Antimelanogenic Effects of Picrionoside A Isolated from the Leaves of Korean Ginseng

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This study was initiated to isolate active metabolites from the leaves of Panax ginseng. Among them, picrionoside A, a megastigmane glucoside, was isolated from the leaves of P. ginseng C. A. Meyer and its chemical structure was determined based on spectroscopic methods, including FAB-MS, one-dimensional (1D)-NMR, 2D-NMR, and IR spectroscopy. Picrionoside A from P. ginseng has not been investigated previously, and its biological or pharmaceutical activities have not been reported elsewhere. The IC50 value of mushroom tyrosinase-inhibitory activity of picrionoside A was 9.8 μM, and the rate of inhibition of synthesized melanin content in melan-a cells was 17.1% at a concentration of 80 μM without cytotoxicity. Furthermore, picrionoside A dramatically reduced body pigmentation in the zebrafish model. Taken together, the results suggest that picrionoside A isolated from the leaves of P. ginseng may be an effective skin-whitening agent that could be a potent candidate material in the cosmetic industry.

Key words Panax ginseng; picrionoside A; melanogenesis; tyrosinase

Panax ginseng C. A. Meyer is a very famous traditional medicinal herbs and many studies have reported the chemical constituents of the root of P. ginseng. The more than 120 kinds of ginsenosides have been isolated and studied the various beneficial bioactive effects such as a immunomodulatory, nutritional fortification, anti-diabetic, anti-carcinogenic as well as anti-oxidant activities.1–5) Although the ginsenoside has a major compounds of P. ginseng and represents the most therapeutic values of P. ginseng, recent attentions are given into the therapeutic values of P. ginseng leaves constituents, the usefulness of P. ginseng leaves will be evaluated for 4 months in an aeroponic system were supplied by the Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, RDA, Eumseong, Korea.

Materials The leaves of hydroponic P. ginseng cultivated for 4 months in an aeroponic system were supplied by the Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, RDA, Eumseong, Korea.

Materials Mushroom tyrosinase, l-tyrosine, 12-o-tetradecanoylphorbol-13-acetate (TPA) and 1-phenyl-2-thiourea (PTU) were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The plastics used in the tissue culture were purchased from Falcon (Becton Dickinson, Franklin Lakes, NJ, U.S.A.), and the media and additives were obtained from Gibco (Grand Island, NY, U.S.A.). Kieselgel 60 and LiChroprep RP-18 resins were used for column chromatography (Merck, Darmstadt, Germany), Kieselgel 60 F254 (Merck) and RP-18 F254S (Merck) were used as solid phases for TLC experiment.

Note

Panax ginseng C. A. Meyer is a very famous traditional medicinal herbs and many studies have reported the chemical constituents of the root of P. ginseng. The more than 120 kinds of ginsenosides have been isolated and studied the various beneficial bioactive effects such as a immunomodulatory, nutritional fortification, anti-diabetic, anti-carcinogenic as well as anti-oxidant activities.1–5) Although the ginsenoside has a major compounds of P. ginseng and represents the most therapeutic values of P. ginseng, recent attentions are given into the therapeutic values of P. ginseng leaves constituents, the usefulness of P. ginseng leaves will be increased. It has been reported that the functional constituents of leaves of P. ginseng are major ginsenosides, minor ginsenosides, polysaccharides, and glycosyl glycerides which have anti-cancer, immunostimulating effects, whitening activity, and anti-inflammatory effects.7–11) Especially, megastigmane glucosides have never been investigated from the leaves of P. ginseng.

Melanin has a various important physiological function such as the major defense mechanism against visible as well as ultraviolet radiation. However, overexpressed melanin caused the freckles, senile lentigines and other forms of melanin hyperpigmentation which are serious aesthetic problems in human body.12) Melanogenesis is regulated by three major melanocyte specific enzyme, tyrosinase, tyrosinase-related protein 1 and tyrosinase related protein 2.13) Notably, tyrosinase plays a key role in melanogenesis and it has an attractive target in the development for new depigmentation agents.12) Therefore, melanin production is mainly controlled by tyrosinase expression and activation.14)

In this study, the active compound was isolated from the leaves of P. ginseng and identified picrionoside A, megastigmane glucosides, which was previously doesn’t reporting any biological activities elsewhere. Uchiyama et al. isolated the picrionoside A and B; megastigmane glycoside, from the whole plants of Picris hieracioides var.5) However, these biological functions are also not studied. Therefore, we firstly evaluated the anti-melanogenic activities of picrionoside A on melan-a cells and zebrafish by regulating the expression of melanogenic enzyme, tyrosinase.

