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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.

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data of **1–3** in $CDCl_3$ (δ in ppm).[a]

Position	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1a	44.0	2.15, dd (11.0, 4.5)	46.0	2.39, dd (12.3, 6.2)	46.5	2.63, d (16.7)
1b		1.16, overlap		1.63, overlap		1.90, d (16.7)
2	74.3	5.23 td (11.0, 4.5)	72.4	5.88, dd (12.3, 6.2)	170.8	
3	81.2	3.41 d (11.0)	209.1			
4	40.1		49.1		86.1	
5	55.4	0.96, overlap	57.3	1.29, overlap	52.4	1.47, overlap
6a	18.6	1.62, overlap	19.4	1.59, overlap	21.4	1.48, overlap
6b		1.44, overlap				
7a	32.9	1.54, overlap	32.7	1.58, overlap	31.9	1.60, overlap
7b		1.36, overlap		1.40, overlap		1.40, overlap
8	40.3		40.3		39.9	
9	47.4	1.72, overlap	47.2	1.81, overlap	44.7	1.72, overlap
10	38.6		38.3		36.5	
11	24.0	2.01, m	24.1	2.11, m	23.3	2.02, overlap
12	129.2	5.34, brt (3.7)	128.7	5.37, brt (3.6)	128.5	5.36, brt (3.5)
13	138.2		138.5		138.4	
14	41.4		41.6		41.6	
15a	28.4	1.74, overlap	28.5	1.76, overlap	28.3	1.74, overlap
15b		1.07, overlap		1.08, overlap		1.10, overlap
16a	25.6	2.55, overlap	25.6	2.57, overlap	25.5	2.53, dd (13.4, 5.5)
16b		1.60, overlap		1.61, overlap		1.60, overlap
17	47.9		47.9		48.0	
18	53.1	2.54, s	53.2	2.56, s	53.2	2.56, s
19	73.3		73.3		73.3	
20	41.3	1.41, overlap	41.3	1.42, overlap	41.3	1.42, overlap
21a	26.2	1.70, overlap	26.2	1.72, overlap	26.1	1.68, overlap
21b		1.31, overlap		1.32, overlap		1.31, overlap
22a	37.7	1.80, m	37.6	1.83, overlap	37.5	1.80, m
22b		1.67, overlap		1.69, overlap		1.67, overlap
23	28.8	1.12, s	25.1	1.17, s	24.8	1.34, s
24	17.0	0.93, s	21.5	1.22, s	33.0	1.44, s
25	16.6	1.12, s	16.2	1.38, s	16.1	1.07, s
26	17.2	0.75, s	17.3	0.83, s	16.5	0.78, s
27	24.7	1.27, s	24.7	1.27, s	24.4	1.29, s
28	183.7		183.3		183.3	
29	16.3	0.95, d (6.7)	16.3	0.96, d (6.7)	16.3	0.95, d (6.7)
30	27.6	1.19, s	27.6	1.21, s	27.6	1.22, s
1′	130.6		130.1			
2′	129.8	8.04, dd (8.2, 1.1)	130.0	8.08, dd (8.4, 1.3)		
3′	128.6	7.45, brt (7.8)	128.5	7.44, dd (8.4, 7.6)		
4′	133.2	7.57, brt (7.8)	133.3	7.57, brt (7.6)		
5′	128.6	7.45, brt (7.8)	128.5	7.44, dd (8.4, 7.6)		
6′	129.8	8.04, dd (8.2, 1.1)	130.0	8.08, dd (8.4, 1.3)		
7′	167.2		166.1			

[a] Assignments were based on 2D NMR including HSQC and HMBC. Well-resolved couplings are expressed with coupling patterns and coupling constants in Hz in parentheses.

2.6. NGF and cell viability assays

The C6 glioma cells (the Korean Cell Line Bank, Seoul, Republic of Korea) were used to measure the induction of NGF release into the culture medium. The test cells were seeded onto 24-well plates at a density of 1×10^5 cells/well, and after 24 h, the cells were treated with serum-free DMEM containing different concentrations of compound for an additional 24 h. The medium was collected from the cultured plates, and NGF levels were measured using an ELISA kit. Cell viability was also measured using an MTT assay, in which the results were expressed as a percentage of the control group (untreated cells). 6-Shogaol was used as the positive control.

