BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 23(3), 283-289 (2015)

Phenolic Compounds from the Leaves of *Stewartia pseudocamellia* Maxim. and their Whitening Activities

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Abstract

The half-dried leaves of *Stewartia. pseudocamellia* were extracted with hot water (SPE) and partitioned with *n*-hexane (SPEH), dichloromethane (SPED), and ethyl acetate (SPEE) successively. SPE and SPEE showed significant inhibitory effects against melanogenesis and tyrosinase activities. By bioassay-guided isolation, ten phenolic compounds were isolated by column chromatography from SPEE. The whitening effect of the isolated compounds from SPEE were tested for the inhibitory activities against melanogenesis using B16 melanoma cells, *in vitro* inhibition of tyrosinase, and L-3,4-dihydorxy-indole-2-carboxylic acid (L-DOPA) auto-oxidation assay. A cytotoxic activity assay was done to examine the cellular toxicity in Raw 264.7 macrophage cells. Of the compounds isolated, gallic acid and quercetin revealed significant inhibitory activities against melanogenesis compared to arbutin. In particular, quercetin exhibited similar inhibitory activities against tyrosinase and L-DOPA oxidation without cytotoxicity. These results suggested that SPE could be used as a potential source of natural skin-whitening material in cosmetics as well as in food products.

Key Words: *Stewartia pseudocamellia* Maxim., Quercetin, Melanogenesis, Tyrosinase, Skin whitening

INTRODUCTION

Melanin is a pigment of skin which is synthesized by melanocytes in melanosomes. It is distributed in various organs of the human body, including skin, hair, eyes, and brain (Ko *et al*., 2014). Melanin plays diverse physiological roles in the human body, such as maintaining body temperature, protecting the skin against UV radiation, and scavenging toxic substances and free radicals (Solano *et al*., 2006). The abnormal formation of melanin leads to serious dermal problems such as age spot, melasma, freckles and even skin-cancer (Stefania *et al*., 2003). The hydroxylation of tyrosine to L-DOPA (3,4-dihydroxyphenylalanine) and oxidation of L-DOPA to DOPA quinone are the first two steps in the biosynthesis of melanin, and tyrosinase is involved in this pathway. Tyrosinase, a multifunctional copper-containing enzyme that is present in microorganism, plants, and animals, is the key enzyme in melanogenesis (Kim *et al*., 2008; Liang *et al*., 2011; Park *et al*., 2010). Thus, the ability to control the activity of tyrosinase is an important in inhibiting melanogenesis. Several tyrosinase inhibitors such as arbutin, kojic acid, glabridin, oxyresveratrol, hydroquinone, and vitamin C have been reported, and arecommonly used in cosmetics and medicines (Maeda and Fukuda, 1991; Seo *et al*., 2003; Ohad *et al*., 2004). However, many studies on skin whitening products are always being conducted, since some of these inhibitors have side effects or limitations, including insufficient penetrative ability, high toxicity, and low activity (Ohad *et al*., 2004). Therefore, it is important to find whitening agents that overcome these disadvantages.

Stewartia pseudocamellia Maxim. belongs to the Theaceae-family. It grows throughout Korea (Lee *et al*., 2012a), and has long been used as a traditional medicine to treat liver disease, neuralgia, and quadriplegia. Several studies have reported the various biological activities of *S. pseudocamellia*, including anti-inflammatory, angiogenic (Lee *et al*., 2010a), antioxidant (Lee *et al*., 2010b), osteoclastic differentiation, and inhibitory against bone resumption (Park *et al*., 2007). A phytosterol derivative, 3-*O*-*β*-D-glucopyanosylspinasterol, which showed anti-

Open Access **http://dx.doi.org/10.4062/biomolther.2014.140**

Received Dec 11, 2014 **Revised** Jan 15, 2015 **Accepted** Jan 15, 2015
Published online May 1, 2015

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inflammatory activities (Jung *et al*., 2012), was isolated from the leaves of *S. pseudocamellia*. Other constituents including phenolic compounds, lignans, and flavonoids (Lee *et al*., 2010b; Kim *et al*., 2014) were also isolated from stems and twigs of this plant.

The aim of this study is to identify the compounds which revealed the whitening activity from the water extracts of *S. pseudocamellia*. The whitening activities were determined by measuring the melanin contents using B16 melanoma cells and the inhibitory activity of tyrosinase during melanogenesis. Ten compounds (1-10) were isolated from the ethyl acetate fraction of *S. pseudocamellia*. The structures of isolated compounds were characterized by spectroscopic experiments such as ^{1}H NMR, ^{13}C NMR, and LC/MS.

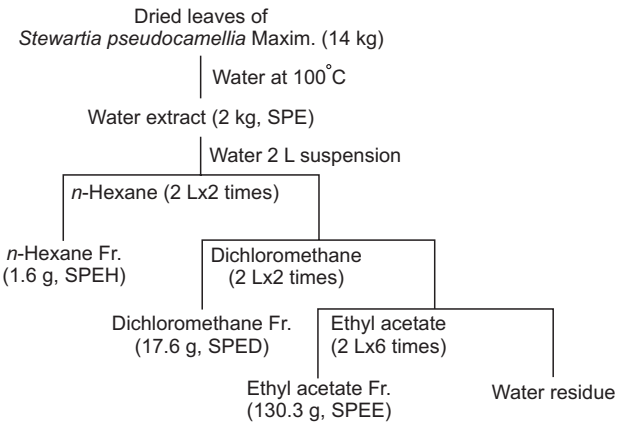


Fig. 1. Extraction and solvent partition of *S. pseudocamellia* Maxim. leaves.

