J. Cosmet. Sci., **65,** 377–388 (November/December 2014)

The mixture of different parts of *Nelumbo nucifera* and two bioactive components inhibited tyrosinase activity and melanogenesis

SU-YOUNG JUNG, WON-SEOK JUNG, HO-KYUNG JUNG, GYEONG-HWAN LEE, JUNG-HEE CHO, HYUN-WOO CHO, and IN-YOUNG CHOI, *Clinical Trial Center for Functional Foods, Chonbuk National University Hospital, Jeonju (S.-Y.J.), and Jeonnam Development Institute for Korean Traditional Medicine, Jangheung-gun, Jeonnam 529-851 (W.-S.J., H.-K.J., G.-H.L., J.-H.C., H.-W.C., I.-Y.C.), Republic of Korea.*

Accepted for publication August 17, 2014.

Synopsis

Melanin is the pigment responsible for the color of the eyes, hair, and skin in humans. Tyrosinase is well known to be the key enzyme in melanin biosynthesis. JKTM-12 is composed of the flowers, roots, seeds, and receptacles of *Nelumbo nucifera* (lotus). In this study, JKTM-12 was investigated for its inhibitory effects on tyrosinase activity and melanin biosynthesis in B16F10 melanoma cells. Moreover, two main bioactive compounds (hyperoside and astragalin) were found from the receptacles of *N. nucifera*, which are used as the main material of JKTM-12. JKTM-12 was shown to inhibit tyrosinase activity and melanin biosynthesis in alpha-melanocyte-stimulating hormone–stimulated B16F10 melanoma cells. Hyperoside and astragalin, which are the main bioactive compounds of JKTM-12, not only inhibited tyrosinase activity and melanogenesis but also tyrosinase-related protein 1 and tyrosinase-related protein 2 mRNA expression without cytotoxicity at various experiment doses (0.1, 1, and 10 μg/ml). These results suggest that JKTM-12 has the potential for skin whitening with hyperoside and astragalin as the main bioactive compounds.

INTRODUCTION

Melanogenesis is the process primarily responsible for the production of melanin, which is related to the color of the skin and hair, and for its role of protection against ultraviolet radiation. However, excessive accumulation of melanin causes hyperpigmentation such as melisma, post-inflammatory melanoderma, and solar lentigo, which result in various clinical and cosmetic concerns (1–3). Melanin synthesis is regulated by melanogenic enzymes including tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2) (4,5). Tyrosinase is the rate-limiting enzyme for melanogenesis and catalyzes the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the

Address all correspondence to In-Young Choi at iychoi@wonkwang.ac.kr.

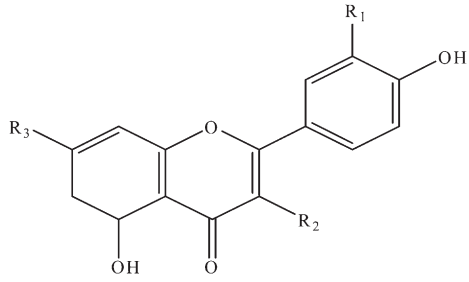
oxidation of DOPA to dopaquinone, both of which comprise the first stages of melanin synthesis (6). TRP-2 catalyzes the conversion of dopachrome to 5,6-dihydroxylindole-2 carboxylic acid (DHICA), and TRP-1 oxidizes the DHICA to indole-5,6-quinone carboxylic acid and subsequently produces melanin (7). Therefore, not only tyrosinase inhibitors but also TRP-1 or TRP2 inhibitors are of interest in the cosmetic industry for the treatment of hyperpigmentation and as skin-whitening agents (8,9).

JKTM-12 is composed of the flowers, roots, seeds, and receptacles of *Nelumbo nucifera* (Nymphaeaceae). *N. nucifera*, known as the sacred lotus, is used as food and medicine in East Asia and India (10). Various pharmacologically active substances, including alkaloids, flavonoids, triterpenoids, polyphenols, steroids, and glycosides, have been extracted from different parts of *N. nucifera* (10). In this study, we investigated the inhibitory effects of JKTM-12 on tyrosinase and melanin biosynthesis. Moreover, hyperoside and astragalin (Figure 1), which are the active compounds of JKTM-12 are shown to inhibit melanin biosynthesis, TRP-1 and TRP-2 mRNA expression, and cellular tyrosinase activity in B16F10 murine melanoma cells.

