Investigation of chemical constituents from *Spiraea prunifolia* var. *simpliciflora* and their biological activities

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**A R T I C L E   I N F O**

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Neuroprotection

**A B S T R A C T**

Quantum mechanics (QM)-based calculations for elucidating full structures of natural compounds are growing in importance and reliability. Two new phenolic glycosides (1 and 2) and 11 known compounds were isolated from the twigs of *Spiraea prunifolia* var. *simpliciflora*. The chemical structures of the new compounds (1 and 2) were initially established through different NMR techniques (\(^1\)H and \(^13\)C NMR, COSY, HSQC, and HMBC), HRMS data analysis, and chemical hydrolysis. These structure assignments were further verified by QM-based NMR chemical shift calculations. All of the purified compounds (1–13) were evaluated for their cytotoxicity against four human cancer cell lines (A549, SK-OV-3, SK-MEL-2, and BT549). Those phytochemicals were also evaluated for both anti-inflammatory activity through the measurement of nitric oxide (NO) production levels in lipopolysaccharide (LPS)-stimulated murine microglia BV-2 cell lines and neuroprotective effects via induction of nerve growth factor (NGF) in C6 glioma cells.

1. Introduction

*Spiraea prunifolia* var. *simpliciflora* Nakai (Rosaceae), commonly called “bridal wreath,” is a deciduous shrub widely distributed in Korea. The roots of this plant have been used as Korean traditional medicine to treat malaria, fever, and emetic conditions, and its young leaves have been consumed as a salad (Oh et al., 2001). Previous investigation reported that the extracts of *S. prunifolia* var. *simpliciflora* show anti-inflammatory and antipyretic activities (So et al., 1999), and possess terpenoids, flavonoids, and phenolic compounds (Oh et al., 2001; Park et al., 2013; Yean et al., 2014; Youn and Chung, 1987). On the contrary to numerous studies on the roots, there have only been a few investigations of bioactive phytochemicals of the twigs.

Quantum mechanics (QM)-based predictions, for the identification and/or verification of full structures of natural product-originating compounds, are growing in significance and reliability (Lodewyk et al., 2011). These approaches are of particular use in verifying structural assignments, considering erroneous assignment due to a high degree of molecular complexity, human errors, and NMR correlation ambiguities (Grimblat et al., 2015). Despite the utility of synthesis and X-ray crystallography in structural validation and corrections (Nicolaou et al., 2000), such methods can be impractical in terms of costs and unfavorable molecular properties. Alternatively, QM-based calculations of NMR properties, using gauge-including atomic orbitals (GIAO) NMR chemical shift calculations, have been spotlighted as effective protocols for the verification of chemical structure assignments made based upon conventional methods (Grimblat et al., 2015; Kim et al., 2017a; Lodewyk et al., 2011).

In a continuing search for bioactive scaffolds from Korean medicinal plants, we recently reported cytotoxic phenolic compounds from *S. prunifolia* var. *simpliciflora* (Jang et al., 2015). In this study, we isolated and identified two new phenolic compounds, trans- and cis-pruspirides (1 and 2), along with 11 known compounds from this plant (Fig. 1). The chemical structures of purified compounds were determined by 1D and 2D NMR (\(^1\)H and \(^13\)C NMR, COSY, HSQC, and HMBC), HRMS, and LC/MS data analysis. Notably, the initial structural assignment of compound 1 was verified utilizing GIAO-based NMR chemical shift calculations. These phytochemicals (1–13) were also assessed for their cytotoxic, anti-inflammatory, and neuroprotective activities employing relevant bioassay approaches.
2. Results and discussion