MATERIALS AND METHODS

General experimental procedures

The structures of isolated compounds were determined through various spectroscopic experiments, i.e., ^{1}H NMR, ^{13}C NMR, and mass spectrometry. NMR spectra were conducted on Varian and Bruker 500 and 700 MHz spectrometers, respectively. Mass spectrometry was performed using a triple quadrupole LC-Mass with electrospray ionization (ESI) source in negative ion mode. RP C_{18} prep-HPLC was carried out on Spot Prep II (Armen-instrument, France) with a C_{18} column (watchers 120 ODS-BP, 20×210 mm, 10 μm). The resins, including silica gel (40-100 μm, Kanto Chemical Co., Japan), RP-C18 silica gel (70-230 mesh, Merck Ltd., Germany), and Sephadex LH-20 (25-100 μm, Merck Ltd., Germany), were used for column chromatography. *In vitro* assays were conducted in 96-well microplate using Multiskan GO ELISA microplate reader (Thermo Fisher Scientific, USA) to measure absorbance at various wavelengths.

Plant material

The leaves of *S. pseudocamellia* Maxim. were collected from Wando-gun, Jeollanam-do, Korea, in August, 2013 and identified by Lifetree Co., Korea. Samples were dried at room temperature in the shade.

Extraction and isolation

The dried leaves of *S. pseudocamellia* Maxim. (14 kg) were extracted with water at 100°C and filtered with filter paper (Advantec No. 13). The resultant water extract (2 kg, SPE) was evaporated under reduced pressure at 45°C using a Hei-VAP rotary evaporator (Heidolph, Germany) and then dissolved in water. The water soluble portion was partitioned consecutively with *n*-hexane (2L×2 times, SPEH), dichloromethane (2L×2 times, SPED), and ethyl acetate (2L×6 times, SPEE) as shown in Fig. 1. SPEE (130 g) demonstrated significantly higher whitening activity compared to other fractions,and was separated over silica gel column with a solvent system of $CHCl_3$-MeOH (7:1→0:1) as the eluent to give nine fractions (SPEE1–SPEE9). Fraction SPEE2 (8.3 g) was subjected to silica gel column chromatography ($CHCl_3$-MeOH, 50:1→0:1) to give five subfractions (SPEE2-1–SPEE2-5). Subfraction SPEE2-1 (2.1 g) was further chromatographed on a silica gel column ($CHCl_3$-MeOH, 50:1→0:1) and purified by RP C_{18} prep-HPLC (20% ACN) with a C_{18} column to yield compounds 1 (18 mg) and 2 (60 mg). Fraction SPEE3 (6.7 g) was chromatographed on a silica gel column ($CHCl_3$-MeOH, 10:1→0:1) to give five subfractions (SPEE3-1–SPEE3-5). Subfraction SPEE3-2 (1.2 g) was purified by sephadex LH-20 column chromatography (60→100% MeOH) to yield compound 3 (870 mg). Subfraction SPEE3-5 (0.8 g) was subjected to RP-18 column chromatography (20→100% MeOH) and purified by RP C_{18} prep-HPLC (50→100% MeOH) with a C_{18} column to yield compound 5 (300 mg). Fraction SPEE6 (11.0 g) was separated over a silica gel column with a solvent system of $CHCl_3$-MeOH (7:1) as the eluent to give four subfractions (SPEE6-1–SPEE6-4). Subfraction SPEE6-1 (0.5 g) was purified by preparative HPLC, using a gradient solvent system that varied from 10% to 100% MeOH, to yield compound 4 (45 mg). Fraction SPEE7 (4.9 g) was separated over a silica gel column with a solvent system of $CHCl_3$-MeOH (7:1) as the eluent to give four fractions (SPEE7-1–SPEE7-4). Fraction SPEE7-4 (2.2 g) was separated over RP-18 column with a solvent system of 20% MeOH as the eluent to give five subfractions (SPEE7-4-1–SPEE7-4-5). Each of the subfractions; SPEE7-4-2 (0.2 g), SPEE7-4-4 (0.4 g), and SPEE 7-4-5 (0.2 g) was purified by RP-C_{18} prepHPLC (40% ACN) with a C_{18} column to yield compounds 6 (6 mg), 7 (6 mg), 8 (17 mg), and 9 (23 mg). Fraction SPEE8 (18.0 g) was subjected to an MPLC (0→100%MeOH) solvent system to yield two fractions (SPEE8-1 and SPEE8-2). Fraction SPEE8-2 (2.2 g) was purified with silica gel chromatography ($CHCl_3$-MeOH, 1:0→ 0:1) to yield compound 10 (18 mg).