MATERIALS AND METHODS

MATERIALS

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (Grand Island, NY). Glycerol tributyrate, L-tyrosine, mushroom tyrosinase, phenylmethanesulfonyl fluoride (PMSF), kojic acid, and alpha-melanocyte-stimulating hormone (α-MSH) were purchased from Sigma-Aldrich (Steheim, United Kingdom). Monoclonal tyrosinase and GAPDH antibodies



	R_1	R_2	R_3
Hyperoside	OH	O-galactose	OH
Astragalin	H	O-glucose	OH

Figure 1. Chemical structure of astragalin and hyperoside.

were purchased from Cell Signaling Technology (Denver, MA). Anti-mouse horseradish peroxidase-conjugated immunoglobulin G (IgG) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ECL solution was purchased from Millipore Corporation (Billerica, MA). All other chemicals and solvents used in this study were of the analytical grade.

PREPARATION OF JKTM-12

N. nucifera was harvested from the Muan-Gun area, South Korea, in August 2010 to February 2011. JKTM-12 (240 g) was composed of the flowers (46 g), seeds (54 g), roots (60 g), and receptacles (80 g) of *N. nucifera*, respectively. JKTM-12 was prepared by boiling at 70°C with 70% EtOH for 3 h three times. It was then filtered and concentrated with a vacuum evaporator (N-3000, EYELA, Tokyo, Japan). After freeze-drying, the powder was stored at −4°C. The weight of the EtOH extract powder of JKTM-12 was 24.2 g (yield: 10.1%). JKTM-12 (EtOH extract powder) was dissolved in DMSO when used.

MUSHROOM TYROSINASE INHIBITORY ASSAY

The assay was performed using relevant methods (9). JKTM-12 each sample was dissolved in DMSO to a final concentration of 200 mg/ml. This extract stock solution was then diluted to 100 μg/ml in 100 m*M* potassium phosphate buffer (pH 6.8). Serial dilutions were made to attain five concentrations. Kojic acid was used as a positive control. In a 96-well plate, 20 μl of each extract serial dilution was combined with 20 μl of mushroom tyrosinase (250 Units/ml in phosphate buffer) and 100 μl of 100 m*M* potassium phosphate buffer in triplicate. After incubation at room temperature for 5 min, 40 μl of the substrate (100 unit L-DOPA) was added to each well. The final concentrations of the extract samples ranged from 100 to 2000 μg/ml. After incubation at 37°C for 15 min, optical densities of the reaction mixtures in the wells were recorded at 490 nm using a BIO-TEK Power Wave XS multi-well plate reader (Bio-Teck Instruments, Winooski, VT). The activity rate was calculated by the following equation:

Mushroom tyrosinase activity (%) = Absorbance of sample/Absorbance of control × 100.

CELL CULTURE

B16F10 murine melanoma cells were supplied by KCLB (Seoul, South Korea). They were cultured in DMEM supplemented with 10% heat-inactivated FBS, 10 U/ml of penicillin, 10 μg/ml of streptomycin in a 37°C, 5% CO_2, 95% air humidified atmosphere.

ASSAY OF CELL VIABILITY

B16F10 cell viability by the samples was measured by MTS assay according to the instructions provided by the manufacturer. Briefly, B16F10 cells were plated in 96-well culture plates (1×10^4 cells per well). After 24 h, the media were changed and the cells were treated with the samples at various concentrations for 24 h. Ten microliters of MTS (5.0 μg/μl) was added to each well for an additional 4 h of incubation at 37°C.

Absorbance was read with a microplate reader (MultiskanMK3, Thermo Scientific, Waltham, MA) at 490 nm. Cell viability rate (%) of the samples against the proliferation of B16F10 was calculated using the following equation

Cell viability rate (%) = Absorbance of well with samples/Absorbance of well without samples × 100

MELANIN ASSAY

B16F10 cells (2×10^5 cells) were cultured in DMEM with 10% FBS. After 12 h, the cells were treated with various concentrations of the samples or media only as a blank for 1 h. Following treatment, 100 nM α-MSH was added to the cells and incubated at 37°C with 5% CO_2 in a humidified atmosphere. After 48 h, the cells were washed with phosphate-buffered saline (PBS) and harvested (5000 rpm × 10 min). The pellets containing a known number of cells were dissolved in 1 N NaOH solution containing 10% DMSO and sonicated for 1 h. The amount of melanin content was then monitored by a microplate reader at 490 nm. Data are expressed in terms of melanin synthesis inhibitory activity compared to the control. Inhibitory activity (%) was calculated using the following equation.