Compound 1 was purified as a colorless gum and its molecular formula was confirmed as C_{20}H_{24}O_{10} based on the protonated HRFABMS molecular ion [M + H]^+ at m/z 425.1450 (calcd for C_{20}H_{25}O_{10}, 425.1448). The ^1H NMR spectrum of 1 exhibited the presence of a trans-coumaroyl group ([δ_H 7.76 (1H, d, J = 15.9 Hz), 7.51 (2H, d, J = 8.7 Hz), 6.84 (2H, d, J = 8.7 Hz), and 6.40 (1H, d, J = 15.9 Hz)], a glucopyranosyl moiety ([δ_H 5.50 (1H, d, J = 8.1 Hz), 4.51 (1H, dd, J = 12.1, 2.1 Hz), 4.31 (1H, dd, J = 12.1, 5.7 Hz), 3.69 (1H, ddd, J = 9.7, 5.7, 2.1 Hz), 3.50 (1H, t, J = 9.0 Hz), 3.46 (1H, dd, J = 9.1, 8.1 Hz), and 3.43 (1H, dd, J = 9.7, 9.0 Hz)], two olefinic protons ([δ_H 6.27 (1H, d, J = 1.5 Hz) and 5.72 (1H, dd, J = 1.5, 1.1 Hz)], and two methylenes ([δ_H 3.69 (2H, t, J = 6.6 Hz), 2.56 (2H, td, J = 6.6, 1.1 Hz)]) in 1-caffeoyl-6-tuliposide A. The ^13C NMR spectrum of 1 showed 20 resonances characteristic for an ester-like carbonyl (δ_C 168.4), an 1,4-disubstituted aromatic ring (δ_C 148.1, 138.8, 127.7, and 114.6), a glucopyranosyl group (δ_C 95.9, 78.2, 76.5, 74.2, 71.3, and 64.9), an oxygenated carbon (δ_C 61.7), and a methylene carbon (δ_C 36.4). These spectroscopic data (Table 1) were similar to those of 1-cafeoyl-6-tuliposide A (Park et al., 2013), except for the presence of resonances for an 1,4-disubstituted aromatic ring in 1 (see above) instead of those for an 1,2,4-trisubstituted aromatic ring (δ_C 148.2, 145.4, 126.3, 121.5, 115.1, and 113.8; δ_H 7.06 (1H, d, J = 1.8 Hz), 6.97 (1H, dd, J = 8.4, 1.8 Hz), and 6.78 (1H, d, J = 8.4 Hz)) in 1-cafeoyl-6-tuliposide A.

The 2D structure of 1 was established by analyses of 2D NMR data including COSY, HSQC, and HMBC, and the connectivities among the trans-coumaroyl group, glucopyranose, and 4-hydroxy-2-methylenebutanoyl moiety were established via the HMBC cross peaks of H-1′/C-9 and H-6′/C-1″ (Fig. 2). The monosaccharide unit was assumed to be β-glucopyranose by the associated chemical shifts of the ^1H and ^13C resonances, which was confirmed by the relatively large coupling constants between H-1′/H-2′ (8.1 Hz), H-2′/H-3′ (9.1 Hz), H-3′/H-4′ (9.0 Hz), and H-4′/H-5′ (9.7 Hz). To establish the absolute configuration of the glucopyranosyl motif, compound 1 was hydrolyzed and

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derivatized with l-cysteine methyl ester hydrochloride and o-tolyl isothiocyanate (Tanaka et al., 2007). The LC/MS analysis was performed for the derivative of 1 with those of authentic n- and l-glucopyranose samples. The results revealed that retention time of the glucopyranose derivative from compound 1 was identical to that from the standard n-glucopyranose, confirming the presence of β-n-glucopyranose moiety in 1. Consequently, the structure of 1 was determined as 1-O-trans-p-coumaroyl-6-O-(4-hydroxy-2-methylebutanoyl)-β-n-glucopyranose.

The trivial name trans-pruspiride was assigned to 1.