2.7. Measurement of nitric oxide production and cell viability

BV-2 cells were stimulated with 100 ng/mL LPS in the presence and absence of samples for 24 h. Control cultures received the carrier solvent (0.1% dimethyl sulfoxide). Nitrite in the culture media, a soluble oxidation product of NO, was measured by the Griess reaction. The supernatant (50 μL) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-1-napthylethylenediamine dihydrochloride in 5% phosphoric acid). The absorbance at 570 nm was measured after 10 min using a microplate reader. Sodium nitrite was used as a standard to calculate the NO_2^- concentration. N^G–monomethyl-L-arginine (NMMA, Sigma, St. Louis, MO, USA), a well-known NO synthase inhibitor, was tested as a positive control.

2.8. Statistical analysis

All results are presented as the mean ± standard error of the mean (S.E.M.). Significant differences between experimental groups were determined using one-way ANOVA followed by a Newman–Keuls *post hoc* test using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, U.S.A.). $p < 0.05$ was considered statistically significant.

3. Result and discussion

3.1. Isolation of compounds

MeOH extract of *B. schmidtii* twigs was subjected to liquid-liquid solvent-partitioning to yield *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH soluble fractions. Repeated column chromatographic

purification of the $CHCl_3$-soluble and EtOAc-soluble fractions afforded two new triterpenes (**1**,**2**), a new nor-triterpene (**3**), and ten known ones (**4–13**) (Fig. 1).

3.2. Structure elucidation of isolated compounds

Compound **1** was isolated as a white gum, and its molecular formula was determined to be $C_{37}H_{52}O_6$ based on the HRESIMS ion signal $[M+Na]^+$ observed at *m/z* 615.3658 (calcd. for $C_{37}H_{52}O_6Na$, *m/z* 615.3662). The IR absorption spectrum suggested the presence of ester (1715 cm^{-1}), and hydroxyl (3336 cm^{-1}) groups. The ^1H NMR spectrum of **1** showed resonances for six singlet methyl protons [δ_H 1.27, 1.19, 1.12 (×2), 0.93, and 0.75], a doublet methyl proton [δ_H 0.95 (3H, d, *J* = 6.7 Hz)], an olefinic proton [δ_H 5.34 (1H, brt, *J* = 3.5 Hz)], two oxygenated methine protons [δ_H 5.23 (1H, td, *J* = 11.0, 4.5 Hz) and 3.41 (1H, d, *J* = 11.0 Hz)], and aromatic ring protons [δ_H 8.04 (2H, dd, *J* = 8.2, 1.1 Hz), 7.57 (1H, brt, *J* = 7.8 Hz) and 7.45 (2H, brt, *J* = 7.8 Hz)]. The ^{13}C NMR spectrum of **1** displayed 37 resonances including two carbonyl carbons (δ_C 183.7 and 167.2), olefinic and aromatic carbons [δ_C 138.2, 133.2, 130.6, 129.8 (×2), 129.2, and 128.6 (×2)], two oxygenated methine carbons (δ_C 81.2 and 74.3), and an oxygenated quaternary carbon (δ_C 73.3). These ^1H and ^{13}C NMR data of **1** (Table 1) were similar to those of tormentic acid [6], except for the presence of benzoyl group. The benzoyl group was located at C-2 based on the HMBC cross-peak from H-2 [δ_H 5.23 (1H, td, *J* = 11.2, 4.5 Hz)] to C-7′ (δ_C 167.2) (Fig. 2). The planar structure of **1** was elucidated through 2D NMR analysis, including the COSY, HSQC, and HMBC spectra (Fig. 2). The relative configuration of **1** was determined to be identical to that of tormentic acid (**1a**) by the NOESY correlations (Fig. 2) and their ^1H, ^{13}C NMR data [6]. Alkaline hydrolysis of **1** afforded tormentic acid (**1a**), which was identified by comparison of the ^1H NMR data with the reported data [6]. Thus, compound **1** was assigned as 2α-*O*-benzoyl-3β,19α-dihydroxy-urs-12-en-28-oic acid.

