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Inhibitory effect of berberine from *Coptidis rhizoma* on melanin synthesis of murine malignant melanoma

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Berberine has abundant beneficial properties including anti-cancer effects. In the present study, we examined the inhibitory effect of berberine on α-melanocyte-stimulating hormone (α-MSH)-induced melanogenesis in B16F1 melanoma cells. The results showed that berberine decreased the expression of tyrosinase and microphthalmia-associated transcription factor (MITF) in a dose-dependent manner. In order to observe the potential target for the inhibitory effect of berberine, we examined the effect of berberine on TRP-1 and TRP-2. The results showed that berberine led to a reduction of TRP-1, while the change of TRP-2 was inconspicuous. In the end, we observed the specific effect of berberine on zebrafish skin pigmentation. Overall, the results suggested that berberine inhibits tyrosinase activity and melanin synthesis by attenuating the expression of tyrosinase and MITF. Therefore, these findings may contribute to the potential application of berberine in medicinal or cosmetic products.

1. Introduction

Melanoma, a highly malignant subtype of skin tumor, is the primary cause of death from skin diseases due to its tendency to metastasize (Mittal et al. 2014; Uong and Zon 2010; Batus et al. 2013). Melanoma contributes only 4 % to total skin cancer cases, however, it is responsible for about 80 % of all skin cancer-related deaths. Patients diagnosed with malignant melanoma usually survive for no more than two years (Balch et al. 2000; Bhatia et al. 2009), only 14 percent of them can survive for more than five years (Miller and Mihm 2006; Delyon et al. 2015).

High incidence of melanoma has been reported in many countries, such as Australia and United States of America (Hayat et al. 2007; Ferguson 2005; Siegel et al. 2015; Erdmann et al. 2013), and the rate is getting higher and higher. The current treatments are dominated by surgery, chemotherapy, and radiotherapy. In order to find more chemotherapy drugs, many compounds which have the ability to inhibit melanin synthesis have been isolated from natural plants, such as flavonoids, kojic acid, hydroquinone, and arbutin (Song et al. 2015; Lizuka et al. 2000; Choi et al. 2002; Kang et al. 2003).

Berberine, a major active component of *Coptidis rhizoma*, has been clinically used in certain formulae for treating some diseases, such as inflammation, bacterial infections, and cancers including skin cancer (Singh et al. 2011; Kuo et al. 2004). Although berberine has extensive pharmacological effects, the mechanisms involved still need to be determined. In this study, we investigated the inhibitory effects of berberine on α-melanocyte stimulating hormone (α-MSH)-induced melanogenesis in B16F1 melanoma cells, analyzed the underlying mechanism of action and further observed the effect on skin pigmentation.

2. Investigations, results and discussion

2.1. Identification of berberine from Coptidis rhizoma

The isolated single compounds were analyzed by HPLC and UPLC-ESI-MS after *Coptidis rhizoma* extracts were separated. As shown in Fig. 1, the retention time of spot 1 was the same to that of berberine standard. In order to further confirm if the spot 1 is berberine, we continue to use the method of UPLC-ESI-MS analysis. In comparison with the data of Fig. 2, compound 1 and berberine standard have the same molecular weight. Therefore, we can conclude that compound 1 is berberine.