The structural assignment of compound 1 was further confirmed by the comparison of the experimental and computed 13C NMR chemical shift values (Kim et al., 2017a, 2017b; Lodewyk et al., 2011; Smith and Goodman, 2010; Waters et al., 2015). Major conformers of 1 were identified upon conformational searches utilizing Macromodel (Schrodinger LLC) and further optimized using the B3LYP hybrid density-functional theory (DFT) method with the 6–31+G(d,p) basis set (Fig. 3). The NMR shielding properties of those conformers were calculated employing GIAO-based NMR chemical shift calculations using the identical basis set for optimization. The computed 13C NMR chemical shift data for 1 (Table 1) were plotted with the experimental data (Fig. 4). The statistical analysis of the experimental and the calculated 13C NMR chemical shift values exhibited an excellent correlation slope with the R2 value of 0.9954, verifying our initial structural establishment of 1.

Compound 2 was obtained as a colorless gum with the identical molecular formula to that of 1 (C20H25O10, 425.1448), given its HRFABMS data displaying the protonated ion [M + H]+ at m/z 425.1445 (calcd for C20H25O10, 425.1448). Inspection of the 1H and 13C NMR data of 2 (Fig. 2) was established as 1-O-cis-p-coumaroyl-6-O-(4-hydroxy-2-methylebutanoyl)-β-n-glucopyranose, and the trivial name cis-pruspiride was given to 2.

Other 11 compounds were identified as 2-(2-hydroxy-5-methoxyphe)- ethanol (3) (Lal et al., 1987), vanillin (4) (Kim et al., 2003), syringaldehyde (5) (Kim et al., 2003), quercetin 3-O-β-n-glucopyranoside (6) (Markham et al., 1978), taraxerol (7) (Sakurai et al., 1986), β-amyrin (8) (Lima et al., 2004), germanicol (9) (Kim et al., 2005), germacrene (10) (Frontana et al., 1994), methyl 3-O-acetylbutelinate (11) (Krommer et al., 2010), β-sitosterol (12) (Ahmad et al., 2010), and sitosterol-3-O-β-n-glucopyranoside (13) (Kojima et al., 1990) based on their observed and reported spectroscopic data.

The cytotoxicity of the isolates (1–13) was evaluated against four human cancer cell lines, A549, SK-OV-3, SK-MEL-2, and BT549. As shown in Table 2, compounds 8, 11, and 12 exhibited weak cytotoxicity possessing IC50’s ranging from 23.59 to 29.71 μM. Among four lupane-type triterpenoids (7–10) only compound 8 (β-amyrin) displayed cytotoxicity against SK-OV-3 cell line (IC50 29.71 μM), while the others were inactive (IC50 > 30 μM). Interestingly, compound 9 (germanicol), a regiosomer of 8, with different double bond positioned between C-18 and C-19, was not active, demonstrating that the olefinic bond location at C-12 and C-13 appears to play an important role in exerting cytotoxic activity of lupane-type triterpenoids. Moreover, si-tosteryl-3-O-β-n-glucopyranoside (13) displayed no activity whereas its aglycone 12 showed cytotoxicity against A549 cell line (IC50 29.02 μM), implying that such monosaccharide moieties might be involved in nullifying cytotoxicity.

The anti-inflammatory effects on the purified phytochemicals (1–13) were tested through the measurement of NO production levels in the LPS-stimulated murine microglia BV-2 cell line (Table 3). Compounds 4 and 11 exerted moderate inhibition of NO production with IC50’s of 88.12 and 93.10 μM without significant cell toxicity.

Figure 3: 3D structure of compound 1 and its surface model minimized at the MMFF force field and subsequently optimized at the B3LYP/6-31+G(d,p) level. Yellow dashed lines represent hydrogen bonding, and red represents oxygen and green and white represent carbon and hydrogen, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Figure 4: Statistical analysis of experimental and computed 13C NMR chemical shift values of 1.

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

<table>
<thead>
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<th>Comp.</th>
<th>IC50 (μM)*</th>
<th>A549</th>
<th>SK-OV-3</th>
<th>SK-MEL-2</th>
<th>BT549</th>
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<td>&gt; 30.0</td>
<td>&gt; 30.0</td>
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<td>23.59</td>
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<td>&gt; 30.0</td>
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<td>29.02</td>
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* 50% inhibitory concentration; the concentration of compound that caused a 50% inhibition in cell growth.