3-Hydroxybenzoic acid (1)

White amorphous powder; mp200-203°C; ESI-MS *m/z* 137 $[M-H]^-$, ^{1}H NMR (700 MHz, CD_3OD) δ 7.50 (1H, dd, *J*=1.8, 7.1 Hz, H-6), 7.40 (1H, d, *J*=2.8 Hz, H-2), 7.30 (1H, d, *J*=7.8 Hz, H-5), 7.00 (1H, dd, *J*=1.9, 7.1 Hz, H-4); ^{13}C NMR (175 MHz, CD_3OD) δ 170.1 (C-7), 158.9 (C-3), 133.4 (C-1), 130.6 (C-5), 122.0 (C-6), 121.2 (C-4), 117.4 (C-2).

4-Hydroxybenzoic acid (2)

White amorphous powder; mp213-215°C; ESI-MS *m/z* 137 $[M-H]^-$; ^{1}H NMR (700 MHz, CD_3OD) δ 7.78 (2H, d, *J*=8.2 Hz, H-2, 6), 7.44 (2H, d, *J*=8.2 Hz, H-3, 5); ^{13}C NMR (175 MHz, CD_3OD) δ 170.2 (C-7), 163.5 (C-4), 133.2 (C-2, 6), 122.9 (C-1), 116.2 (C-3, 5).

Protocatechuic acid (3)

White amorphous powder; mp200-202°C; ESI-MS *m/z* 153 $[M-H]^-$; ^{1}H NMR (700 MHz, CD_3OD) δ 7.47 (1H, s, H-2), 7.44 (1H, dd, *J*=2.0, 8.1 Hz, H-6), 6.82 (1H, d, *J*=8.1 Hz, H-5); ^{13}C NMR (175 MHz, CD_3OD) δ 170.4 (C-7), 151.7 (C-4), 146.2 (C-3), 124.0 (C-6), 123.2 (C-1), 117.9 (C-5), 115.9 (C-2).

Gallic acid (4)

Pale brown powder; mp250-251 °C; ESI-MS *m/z* 169 $[M-H]^-$; ^{1}H NMR (700 MHz, CD_3OD) δ 7.90 (2H, s, H-2, 6);^{13}C NMR (175 MHz, CD_3OD) δ 170.6 (C-7), 146.5 (C-3, 5), 139.7 (C-4), 122.1 (C-1), 110.5 (C-2, 6).

Quercetin (5)

Yellow powder; mp316-317°C; ESI-MS *m/z* 301 $[M-H]^-$; ^{1}H NMR (700 MHz, CD_3OD) δ 7.75 (1H, d, *J*=1.4 Hz, H-2′), 7.60 (1H, dd, *J*=1.4, 8.4 Hz, H-6′), 6.90 (1H, d, *J*=8.4 Hz, H-5′), 6.40 (1H, brs, H-8), 6.10 (1H, d, *J*=1.4 Hz, H-6); ^{13}C NMR (175 MHz, CD_3OD) δ 175.9 (C-4), 164.0 (C-7), 161.1 (C-5), 161.1 (C-5), 156.0 (C-9), 147.3 (C-4′), 146.5 (C-2), 144.8 (C-3′), 135.8 (C-3), 122.7 (C-1′), 120.2 (C-6′), 114.8 (C-5′), 114.5 (C-2′), 103.1 (C-10), 97.8 (C-6), 92.9 (C-8).

Quercetin-3-*O*-(6''-*O*-galloyl)-*β*-glucopyranoside (6)

Pale brown amorphous powder; mp202-205°C; ESI-MS *m/z* 616 $[M-H]^-$; ^{1}H NMR (700MHz, CD_3OD) δ 7.81 (1H, d, *J*=2.2 Hz, H-2'), 7.59 (1H, dd, *J*= 2.2, 8.5 Hz, H-6'), 6.91 (2H, s, H-6‴, 2‴), 6.84 (1H, d, *J*=8.5 Hz, H-5'), 6.40 (1H, d, *J*=2.0 Hz, H-8), 6.21 (1H, d, *J*=2.0 Hz, H-6), 5.14 (1H, d, *J*=7.8 Hz, H-1″), 4.30-3.60 (6H, m, H-6″, 5″, 4″, 3″, 2″); ^{13}C NMR (175 MHz, CD_3OD) δ 179.6 (C-4), 168.1 (C-7‴), 166.2 (C-7), 163.0 (C-5), 159.1 (C-2), 158.5 (C-9), 150.1 (C-4′), 146.5 (C-3‴, 5‴), 145.9 (C-3′), 139.9 (C-4‴), 135.8 (C-3), 123.2 (C-1′), 122.9 (C-6′), 121.2 (C-1‴), 117.9 (C-5′), 116.2 (C-2′), 110.2 (C-2‴, 6‴), 105.7 (C-1″), 100.1 (C-6), 95.0 (C-8), 79.6 (C-3″), 75.1 (C-5″), 74.6 (C-2″), 70.2 (C-4″), 63.9 (C-6″).