MATERIALS AND METHODS

Plant Materials The leaves of hydroponic P. ginseng cultivated for 4 months in an aeroponic system were supplied by the Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, RDA, Eumseong, Korea.

Materials Mushroom tyrosinase, l-tyrosine, 12-o-tetradecanoylphorbol-13-acetate (TPA) and 1-phenyl-2-thiourea (PTU) were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The plastics used in the tissue culture were purchased from Falcon (Becton Dickinson, Franklin Lakes, NJ, U.S.A.), and the media and additives were obtained from Gibco (Grand Island, NY, U.S.A.). Kieselgel 60 and LiChroprep RP-18 resins were used for column chromatography (Merck, Darmstadt, Germany), Kieselgel 60 F254 (Merck) and RP-18 F254S (Merck) were used as solid phases for TLC experiment.

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Extraction and Isolation of Picrionoside A  The dried and powdered leaves of *Panax ginseng* C. A. MEYER (6.27 kg, National Institute of Horticultural and Herbal Science, Eumsung, Korea) were extracted with 80% methanol (MeOH) (30 L×3) at room temperature for 24 h. The extracts were filtered and evaporated under reduced pressure at 45°C to yield 1.4 kg of extract. The extract was poured into H2O and extracted with ethyl acetate and n-butanol, successively. The ethyl acetate (EtOAc) fraction (75 g) was applied to a silica gel column (ϕ 14×16 cm) and eluted with chloroform (CHCl3)–MeOH (30 : 1, 60 L) and CHCl3–MeOH–H2O (15 : 3 : 1, 136 L) to yield 24 fractions (PGLE1 to PGLE24). Fraction PGLE13 (1.53 g, Ve/Vt = 0.34–0.36, where Ve refers to the volume of eluent for the corresponding fraction and Vt represents the total elution volume) was subjected an ODS column (ϕ 4×12 cm, MeOH–H2O = 3 : 1, 10 : 1, 30 : 1, each 1.5 L) to yield 16 fractions (PGLE13-1 to PGLE13-16). Fractions PGLE13-1 and PGLE13-2 were combined (463 mg, Ve/Vt = 0.00–0.07) and were further fractionated over the SiO2 column (ϕ3×15 cm CH3Cl–MeOH–H2O=20:3:1, 3.2L) to yield 11 fractions (PGLE13-1 to PGLE13-11). Fraction PGLE13-1-7 (111 mg, Ve/Vt = 0.49–0.62) was further fractionated over the ODS column (ϕ2.5×7 cm MeOH–H2O=4:1, 100 mL) to yield 16 fractions (PGLE13-1-7-1 to PGLE13-1-7-16), including picrionoside A [PGLE13-1-7-4, 10 mg, Ve/Vt = 0.05–0.07, TLC *Rf* = 0.50 (RP-18 F254S, MeOH–H2O=2:1)].

Instrumental Analysis  FAB-MS spectrum was recorded on a JEOL JMS-700 (Tokyo, Japan). NMR spectra were recorded on a Varian Inova AS 400 spectrometer (400 MHz, Varian, Palo Alto, CA, U.S.A.).

Mushroom Tyrosinase Inhibitory Activities  The reaction mixture for mushroom tyrosinase (EC 1.14.18.1, Sigma) activity consisted of 150 µL of 0.1 M phosphate buffer (pH 6.5), 3 µL of sample solution, 8 µL of mushroom tyrosinase (2100 unit/mL, 0.05 M phosphate buffer at pH 6.5), and 36 µL of 1.5 mM L-tyrosine. Tyrosinase activity was determined by reading the optical density at 475 nm on a microplate reader (Bio-Rad 3550, Richmond, CA, U.S.A.) after incubation at 37°C for 20 min. The inhibitory activity of the sample was expressed as the concentration that inhibits 50% of the enzyme activity (IC50).

Cell Cultures  The melan-a cell was received as a gift from Prof. Dorothy C. Bennett (St. George’s Hospital Medical School, London, U.K.). This cell line was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), streptomycin-penicillin (100 µg/mL each) and 200 nM TPA, a potent tumor promoter, at 37°C in 5% CO2. Cell viability was determined using the CCK8 cells viability assay kit through the supplier manuals.

