

## Note

## Antineuroinflammatory and Antiproliferative Activities of Constituents from *Tilia amurensis*

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As part of our ongoing search for bioactive constituents of natural Korean medicinal resources, we found in a preliminary study that the methanol (MeOH) extract from the trunks of *Tilia amurensis* RUPR. showed an inhibitory effect on nitric oxide (NO) production in an activated murine microglial cell line. A bioassay-guided fractionation and chemical investigation of the MeOH extract resulted in the isolation and identification of a new isoflavonoid glycoside, orobol 4'-*O*- $\beta$ -D-apiofuranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside (**1**) and 16 known compounds (**2**–**17**). The structure of the new compound was determined by spectroscopic methods, *i.e.*, one-dimensional (1D) and two-dimensional (2D)-NMR techniques and high resolution (HR)-MS, and chemical methods. The antineuroinflammatory activities of the isolated compounds were determined by measuring NO levels in the medium using murine microglial BV-2 cells. Among them, 12 compounds, including compound **1** (most active with an IC<sub>50</sub> value of 23.42  $\mu$ M), inhibited NO production in lipopolysaccharide-stimulated BV-2 cells. Moreover, compounds **1**–**4** showed moderate antiproliferative activities against the SK-MEL-2 cell line, with IC<sub>50</sub> values ranging from 12.31 to 19.67  $\mu$ M.

**Key words** *Tilia amurensis*; Tiliaceae; isoflavonoid glycoside; nitric oxide; antineuroinflammation; antiproliferation

Microglia are resident immune cells in the central nervous system (CNS). These cells are rapidly activated by pathogenic stimuli and consequently produce various proinflammatory mediators and cytokines.<sup>1,2</sup> In particular, excessive nitric oxide (NO) produced from activated microglia has been known to induce neuronal cell death through many *in vitro* and *in vivo* studies.<sup>3,4</sup> Therefore, discovering compounds that inhibit NO production in activated microglia is an important strategy to prevent progressive neuronal damage. As part of our ongoing search for bioactive constituents from natural Korean medicinal resources, we found that the methanol (MeOH) extract from the trunks of *Tilia amurensis* RUPR. exhibited inhibitory effect on NO production using an activated murine microglial cell line BV-2 in the screening study.

*T. amurensis* belongs to the family of Tiliaceae and is commonly known as bee tree. It is mostly found near Russia and areas in East Asia, such as China, Korea, and Japan. Its leaves have been used as traditional Korean medicine to treat cancer and rheumatoid arthritis.<sup>5</sup> In addition, tea made from the flowers of the plant have common medicinal uses, such as antispasmodic, diaphoretic, and sedative.<sup>6</sup> Earlier pharmacological study on DNA topoisomerase inhibitory activity of *T. amurensis* has indicated anticancer properties.<sup>7</sup> Recently, neuroprotective compounds such as (–)-epicatechin, scopoletin, nudiposide and lyoniside, were isolated from *T. amurensis* and quality-evaluated.<sup>8</sup> However, with the exception of our previous study, not much research has been conducted on this plant. We have previously reported the anti-inflammatory and antitumor activities of lignan constituents and a novel flavan-3-ol dimer in *T. amurensis*.<sup>9,10</sup>

Our continuing interest in bioactive constituents from *T. amurensis* led us to further investigate anti-neuroinflammatory

metabolites of *T. amurensis* trunk since its MeOH extract showed anti-neuroinflammatory activity by inhibiting lipopolysaccharide (LPS)-stimulated NO production. A bioassay-guided fractionation and chemical investigation of the MeOH extract resulted in the isolation and identification of a new isoflavonoid glycoside, orobol 4'-*O*- $\beta$ -D-apiofuranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside (**1**) and 16 known compounds (**2**–**17**). Here, we describe the isolation, structural elucidation, and the anti-neuroinflammatory and antiproliferative activities of the isolated compounds from the *T. amurensis* trunk.

### Results and Discussion

The MeOH extract of *T. amurensis* trunks was subjected to liquid–liquid solvent-partitioning to yield *n*-hexane, chloroform (CHCl<sub>3</sub>), ethyl acetate (EtOAc), and *n*-butyl alcohol (*n*-BuOH) soluble fractions. Among them, the active EtOAc-soluble fraction with anti-neuroinflammatory effect was further separated by silica gel or Sephadex LH-20 column chromatography, and subsequent HPLC purification to obtain one new isoflavonoid glycoside (**1**) and 16 known compounds (**2**–**17**) (Fig. 1). To the best of our knowledge, the presence of isoflavonoid glycoside is the second example from the genus *Tilia*. The first isoflavonoid glycoside, orobol 4'-*O*- $\beta$ -glucopyranoside, was isolated from *T. taquetii* SCHNEIDER.<sup>11</sup>

Compound **1** was isolated as a yellowish gum. The molecular formula was established as C<sub>26</sub>H<sub>28</sub>O<sub>15</sub>, based on the positive ion peak at *m/z* 603.1323 [M+Na]<sup>+</sup> in the high resolution-electrospray ionization (HR-ESI)-MS (Calcd for C<sub>26</sub>H<sub>28</sub>O<sub>15</sub>Na, 603.1326) with <sup>13</sup>C-NMR spectroscopy. The IR absorption spectrum suggested the presence of phenyl (2946 and 1450 cm<sup>-1</sup>), carbonyl (1656 cm<sup>-1</sup>), and hydroxyl (3354 cm<sup>-1</sup>) groups. The UV spectrum ( $\lambda_{\max}$  258 nm) of **1** was typical of

