Effect of Ikarisoside A Isolated from *Epimedium Koreanum* on Melanogenesis

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Melanin plays an important role in protecting human skin from the damaging effects of ultra-violet radiation, in the determination of phenotypic appearance, and in absorption of toxic drugs and chemicals.[1-3] Existing in skin, hair and some sensorial tissues such as eyes and inner ear, its pigment is a heterogeneous biopolymers synthesized from various intermediate products derived from dihydroxyphenylalanine (DOPA) and DOPAquinone.[4,5] Melanocytes are specialized cells located at the basal layer of the epidermis and melanin pigment is the most important characteristic of differentiation in melanocytes or melanoma cells.

Recently many efforts are focused to understand the mechanical insights of melanogenesis to develop the therapeutic agents for hyper- and hypo-pigmentation. The pathological role of pigmentation originates mainly from the fact that melanin pigments serve not only as the major determinant of the skin color but also as the major source of skin protectant against ultraviolet radiation, sun-induced skin damage as well as skin carcinogenic effects.[6] B16/F10 murine melanoma cells have been widely used to elucidate the regulatory mechanisms of melanogenesis and pigment cell proliferation.

Epimedium koreanum (hereafter: EK) belonging to the genus *Epimedium* is an important oriental herbal medicine. The aerial part of EK has been commonly used as a tonic to stimulate hormone secretion, to cure impotence and forgetfulness, whereas the underground parts are used successfully for the treatment of asthmatic attacks and menstrual irregularity.[7]

The water extract of this plant was used with combination of a mixture of crude extracts from other medicinal plants to develop and apply as new therapeutic medicines. Although the constituents of this plant were investigated by some researchers,[8-10] these studies did not report the biological activities of the isolated compounds or crude extracts. The study on melanogenesis in B16/F10 melanoma cells by this agent was also still not investigated. In this paper, we are reporting the role of ikarisoside A extracted from EK on the regulation of melanogenesis in B16/F10 melanoma cells.

Experimental Section

Extraction and isolation. The aerial parts of EK (4.5 kg) were extracted with MeOH (15 L × 3) at room temperature and the total extracts were concentrated. The concentrated residue suspended in H_2O and extracted successively with CH_2Cl_2 and EtOAc. A portion (382 g) of the EtOAc soluble fraction was separated first by column chromatography (eluent; $CHCl_3$-MeOH) on a highly porous synthetic resin, Diaion HP-20 (Mitsubishi Kasei, Tokyo) to obtain 8 fractions. Fr. 3 (31.8 g) was rechromatographed over a silica gel column eluted with $CHCl_3$-MeOH (100 : 1, 50 : 1, 25 : 1, 10 : 1 and 5 : 1) as eluent, yielding subfractions 3A-3H. Subfraction 3G eluted with $CHCl_3$-MeOH (4 : 1) was evaporated to give 3.4 g of residue. After the MeOH solution of the residue was allowed to stand, the yellow precipitate was collected and recrystallized from MeOH to give compound 1 (*ikarisoside A*., 730 mg).

Ikarisoside A. $[\alpha]_D^{16} = -79$(c 0.99, C_5H_5N); EIMS (70 eV) m/z 370(2), 355(31), 354(93), 339(100), 337(8), 325(7), 311(11), 299(86), 286(74), 270(10), 233(3), 165(24), 128(16), 121(54), 69(18), (UV λ_{max}^{EtOH} nm (logε): 224 (sh 4.36), 271 (4.33), 310 (4.10), 354 (sh 4.03) ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 0.83 (3H, *d*, J = 6 Hz, Me-5''), 1.63-1.69 (6H, *brs*, Me-12, 13), 3.18 (2H, *m*, H-4'' and H-5''), 3.36 and 3.43 (2H, *br. dd*, J = 7, 14 Hz, H-9), 3.51 (1H, *br dd*, J = 4, 8 Hz, H-3''), 4.01 (1H, *br s*, H-2''), 5.17 (1H, *qt*, J = 1, 7 Hz, H-10), 5.30 (1H, *d*, J = 1.5 Hz, H-1''), 6.32 (1H, *s*, H-6), 6.93 (2H, *d*, J = 9 Hz, H-3' and H-5'), 7.77 (2H, *d*, J = 9 Hz, H-2' and H-6'), 12.54 (1H, *s*, OH-5); ^{13}C-NMR (500 MHz, DMSO-d_6) δ: 156.3 (C-2), 133.6 (C-3), 177.2 (C-4), 158.2 (C-5), 97.9 (C-6), 160.8 (C-7), 105.5 (C-8), 156.1 (C-9), 103.7 (C-10), 21.7 (C-11), 121.8 (C-12), 130.3 (C-13), 17.6 (C-14), 25.2 (C-15), 120.3 (C-1'), 129.8 (C-2', 6'), 114.9 (C-3', 5'), 159.2 (C-4'), 101.5 (C-1''), 69.8 (C-2''), 70.2 (C-3''), 70.3 (C-4''), 71.0 (C-5''), 17.3(C-6'').

