Depigmentation of Melanocytes by (2Z,8Z)-Matricaria Acid Methyl Ester Isolated from *Erigeron breviscapus*

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To discover an active skin depigmenting agent, we isolated a novel inhibitor of melanin biosynthesis from the methanol extract of *Erigeron breviscapus* using a bioactivity-guided fractionation and identified it as (2Z,8Z)-matricaria acid methyl ester by means of spectroscopic analysis. The compound showed strong whitening activity in melan-a cell. Compared with arbutin (IC₅₀ = 4.0 μM) as a positive control, the depigmentation IC₅₀ value for (2Z,8Z)-matricaria acid methyl ester was 25.4 μM in B16F10 melanoma cell. Moreover, its inhibitory effect on tyrosinase, the key enzyme of melanogenesis, was examined by *in vivo* and *in vitro* tyrosinase assay and Western blot. The results indicate that (2Z,8Z)-matricaria acid methyl ester isolated from *Erigeron breviscapus* is a promising compound that could be useful for treating hyper-pigmentation as skin-whitening agents.

Key words melanin; *Erigeron breviscapus*; (2Z,8Z)-matricaria acid methyl ester; skin whitening

Melanin is a phenolic biopolymer that is responsible for pigmentation.1) Various skin colors for individuals are mostly determined by types, amount, and arrangement of melanin, which is synthesized by epidermal melanocytes.2,3) Although melanin plays a crucial role in absorbing free radicals from the cytoplasm and shielding from UV light, the overproduction and accumulation of melanin in the skin could lead to a serious skin disorder.4) Current therapies for skin pigmentation diseases are unsatisfactory because of their low activity and cell toxicity.5) Thus, research on *E. breviscapus* has been studied and its neuroprotective actions, antibacterial, and antifungal activity have been reported.18–21) However, its depigmenting effect has not yet been examined.

In this study, we isolated and identified the active compound, and investigated its depigmenting activity.

MATERIALS AND METHODS

**Materials** Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), trypsin EDTA, phosphate buffered saline (PBS), penicillin/streptomycin were purchased from Invitrogen Corp. (CA, U.S.A.). Arbutin, PTU (N-phenylthiourea), TPA (12-O-tetradecanoylphorbol-13-acetate), Mushroom tyrosinase, L-DOPA (3,4-dihydroxy-L-phenylalanine), DMSO (dimethyl sulfoxide), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Goat polyclonal tyrosinase antibody and anti-goat HRP-conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc. Analytical grade HPLC solvents were purchased from J.T. Baker U.S.A. Silica gel 60 (0.063—0.2 mm) was purchased from Merek. *Erigeron breviscapus* was purchased from Houbu Pharmaceutical Co., Ltd. in Anhui Province, China.

**Isolation of (2Z,8Z)-Matricaria Methyl Ester** To prepare the melanin inhibitory compound, extraction and isolation of *E. breviscapus* was carried out based on activity-guided fractionation as described in Fig. 2. The dried *E. breviscapus* (70 g) was extracted four times with 99.5% methanol for 10 h at 40 °C. The resulting mixtures were fil-
trated and concentrated to dryness at 40 °C under vacuum to produce a methanol extract (14 g). Subsequently, the extract was suspended in water, and re-extracted by hexane to yield the non-polar fraction (1 g). The hexane fraction was column chromatographed on a silica gel and eluted with hexane–chloroform 55 : 45 where 117.5 mg of active compound was obtained. Examination of spectroscopic data such as 1D and 2D (HMQC, HMBC) NMR, and EI-MS confirmed the chemical structure of the compound as (2Z,8Z)-matricaria acid methyl ester (Fig. 3).22)

**Cell Culture and Inhibition Assay** B16F10 cell culture: B16F10 murine melanoma cells were purchased from ATCC (American Type Culture Collection) and cultured in DMEM medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO2. The cells were seeded into a 6 well plate (Falcon, U.S.A.) at a concentration of 0.3×10^5 cells/ml and 96 well plate with 1.25×10^4 cells/ml.23)

Melan-a cell culture: Murine melan-a melanocytes, originally derived from C57BL/6J mice, were purchased from ATCC (American Type Culture Collection) and cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin and 200 mM TPA at 37 °C in a humidified atmosphere with 10% CO2. The cells were then seeded into a 6 well plate at a concentration of 0.8×10^5 cells/ml and 24-well plate with 0.4×10^4 cells/ml.13)

MTT assay was performed to examine the viability of cells. Briefly, B16F10 cells or melan-a cells were seeded at an appropriate cell density. After 24 h incubation, cells were treated with various concentrations of sample for 48 h. After incubation with MTT solution for 4 h, the optical density of each well was read at 540 nm in an ELISA reader.24)

For cellular melanin measurement, the cells were seeded into a 6-well plate at an appropriate density. After 24 h of cultivation, the cells were treated with various concentrations of sample for 48 h. The harvested cells were washed twice with cold phosphate-buffered saline (PBS). A lysis buffer was prepared. Cells were disrupted by sonication at 4 °C and separated by centrifugation at 16000 rpm for 20 min. After quantifying protein content using a protein assay kit (Bio-Rad, U.S.A.), the cell lysates were adjusted to the same concentration of protein with a lysis buffer.25) The reaction mixtures including cellular extracts, 5 mM L-DOPA, and 0.1 M sodium phosphate buffer (pH 6.8) were incubated in a 96-well plate at 37 °C for 60 min. Absorbance was measured at 475 nm using an ELISA reader.26) This data was expressed as percent of control (%)=\((A_{\text{test sample}}/A_{\text{control}})\)·100, where, A is absorbance.