Trifolin (7)

Yellowish powder; mp235-237°C; ESI-MS *m/z* 447[M-H]$^-$; ^{1}H NMR (700 MHz, CD_3OD) δ 8.10 (2H, d, *J*=8.8 Hz, H-2', 6'), 6.92 (1H, d, *J*=8.8 Hz, H-3', 5'), 6.41 (1H, d, *J*=2.1 Hz, H-8), 6.20 (1H, d, *J*=2.1 Hz, H-6), 5.11 (1H, d, *J*=7.8 Hz, H-1"), 3.70-3.80 (6H, m, H-2", 3", 4", 5", 6"); ^{13}C NMR (175 MHz CD_3OD) δ 179.8 (C-4), 167.0 (C-7), 163.2 (C-5), 161.8 (C-4'), 159.1 (C-9), 158.8 (C-2), 135.7 (C-)3, 132.5 (C-2', 6'), 122.9 (C-1'), 116.3 (C-3', 5'), 105.6 (C-10), 105.2 (C-1"), 100.3 (C-6), 95.1 (C-8), 77.3 (C-5"), 75.2 (C-3"), 73.2 (C-2"), 70.2 (C-4"), 62.1 (C-6").

Quercitrin (8)

Yellowish powder; mp170-172°C; ESI-MS *m/z* 447[M-H]$^-$; ^{1}H NMR (700 MHz, CD_3OD) δ 7.36 (1H, d, *J*=1.6 Hz, H-2'), 7.32 (1H, dd, *J*=1.6, 8.2 Hz, H-6'), 6.94 (1H, d, *J*=8.2 Hz, H-5'), 6.39 (1H, s, H-8), 6.22 (1H, s, H-6), 5.37 (1H, s, H-1"), 4.24-3.27 (4H, m, H-2", 3", 4", 5"), 0.90 (3H, d, *J*=6.2 Hz, H-6"); ^{13}C NMR (175 MHz, CD_3OD) : δ 179.8 (C-4), 166.1 (C-7), 163.4 (C-5), 159.5 (C-2), 158.7 (C-9), 150.0 (C-4'), 146.6 (C-3'), 136.4 (C-3), 123.1 (C-6'), 123.0 (C-1'), 117.1 (C-5'), 116.5 (C-2'), 106.0 (C-10), 103.7 (C-1"), 100.0 (C-6), 94.9 (C-8), 73.4 (C-2"), 72.3 (C-3"), 72.2 (C-4"), 72.1 (C-5"), 17.8 (C-6").

Quercetin-3-*O*-(2''-*O*-galloyl)-α-rhamnopyranoside (9)

Yellow amorphous powder; mp198-200°C; ESI-MS *m/z* 599 $[M-H]^-$; ^{1}H NMR (700 MHz, CD_3OD) δ 7.26 (1H, brs, H-2'), 7.25 (1H, dd, *J*=1.8, 8.3 Hz, H-6'), 6.97 (2H, s, H-6‴, 2‴), 6.84 (1H, d, *J*=8.3 Hz, H-5'), 6.30 (1H, brs, H-8), 6.10 (1H, d, *J*=1.3 Hz, H-6), 5.53 (1H, d, *J*=1.2 Hz, H-1″), 3.90-3.20 (4H, m, H-5″, 4″, 3″, 2″), 0.93 (3H, d, *J*=5.4 Hz, H-6″);^{13}C NMR (175 MHz, CD_3OD) δ 179.5 (C-4), 167.6 (C-7‴), 166.0 (C-7), 163.4 (C-5), 159.4 (C-2), 158.7 (C-9), 150.0 (C-4′), 146.6 (C-3‴), 146.6 (C-5‴), 140.1 (C-4‴), 135.8 (C-3), 123.0 (C-1′), 123.0 (C-6′), 121.4 (C-1‴), 117.0 (C-2′), 116.6 (C-5′), 110.5 (C-2‴, 6‴), 106.0 (C-10), 100.7 (C-1‴), 100.0 (C-6), 94.9 (C-8), 73.9 (C-4″), 93.6 (C-2″), 72.3 (C-5″), 70.9 (C-3″), 18.0 (C-6″).

Rutin (10)

Yellow amorphous powder; mp188-192°C; ESI-MS *m/z* 609 $[M-H]^-$; ^{1}H NMR (700 MHz, DMSO-d_6) δ 7.55 (1H, d, *J*=2.0 Hz, H-2'), 7.52 (1H, dd, *J*=2.0, 8.1 Hz, H-6'), 6.84 (1H, d, *J*=8.1 Hz, H-5'), 6.38 (1H, d, *J*=2.8 Hz, H-6), 6.19 (1H, d, *J*=2.8 Hz, H-8), 5.33 (1H, d, *J*=7.8 Hz, H-1″), 5.10 (1H, d, *J*=1.2 Hz, H-1‴), 3.71-3.04 (10H, m, H-2″, 3″, 4″, 5″, 6″, 2‴, 3‴, 4‴, 5‴), 0.99 (1H, d, *J*=6.0 Hz, H-6‴). ^{13}C NMR (175 MHz, DMSO-d_6) δ 177.8 (C-4), 164.5 (C-7), 161.7 (C-5), 157.0 (C-9), 156.9 (C-2), 148.9 (C-4′), 145.2 (C-3′), 133.5 (C-3), 122.0 (C-6′), 121.7 (C-1′), 116.7 (C-5′), 115.7 (C-2′), 104.4 (C-10), 101.6 (C-1″), 101.2 (C-1‴), 99.1 (C-6), 94.0 (C-8), 76.9 (C-3″), 76.4 (C-5″), 74.5 (C-2″), 72.3 (C-4‴), 71.0 (C-2‴), 70.8 (C-3‴), 70.7 (C-4″), 68.7 (C-5‴), 67.4 (C-6″), 18.2 (C-6‴).