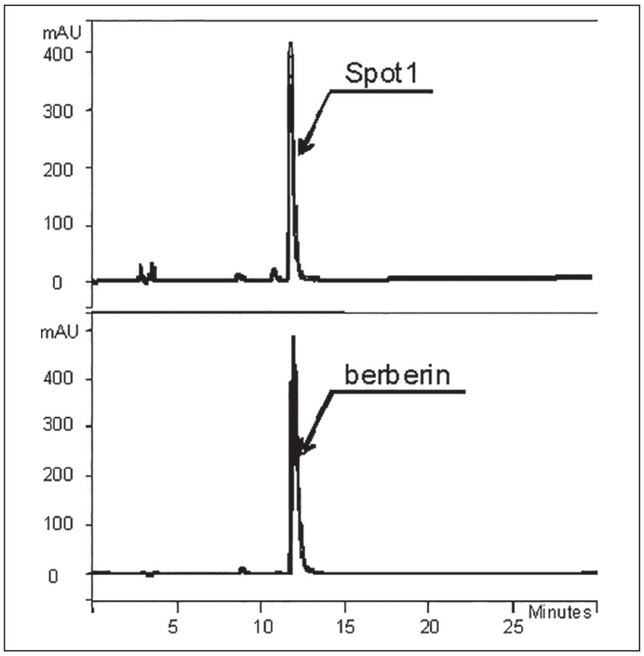

Fig. 1: HPLC chromatogram of *Coptidis rhizoma* extract and berberine standard.

2.2. Effects of berberine from Coptidis rhizoma on viability and melanin content in B16F1 melanoma cells

Prior to examining the effect of berberine on melanin content, we examined the effect of berberine on cell viability. The results showed that berberine did not have significant cytotoxicity to B16F1 melanoma cells at the concentration of 50 µM. In the subse-

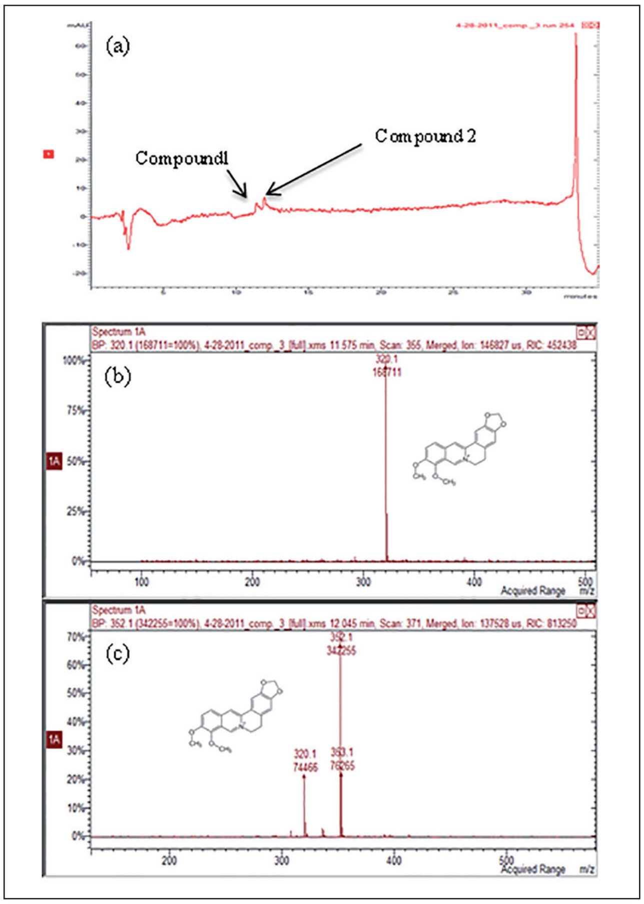

Fig. 2: UPLC-ESI-MS chromatogram. (a) LC analysis of *Coptidis rhizoma* extract. (b) Mass analysis of berberine standard. (c) Mass analysis of *Coptidis rhizoma* extract.

quent experiments, the change of melanin content in B16F1 melanoma cells treated with berberine was evaluated. The inhibition rate of melanin synthesis was significantly increased by berberine in a dose-dependent manner (Fig. 3). The IC_{50} value of berberine was 26.47 μM.