Inhibitory activity (%) = [1 − (Absorbance of sample − Absorbance of blank)/Absorbance of control] × 100.

CELLULAR TYROSINASE ACTIVITY ASSAY

Cellular tyrosinase activity was determined based on a modification of a previously described method (8). As many as 2×10^4 cells/cm^2 were treated with the samples for 1 h, followed by addition of 100 nM α-MSH. After 48 h, the incubated cells were harvested and washed twice with ice-cold PBS by centrifugation at 1000 × *g* for 5 min. The harvested cells were lysed in 1% Triton X-100 and 0.1 m*M* PMSF in PBS. The total protein was collected by centrifugation at 10,000 × *g* for 25 min at 4°C. The reaction mixture consisted of 100 µl 1 mg/ml L-DOPA solution and 50 µl cell-extracted protein. Dopachrome formation at 37°C for 30 min was measuring the absorbance at a wavelength of 475 nm using a microplate reader.

WESTERN BLOT ASSAY

B16F10 cells were treated with the samples for 1 h, and α-MSH was added. After 48 h, cells were collected and lysed in a RIPA cell lysis buffer 3 containing 50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1.2% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS (Enzo Life Sciences, Inc., Plymouth, PA). The lysates were denatured at 95°C for western blot assay. Proteins were separated using 10% SDS-polyacrylamide gel electrophoresis running gel. The resolved proteins were transferred to nitrocellulose membranes and then were blocked using 5% skim milk in Tris HCl buffer. Membranes were incubated with tyrosinase and GAPDH antibodies. And the membranes were washed three times every 15 min then incubated with anti-mouse horseradish

peroxidase antibody for 30 min. After the membrane was washed four times every 15 min, the bends of bound antibodies were detected by enhanced chemiluminescence reagents, and the images of protein expression were obtained using imaging system (Li-Cor Inc., Lincoln, NE).

TRP-1, TRP-2 mRNA ASSAY

Total RNA was isolated with the Trizol-Reagent (Invitrogen, Carlsbad, CA) according to the instructions provided by the manufacturer. Reverse transcription reactions were performed with SuperScript III reverse transcriptase (Invitrogen) following the manufacturer's instructions, using 2 μg of total RNA. Real-time PCR reactions were performed using Taqman gene expression assays by the 7500 Real Time PCR system (AB Applied Biosystems, Foster City, CA). The mRNA expression levels of TRP-1 (assay ID: Mm00453201_m1) and TRP2 (assay ID: Mm01225584_m1) were normalized to Hprt1 (assay ID: Mm00446968_m1) as loading controls.

ANALYTICAL HPLC OF JKTM-12

The purity of the two compounds was confirmed by analytical HPLC using a diode array detector. Analytical HPLC was run on a Supelco discovery C18 reverse phase column, 25 cm × 4.6 mm, 5 μm. The mobile phase consisted of two solvents, water (0.05% formic acid) and acetonitrile. The run started with 100% water and then increased to 100% acetonitrile in 35 min; the flow rate was 1 ml/min.

STATISTICAL ANALYSIS

The results were analyzed statistically using one-way analysis of variance (ANOVA), and the least significant differences ($p < 0.05$) were determined according to Duncan's t-test.

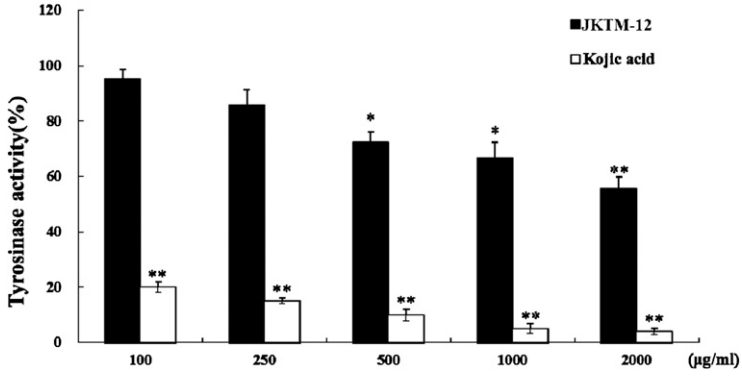


Figure 2. Effect of JKTM-12 on tyrosinase activity. Mushroom tyrosinase was incubated with various concentrations of JKTM-12 and L-DOPA as substrates. Kojic acid was used as positive control. Data are presented as the mean ± SEM of three individual experiments, performed in triplicate. Values are significantly different by comparison with untreated control. *$p < 0.05$, **$p < 0.001$.