(115.33 ± 6.73 and 82.08 ± 4.42%). Compound 5, a C-5 methoxy-derivatized derivative of 4, exhibited poor activity \((\text{IC}_{50} 430.89 \mu M)\), which was indicative of the methoxy group at C-5 in benzaldehyde being a diminishing factor in the production of NO in BV-2 cell. The other compounds showed weak or no activity.

The neuroprotective activities of the isolated compounds (1–13) were also evaluated by assessing their induction potentials on NGF secretion in C6 cells (Table 4). All tested compounds displayed moderate to weak activities with stimulatory levels ranging from 88.40 ± 8.71 to 138.98 ± 9.49% without substantial cell toxicity at concentrations ranging from 20 μM. Results are expressed as the mean ± standard deviation (SD) of three independent experiments.

### 3. Experimental

#### 3.1. General experimental procedures

Optical rotation data were recorded utilizing a JASCO P-1020 polarimeter (JASCO, Easton, MD, USA). Ultraviolet (UV) spectra were garnered using a Shimadzu UV-1601 UV–vis spectrophotometer (Shimadzu, Tokyo, Japan). The NMR studies were accomplished employing a Bruker AVANCE III 700 NMR spectrometer and resultant spectra were processed using MestReNova (Mnova) 10.0 with default weighting functions. HRFABMS data were acquired on a Waters SYNAPT G2 (Milford, MA, USA). The HPLC-DAD-MS data were measured using an Agilent 1260 Infinity HPLC system (Agilent, Santa Clara, CA, USA) using a Luna C18 10 μm column (250 mm length × 4.6 mm i.d.; Phenomenex, Torrance, CA, USA). Purification was achieved using a semi-preparative HPLC system equipped with a Gilson 306 pump (Middleton, WI, USA), a Shodex refractive index detector (New York, NY, USA), and a Luna C18 10 μm column (250 mm length × 10 mm i.d.; Phenomenex, Torrance, CA, USA) or an Apollo silica 5 μm column (250 mm length × 10 mm i.d.; Apollo, Manchester, UK). Open columns packed with silica gel 60 (70–230 and 230–400 mesh; Merck) or RP-C18 gel (230–400 mesh; Merck, Darmstadt, Germany) were implemented for crude fractionation and separation. Precoated silica gel F254 plates and RP–18 F254 plates (Merck) were utilized for thin-layer chromatography (TLC).

#### 3.2. Plant material

The twigs of *S. prunifolia var. simpliciflora* were collected from Goesan, Republic of Korea in March 2013, and the plant material was authenticated by one of the authors (K. R. L.). A voucher specimen, coded SKKU-NPL 1301, was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea.

#### 3.3. Extraction and isolation

The dried twigs of *S. prunifolia var. simpliciflora* (7 kg) were extracted with 80% aqueous MeOH under reflux and crude extract was filtered. The filtrate was evaporated to acquire the crude extract (380 g). The crude was suspended in H2O, and partitioned with n-hexane, CHCl3, EtOAc, and n-butanol, yielding 28, 29, 12, and 47 g of the respective residues. The n-hexane-soluble fraction (15 g) was subjected to a normal phase open column chromatography (n-hexane:EtOAc, 10:1 → 1:1) to generate seven fractions (H1–H7). Fraction H1 (1.2 g) was chromatographed on a silica gel open column (n-hexane:EtOAc, 40:1) and further purified by semi-preparative silica gel HPLC (2 mL/min; n-hexane:EtOAc, 25:1) to obtain 11 (20 mg). Compound 10 (12 mg) was furnished upon the purification of fraction H2 (2.3 g) with a silica gel open column (n-hexane:EtOAc, 40:1) followed by semi-preparative silica gel HPLC (2 mL/min; n-hexane:EtOAc, 25:1). Fraction H3 (1.4 g) was applied to a normal phase open column chromatography (silica; n-hexane:EtOAc, 40:1) and further purified using semi-preparative silica gel HPLC (2 mL/min; n-hexane:EtOAc, 20:1). The purified compound was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea.
25:1) to give 12 (60 mg). Compound 7 (24 mg) was obtained by separation of fraction H4 (1.5 g) with a silica gel open column (CHCl₃:MeOH, 40:1) followed by the recrystallization in MeOH.