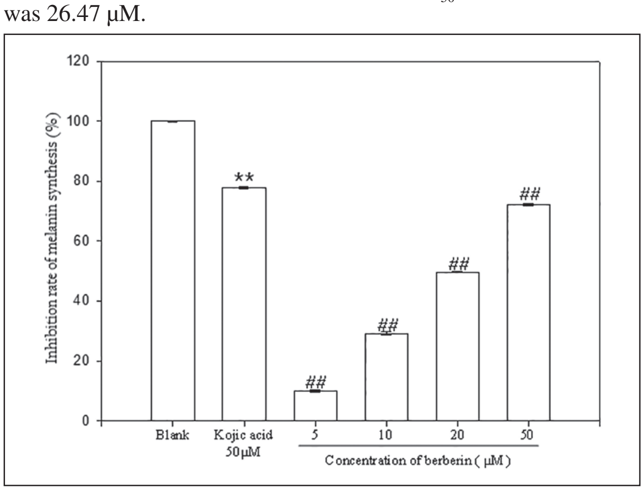

Fig. 3: Effect of berberine on melanin synthesis in B16F1 melanoma cells (* significant compared with the blank, **: p < 0.01, #: significant compared with the kojic acid alone, ##: p < 0.01).

2.3. *Effect of berberine on tyrosinase activity*

B16F1 melanoma cells were stimulated with α-MSH and treated with berberine. Lysates of the cells were subjected to western blotting analysis with anti-tyrosinase antibody. As shown in Fig. 4, tyrosinase expression increased in B16F1 melanoma cells stimulated with α-MSH. On the contrary, berberine decreased tyrosinase expression in a dose-dependent manner. Actin was not affected to the same extent by berberine.

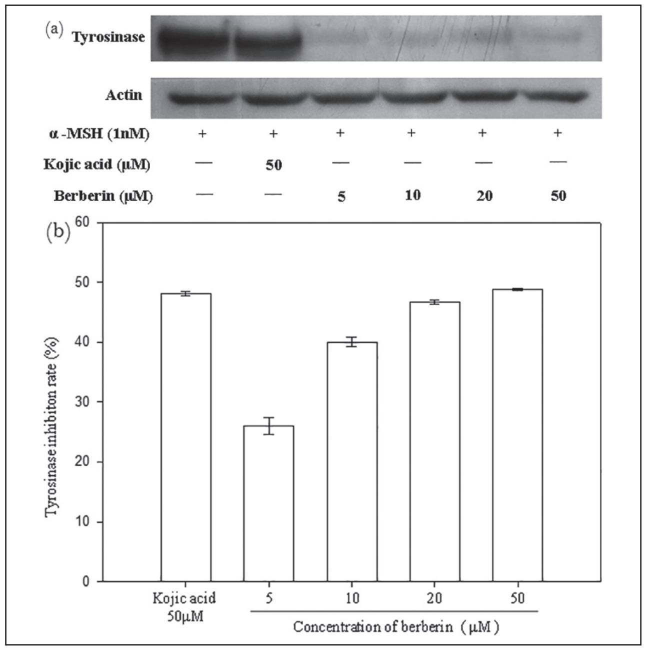

Fig. 4: Effect of berberine on tyrosinase activity. (a) Effect of berberine on tyrosinase and actin protein expression. Cells (5 x 106 cells) were treated with a range of concentrations (5-50 μM). Experiments were performed three times with similar results and the typical one is presented. Lane 1, Blank (DMSO); lane 2, kojic acid (50 μM); lane 3, berberine (5 μM); lane 4, berberine (10 μM); lane 5, berberine (20 μM); lane 6, berberine (50 μM). (b) Tyrosinase inhibition rate of berberine in B16F1 melanoma cells.

2.4. *Effect of berberine on mRNA expression of melanogenesis-related genes*

We further examined the effect of berberine on mRNA level. The cells were treated with 5, 10, 20, and 50 μM berberine for 24 h and total RNA was extracted. Specific mRNA was amplified by RT-PCR using specific primers for tyrosinase, TRP-1, TRP-2, and MITF. As shown in Fig. 5, mRNA levels of tyrosinase, TRP-1, and MITF decreased after treatment with berberine in a dose-dependent manner, whereas mRNA levels of TRP-2 declined inconspicuously. Although we cannot draw the exact conclusion of mechanisms from the results, we observed that the influence of TRP-2 was not obvious.