Measurement of Melanin Content  The cells were incubated in a fresh medium containing various concentrations of compounds for 4 d. After the cells had been washed with phosphate-buffered saline (PBS), they were lysed and transferred to a 96-well plate. The melanin contents were estimated via absorbance measurements at a wavelength of 405 nm.

Tyrosinase Activity  Tyrosinase activity in melan-a cells was determined as described previously. In brief, cell lysate of melan-a cells incubated with the test substance was incubated with 15 mM L-3,4-dihydroxyphenylalanine (L-DOPA) at 37°C, and it measured absorbance every 10 min, for at least 1 h, at a wavelength of 475 nm, using an enzyme-linked immunosorbent assay (ELISA) reader.
Origin and Maintenance of Parental Fish  Adult zebrafishes were obtained from a zebrafish organogenesis mutant bank (ZOMB) in Gyeongbuk National University and embryos were obtained from natural spawning that was induced at the morning by turning on the light. Collection of embryos was completed within 30 min. 17)

Compounds Treatment and Phenotype-Based Evaluation  Synchronized embryos were collected and arrayed by pipette (7–9 embryos per well in 24 well plates containing 1mL of embryo medium). Test compounds were dissolved in 0.1% dimethyl sulfoxide (DMSO), and then added to the embryo medium from 9 to 72h post-fertilization (63h exposure). The effects on the pigmentation of zebrafish were observed under the stereomicroscope.

Statistical Analysis  Data are presented as the mean ± standard deviation (S.D.). Significant differences between results were determined by Williams’ multiple range test. The p-values of <0.05 were taken to be significant.

RESULTS AND DISCUSSION

The Identification of Picrionoside A  Picrionoside A is obtained as amorphous powder from methanol, exhibiting a UV absorption maximum at 225nm (Fig. 1A). The molecular formula was determined to be 371 [M+H]⁺ in the positive FAB/MS. Comparisons of the NMR and MS data of the isolated compound with reported values led to identification of the known compound as picrionoside A. 15)

Physical Properties and NMR Data of Picrionoside A  Picrionoside A: amorphous powder; positive FAB/MS m/z 371 [M+H]⁺; ¹H-NMR (400 MHz, pyridine- d₅) δ_H: 6.63 (1H, dd, J=15.6, 10.4 Hz, H-7), 6.17 (1H, d, J=15.6 Hz, H-8), 5.91 (1H, brs, H-4), 4.96 (1H, d, J=7.6 Hz, H-1'), 4.54 (2H, m, overlapped, H-3, 6'a), 4.37 (1H, m, H-6'b), 4.17–4.27 (2H, m, overlapped, H-3', 4'), 3.94–4.01 (2H, m, overlapped, H-2', 5'), 2.42 (1H, d, J=10.4 Hz, H-6), 2.24 (3H, s, H-10), 1.83 (1H, dd, J=13.6, 5.6 Hz, H-2'a), 1.69 (1H, dd, J=13.6, 5.6 Hz, H-2'b), 1.49 (3H, s, H-13), 1.01 (3H, s, H-12) 0.78 (3H, s, H-11); ¹³C-NMR (100 MHz, pyridine-d₅) δ_C: 197.4 (C-9), 147.1 (C-7), 135.2 (C-5), 133.9 (C-8), 125.8 (C-4), 103.2 (C-1'), 78.7 (C-3'), 78.4 (C-5'), 75.2 (C-2'), 72.3 (C-3), 71.8 (C-4'), 62.9 (C-6'), 54.4 (C-6), 40.0 (C-2), 33.6 (C-1), 29.1 (C-12), 27.0 (C-10), 24.9 (C-11), 22.5 (C-13).

Anti-melanogenic Effect of Picrionoside A  In the course of screening for new melanogenic inhibitor, several kinds of compounds and extract were evaluated mushroom tyrosinase inhibition at the 0 to 30µM concentrations (data not shown).

Among them, picrionoside A showed the highest mushroom tyrosinase inhibitory activity and its IC₅₀ value was 9.8µM which were about 6.8 fold to 10 fold higher than that of kojic acid and arbutin, respectively (Table 1).

