melasma. Thus, the inhibition of melanogenesis has been the focus as the effective method for skin depigmenting and lightening in medicinal and cosmetic applications (Khan, 2012; Liang *et al*., 2013). In order to evaluate anti-melanogenesis efficacy of *O. fruticosa* extracts, we examined its *n*-hexane, EtOAc, *n*-butanol and water fractions on melanin production using activated B16 murine melanoma cells. Among the four fractions, the EtOAc extract (100 μg/mL) markedly inhibited MSH-induced melanin synthesis (data not shown). Therefore, the study was focused on ethyl acetate fractions of *O. fruticosa* branches (OBE) for melanin production and melanogenic protein expression in melanoma cells. To investigate the effects of OBE on melanogenesis, melanin contents were measured using B16F10 murine melanoma cells which were treated with the extracts for 2 days. As shown in Fig. 1A, the melanin contents were reduced by OBE in a concentration-dependent manner. Even though some anti-melanogenic agents such as hydroquinone, kojic acid and arbutin are under clinical use, they have been reported to exhibit side effects such as the skin irritation and cell toxicity (Yoon *et al*., 2010; Kim *et al*., 2013). Therefore, it is necessary to develop novel potent natural products having anti-melanogenic efficacy without side effects. To investigate the cytotoxicity of OBE on cell proliferation, B16 murine melanoma cells were treated with various concentrations (25 – 100 μg/mL) of OBE for 72 hr. As shown in Fig. 1B, there was no significant difference in cell proliferation between control and OBE-treated cells up to 100 μg/mL, suggesting that the inhibitory effects of OBE on melanin biosynthesis were not attributable to its cytotoxicity.

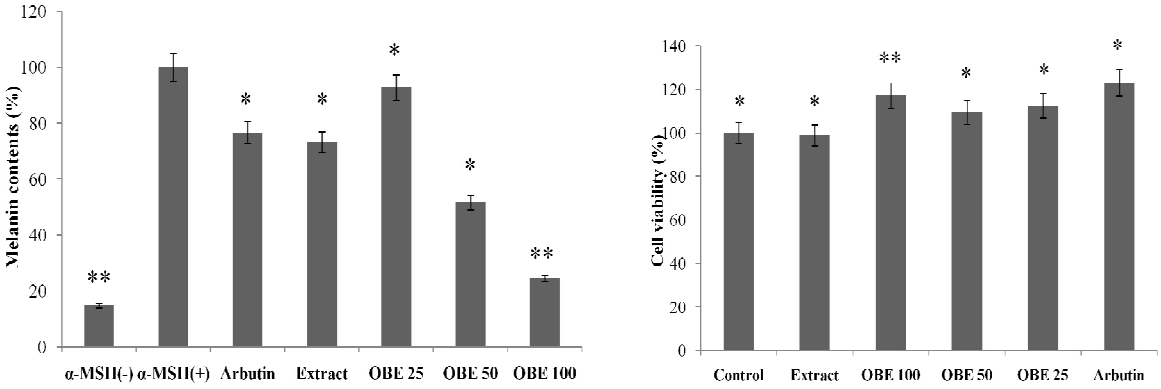

Fig. 1: Inhibitory effect of the OBE on melanin contents (A) and cell viability (B) of the B16F10 cells.
B16F10 cells (2.0×10^4 μg/mL) were pre-incubated for 18 h, and the melanin content was assayed after incubation of the B16F10 cells treated with α-MSH (50 nM) and OBE (25, 50 and 100 μg/mL) for 72 h at 37℃ in a 5% CO_2 atmosphere. The absorbance was measured at 405 nm by an ELISA. MTT assay was performed after incubation of the B16F10 cells treated with varying concentrations (25, 50 and 100 μg/mL) of OBE for 24 h at 37℃ in a 5% CO_2 atmosphere. The absorbance was measured at 570 nm with a spectrophotometer (Power Wave; Bio-tek, Winooski, VT). Values are the mean ± SEM of triplicate experiments. **,P<0.05; **,P<0.01*.