RESULTS

INHIBITION OF TYROSINASE ACTIVITY AND MELANIN BIOSYNTHESIS BY JKTM-12

The effect of the JKTM-12 EtOH extract (JKTM-12) on tyrosinase activity was examined. As shown in Figure 2, JKTM-12 inhibited tyrosinase activity in a dose-dependent manner. Kojic acid is a well known as tyrosinase inhibitor (11), so we used kojic acid as a positive control. Because tyrosinase is the rate-limiting enzyme for melanin, recent studies have demonstrated that the inhibitory activity of compounds toward mushroom tyrosinase is correlated with their inhibition of melanin synthesis in melanocytes *in vitro* (8,9,12). In this study, we examined cell viability and melanin biosynthesis by JKTM-12 in B16F10 murine melanoma cells. The cells were incubated with 50, 100, 500, 1000 μg/ml of JKTM-12 for 48 h. Fifty microgram/milliliter JKTM-12 had no cytotoxicity on B16F10 cells. Although 100 μg/ml JKTM-12 slightly decreased cell viability, it was not significant. However, high concentrations of JKTM-12 (500 and 1000 μg/ml) decreased B16F10 cell viability by 67.83% and 57.57%, respectively (Figure 3A). Next, we investigated whether JKTM-12 prevented melanin biosynthesis by the indicated concentrations

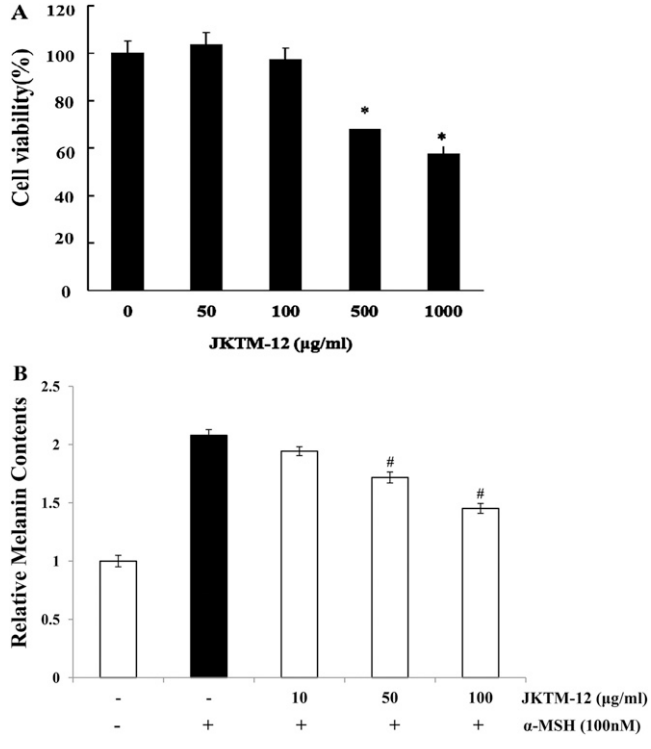


Figure 3. Effects of JKTM-12 on (A) viability and (B) melanin contents in B16F10 cells. (A) Cells were cultured in 96-well plates (1×10^4 cells/well) with the indicated concentrations of JKTM-12 for 24 h and then processed for the analysis of viability. (B) Cells were cultured in 6-well plates (2×10^5 cells/well) with the indicated concentration for 1 h and then exposed to 100 nM α-MSH for 48 h. Data are presented as the mean ± SEM of three individual experiments, performed in duplicate. Values are significantly different by comparison with untreated control (*$p < 0.05$) and only α-MSH-treated control ($^{\#}p < 0.05$).

without cytotoxicity. Indicated concentrations of JKTM-12 (10, 50, and 100 μg/ml) were pretreated in B16F10 cells for 1 h, and then 100 nM α-MSH was added to the cells to stimulate melanin biosynthesis in the cells. After 48 h, melanin level was detected using melanin assay described in the experimental section. Melanin was increased by 100 nM α-MSH treatment in B16F10 cells; however, pretreatment of 50 μg/ml and 100 μg/ml JKTM-12 inhibited the melanin level (Figure 3B). Moreover, 100 μg/ml JKTM-12 inhibited the cellular tyrosinase activity and tyrosinase protein expression by western blotting (Figure 6). These result demonstrated that JKTM-12 inhibits melanin biosynthesis without cell cytotoxicity.