Reagents and chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Gibco Laboratories (Carlsbad, CA, USA). 1,1-diphenyl-2-picrylhydraxyl (DPPH), arbutin, L-tyrosine, mushroom tyrosinase, and L-3,4-dihydroxyphenylalanin (L-DOPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Japan). All solvents used in this study were of analytical and HPLC grade.

Cell culture

The B16F1 melanoma and Raw 264.7 macrophage cells, a murine cell line, were obtained from Korean Cell Line Bank (Seoul, Korea), and cultured in DMEM containing 10% heat-inactivated fetal bovine serum, 100 μg/mL streptomycin, and 100 units/mL penicillin in an incubator at 37°C with a humidified atmosphere of 95% air and 5% CO_2.

Measurement of melanin contents

The method as described by Hosoi (Hosoi *et al*., 1985) was slightly modified. Briefly, melanoma B16F1 cells were seeded at a density of 1×10^5 cells/well in culture plates and incubated at 37°C with 5% CO_2 for 24 h. The cells were then treated with SPE, its fractions, and compounds at different concentrations (SPE, 10, 50, 100 and 500 μg/mL; fractions, 10, 50 and 100 μg/mL; compounds, 1, 10 and 20 μg/mL) by the stimulant of 3-isobutyl-1-methylxanthine (IBMX, 100 μM) for 3 days. Then, the cells were washed with PBS, collected with trypsin and centrifuged at 3,000 rpm for 5 min. To obtain the cell pellets, the supernatant was discarded and 1N NaOH containing 1% DMSO was added. After incubating at 85°C for 1 hour, the absorbance was determined at 405 nm with the microplate reader.

Assay for tyrosinase inhibition activity

The method as described by Momtaz and Nerya was slightly modified (Momtaz *et al*., 2008). Two assays were performed to measure tyrosinase inhibition; one measured tyrosinase activity and the other measured L-DOPA oxidation. The SPE, its fractions, and compounds were diluted with 0.05M potassium phosphate buffer (pH 6.5) at different concentrations (SPE and fractions, 10, 50, 100, 200 and 500 μg/mL; compounds, 1, 10 and 20 μg/mL). Then 140 μL of test samples were added into a 96-well micro plate containing 20 μL of 2000 units/mL tyrosinase. After incubation at 37°C for 10 min, the absorbance was determined at 490 nm with the microplate reader (Background). Then 40 μL of 2.5 mM mushroom tyrosinase was added and mixed well. After incubation at 37°C for 15 min, the absorbance was performed at 490 nm again (Result). L-DOPA oxidation inhibition assay was similar to previous method using tyrosine, but SPE, its fractions, and compounds were diluted with 0.05 M potassium phosphate buffer at pH 7.0 and 6 mM L-DOPA was added. Arbutin was used as a positive control. The results were compared to the control.

Assay for DPPH radical scavenging activity

SPE, its fractions, and compounds were prepared at various concentrations (SPE and fractions, 1, 10, 20, 50, 100 and 200 μg/mL; compounds, 1, 10, 20 and 50 μg/mL) and added into a 96-well plate with 0.4 mM DPPH. The reaction mixtures were incubated at room temperature for 30 min in the dark, and the absorbance was measured at 517 nm on a microplate reader.

Cell viability

The cell viability of SPE, its fractions, and compounds were evaluated using Raw 264.7 murine macrophage cells. The cells (2×10^5 cells/well) were seeded in a 96-well plate, grown for 24 h, and then 100 μL of each sample (SPE and its fractions, 10, 20, 50, 100, 200 and 500 μg/mL; compounds, 1, 10 and 20 μg/mL) were treated at 37°C in the humidified atmosphere containing 5% CO_2. After 24 h, 10 μL of cell counting kit-8 (CCK-8) solution was directly added to wells and incubated for an additional 4 h. The absorbance was measured at a wavelength of 570 nm using the microplate reader.

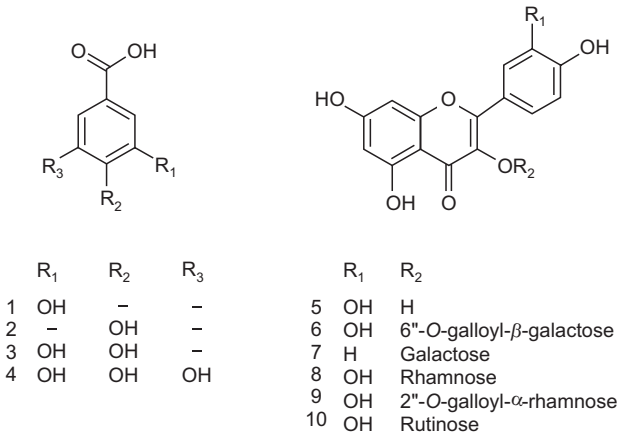
Statistical analysis

The experiment was performed in triplicate and results were expressed as mean ± SD. The statistical significance of the differences between controls and samples were performed by unpaired *t*-test, and *p*-values less than 0.05 were considered to be significant.

