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Bioactive triterpenoids from twigs of *Betula schmidtii*

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ABSTRACT

Investigation of the MeOH extract of *Betula schmidtii* twigs resulted in the isolation and identification of three new triterpenoids (**1–3**), along with ten known ones (**4–13**). The structures of new compounds (**1–3**) were elucidated by spectroscopic methods, including 1D, 2D NMR (^{1}H and ^{13}C NMR, COSY, HSQC, HMBC, and NOESY), HR-MS, and chemical methods. All the isolated compounds were evaluated for their cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines. Compound **11** exhibited potent cytotoxic activities against four cell lines, and compounds **5** and **13** significantly induced nerve growth factor secretion in a C6 rat glioma cell line. Their anti-inflammatory effects were also assessed by measuring nitric oxide production in lipopolysaccharide–activated BV-2 cells. Compounds **7** and **12** displayed potent inhibition of nitric oxide production, without significant cell toxicity.

1. Introduction

Betula schmidtii Regel is a deciduous tree belonging to the family Betulaceae and is widely distributed throughout Korea, Japan, and China. This plant source has been used as Korean traditional medicine for the treatment of stomach disorder [1]. This plant has the appearance of dark brown bark unlike white birches such as *B. platyphylla* var. *japonica* and *B. ermanii* that are plants of the same genus. Previous phytochemical research on *B. schmidtii* reported triterpenes, lignans, diarylheptanoids, and flavonoids [2]. According to a reported study, phytochemicals isolated from *Betula* species showed the biological activities such as immunomodulation, anti-inflammation, antioxidation, hepatoprotection, and anticancer effects. Mostly the anti-arthritic and anticancer effects of these phytochemicals have been focused [3]. The anti-inflammatory and anticancer effects of phytochemicals from *Betula* species have attracted our attention to evaluate the role of the compounds isolated from *B. schmidtii* for their anti-neuroinflammatory, neuroprotective, and anticancer effects.

As a part of our ongoing search for bioactive constituents from Korean medicinal sources, chemical investigations of *B. schmidtii* twigs were carried out, leading to the isolation and characterization of three new (**1–3**) and ten known triterpenoids. The structures of the new compounds (**1–3**) were elucidated by spectroscopic methods, including 1D, 2D NMR (^{1}H and ^{13}C NMR, COSY, HSQC, HMBC, and NOESY), HR-MS, and chemical methods. The isolated compounds (**1–13**) were evaluated for their cytotoxic, nerve growth factor (NGF)-potentiating, and anti-neuroinflammatory activities. Herein, we report the isolation and structural elucidation of the isolates and their biological activities.

2. Experimental

2.1. General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter. Infrared (IR) spectra were recorded on a JASCO FT/IR-4600 spectrometer, and Ultarviolet (UV) spectra were recorded with a Shimadzu UV-1601 UV–Visible spectrophotometer. NMR spectra were recorded on a Varian unity INOVA 500 NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C) with chemical shifts given in ppm (δ). HRESI mass spectra were obtained on a Waters SYNAPT G2 Q-TOF mass spectrometer, and the preparative high performance liquid chromatography (HPLC) was performed using a Gilson 306 pump with a Shodex refractive index detector and a Phenomenex Luna 10 μm column (250 × 10 mm). Silica gel 60 (Merck, Darmstadt, 70–230 mesh, and 230–400 mesh) and RP-C_{18} silica gel (Merck, 230–400 mesh) were used for column chromatography. Low performance liquid chromatography (LPLC) was performed over Merck LiChroprep Lobar-A Si gel 60 (240 × 10 mm) with an FMI QSY-0 pump (ISCO). Ion exchange resin (Dowex®

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50WX8 hydrogen form, SIGMA-ALDRICH) was used for alkali elimination. TLC was performed using Merck pre-coated silica gel F_{254} plates and RP-18 F_{254s} plates. Spots were detected on TLC under UV light or by heating after spraying the samples with anisaldehyde-sulfuric acid.

2.2. Plant material

The twigs of *B. schmidtii* were collected at Goesan, Korea in March 2013. The plant was identified by one of the authors (K.R. Lee). A voucher specimen (SKKU-NPL 1303) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea.