Compound **2** was obtained as a colorless gum. The molecular formula of **2** was determined to be $C_{37}H_{50}O_6$ by positive mode HRESIMS data at *m/z* 613.3499 $[M+Na]^+$ (calcd. for $C_{37}H_{50}O_6Na$, 613.3505). ^1H and ^{13}C NMR data of **2** were quite similar to those of **1**, except for the presence of a carbonyl signal (δ_C 209.1) and the absence of an oxygenated carbon signal (δ_C 81.2). Moreover, the HMBC correlation from H-1, H-23, and H-24 to C-3 deduced the carbonyl carbon to be located at C-3 (Fig. 2). The relative stereochemistry was identical to those of **1** based on the NOESY correlations and coupling constants displayed in the ^1H NMR spectrum. Alkaline hydrolysis of **2** gave 2α, 19α-dihydroxy-3-oxo-urs-12-en-28-oic acid (**2a**), which was identified by comparing ^1H NMR spectrum with previously reported data [7]. Therefore, the structure of

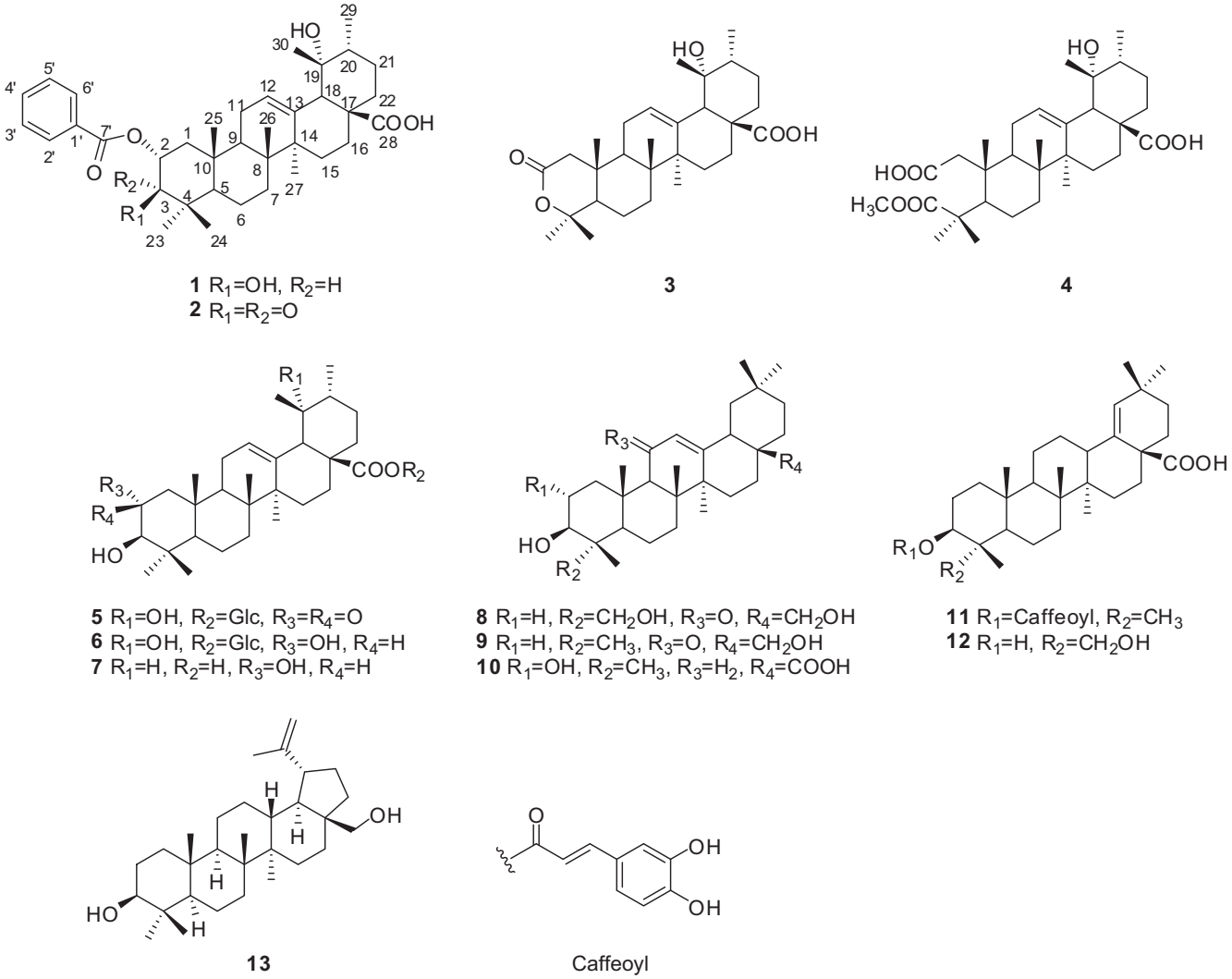

Fig. 1. Chemical structures of compounds **1–13**.