INHIBITION OF MELANIN BIOSYNTHESIS AND TRP-1, TRP-2 mRNA EXPRESSION BY HYPEROSIDE AND ASTRAGALIN

We tested tyrosinase activity using the components of JKTM-12 ethanol extract. Interestingly, the receptables of *N. nucifera* inhibited tyrosinase activity more strongly than the

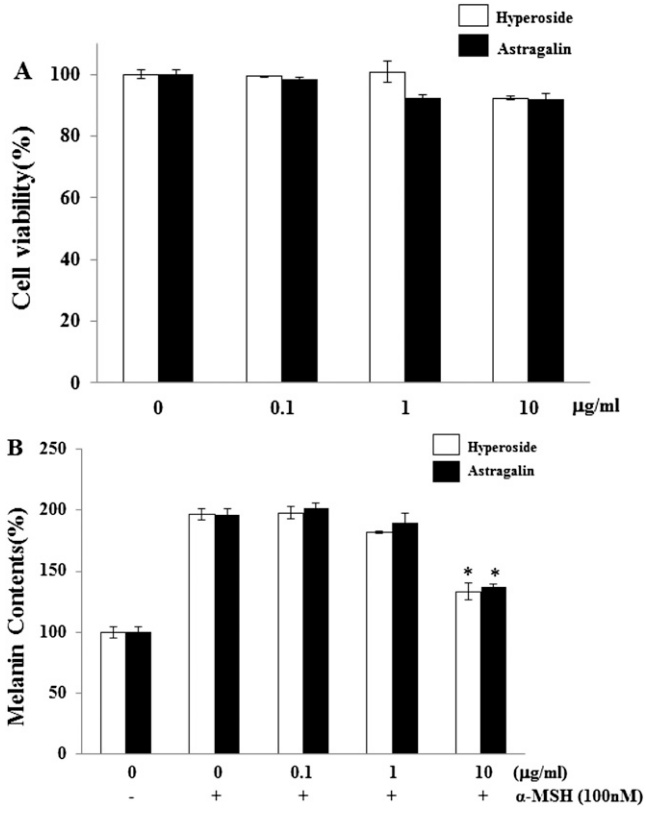


Figure 4. Effects of hyperoside and astragalin on (A) viability and (B) melanin contents in B16F10 cells. Cells were cultured with the indicated concentrations of hyperoside and astragalin and then processed for the analysis of viability and melanin contents. Data are presented as the mean ± SEM of three individual experiments, performed in duplicate. *$p < 0.05$ versus the only α-MSH-treated control values.

flowers, roots, and seeds of *N. nucifera* (data not shown). In our previous study, we isolated two bio-active compounds (hyperoside and astragalin) from an n-EtOAc fraction using the receptacles of *N. nucifera*. Hyperoside (IC50 = 15.67 μg/ml) and astragalin (IC50 = 21.22 μg/ml) were shown to inhibit tyrosinase activity (13). In this study, we demonstrated that hyperoside and astragalin inhibit melanine synthesis, TRP-1 mRNA, TRP-2 mRNA expression, cellular tyrosinase activity, and tyrosinase protein level. Hyperoside and astragalin were not cytotoxic to melanoma cells at the indicated concentrations (Figure 4A). However, pretreatment of hyperoside and astragalin suppressed melanin biosynthesis overexpressed by α-MSH in B16F10 cells in a dose-dependent manner (Figure 4B). Because TRP-1 and TRP-2 are known as melanogenic enzymes, we investigated whether hyperoside and astragalin regulated TRP-1 and TRP-2 mRNA expression. As shown in Figure 5, TRP-1 and TRP-2 mRNA were overexpressed by 100 nM α-MSH in 48 h. However, pretreatment of hyperoside and astragalin inhibited TRP-1 and TRP-2 mRNA expression. Cellular tyrosinase and tyrosinase protein level were activated by 100 nM α-MSH, but pretreatment of hyperoside (10 μg/ml) and astragalin (10 μg/ml) inhibited