2.3. Extraction and isolation

Twigs of *B. schmidtii* (7 kg) were extracted three times using 80% aqueous MeOH for 1 day under reflux, and filtered. The resultant MeOH extract (410 g) was suspended in distilled water (2.4 L) and then successively partitioned with *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH, yielding 15, 18, 19 and 116 g, respectively. The $CHCl_3$-soluble phase (10 g) was separated over a silica gel column (230–400 mesh, 350 g) eluted with $CHCl_3$-MeOH [40:1 (1.0 L), 30:1 (1.0 L), 15:1 (0.6 L), 9:1 (1.0 L), 4:1 (1.0 L) and 1:1 (1.0 L)] to afford six fractions [Fr. A, 40:1, 1.0 L; Fr. B, 30:1, 1.0 L; Fr. C, 15:1, 0.6 L; Fr. D, 9:1, 1.0 L; Fr. E, 4:1, 1.0 L; Fr. F, 4:1, 1.0 L]. Fr. B (1.0 g) was chromatographed on an RP-C_{18} silica gel column (230–400 mesh, 80 g, 75% aqueous MeOH – 100% MeOH) to give 10 subfractions [Fr. B1–B10 (each 0.5 L)]. Fr. B5 (138 mg) was subjected to separation on a Lobar-A Si 60 (240 × 10 mm) column, eluting with Hexane – EtOAc (4:1), to give 2 subfractions (B51–B52). Fr. B52 (64 mg) was purified by semi-preparative reversed-phase HPLC (80% aqueous MeOH, flow rate of 2.0 ml/min) to acquire compounds **3** (5 mg, t_R = 22.3 min) and **4** (11 mg, t_R = 26.6 min). Fr. B6 (65 mg) was purified by semi-preparative normal-phase HPLC ($CHCl_3$ – MeOH, 40:1) at a flow rate of 2.0 ml/min to yield compounds **1** (7 mg, t_R = 8.5 min) and **9** (10 mg, t_R = 12.3 min). Compound **2** (4 mg, t_R = 10.1 min) was obtained from fr. B7 (37 mg) employing semi-preparative normal-phase HPLC (flow rate of 2.0 ml/min) with a solvent mixture of $CHCl_3$-MeOH (100:1). Fr. B9 (26 mg) was purified with semi-preparative reversed-phase HPLC at a flow rate of 2.0 ml/min, eluting with 85% aqueous CH_3CN to yield compound **13** (3 mg, t_R = 37.0 min). Fr. D (2 g) was fractionated into 9 subfractions [Fr. D1–D9 (each 1.0 L)] using an RP-C_{18} silica gel open column (230–400 mesh, 120 g) eluting with 60% aqueous MeOH. Fr. D4 (40 mg) separated using semi-preparative reversed-phase HPLC (60% aqueous CH_3CN, flow rate of 2.0 ml/min) to yield compound **8** (4 mg, t_R = 36.8 min). Fr. D7 (87 mg) was purified with semi-preparative reversed-phase HPLC at a flow rate of 2.0 ml/min, eluting with 82% aqueous MeOH to afford compound **12** (4 mg, t_R = 35.2 min). Fr. D8 (202 mg) was separated by semi-preparative reversed-phase HPLC (85% aqueous MeOH) at a flow rate of 2.0 ml/min to obtain compound **10** (6 mg, t_R = 37.0 min). Compounds **7** (4 mg, t_R = 22.3 min) and **11** (11 mg, t_R = 48.8 min) were isolated upon purification of fr. D9 (87 mg) by semi-preparative reversed-phase HPLC (90% aqueous MeOH) at a flow rate of 2.0 ml/min. Fr. E (2.4 g) was fractionated into 6 subfractions [Fr. E1–E6 (each 1.0 L)] using an RP-C_{18} silica gel open column (230–400 mesh, 130 g) eluting with 60% aqueous MeOH. Compound **5** (3 mg, t_R = 22.7 min) was afforded from fr. E5 (28 mg) by semi-preparative HPLC (42% aqueous CH_3CN) at a flow rate of 2.0 ml/min. Another fraction, EtOAc-soluble phase (11 g) was separated over a silica gel column (230–400 mesh, 350 g) eluted with EtOAc-MeOH-H_2O [10:1:0.1 (1.3 L), 8:1:0.1 (1.5 L), 5:1:0.1 (1.5 L) and 1:1:0.1 (1.5 L)] to afford three fractions [Fr. G, 10:1:0.1, 1.3 L; Fr. H, 8:1:0.1, 1.5 L; Fr. I, 5:1:0.1, 1.5 L]. Fr. H (0.7 g) was chromatographed on an RP-C_{18} silica gel column (230–400 mesh, 80 g, 40% aqueous MeOH – 100% MeOH) to give 7 subfractions [Fr. H1 – H7 (each 0.5 L)]. Fr. H7 (26 mg) separated using semi-preparative reversed-phase HPLC (40% aqueous CH_3CN, flow rate of 2.0 ml/min) to yield compound **6** (8 mg, t_R = 20.8 min).