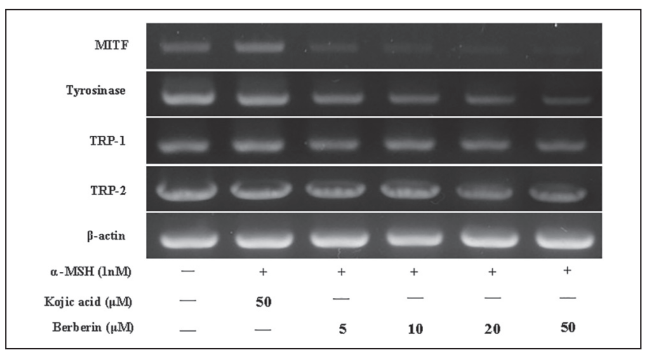

Fig. 5: Effect of berberine on mRNA expression of melanogenesis-related genes. The sizes amplified gene products were 528 bp for β-actin, 477 bp for tyrosinase, 268 bp for TRP-1, 1044 bp for TRP-2, and 910 bp for MITF.

2.5. *Effect of berberine on zebrafish pigmentation*

In order to further observe the effect of berberine on skin pigmentation, we treated zebrafish with berberine. Kojic acid (50 μM), as a positive control, was used routinely to inhibit pigmentation in zebrafish body. As shown in Fig. 6, berberine showed inhibitory effects on zebrafish pigmentation, especially the dorsal pigmentation.

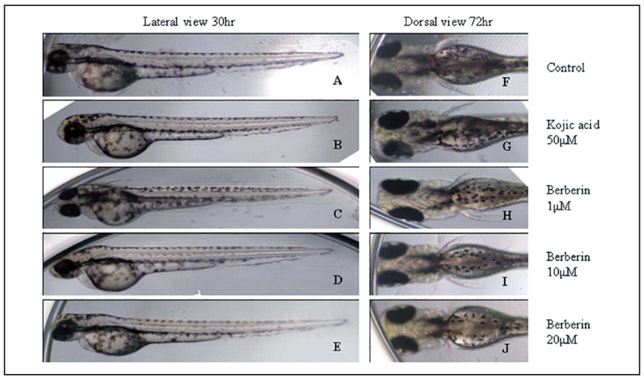

Fig. 6: Effect of berberine on melanogenesis of zebrafish. Synchronized embryos were treated with berberine at the different concentration. Test compounds were dissolved in water, and then mixed to the embryo medium. The effects on the pigmentation of zebrafish were observed under the microscope. (A-E) Leateral view of embryos at 30 h. (F-J) Dorsal view of embryos at 72 h. (A, F) Untreated zebrafish embryos as a water control. (B, G) Kojic acid at concentration of 50 μM as a positive control. (C-J) Berberine at concentrations of 1, 10, 20 μM as a treated group.

2.6. *Conclusion*

Through the above experiments, we can conclude that berberine obviously inhibits melanogenesis in B16F1 melanoma cells. Furthermore, the experiment of the influence of berberine on zebrafish pigmentation indicated that berberine has a certain effect on pigmentation of the skin surface. These findings strongly suggest that berberine can become a beneficial therapeutic agent for clinical use, or an ingredient for skin-whitening products. However, further studies are required to evaluate the efficacy and safety of berberine.

3. Experimental

3.1. *Materials*

Coptidis rhizoma was purchased from Kyung Dong Pharm Co. (Seoul, Korea). The standard substance of berberine was obtained from Sigma-Aldrich Co. (St. Louis, Mo, USA). Folin-Ciocalteu's reagent was supplied by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Propidium iodide was purchased from Thermo Fisher Scientific Inc. (New York, USA). Catechin, ascorbic acid, mushroom tyrosinase, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4), trichloroacetic acid, dimethyl sulfoxide (DMSO), and all other chemicals were bought from Sigma Chemical Co. (USA). All solvents used in spectrophotometric analysis were of HPLC grade (J.T. Baker, USA).