RESULTS

Isolated compounds

Ten compounds, including four simple phenolic derivatives, one flavonoid, and five flavone glycosides, were isolated from ethyl acetate fraction of SPE (Fig. 2). They were identified as 3-hydroxybenzoic acid (1), 4-hydroxybenzoic acid (2) (Pyo *et al*., 2002), protocatechuic acid (3) (Lee *et al*., 2012b), gallic acid (4) (Meshram *et al*., 2011), quercetin (5) (Güvenalp and Demirezer, 2005), quercetin-3-*O*-(6″-*O*-galloyl)-β-glucopyranoside(6) (Didem *et al*., 2009), trifolin (7) (Susanne *et al*., 2004), quercitrin (8) (Pyo *et al*., 2002), quercetin-3-*O*-(2″-*O*-galloyl)-α-rhamnopyranoside (9) (Lee *et al*., 2013), and rutin (10) (Pyo *et al*., 2002) by comparing their physical data such as ^{1}H NMR,^{13}CNMR, and MS spectra in the literature. Compounds 1-4 and 6-10 were isolated for the first time from this plant.



	R_1	R_2	R_3
1	OH	–	–
2	–	OH	–
3	OH	OH	–
4	OH	OH	OH

	R_1	R_2
5	OH	H
6	OH	6"-*O*-galloyl-β-galactose
7	H	Galactose
8	OH	Rhamnose
9	OH	2"-*O*-galloyl-α-rhamnose
10	OH	Rutinose

Fig. 2. Structures of compounds 1-10 isolated from ethyl acetate fraction of *S. pseudocamellia* leaves.

Table 1. Inhibitory effects of SPE and its fractions on melanogenesis in B16F1 melanoma cells

Sample conc.	Inhibition (%)[a,b]		
	10 μg/mL	50 μg/mL	100 μg/mL
IBMX control	0.0 ± 1.4		
SPE	2.2 ± 0.2	5.3 ± 0.1**	26.2 ± 1.2***
SPEH	1.2 ± 0.6	10.3 ± 4.5**	57.3 ± 1.3***
SPED	1.4 ± 3.4	-0.9 ± 3.1	20.8 ± 3.5***
SPEE	13.2 ± 4.1**	29.3 ± 1.6***	57.1 ± 3.7***
Arbutin	11.8 ± 3.6**	28.2 ± 0.9***	31.3 ± 0.3***

[a]Each value was expressed as the mean ± SD from the three independent experiments, [b]**p<0.01, ***p<0.001 compared with IBMX control values.

Inhibition of melanogenesis

The melanogenesis inhibitory activity of SPE and its constituents was examined in B16F1 melanoma cells, with arbutin used as a positive control. Treatment with 50-500 μg/mL of SPE reduced the melanin content in a dose-dependent manner without cytotoxicity. SPE inhibited melanogenesis in B16F1 cells by approximately 34.0% at 500 μg/mL, compared to control cells. Among the fractions, SPEH and SPEE showed significant anti-melanogenesis activity, but SPEH exhibited cytotoxicity at 100 μg/mL. Meanwhile, SPEE reduced the melanin content by approximately 30% and 57% at 50 and 100 μg/mL, respectively, whencompared to control without cytotoxicity (Table 1). Compounds 4 and 5 significantly decreased melanin significantly byapproximately16% and 23% when treated with 10 μg/mL.

Inhibition of tyrosinase activity and L-DOPA oxidation

The inhibitory activity of SPE, its fraction and constituents

Table 2. Inhibitory effects of SPE, its fractions and isolated compounds from SPEE on the activity of mushroom tyrosinase

Sample conc.	Inhibition (%)[a,b,c]		
	100 μg/mL	200 μg/mL	500 μg/mL
NC	0.0 ± 0.1		
SPE	-4.7 ± 0.6	-1.1 ± 2.8	18.3 ± 2.6**
SPEH	-3.9 ± 2.8	-2.5 ± 2.9	3.4 ± 2.8
SPED	19.4 ± 0.6***	24.1 ± 0.3***	33.2 ± 2.9***
SPEE	30.9 ± 1.0***	38.1 ± 2.1***	65.4 ± 1.8***
	Inhibition (%)[a,b,c]		
	1 μg/mL	10 μg/mL	20 μg/mL
1	18.2 ± 1.6***	17.0 ± 0.7***	17.1 ± 1.3***
2	17.2 ± 1.1***	21.2 ± 1.0***	21.8 ± 1.5***
3	12.4 ± 1.8**	14.5 ± 3.3**	18.2 ± 3.8**
4	11.6 ± 3.2*	10.1 ± 2.5*	8.0 ± 1.0**
5	9.4 ± 1.2***	23.3 ± 2.2***	24.5 ± 3.5***
6	9.2 ± 1.8**	13.6 ± 4.7**	17.4 ± 36***
7	8.8 ± 2.1**	15.3 ± 0.9***	15.5 ± 2.4***
8	14.5 ± 0.1**	14.6 ± 0.8**	17.0 ± 1.7***
10	4.3 ± 3.1*	7.0 ± 3.4*	8.4 ± 2.0**
	10 μg/mL	100 μg/mL	500 μg/mL
Arbutin	8.3 ± 1.8**	21.9 ± 1.4***	43.8 ± 1.3***

[a]The inhibitory activity was determined spectrophotometrically using tyrosinase as the substrate, [b]Each value was expressed as the mean ± SD from the three independent experiments, [c]**p<0.01, ***p<0.001 compared with negative control.