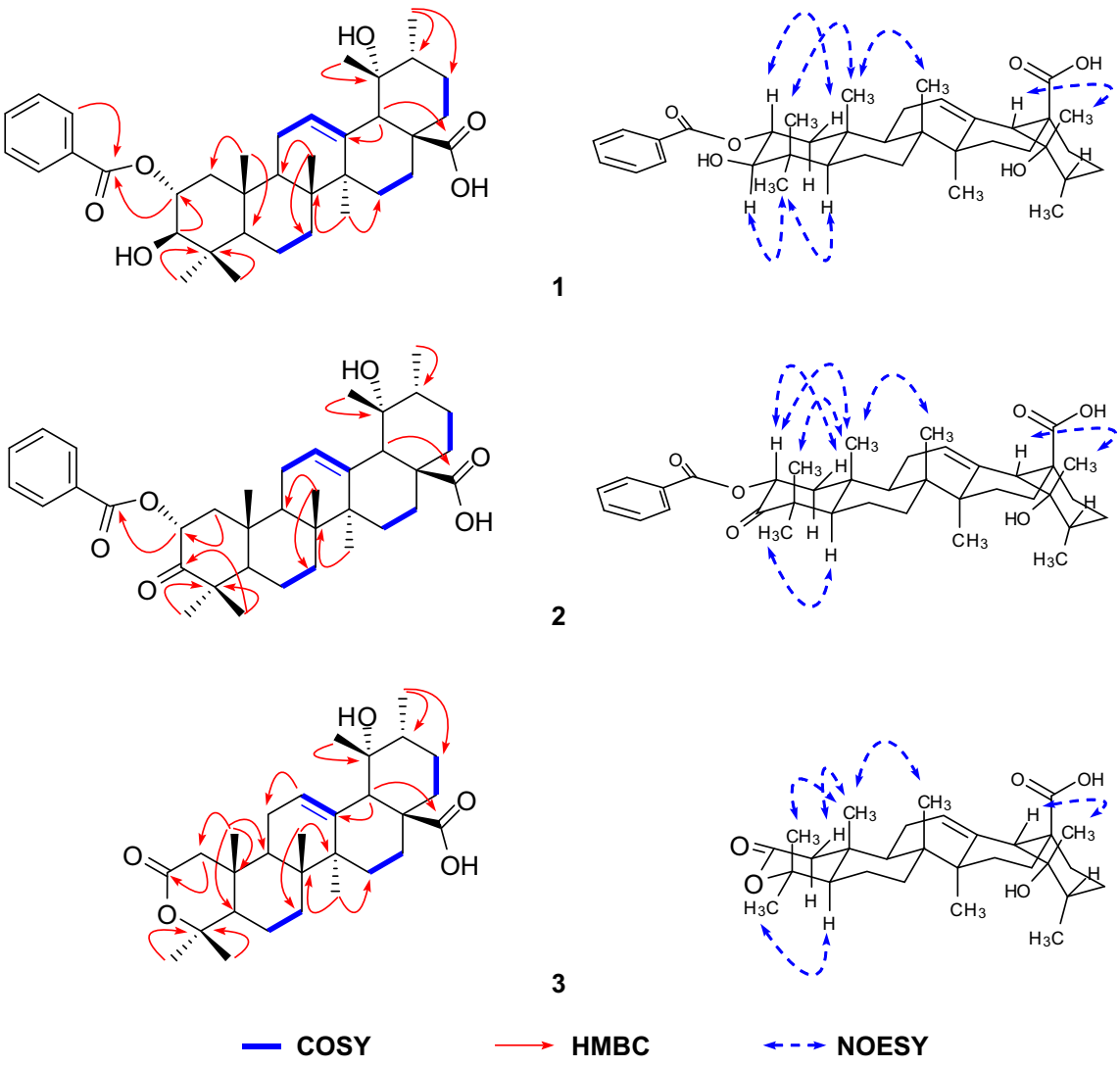

Fig. 2. Key ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of **1–3.**

2 was determined to be 2α-*O*-benzoyl-19α-hydroxy-3-oxo-urs-12-en-28-oic acid.

Compound **3**, a white gum, had a molecular formula of $C_{29}H_{44}O_5$ as established by the $[M+Na]^+$ peak at *m*/*z* 495.3091 (calcd. for $C_{29}H_{44}O_5Na$, 495.3086) in the positive-ion HRESIMS. The IR spectrum showed peaks at 3397, 1708, and 1656 cm^{-1} assigned hydroxyl, δ-lactone, and carboxylic acid groups, respectively [8]. The ^{1}H NMR spectrum exhibited characteristic resonances for six singlet methyl protons [δ_H 1.44, 1.34, 1.29, 1.22, 1.07, and 0.78], a doublet methyl proton [δ_H 0.95 (3H, d, J = 6.7 Hz)], and an olefinic proton [δ_H 5.36 (1H, brt, J = 3.5 Hz)]. The ^{13}C NMR spectrum of **3** displayed a total of 29 carbon signals including two carbonyl carbons at δ_C 183.3 and 170.8, two olefinic carbons at δ_C 138.4 and 128.4, and an oxygen-bearing carbon at δ_C 86.1. Analysis of the 1D and 2D NMR spectra indicated that **3** revealed similarities to those of cecropiacic acid 3-methyl ester (**4**), except for the A ring part (Table 1) [9]. The presence of lactone carbonyl group (δ_C 170.8) in **3** instead of a carboxylic acid (δ_C 177.8) and a carboxylic ester (δ_C 184.4) in **4** implied that carboxylic acid and carboxylic ester groups in **4** were condensed to lactone ring. In addition, the ^{13}C NMR values of A ring part in **3** were matched with those previously reported for diterpenoids, and methyl signals were also similar to those of the literatures (Table 1) [8,10,11]. The lactone ring in **3** was supported by the HMBC correlations from δ 2.63 (H-1a) and δ 1.90 (H-1b) to δ 170.8 (C-2), from δ 1.07 (H-25) to δ 46.5 (C-1), δ 52.4 (C-5), and δ 36.5 (C-10), and from δ 1.34 (H-23) and δ 1.44 (H-24) to δ 86.1 (C-4) (Fig. 2). The relative configuration of **3** was confirmed as being the same as those of **4** through the NOESY correlations (Fig. 2), and their ^{13}C NMR data closely similar to those of the reported ursolic acid derivatives (Table 1) [9]. Thus, the structure of **3** was established as shown in Fig. 1, and named as schmidic acid.