The mouse skin cancer B16F1 melanoma cells (KCLB 80007) were purchased from Korean Cell Line Bank (Seoul, Korea). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum, Dulbecco's phosphate buffered saline (DPBS), trypsin, fetal calf serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin were obtained from Thermo Fisher Scientific Inc. (Utah, USA). The goat polyclonal anti-tyrosinase antibody (1:500 dilution) and horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). The enhanced chemiluminescence kit was purchased from Amersham Biosciences (Little Chalfont, UK). The trizol reagent was obtained from Intron Biotechnology (Seongnam-Si, Korea).

3.2. *Extraction and fractionation*

The plant material was extracted two times for 24 h at room temperature using ethanol as a solvent, with occasional shaking (Shi et al. 2014). After that, the extract was filtered and the residue was extracted again. Each experiment was repeated three times. The extract was evaporated to dryness using a rotary evaporator (Eyela, Japan), and then the extract powder was dissolved in ethanol and stored at -20 °C until it was used. The concentrated extract was divided into 150 fractions by using Silica gel 60 for column chromatography (Merck, Darmstadt, Germany), the solvent system was butanol/acetic acid/water (7:2:1, v/v). Each fraction was filtered through 0.45 μm filter paper and concentrated in vacuum dryer. The powder was dissolved in DMSO, and then further diluted in distilled water to an appropriate concentration.

3.3. *TLC analysis*

Each fraction was dissolved in ethanol, and then thin layer chromatography (TLC) was performed on silica gel 60 F254 plates (20 × 10 cm, 0.2 mm thickness, Merck, Germany). The development of the plate was carried out by allowing the solvent to saturate for 9 h at room temperature. The solvent system consisted of butanol/acetic acid/water (7:2:1, v/v). Total volume of solvent mixture was 30 mL and the migration distance was 80 mm. After removed from the chamber, the plate was air-dried in a fume hood for 30 min and visualized under ultraviolet light at 365 nm.

3.4. *HPLC analysis*

The high performance liquid chromatography (HPLC) system consisted of a ProStar 230 solvent delivery module (Varian, USA), ProStar 410 autosampler (Varian, USA), and ProStar 335 photodiode array detector (Varian, USA). Separation was carried out on a Polaris C18-A column (4.6 × 250 mm, 5 μm, Varian, USA). After injection, the column was eluted with a linear gradient of 25-55% acetonitrile in water (containing 0.2% KH_2PO_4), lasting more than 30 min and then back to initial conditions. The flow rate was 1.0 mL/min and the detection was performed at 236 nm.

3.5. *Mass spectrometer analysis*

The ultra performance liquid chromatography (UPLC) eluent was introduced directly into an electrospray ionization (ESI) probe. The ESI source was coupled to a hybrid quadrupole orthogonal time-of-flight (Q-TOF) mass spectrometer (Synapt G2, Waters MS Technologies, Manchester, UK). A capillary and cone voltage of ±3.0 kV and 30 V, and capillary temperature of 120 °C were used for both polarities respectively. The desolvation conditions were as follows: desolvation gas flow rate, 800 L/h; desolvation gas temperature, 600 °C. Data acquisition took place over the mass range of 50-1200 m/z. The sample was introduced into the ESI source at a constant flow rate of 20 μL/min by using an external syringe pump (Harvard Apparatus 11 Plus, Holliston, MA, USA).

3.6. *Cell culture*

Cells were cultured in DMEM, containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Incubation conditions were maintained with 95% relative humidity and 5% CO_2 at 37 °C. Cells were harvested after 24 h incubation.

3.7. *Cell viability assay*

Cell viability was assessed by MTT method (Liu et al. 2016). After 4 to 5 days culture, cells were seeded at a density of 5×10^4 cells/well in 24 well plates. The volume of culture medium per well was 1 mL. In control experiment, cells were grown in the same media containing drug-free vehicle. After incubating with drug for 48 h, 100 μL MTT (5 mg/mL) was added and then cells were incubated for 4 h. To dissolve the formazan crystals, 200 μL DMSO was added to each culture medium and mixed by pipetting. The absorbance was measured at 570 nm. Relative cell viability was obtained by scanning with the microplate reader (Benchmark Plus, Bio-Rad, USA).