Cell culture. The murine B16/F10 melanoma cells were purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine, supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Biofluids, Rockville, MD) in a humidified atmosphere containing 5% CO_2 in air at 37 °C.

MTT assay. Growth of B16/F10 melanoma cells in the presence of *ikarisoside A* was determined by MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) assay. This assay was performed as described in the modified method of Mosmann.[11] Cells were seeded in 96 well microculture plate at 2×10^3 cells per well and allowed to adhere overnight. Cells were exposed to 1, 2.5, 5, 10, 50, 100, and 200 μg/mL of *ikarisoside A* for 48 h.

MTT was prepared at a concentration of 5 mg/mL in sterile phosphate buffered saline system (PBS). A 20 μL aliquot of the stock solution of MTT was added to each well. After 3 h of incubation at 37 °C, 150 μL of DMSO was added to each well in order to dissolve the formazan crystals. Optical absorbancy were measured at 570 nm using a 96 well multiscanner (Dynatech Instruments, Torrance, CA). A well containing DMEM medium, MTT, and extraction buffer in the absence of B16/F10 melanoma cells was used as the blank. The results obtained were calculated from 3 experiments and presented as a percentage of control values.

Trypan blue exclusion test. The cells were seeded at a density of 1×10^5 cells/well. After treating with ikarisoside A for 48 h, the detached and adhering cells were harvested and suspended in PBS. One volume of 0.4% trypan blue solution (Sigma Chemical Co., St. Louis, MO., USA) was added to two volumes of the cell suspension, and the dye-excluding cells were counted using a light microscope in a hemocytometer.

Assay of melanin content. Melanin content was determined according to modification of the procedure described by Hosoi.[12] Cells were harvested with 0.25% trypsin-EDTA, washed twice with cold-PBS, and solubilized in 1 mL of 1 N NaOH for 1 h at 80 °C. Lysates were centrifuged at 12,000 × g for 10 min. The absorbance of the supernatant was measured at 405 nm. Melanin content was calculated by comparison with a standard curve using a synthetic melanin (Sigma Chemical Co., St. Louis, MO., USA) and was expressed as a percentage of control value.

Determination of tyrosinase activity. Tyrosinase activity was determined by the method of Matinez-Esparza *et al.*[13] For this experiment, cells were incubated with the different concentrations of ikarisoside A for 48 hrs. The cells were harvested, washed with cold-PBS, and lysed in 100 μL of lysis buffer (0.1 M phosphate buffer, pH 6.8, containing 1% (w/v) Triton X-100) supplemented with protease inhibitors (0.1 mM sodium orthovanadate, 2 g/mL aprotinin, 2 g/mL leupeptin, and 0.1 mM PMSF) for 30 min on ice. Cellular extracts were clarified by centrifugation at 9,000 × g for 20 min. Protein concentration was determined by the Bradford method (Bio-Rad) using BSA as a standard. A 50 μL of each extract was mixed with 100 μL of 0.1 M sodium phosphate (pH 7.0) at 37 °C for 5 min, and then added 50 μL of 10 mM catechol. Activity was assayed at 405 nm for 1 h using ELISA reader.

Statistical analysis. Data are expressed as means standard deviation. Student's t-test was used to determine the statistical differences between mean values. Differences were considered significant when the P value was less than 0.05.

Results and Discussion

The structure of *ikarisoside A*. The ^{13}C-NMR spectrum of compound 1 (Figure 1) was analysed by the off-resonance decoupling technique as well as by comparing it with the ^{13}C-NMR spectra of model compounds, such as kaempferol,[14] epimedoside A.[15] and other flavonol glycosides.[16] In these spectra, the signal for the C-2 of **1** appeared to be a more downfield shift than that for C-2 of kaempferol, whereas the C-3 of **1** appeared to be an upfield shift. These results suggest that the **1** is a flavonol 3-glycoside. The location of the prenyl group at the C-8 position was depended by the chemical shift value of the carbon atom at the C-6 position (δ 97.9).[16] The sugar moiety of **1** was identified as rhamnose by comparative examination of the ^{13}C-NMR spectra of **1** and model compounds, such as quercetin 3-o-rhamnoside. From the above results and consideration of ^{1}H-NMR spectrum of **1**, *ikarisoside A*[17] may be characterized as 8-prenylkaempferol 3-o-rhamnopyranoside.