**Western Blot Analysis** Protein samples extracted from melan-a cells were subjected to SDS/PAGE (12% gel) and transferred to a PVDF membrane electrophoretically. The membrane was incubated in a blocking solution (5 g of non-fat dry milk in 100 mL of TBS-T (TBS consisting 1% Tween-20)) for 2 h at room temperature with shaking. The membrane was then incubated for 2 h with goat polyclonal tyrosinase antibody or β-actin antibody and further incubated with an anti-goat horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia, Piscataway, NJ, U.S.A.).

All the values were expressed as mean±standard deviation (S.D.). The statistically significant difference was examined by the Student’s t-test.

**RESULTS**

**Isolation of Active Compound and Structure Determination** The melanin inhibition by the methanol extract of *E. brevicaus* was observed at 50 µg/ml (Fig. 1). From this methanol extract, a compound with melanin biosynthesis-inhibitory activity was isolated by hexane fractionation and subsequent silica gel column chromatography (Fig. 2). As shown in the fractionation scheme, 117.5 mg of a pure (2Z,8Z)-matricaria acid methyl ester was obtained. The compound is a white powder, soluble in methanol. The molecular formula is C_{11}H_{15}O_{2} based on EI-MS analysis (M+, m/z 177.0). In the 1H-NMR in DMSO-d_{6}, four olefinic protons (δ 6.56, 1H, d, J=11.6 Hz; 6.40, 1H, d, J=11.6 Hz; 6.36, 1H, dq, J=10.8, 6.8 Hz; 5.78, 1H, dq, J=10.8, 1.2 Hz) as well as one vinyl methyl (δ 1.88, 3H, dd, J=6.8, 1.2 Hz) and a methoxy group (δ 3.68, 3H, s) were observed. Coupling constant analysis confirmed the olefins were in cis-configurations.13) C and DEPT NMR identified that 11 signals are corresponding to one carbonyl (δ 163.9), four olefinic (δ 145.1, 131.6, 122.1, 108.4), four alkynyl (δ 84.2, 83.4, 78.7, 77.3) protons.
and two methyl (δ 51.5, 16.6) carbons. From these data, the compound was identified as methyl 2-cis-8-cis-deca-2,8-diene-4,6-dyynoate [(2Z,8Z)-matricaria acid methyl ester] (Fig. 3).22)

Inhibition of Melanin Biosynthesis by (2Z,8Z)-Matricaria Acid Methyl Ester

To determine depigmenting activity, the change in the melanin contents in the cells treated with (2Z,8Z)-matricaria acid methyl ester was evaluated. In B16F10 melanoma cells, the IC_{50} value of (2Z,8Z)-matricaria methyl ester was 25.4 μM, while that of arbutin, a positive control, was 4.0 μM. Thus, the compound is approximately 160 times more potent than arbutin, a widely used lightening ingredient in medicated cosmetics. In addition, the melanin contents of melan-a cell were also significantly attenuated by the compound without cytotoxicity (Fig. 4).

Analysis of the Inhibitory Mechanism of Melanin Biosynthesis by (2Z,8Z)-Matricaria Acid Methyl Ester

The effect of (2Z,8Z)-matricaria acid methyl ester on the catalytic activities of tyrosinase is shown in Fig. 5. The compound had no effect on the oxidation activity of mushroom tyrosinase (Fig. 5A) whereas kojic acid completely inhibited mushroom tyrosinase activity. However, in the cell-based tyrosinase assay, inhibition of the tyrosinase activity by (2Z,8Z)-matricaria acid methyl ester was displayed in dose-dependent manner in both B16F10 melanoma cells (Fig. 5B) and melan-a cells (Fig. 5C). It should be noted that the reduced melanin contents were attributed to the suppression of tyrosinase expression, rather than the direct inhibition of tyrosinase activity. Thus, to characterize the expression of tyrosinase protein, Western blot analysis was performed. As shown...
in Fig. 6, melan-a cells treated with 9 μM and 4.5 μM of (Z,Z,8Z)-matricaria acid methyl ester had significantly decreased tyrosinase protein levels compared with the PTU-treated cells as a positive control.

DISCUSSION

E. breviscapus is a medical herb that has often been used by the Chinese for expelling the cold, relieving exterior syndrome and pain. The plant had been first recorded in an old traditional medicinal text entitled “Dian Nan Ben Cao” written by Lan Mao in the middle of the fifteenth century and has gained notoriety for treating cardiovascular disease since the 1970’s in China. We found that this plant contains a melanin biosynthesis inhibitor isolated from plants.

To find the mechanism of melanin inhibition, tyrosinase, the most important enzyme on melanogenesis was investigated for its activity (Fig. 5) and expression (Fig. 6). No inhibition of in vitro mushroom tyrosinase activity was observed by the isolated compound. However, a significant decrease in cellular tyrosinase activity was observed in the compound-treated cells. Also a decreased tyrosinase protein level was found by western blot analysis. Therefore, it is suggested that isolated (Z,Z,8Z)-matricaria acid methyl ester from E. breviscapus inhibits melanin synthesis by down-regulation of tyrosinase expression (Fig. 6). Numerous factors are involved in melanogenesis. The depigmenting mechanism of (Z,Z,8Z)-matricaria acid methyl ester should be examined more and is currently in progress.

These results showed that (Z,Z,8Z)-matricaria acid methyl ester isolated from Erigeron breviscapus can be a promising compound that could be used as skin-whitening agent for treating hyperpigmentation.

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