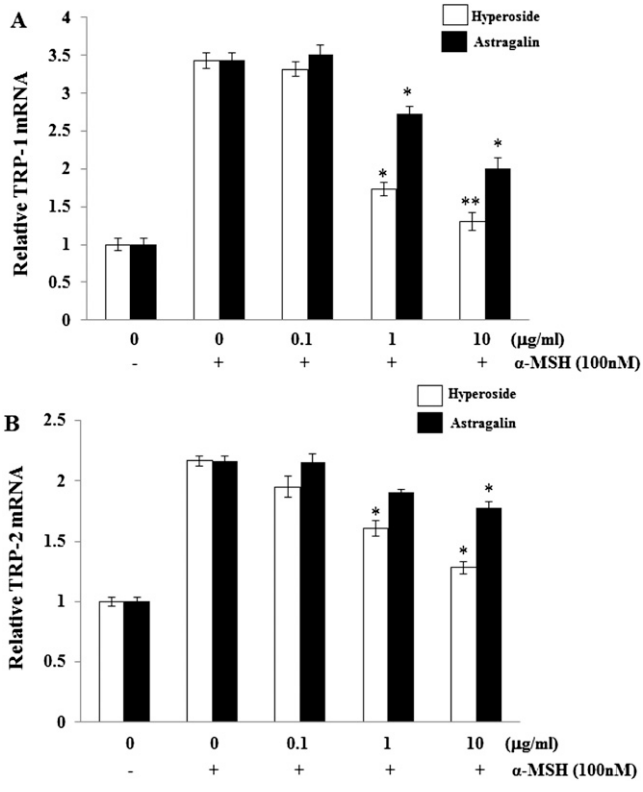


Figure 5. Effects of hyperoside and astragalin on the mRNA expresstion of (A) TRP-1 and (B) TRP-2 in B16F10 cells. Cells were incubated in 6 well plates (2×10^5 cells/well) with the indicated concentrations of hyperoside or astragalin for 1 h and then exposed to 100 nM α-MSH for 48 h. mRNA expression of TRP-1 and TRP-2 was detected by real-time PCR. Hprt1 was used as a loading control. Data are presented as the mean ± SEM of three individual experiments, performed in triplicate. *$p < 0.05$ and **$p < 0.01$ versus the only α-MSH-treated control values.

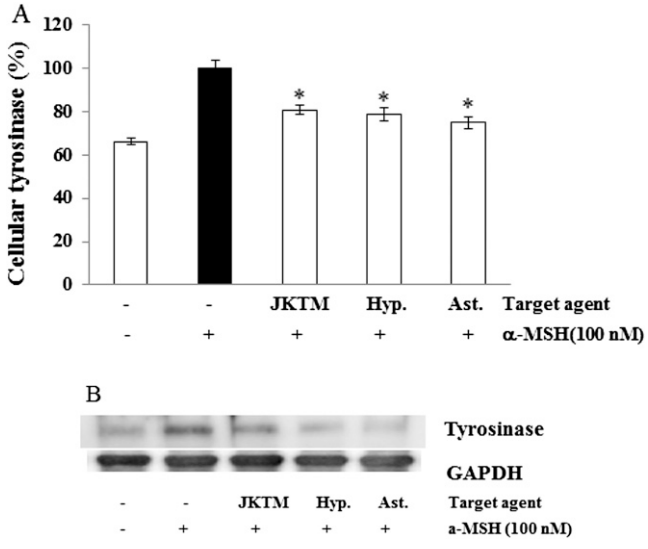


Figure 6. Effects of JKTM-12, hyperoside, and astragalin on the (A) cellular tyrosinase and (B) tyrosinase protein expression in B16F10 cells. Cells were incubated in 6-well plates (2×10^5 cells/well) with JKTM-12 (JKTM, 100 μg/ml), hyperoside (Hyp., 10 μg/ml), or astragalin (Ast., 10 μg/ml) for 1 h and then exposed to 100 nM α-MSH for 48 h. Data are presented as the mean ± SEM of three individual experiments, performed in duplicate. *$p < 0.05$ versus the only α-MSH treated control values.

the cellular tyrosinase activity and tyrosinase protein expression (Figure 6). Hyperoside and astragalin were identified in JKTM-12 by HPLC (Figure 7). The retention times of hyperoside and astragalin were 20.519 and 22.902 min and the concentration of hyperoside and astragalin in JKTM-12 was 5.41 and 49.02 μg/g, respectively.