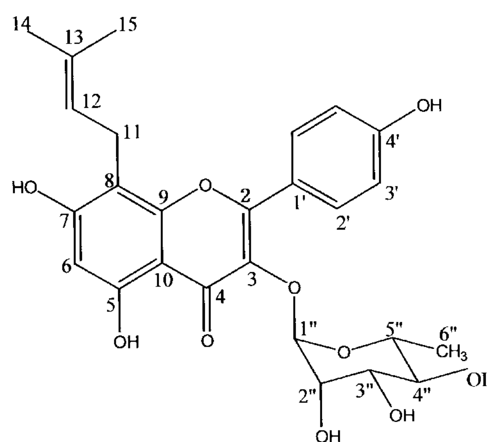

Figure 1. The Structure of Ikarisoside A (8-prenylkaempfenol 3-o-rhamnopyranoside) isolated from Epimedium koreanum.

Effect of ikarisoside A on cell viability of B16/F10 melanoma cells. Many laboratories worldwide have adopted a microculture assay based on metabolic reduction of MTT for automated assay of drug effects on cell viability or growth. This assay is a method to examine the level of cytotoxicity by surveying mitochondria activity in cells. The effect of the exposure to increasing concentrations of *ikarisoside A* on the cell viability of B16/F10 melanoma cells, as assessed by the MTT test is shown in Figure 2. Cells were incubated with the concentrations of 1, 2.5, 5, 10, 50, 100, and 200 μg/mL *ikarisoside A* for 48 h. *Ikarisoside A* had no significant effect on the cell viability in all treatment groups as well as morphological change by itself, but a little decrease in a concentration of 200 μg/mL without a statistical significance. We further investigated the number of viable cells by means of the trypan blue exclusion test to examine if there was any difference between control group and *ikarisoside A* treated groups. As for Figure 3, the percentage of cells excluding trypan blue did not show any significant decrease after *ikarisoside A* treatment for 48 h. Together all data, the results showed that cells treated with *ikarisoside A* in the range of 1-100 μg/mL for 48 h did not exhibit cytotoxicity and therefore, further studies in this paper were estimated within this range of *ikarisoside A*.

Effect of ikarisoside A on melanogenesis in B16/F10 melanoma cells. EK is one of the important Chinese herbal

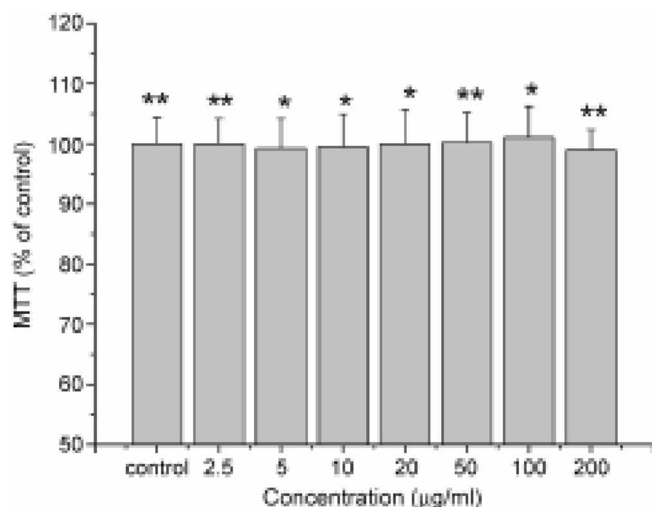

Figure 2. Effect of ikarisoside A on the viability of B16/F10 melanoma cells. The cells were cultured in the presence of various concentrations of ikarisoside A for 48 h. The viability of the cell was measured by MTT assay. Results were expressed as absorbance and data were mean ± SD of at least five determinations. (*p<0.05, **p<0.01).

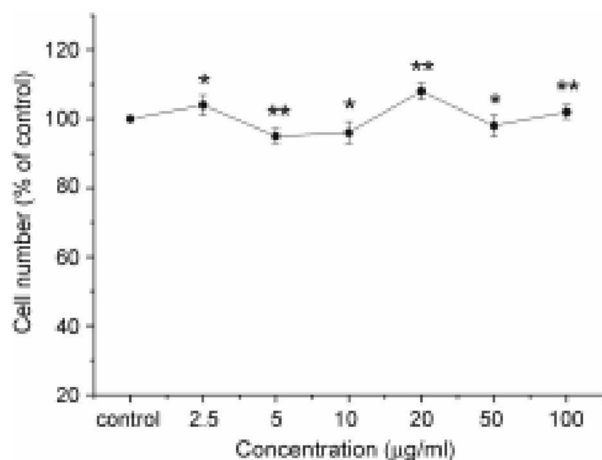

Figure 3. Effect of ikarisoside A on the viability of B16/F10 melanoma cells. The cells were cultured in the presence of various concentrations of ikarisoside A for 48 h. The viability of the cells was measured by Trypan blue test. Results were expressed as absorbance and data were mean ± SD of at least five determinations. (*p<0.05, **p<0.01).