2.3.1. 2α-O-Benzoyl-3β,19α-dihydroxy-urs-12-en-28-oic acid (*1*)

White amorphous gum $[\alpha]_D^{25}$ + 3.3 (*c* 0.06, MeOH); IR (KBr) ν_{max} 3696, 3336, 2941, 2830, 1715, 1546, 1453 cm^{-1}; UV λ_{max} (MeOH) 274, 228, 210 (sh) nm; ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; HRESIMS (positive-ion mode) *m*/*z* 615.3658 $[M+Na]^+$ (calcd. for $C_{37}H_{52}O_6Na$, 615.3662).

2.3.2. 2α-O-Benzoyl-19α-hydroxy-3-oxo-urs-12-en-28-oic acid (*2*)

Colorless amorphous gum $[\alpha]_D^{25}$ + 34.0 (*c* 0.05, MeOH); IR (KBr) ν_{max} 3707, 3370, 2942, 2830, 2048, 1713, 1546, 1453 cm^{-1}; UV λ_{max} (MeOH) 273, 229, 210 (sh) nm; ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; HRESIMS (positive-ion mode) *m*/*z* 613.3499 $[M+Na]^+$ (calcd. for $C_{37}H_{50}O_6Na$, 613.3505).

2.3.3. Schmidic acid (*3*)

White amorphous gum $[\alpha]_D^{25}$ + 58.4 (*c* 0.06, MeOH); IR (KBr) ν_{max} 3706, 3397, 2945, 2868, 2832, 1708, 1656, 1459 cm^{-1}; UV λ_{max} (MeOH) 254, 209 nm; ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; HRESIMS (positive-ion mode) *m*/*z*: 495.3091 $[M+Na]^+$ (calcd. for $C_{29}H_{44}O_5Na$, 495.3086).

2.4. Alkaline hydrolysis of *1* and *2*

Compounds **1** and **2** (each 1.0 mg) were hydrolyzed with 0.1 N KOH (1 ml) at room temperature for 4 h. The reaction mixture was subsequently eluted using an ion exchange column (Dowex® 50WX8 hydrogen form, SIGMA-ALDRICH) in distilled water to remove KOH. A portion of the reaction product was partitioned between $CHCl_3$ – H_2O (each 1.0 ml). The $CHCl_3$-soluble phase was isolated through semi-preparative HPLC (85% aqueous MeOH) to give the aglycones **1a** and **2a**, which were identified as tormentic acid (**1a**) and 2α, 19α-dihydroxy-3-oxo-urs-12-en-28-oic acid (**2a**) by comparison of ^1H NMR and MS data.

2.5. Cytotoxicity assessment

The cytotoxicity of the compounds against four cultured human cancer cell lines was evaluated by the sulforhodamine B (SRB) assay [4,5]. Each cell line was inoculated over standard 96-well flat-bottom microplates and incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. The attached cells were incubated with the serially diluted samples. After continuous exposure to the compounds for 48 h, the culture medium was removed from each well and the cells were fixed with 10% cold trichloroacetic acid at 4 °C for 1 h. After washing with tap water, the cells were stained with 0.4% SRB dye and incubated for 30 min at room temperature. The cells were washed again and solubilized with 10 mM unbuffered Tris base solution (pH 10.5). The absorbance was measured spectrophotometrically at 520 nm with a microtiter plate reader. The cell lines (National Cancer Institute, Bethesda, MD, USA) used were A549 (non-small cell lung adenocarcinoma), SK-OV-3 (ovarian malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma). Etoposide (Sigma Chemical Co., ≥98%) was used as a positive control. The assays were performed at the Korea Research Institute of Chemical Technology.