DISCUSSION

JKTM-12 is composed of the flowers, roots, seeds, and receptacles of *N. nucifera* (the sacred lotus). JKTM-12 inhibited mushroom tyrosinase activity in a dose-dependent

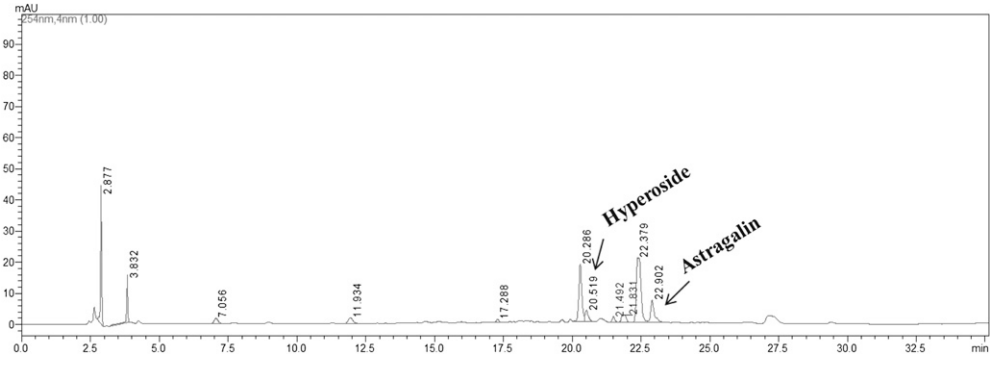


Figure 7. Identification of hyperoside and astragalin in JKTM-12. Hyperoside and astragalin in JKTM-12 were identified by HPCL following the protocols in the experimental section. The retention times of hyperoside and astragalin were 20.519 and 22.902 min, respectively.

manner. When B16F10 murine melanoma cells were cultured with JKTM-12, it suppressed melanin production, cellular tyrosinase activity, and tyrosinase protein level. We identified two compounds (hyperoside and astragalin) and tested the activity in the B16F10 cells stimulated by α-MSH. Hyperoside, quercetin-3-O-β-D-galactoside pyranose, is a flavonoid compound identified from various plants such as *Hypericum perforatum* (14), *Launaea procumbens* (15), *Zanthoxylum bungeanum* (16), and *Rhododendron ponticum* (17). There are evidences that hyperoside has remarkable anti-inflammatory properties (18,19) as well as anti-oxidative effects (17,20–22). Astragalin is extracted from many herbs such as *Morrus alba* (23), *Allium ampeloprasum* var. *porrum* (24), Fuzhuan brick tea (25), and *Cassia alata* (26). Astragalin has antioxidant activity (23,24) and anti-inflammatory effects (27). Hyperoside and astragalin were also isolated from the leaves of *N. nucifera* by high-speed counter-current chromatography (28). Ohkoshi *et al*. (29) reported that a 50% EtOH extract from the leaves of *N. nucifera* stimulated lipolysis in the white adipose tissue of mice, and they identified various flavonoids including rutin, (+)-catechin, hyperoside, isoquercitrin, quercetin, and astragalin. Nakamura *et al*. reported that a MeOH extract of the flower buds and leaves of *N. nucifera* inhibited melanogenesis in B16 melanoma 4A5 cells. They also showed that 100 μg/ml flower buds of *N. nucifera* inhibited melanogenesis by 91 ± 1.8%. This research team found that the inhibitory effect of melanogenesis by the stamen and seeds of *N. nucifera* was lower than that by the flower buds and leaves of this plant (30). In the other report, hyperoside isolated from receptaculum nelumbinis showed obvious DPPH radical scavenging activity and ABTS radical scavenging activity (31). According to this finding, we isolated hyperoside and astragalin from receptaculum nelumbinis and investigated its anti-tyrosinase activity (13). Even though components of JKTM-12 display different activities on melanogenesis in B16F10 cells, JKTM-12 inhibited tyrosinase activity and melanin biosynthesis overexpressed by α-MSH in B16F10 cells. These results were partly caused by hyperoside and astragalin that inhibit tyrosinase activity, melanogensis, TRP-1 mRNA, TRP-2 mRNA expression, cellular tyrosinase, and tyrosinase protein level. Thus, our results indicate that JKTM-12 containing hyperoside and astragalin may prove useful in the development of cosmetic source for preventing hyperpigmentation. However, it needs further studies on normal human melanocytes.

ACKNOWLEDGMENTS

This work was supported by Ministry of Health & Welfare "The Traditional Korean Medicine Industry Development Product R&D Program (C100001)."

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