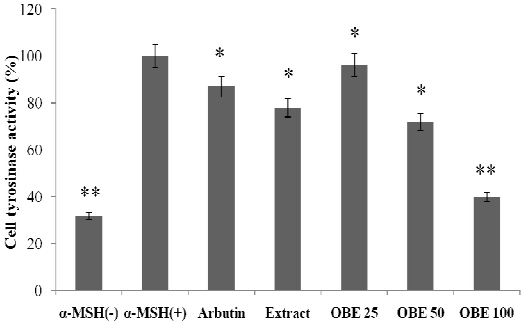

Fig. 2: Inhibitory effect on tyrosinase activity of OBE in B16F10 cells.
B16F10 cells (2.0×10^4 μg/mL) were pre-incubated for 18 h and tyrosinase activity was performed after incubation of B16F10 cells treated with α-MSH (50 nM) and OBE (25, 50 and 100 μg/mL) for 72 h at 37℃ in a 5% CO_2 atmosphere. Absorbance was measured at 405 nm with a ELISA. Values are the mean ± SEM of triplicate experiments. *,P<0.05; **,P<0.01

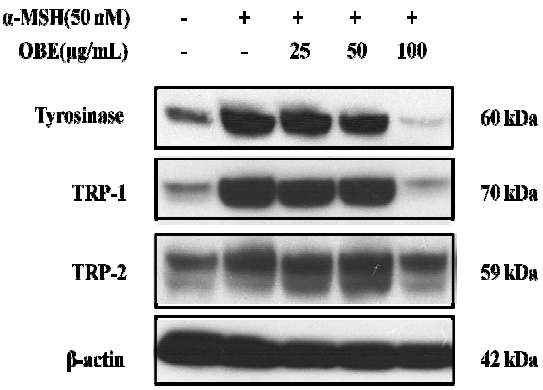

Fig. 3: Inhibitory effect of the OBE on the protein level related to melanogenic factors in the B16F10 cells.
B16F10 cells (1.0×10^5 cells/mL) were pre-incubated for 18 h and were stimulated with α-MSH (50 nM) in the presence of the OBE (25, 50 and 100 μg/mL) for 24 h. The protein level was determined by immunoblotting.

Since cellular tyrosinase activity is also the major factor that leads to melanin synthesis, we determined its enzyme activity on B16 murine melanoma cells. As a result, OBE treatment significantly reduced the cellular tyrosinase activity in a dose-dependent manner compared to the control (Fig. 3).

As a following study, we examined the expression of melanogenic proteins by Western blotting analysis. Tyrosinase, the enzyme catalyzing the rate-determining step of melanogenesis, is known to be a well-characterized marker of differentiation in melanocytes and melanoma cells. Tyrosinase related protein (TRP-1) and TRP-2 eventually are incorporated in the transformation of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) into indole-5,6-quinone-2-carboxylic acid (IQCA) (Wu et *al*., 2012; Yen *et al*., 2012; Chai *et al*., 2013). In this study, the levels of protein

expression of tyrosinase, TRP-1 and TRP-2 were found to be inhibited by OBE treatment. In summary, the present study indicated that OBE inhibited melanogenesis in B16F10 murine melanoma cells. Moreover, major melanogenic signaling factors such as tyrosinase, TRP-1 and TRP-2 were reduced by OBE. However, the inhibitory mechanism of melanin production in B16 murine melanoma cells by OBE remains unclear. The chemical constituents responsible for the activities should be also identified in due course.

The investigations of the exact mechanisms as well as chemical constituents are needed to evaluate the possible use of OBE as a natural skin-whitening agent.

ACKNOWLEDGMENTS

This research was financially supported by the Ministry of Trade, Industry and Energy (MOTIE) and Korea Institute for Advancement of Technology (KIAT) through the Research and Development for Regional Industry

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How to cite this article:

Jung Hwan Han, Sang-Hee Byeon, Chang-Gu Hyun and Nam Ho Lee. Melanogenesis Inhibitory Activity in the Extracts of *Oreocnide fruticosa* (Gaudich.) Hand.-Mazz. Branches. J App Pharm Sci, 2014; 4 (01): 166-169.