3.8. *Measurement of melanin content*

Melanin content of the cultured B16F1 cells was measured as described previously (Yang et al. 2006). The cells were washed twice with PBS and lysed with 20 mM Tris-buffer containing 0.1% Triton X-100 (pH 7.5). Cell lysates were precipitated with the same amount of 20% trichloroacetic acid (TCA). After being washed twice with 10% TCA, the pellets were treated with ethyl alcohol/diethyl ether (3:1). Samples were air-dried, dissolved in 1 mL of 0.85 M KOH, and boiled for 15 min. After cooling, the absorbance was measured at 440 nm. The melanin content of cells was calculated according to the Bradford method (Bradford 1976).

3.9. *Western blotting analysis*

To determine whether berberine showed an inhibitory effect on tyrosinase activity, western blotting analysis was performed (Ponnazhagan et al. 1994). After being treated with berberine for 48 h, B16F1 melanoma cells were collected and lysed with cell lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). Cell lysates (5×10^4 cells/dish) were separated by 8% SDS-polyacrylamide gel electrophoresis. Subsequently, protein was transferred to a PVDF membrane (Laura et al. 1982; Schallreuter et al. 2003; Amae et al. 2000). The membranes were pretreated with 5% skim milk in DPBS containing 0.01% Tween-20. The tyrosinase was detected with goat polyclonal anti-tyrosinase antibody. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence kit according to the manufacturer's instruction. Loading amount per well was normalized using β-actin antibody.

3.10. *Reverse transcription polymerase chain reaction (RT-PCR)*

Total cellular RNA was prepared by using Trizol reagent according to the manufacturer's manual. cDNA was synthesized by the use of 0.1 mM oligo (dT) 16, 10 mM dNTPs, one unit of reverse transcriptase, and 1 μg total RNA. PCR was performed by using HotStarTaq kit (Qiagen, USA) according to the manufacturer's manual. The use of PCR primers was as follows: tyrosinase forward, GGC CAG CTT TCA GGC AGA GGT and reverse, TGG TGC TTC ATG GGC AAA ATC; TRP-1 forward, GCT GCA GGA GCC TTC TTT CTC and reverse, AAG ACG CTG CAC TGC TGG TCT; TRP-2 forward, GAT GAC CGT GAG CAA TGG CC and reverse, CGG TTG TGA CCA ATG GGT GCC; MITF forward, GTA TGA ACA CGC ACT CTC TCGA and reverse, CTT CTG CGC TCA TAC TGC TC; and β-actin forward, ACC GTG AAA AGA TGA CCC AG and reverse, TAC GGA TGT CAA CGT CAC AC. 25 cycles of

PCR were performed. PCR product was loaded on 1% agarose gel and stained with ethidium bromide (EtBr).

3.11. Maintenance of zebrafish and berberine treatment

Adult zebrafish were obtained from a commercial dealer, 15 zebrafish were kept in a 10 L acryl tank at 28.5 °C with a 14/10 h light/dark cycle. Zebrafish were fed three times a day, the tetramin flake food was supplemented with live brine shrimps (Artemia salina). Embryos were obtained from natural spawning, which were induced in the morning by turning on the light. Collection of embryos was completed within 30 min (Love et al. 2004).

Synchronized embryos were collected and arrayed by pipette, and then dispensed in 96 well plates containing 200 µL embryo medium, three or four embryos per well. Berberine was dissolved in distilled water, and then added to the embryo medium from 9 to 72 hpf (63 h exposure). Occasional stirring as well as replacement of the medium was done daily to ensure distribution of berberine. In all experiments, 50 µM kojic acid was used as a positive control. For observation, embryos were dechorionated by forceps, anesthetized in tricaine methanesulfonate solution, and photographed by using the microscope (Nikon, Eclipse TS100, Japan).

3.12. Statistical analysis

All data were a summary of the results from at least three independent experiments. The values were presented as the mean±SD. The statistical analysis of the results was performed by One-way analysis of variance (ANOVA). The results were considered statistically significant if $p < 0.05$.

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Conflicts of interest: None declared.

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