To ascertain the depigmentation activity of the picrionoside A in vitro system, the melan-a cells were treated with 0 to 80µM of picrionoside A. After treated picrionoside A, cells were incubated for three days, and cell viability was assessed using CCK-8 cell viability assay kit. The PTU, a sulfur-containing tyrosinase inhibitor, used as positive control, and picrionoside A doesn’t show the cytotoxicity on the melan-a cell at the all tested concentration (Fig. 1B).

As shown in the Fig. 1D, picrionoside A reduced the melanin content in a dose-dependent manner between 20 to 80µM concentrations. Especially, at the 80µM concentration, 17.1% of melanin synthesis was significantly inhibited versus vehicle control. In the previous report, cinnamic acid extracted from P. ginseng exhibited inhibitory effect of 15.7% on the melanin synthesis at 50µM in the melan-a cell. Compared with cinnamic acid, picrionoside A showed 17.1% melanin synthesis inhibitory activity at the 80µM which has comparable activity at the range of 50 to 80µM. Likewise, incorresponsive tyrosinase inhibitory activity was showed the dose-dependent manner (Fig. 1C).

P. ginseng is one of the most widely used medicinal plants in traditional oriental medicine which has a number of active ingredient including ginsenoside, polysaccharide, phytoestrogens, peptides, polyacetylenes, fatty acid and polyacetylenic alcohols. 1, 6) Among the active ingredients of ginseng, the ginsenoside is known to be the major biologically active compound and most widely studied. In the recent years, it has scientific approaches to clarify the efficacy in skin as well as to treat a wide variety of diseases. To date, several reports shown that the anti-melanogenic effect of ginsenoside. 19, 20) Our result showed that the strong mushroom tyrosinase inhibitory activity (IC₅₀=9.8µM) with picrionoside A. However, tyrosinase activity in the melan-a cell showed the weak inhibition rate. Inversely, the p-cumaric acid, isolated from leaves of P. ginseng, showed the weaker inhibition against mushroom tyrosinase but more strongly inhibited human or murine tyrosinase compared with albutin. 21) The tyrosinase has an active site with two copper atoms, CuA and CuB, and tyrosin binds to CuA site and DOPA binds both CuA and CuB sites. 22) These results may be caused by the specific inhibitor for tyrosin or competitive inhibitor for DOPA model.

Recently, many of researchers are looking for new experimental methods to test the cosmetic ingredients because animal ethics concern have been raised in the worldwide. The zebrafish embryo model becomes a popular model for replacing animal experiments. 23) Zebrafish has melanin pigment on the surface which allowing simple observation on the body pigmentation of process without complicated experimental procedure and the PTU is used as the positive control which is widely used in zebrafish research. 24) As shown in Fig. 2A, the normal surface of zebrafish showed the dark melanin spots in the whole body. However, as shown in Fig. 2D, at 80µM picrionoside A treated zebrafish showed a remarkable inhibitory effect on body’s pigmentation. Especially, head (a), yolk (b), and board (c) between yolk and horizontal myoseptum showed a conspicuous inhibitory effect which decreased to a remarkable extent the total melanin content compared with the untreated zebrafish. PTU showed the brilliant anti-melanogenic effect in the zebrafish model. However, PTU showed

Table 1. Inhibitory Effect of Picrionoside A on the Mushroom Tyrosinase Activity

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<tr>
<th>Compounds</th>
<th>Mushroom tyrosinase IC₅₀ (µM)</th>
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<tbody>
<tr>
<td>Picrionoside A</td>
<td>9.8±1.4</td>
</tr>
<tr>
<td>PTU</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>67.5±0.7</td>
</tr>
<tr>
<td>Arbutin</td>
<td>&gt;100</td>
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IC₅₀, half-maximal inhibitory concentration. Tyrosinase was preincubated with test compounds at 25°C for 10min prior to incubation with l-tyrosin for 30min and the absorbance was determined at 490nm.
the toxicity which inhibits the thyroid function similar to methimazole and potassium perchlorate, which are well known goitrogens.²⁵

Synchronized embryos were treated with melanogenic inhibitors at the indicated concentrations. Picrionoside A were dissolved in 0.1% DMSO, then added to the embryo medium. The effects on the pigmentation of zebrafish were observed under the stereomicroscope (A) vehicle control, (B) 80 µM PTU, (C) 40 µM picrionoside A, (D) 80 µM picrionoside A.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