Table 3. The inhibitory effects of SPE, its fractions, and isolated compoundsfrom SPEE on the oxidation activity of L-DOPA

Sample conc.	Inhibition (%)[a,b,c]		
	100 μg/mL	200 μg/mL	500 μg/mL
NC	0.0 ± 0.1		
SPE	16.2 ± 2.1***	21.0 ± 0.3***	21.1 ± 1.4***
SPEH	3.4 ± 1.2	2.8 ± 2.0	5.3 ± 3.5
SPED	15.6 ± 2.5***	16.5 ± 1.1***	17.6 ± 0.3***
SPEE	16.7 ± 1.9*	24.6 ± 1.4***	24.5 ± 1.8***
	Inhibition (%)[a,b,c]		
	1 μg/mL	10 μg/mL	20 μg/mL
1	-0.1 ± 0.5	16.5 ± 0.5***	20.9 ± 1.1***
2	-3.5 ± 1.8	9.6 ± 3.0**	12.2 ± 0.9***
3	-11.2 ± 2.9	-9.3 ± 2.5	-6.1 ± 1.7
4	0.4 ± 0.7	2.6 ± 0.9	1.6 ± 4.3
5	-7.2 ± 1.2	9.4 ± 0.5*	19.3 ± 23.5***
6	-8.3 ± 1.4	13.6 ± 1.3***	21.8 ± 3.4***
7	-1.2 ± 0.7	12.9 ± 3.3**	9.0 ± 2.0**
8	-2.6 ± 1.0	10.9 ± 3.4**	11.7 ± 1.0***
10	0.7 ± 1.1	12.1 ± 3.7**	18.2 ± 1.7***
	10 μg/mL	100 μg/mL	500 μg/mL
Arbutin	1.8 ± 1.5**	12.1 ± 2.9***	16.7 ± 0.7***

[a]The inhibitory activity was determined spectrophotometrically using L-DOPA as the substrate, [b]Each value was expressed as the mean ± SD from the three independent experiments, [c]*p<0.05,**p<0.01, ***p<0.001 compared with negative control.

against melanogenesis were evaluated *in vitro* using the tyrosinase inhibition and L-DOPA oxidation assays. As shown in Table 2, SPE decreased tyrosinase activity by 18% at 500 μg/mL, but did not exhibit significant inhibitory activity between 10-200 μg/mL. SPED and SPEE inhibited tyrosinase activities in a dose-dependent manner, and SPEE showed a lower inhibitory activity than that of arbutin which was used as a positive control. Tyrosinase activity was decreased to 24%, 31%, 38%, and 65% of the negative control at 50, 100, 200 and 500 μg/mL of SPEE, respectively. Among the isolated compounds, compounds 1-3, 5-8 and 10 inhibited tyrosinase activity. However, most of the compounds showed weak inhibitory effects, except compounds 2 and 5, which reduced tyrosinase activity by 22% and 25% of the negative control, respectively, at 20 μg/mL. We examined the effects of the active fraction and constituents on melanin formation from L-DOPA by oxidation (Souichi *et al*., 2010). SPE, SPED and SPEE exhibited significant oxidation inhibitory activities of L-DOPA (Table 3). SPE and SPEE reduced the oxidation activity from L-DOPA by 21% and 25% of the negative control at 200μg/mL, respectively, and they showed the higher inhibitory activities then arbutin (17%) at the same concentration. The compounds, 1, 2, 5, 6, 8, and 10 only weakly inhibited L-DOPA oxidation activity.

Antioxidant activity

SPE, its fractions, and isolated compounds were tested for their antioxidant activity using the DPPH free radical scavenging assay. As shown in Table 4, SPE, SPED, and SPEE significantly scavenged free radicals. SPEE exhibited the strongest

Table 4. DPPH radical scavenging effects of SPE, its fractions, and isolated compounds from SPEE