medicines and widely used in Asia. This plant has been commonly used as a tonic to stimulate hormone secretion, to cure impotence and forgetfulness, and to treat asthmatic attacks and menstrual irregularity.[18] Among the well-known ingredients, icariin was reported as the main effective ingredient. We isolated *ikarisoside A* from EK and investigated the effect of *ikarisoside A* on the melanin biosynthesis in B16/F10 melanoma cells. Cells were incubated with *ikarisoside A* in range of 1-100 μg/mL for 48 h and then melanin content was estimated. In *ikarisoside A*-treated B16/F10 melanoma cells, the melanin content was increased in a dose-dependent manner (Fig. 4). To clarify the influence of *ikarisoside A* on melanogenesis, we also

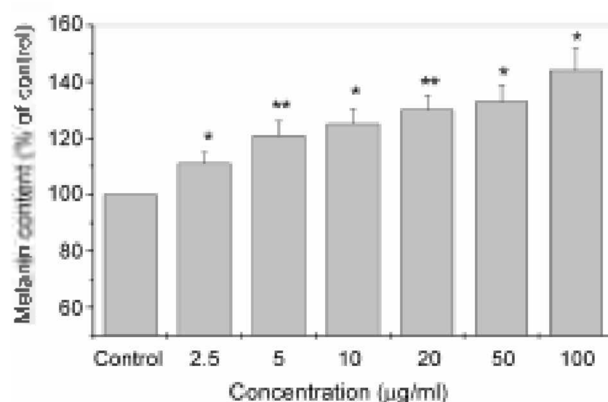

Figure 4. Effect of ikarisoside A on melanin concentration in B16/F10 melanoma cells. The cells were cultured in the presence of various concentrations of ikarisoside A for 48 h. Results were expressed as % control and data were mean±SD of at least five determinations. *significantly different from control group (*p<0.05, **p<0.01)

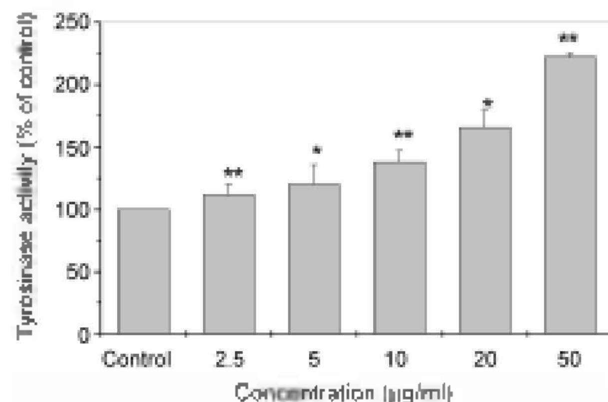

Figure 5. Effect of ikarisoside A on tyrosinase activity in B16/F10 melanoma cells. The cells were cultured in the presence of various concentrations of ikarisoside A for 48 h. Results were expressed as % control and data were mean ± SD of at least five determinations. (*p<0.05, **p<0.01)

measured tyrosinase activity after treatment with *ikarisoside A*. Tyrosinase is the key enzyme in melanin biosynthesis, and a hallmark of differentiation in melanocytes and melanoma cells. Tyrosinase activity was increased in a dose-dependent manner from 2.5 to 50 μg/mL concentration (Fig. 5). These results clearly indicate that *ikarisoside A* stimulates melanin synthesis via activation of tyrosinase in a dose-dependent manner.

Tyrosinase activity was greater when treated with several concentrations of *ikarisoside A* for 48 h, than melanin content. It is considered that the role of tyrosinase is related to the initial phase, the hydroxylation of tyrosine to L-DOPA, in melanogenesis, whereas melanin formation is the final step passing through other numerous pathways and several stages. We found that *ikarisoside A* stimulated the activity of tyrosinase and the up-regulation of the melanin content. These results indicate that *ikarisoside A* regulates melanogenesis by stimulating the activity of tyrosinase, and is sufficient to increase the melanin formation in the B16/

F10 melanoma cells. Although it is necessary to confirm that *ikarisoside A* regulates melanogenesis by tyrosinase in normal melanocytes as in the melanoma cells. these observations add new information to our knowledge of how melanogenesis is regulated. Basic understanding of the regulation of pigment production and distribution could aid in the identification of alternative depigmenting and hyperpigmenting agents.

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