The ten known triterpenoids (**4–13**) were identified by comparison of their spectroscopic data with those in the literatures as cecropiacic acid 3-methyl ester (**4**) [9], 2-oxo-pomolic acid β-D-glucopyranosyl ester (**5**) [12], ursolazuroside 1 (**6**) [13], corosolic acid (**7**) [14], (3β)-3,23,28-trihydroxyolean-12-en-11-one (**8**) [15], 11-oxo-erythrodiol (**9**) [16], maslinic acid (**10**) [7], morolic acid 3-*O*-caffeate (**11**) [17], ambradiolic acid (**12**) [18], and betulin (**13**) [19].

3.3. Antiproliferative activities of the compounds

The cytotoxic activities of the isolated compounds (**1–13**) were evaluated by determining their inhibitory effects on the growth of human non-small cell lung A549, ovarian SK-OV-3, skin SK-MEL-2, and colorectal HCT-15 cancer cell lines by using SRB bioassay

Table 2
Cytotoxicity of selected compounds against four cultured human cancer cell lines in SRB bioassay.

Compound	IC_{50} (μM)[a]			
	A549	SK-OV-3	SK-MEL-2	HCT15
1	7.26	11.75	11.03	3.44
2	5.12	10.71	10.24	3.63
7	23.51	>30.0	>30.0	13.04
8	21.48	>30.0	>30.0	18.23
9	15.40	>30.0	27.56	16.15
10	4.51	20.27	21.04	3.31
11	1.83	5.72	4.08	1.53
12	2.16	24.65	11.06	1.94
13	14.16	11.94	11.27	12.49
Etoposide[b]	0.94	1.65	1.73	0.91

[a] IC_{50} value of compounds against each tumor cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*; [b] Etoposide was used as a positive control.

Table 3
Effects of compounds **3**, **5–8**, and **13** on NGF secretion in C6 glioma cells.

Compound	NGF secretion(%)[a]	Cell viability(%)[b]
1	80.81 ± 8.00	93.75 ± 3.54
3	115.59 ± 1.37	111.14 ± 12.94
5	141.19 ± 5.67	102.40 ± 3.48
6	128.04 ± 3.34	103.92 ± 8.28
7	113.66 ± 4.37	117.85 ± 7.26
8	120.52 ± 3.96	103.31 ± 3.35
13	136.20 ± 4.74	105.06 ± 0.87
6-Shogaol[c]	139.11 ± 8.48	112.13 ± 2.10

[a] C6 cells were treated with 20 μM of compounds. After 24 h, the content of NGF secretion in C6-conditioned media was measured by ELISA. The level of secreted NGF cells is expressed as percentage of the untreated control. The data shown represent the means ± SD of three independent experiments performed in triplicate.
[b] Cell viability after treatment with 20 μM of each compound was determined by MTT assay and is expressed in percentage (%). The results are averages of three independent experiments, and the data are expressed as mean ± SD.
[c] 6-Shogaol was used as positive control.

(Table 2). Among the compounds tested, compound **11** exhibited the most potent cytotoxic activities. The 50% inhibitory concentrations of cell growth (IC_{50}) of compound **11** were 1.83, 5.72, 4.08 and 1.53 μM against A549, SK-OV3, SK-MEL-2 and HCT15 cells, respectively. Compound **11** which contained caffeoyl group at C-3 showed better cytotoxic activities than oleanane-type triterpenoid compounds **8–10** and **12**. In addition, the IC_{50} values of compound **1** were 7.26, 11.75, 77.03 and 3.44 μM, and those of the compound **2** were 5.12, 10.71, 10.24 and 3.63 μM against those cancer cells, respectively. Although the chemical structures of the ursane-type triterpenoids (**1–7**) are relatively similar, compounds **1** and **2** exhibited strong cytotoxicities, suggesting that the presence of a benzoyl group at C-2 could play a key role in their inhibitory effects on cancer cell lines.

3.4. Neuroprotective effects of the compounds

Induction of NGF is a very important benefit not only for the survival and differentiation of sensory neuronal cells, but also for the neuroprotection and the neuronal repair against various neuroinflammatory factors and neurodegenerative conditions [20,21]. Here, we tested the neuroprotective effect of isolated compounds by NGF production assay. The purified compounds (**1–13**) were evaluated for their neuroprotective effects on NGF secretion in C6 glioma cells (Table 3). Compounds **5** and **13** significantly induced NGF secretion with stimulation levels of 141.19 ± 5.67% and 136.20 ± 4.74%, respectively (positive control 6-shogaol was 139.11 ± 8.48%), without any cell toxicity at concentration of 20 μM. And the other tested compounds revealed mild activity (113.66–128.04%).