The CHCl₃-soluble fraction (19 g) was subjected to a normal phase open column chromatography (CHCl₃:MeOH, 30:1 → 1:1) to yield 10 fractions (C1–C10). Fraction C1 (1.0 g) was fractionated utilizing a normal phase open column chromatography (n-hexane:EtOAc:MeOH, 15:1:0.5) and further purified using semi-preparative silica gel or RP-C₁₈ silica gel HPLC (2 mL/min; n-hexane:EtOAc:MeOH, 5:1:1). Fraction C3 (3.4 g) was applied to an RP-C₁₈ silica gel open column (50% → 100% aqueous MeOH) and further purified by semi-preparative silica gel HPLC (2 mL/min; n-hexane:EtOAc:MeOH, 10:1:1) to acquire 13 (16 mg).

The EtOAc-soluble fraction (10 g) was subjected to a normal phase open column chromatography (CHCl₃:MeOH:H₂O, 4:1:0.1) to yield seven fractions (E1–E7). The fraction E3 (0.8 g) was isolated using an RP-C₁₈ silica gel open column (40% → 100% aqueous MeOH) and successively semi-preparative RP-C₁₈ silica gel HPLC (2 mL/min; 20% aqueous MeCN) to obtain 1 (9 mg) and 2 (4 mg). Compound 6 (7 mg) was furnished upon the purification of fraction E4 (1.4 g) with an RP-C₁₈ silica gel open column (40% → 100% aqueous MeOH) followed by the semi-preparative RP-C₁₈ silica gel HPLC (2 mL/min; n-hexane:EtOAc:MeOH, 10:1:1) to acquire 14 (16 mg).

3.3.1. trans-pruspiride (1)

Colorless gum; [α] + 10 (c 0.1, MeOH); IR (KBr) ν max cm⁻¹: 3388, 2940, 1690, 1599, 1156, 1083; UV (MeOH) λ max (log ε) 309 (3.45), 234 (3.30) nm; ¹H (700 MHz) and ¹³C NMR (175 MHz) data, see Table 1; HRFABMS (positive-ion mode) m/z 425.1450 [M + H]⁺ (calcd for C₂₉H₂₅O₁₀, 425.1445).

3.3.2. cis-pruspiride (2)

Colorless gum; [α] + 15 (c 0.1, MeOH); IR (KBr) ν max cm⁻¹: 3389, 2941, 1688, 1601, 1157, 1082; UV (MeOH) λ max (log ε) 290 (3.32), 231 (3.29) nm; ¹H (700 MHz) and ¹³C NMR (175 MHz) data, see Table 1; HRFABMS (positive-ion mode) m/z 425.1445 [M + H]⁺ (calcd for C₂₉H₂₅O₁₀, 425.1448).

3.4. Acid hydrolysis of 1 and 2 and sugar analysis

Compounds 1 and 2 (1 mg) were individually hydrolyzed with 1 N HCl (1 mL) under reflux for 2 h. CHCl₃ was used to extract organic layers from each reaction mixture. The monosaccharide residues were acquired from each reaction mixture of the H₂O-soluble phases upon neutralization using an Amberlite IRA-67 resin. The monosaccharides were added to pyridine (0.5 mL) containing L-cysteine methyl ester hydrochloride (0.5 mg) and the respective reaction mixtures were stirred at 60 °C for 1 h. Then α-tolylisothiocyanate (0.1 mL) was added and stirred at 60 °C for another 1 h. Each reaction mixture was analyzed without purification by LC/MS analysis (0.7 mL/min; 25% aqueous MeCN with 0.1% formic acid for 30 min). The hydrolysatc derivatives of 1 and 2 were detected at 13.6 min and the authentic υ- and χ-gluco-pyranose samples were at 13.6 min and 12.6 min, respectively, verifying that the glycosidic moieties from the two compounds were both υ-configured.