Sample conc.	DPPH radical scavenging activities (%)[a,b]			
	10 μg/mL	20 μg/mL	50 μg/mL	100 μg/mL
NC	0.0 ± 0.7			
SPE	5.4 ± 3.0	13.1 ± 2.6*	31.5 ± 2.3***	72.1 ± 1.0***
SPEH	-1.9 ± 3.4	-2.1 ± 2.1	-0.8 ± 2.8	0.0 ± 1.1
SPED	-2.3 ± 4.1	4.8 ± 3.9*	13.2 ± 3.2***	26.3 ± 2.0***
SPEE	22.2 ± 3.7***	32.7 ± 0.7***	76.1 ± 0.3***	93.0 ± 0.3***
	DPPH radical scavenging activities (%)[a,b]			
	10 μg/mL	20 μg/mL	50 μg/mL	
1	-3.9 ± 2.8	-2.2 ± 2.4	0.8 ± 2.9	
2	-3.6 ± 3.1	7.6 ± 0.8	29.5 ± 0.6***	
3	34.7 ± 1.5***	71.5 ± 1.0***	89.9 ± 0.1***	
4	84.4 ± 0.3***	92.4 ± 0.3***	92.7 ± 0.1***	
5	39.8 ± 1.1***	83.3 ± 0.2***	93.5 ± 0.2***	
6	10.5 ± 2.4*	20.8 ± 2.0***	52.7 ± 1.5***	
7	-2.3 ± 1.2	-0.2 ± 1.1	2.2 ± 3.1	
8	16.5 ± 1.7**	41.8 ± 0.9***	90.8 ± 0.2***	
10	5.6 ± 3.0	16.0 ± 1.7**	41.3 ± 0.7***	
	10 μg/mL	20 μg/mL	50 μg/mL	
Vitamin C	32.1 ± 1.7***	72.6 ± 0.7***	94.1 ± 0.2***	

[a]Each value was expressed as the mean ± SD from the three independent experiments, [b]*p<0.05,**p<0.01, ***p<0.001 compared with negative control, 0.4 mM DPPH only.

scavenging activity, and decreased DPPH free radicals by approximately 76% and 93% compared to the negative control at 50 and 100 μg/mL, respectively. Compounds 2-7 and 10 inhibited DPPH free radical activity and compounds 4 and 5 exhibited similar antioxidant activity as vitamin C, which was used as a positive control at 50 μg/mL.

DISCUSSION

The abnormal formation and overproduction of melanin are potential causes of dermal problems such as freckles, age spot, melasma, vitiligo, and skin cancer. Tyrosinase is a key enzyme in melanogenesis. It is involved in the first two steps of hydroxylation of tyrosine to L-DOPA and the auto-oxidation of L-DOPA to DOPA quinine. Thus, various whitening agents that inhibit the catalysis of tyrosinase, including *Aster ageratoides* Turcz. var. *ageratoides*, *Physalisalkekengivar. francheti* (Mast.) Hort*, Saponaria officinalis*L. (Park *et al*., 2010), *Broussonetiakazinoki* var. *humilis*, *Broussonetia papyrifera*, and *Cornus officinalis* (Hwang and Lee, 2007) have been reported.

In this study, we demonstrated that the leaves of *S. pseudocamellia*, its fractions, and isolated compounds have whitening activities in murine B16 melanoma cells.SPE and SPEE were not toxic to Raw 264.7 macrophage cells, and significantly inhibited IBMX-induced melanin production and tyrosinase activity. However, they may inhibit different steps of the tyrosinase reaction mechanism because they had different effects on the substances of tyrosinase. SPE may inhibit DOPA oxidative reaction of *O*-diphenol to *O*-quinone, but SPEE may inhibit hydroxylation of monophenol to *O*-diphenol by tyrosinase (Hearing and Jimenez, 1987). Among the isolated compounds from SPEE, gallic acid and quercetin decreased the melanin contents without cytotoxicity. In particular, quercetin showed significant inhibitory activity against tyrosinase when both tyrosine and L-DOPA were used as substrates. It was reported that quercetin inhibited melanin formation in B16 melanoma cells with an IC_{50} value of 27 μM compared to arbutin (IC_{50} 198 μM), and also suppressed melanin content, which was observed by reduced intracellular tyrosinase activity and protein expression (Fujii and Saito, 2009; Arung *et al*., 2011). Gallic acid decreased melanin formation, but showed weak inhibitory activities on tyrosinase. This suggests that gallic acid possibility inhibits two other enzymes, tyrosinase-related protein 1 (TRP1) and TRP2, in melanin synthesis. According to previous research, gallic acid exhibit inhibitory activity against other enzymes, such as MITF, TRP1, and DCT (Su *et al*., 2013). Phenolic compounds and flavonoids contain aromatic and pyran rings,and have similar structures to tyrosine, which is oxidized by tyrosinase, and act as substrate analog inhibitors against melanogenesis (Sugumaran, 2002; Boissy and Mange, 2004). Moreover, many investigators reported that plants and its extract containing large amounts of polyphenol compounds have high antioxidant activity. They regulate melanogenesis by inhibiting the oxidative reaction by tyrosinase (Kim and Uyama, 2005). In summary, gallic acid and quercetin isolated from *S. pseudocamellia* decrease melanin production in B16 melanoma cells, and down-regulate the enzymatic activity of tyrosinase. These results suggested that *S. psuedocamella* Maxim. leaves show potent whitening effect and could be used as source of natural skin-whitening materials.

ACKNOWLEDGMENTS

This research was supported by Technology Commercialization Support Program (814003-03-01-SB010), Ministry of Agriculture, Food and Rural Affairs (MAFRA), Ministry of Oceans and Fisheries (MOF), Rural Development Administration (RDA) and Korea Forest Service (KFS).

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