Table 4
Inhibitory effect of selected compounds on NO production in LPS-activated BV-2 cells.

Compound	IC_{50} (μM)[a]	Cell viability (%)[b]
1	4.92	28.22 ± 0.88
2	9.68	68.78 ± 4.79
7	12.93	93.65 ± 15.16
9	16.58	46.61 ± 3.57
10	4.46	17.29 ± 5.12
11	8.62	48.30 ± 1.12
12	15.12	84.94 ± 2.13
l-NMMA[c]	18.48	109.65 ± 4.59

[a] IC_{50} value of each compound was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells.
[b] Cell viability after treatment with 20 μM of each compound was determined by MTT assay and is expressed in percentage (%). The results are averages of three independent experiments, and the data are expressed as mean ± SD.
[c] L-NMMA as positive control.

3.5. Anti-inflammatory activities of the compounds

As nitric oxide act as pro-inflammatory mediator for the induction and pathogenesis of neuroinflammation [22,23], we also screened the role of compounds isolated from *B. schmidtii* against LPS induced NO production. Previous reports have suggested that phytochemicals isolated from *Betula* species possess the anti-inflammatory and anti-arthritic effects [3]. Therefore, we hypothesized that the compounds isolated from *B. schmidtii* might also possess strong anti-inflammatory activity and that can be characterized through the NO production inhibition assay. To prove this notion, we investigated the effect of the isolated compounds (**1–13**) on anti-inflammatory activity via inhibiting nitric oxide (NO) production in LPS–activated BV-2 microglial cells (Table 4). Compounds **7** and **12** displayed potent inhibition of NO production, with IC_{50} values of 12.93 and 15.12 μM, when compared with the positive control N^G-monomethyl-L-arginine (L-NMMA) (18.48 μM), without significant cell toxicity. Although compounds **1**, **2**, **9**, **10**, and **11** also exhibited strong inhibitory activity (4.92, 6.98, 16.58, 4.46, and 8.62 μM), they reduced the cell viability of BV-2 cells with 28.22 ± 0.88%, 68.78 ± 4.79%, 46.61 ± 3.57%, 17.29 ± 5.12%, and 48.30 ± 1.12% at the tested concentration (20 μM) as well. This result implies that the observed inhibitory activity of **1**, **2**, **9**, **10**, and **11** on NO production might be influenced by their cytotoxicity.

4. Conclusions

In the present study, we isolated three new triterpenoids (**1–3**), together with ten known ones (**4–13**) from the twigs of *B. schmidtii*, and all the isolates (**1–13**) were evaluated for their cytotoxicity, neuroprotective, and anti-inflammatory activities. As a results, compound **11** showed significant cytotoxic activities against four cell lines (A549, SK-OV-3, SK-MEL-2, and HCT15), and compounds **5** and **13** exhibited NGF-potentiating activity. Although compounds **1**, **2**, **9**, **10**, and **11** showed strong anti-inflammatory activity, the cell viability was comparably low. Compounds **7** and **12** displayed potent inhibitory activity without significant cell toxicity. The activity of **1**, **2**, **9**, **10**, and **11** on NO production was to be influenced by their cytotoxicity. Compound **1** was weak stimulant of NGF secretion in C6 glioma cells (80.81 ± 8.00%) (Table 3), but tormentic acid (**1a**) exhibited potent NGF secretion in C6 glioma cells (167.37 ± 9.57%) [24], suggesting that the substitution

of benzoyl group decreased NGF secretion in C6 glioma cells. Therefore, this study indicates that these compounds would be potential candidates for treating diseases associated with various cancers, neurodegeneration, and inflammation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bioorg.2018.02.006.

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