3.5. GIAO-based NMR chemical shift calculations of 1 (Lodewyk et al., 2011; Smith and Goodman, 2010; Waters et al., 2015)

GIAO-based chemical shift predictions were performed with reference to our previous communication (Kim et al., 2017a). Conformational searches were carried out using MacroModel in the MMFF force field (gas phase), a 10 kcal/mol upper energy limit, and 0.001 kJ (mol Å)⁻¹ convergence threshold on the rms gradient. The geometries of all the conformers of 1 were optimized utilizing the B3LYP/6-31+G(d,p) level in the gas phase. The GIAO magnetic shielding tensors of those identified conformers were calculated at the B3LYP/6-31+G(d,p) level in the polarizable continuum model (PCM) mode with a dielectric constant representing methanol and averaged based on the Boltzmann populations of each conformer in the associated Gibbs free energy. The chemical shift values were calculated via the following equation: δcalcd = (δav−σav)/(1−σav/δ0), where δcalcd is the calculated NMR chemical shift for nucleus υ and σav are the respective calculated isotropic constants for nucleus υ and tetrалемthylsilane (TMS) calculated at the above-mentioned basis sets.

3.6. Cytotoxicity assessment

The cytotoxicity of purified metabolites were tested against the A549 (non-small cell lung adenocarcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and BT549 (invasive ductal carcinoma), utilizing the sulfhorhodamine B colorimetric (SRB) method. Gisplatin (≥ 98%; Sigma-Aldrich) served as a positive control.

3.7. Assessment of NO generation and cell viability

The BV-2 cells, developed by Dr. V. Bocchini at the University of Perugia (Perugia, Italy), were used for this study (Blasi et al., 1990; Choi et al., 2009). The cells were seeded in a 96-well plate (4 × 10⁴ cells/well) and incubated in the presence or absence of various doses of tested compounds. Lipopolysaccharide (LPS) (100 mg/mL) was added to BV-2 cells and grown for 1 d. The produced levels of nitrite (NO₂⁻) was measured utilizing a nitric oxide synthase (NOS) inhibitor L-NMMA was employed. Graded sodium nitrite solution was utilized to determine nitrite concentrations. An MTT assay was used for the cell viability assay.

3.8. Nerve growth factor (NGF) and cell viability assays

The C6 cells (Korean Cell Line Bank, Seoul, Republic of Korea) were seeded in a 24-well plate at (1 × 10⁵ cells/well). After 1 d, the cells were applied with serum-free DMEM together with various doses of the phytochemicals for another day. From the cultured plates, the medium supernatant was collected and the changes in NGF release were measured utilizing an ELISA kit. The viability of the C6 cells was evaluated via an MTT assay. The outcomes were shown as a percentage compared to the negative control (i.e., untreated cells). The positive control was 6-shogaol.

4. Conclusions

In summary, our current study describes the isolation and characterization of 13 chemical constituents, including two new compounds (1 and 2), from the twigs of S. prunifolia var. simpliciflora. The structures of two new secondary metabolites (1 and 2) were determined via full analysis of NMR (¹H and ¹³C NMR, COSY, HSQC, and HMBC) and HRMS, chemical methods, and NMR chemical shift calculations. In particular, the current study shows how GIAO-based NMR calculations can be of great use in validating NMR-based structural assignments.

The identified molecules (1–13) were assessed for their cytotoxic, anti-inflammatory, and neuroprotective activities utilizing relevant bioassay protocols. Among them, compounds 8, 11, and 12 showed moderate cytotoxic properties against some of four tested human cancer cell lines, compounds 4 and 11 demonstrated mild inhibition of NO.
production in the LPS-stimulated murine microglia BV-2 cell line, and compounds 1, 2, and 11 were weak stimulants of NGF secretion in C6 cells. These results may constitute a basis for follow-up studies with emphasis on medicinal chemical or phytochemical investigations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.phytol.2017.09.014.

References