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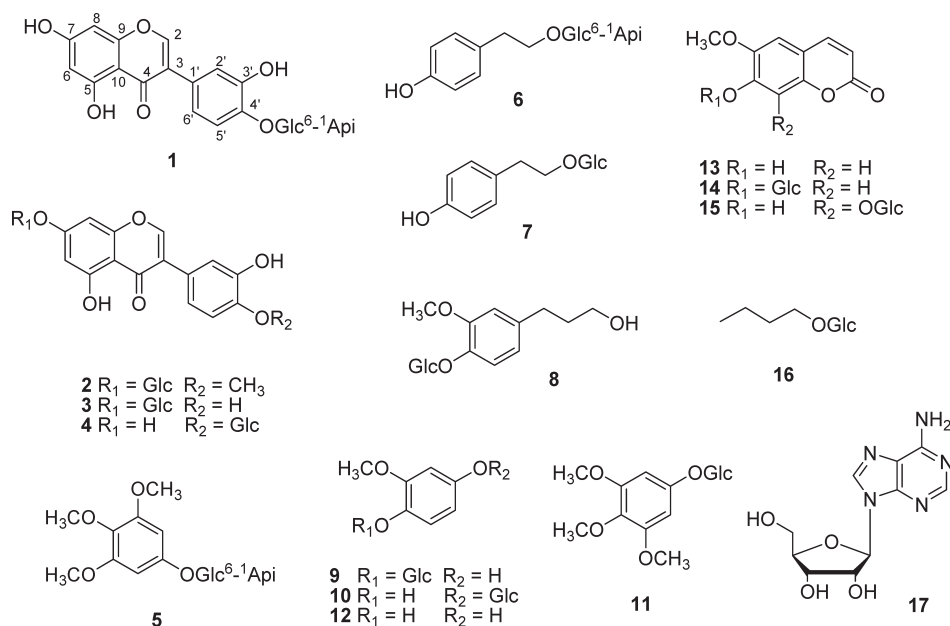


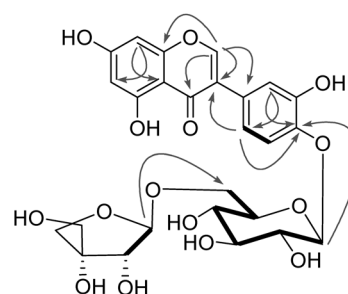
Fig. 1. Chemical Structures of Compounds 1–17

Table 1.  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR Data of Compound 1 in  $\text{CD}_3\text{OD}^a$ 

Position	1	
	$\delta_{\text{H}}$	$\delta_{\text{C}}$
2	8.08 s	154.0
3		123.0
4		180.6
5		162.6
6	6.21 brs	99.3
7		164.8
8	6.32 brs	93.7
9		158.6
10		104.8
1'		126.7
2'	7.09 d (1.5)	116.9
3'		147.2
4'		145.6
5'	7.25 d (8.5)	117.6
6'	6.98 dd (8.5, 1.5)	120.6
Glc 1''	4.83 d (7.5)	102.9
2''	3.53 dd (9.0, 7.5)	73.8
3''	3.48 m	76.4
4''	3.45 m	70.5
5''	3.37 m	76.1
6''	4.03 dd (11.5, 1.5)	68.0
Api 1'''	5.00 d (1.5)	109.9
2'''	3.93 d (1.5)	76.9
3'''		79.3
4'''	3.98 d (10.0)	73.6
	3.76 d (10.0)	
5'''	3.59 s	64.4

<sup>a</sup>)  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data were recorded at 500 and 125 MHz, respectively. Coupling constants (in Hz) are shown in parentheses.

compounds with an isoflavone skeleton,<sup>12</sup> which was also supported by the characteristic resonance for H-2 of an isoflavone observed at  $\delta_{\text{H}}$  8.08 (1H, s) in the  $^1\text{H}$ -NMR spectrum.<sup>13,14</sup> The  $^{13}\text{C}$ -NMR data (Table 1) of 1 showed a total of 26 carbon

Fig. 2.  $^1\text{H}$ - $^1\text{H}$  COSY (Bold Lines) Correlations and Key HMBCs (Arrows) of 1

signals comprising of 15 signals attributable to isoflavone skeleton and the other 11 signals from the two sugars, indicating that compound 1 is isoflavonoid glycoside. The aglycone of 1 was determined as 5,7,3',4'-tetrahydroxyisoflavone, known as orobol by analysis of the  $^1\text{H}$ - $^1\text{H}$  correlation spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), and heteronuclear multiple bond correlation (HMBC) spectra (Fig. 2). The  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra (Table 1) of 1 were very similar to those of orobol 4'-*O*- $\beta$ -glucopyranoside,<sup>11</sup> except for the signals of an additional sugar unit, apiose ( $\delta_{\text{C}}$  109.9, 79.3, 76.9, 73.6, and 64.4).<sup>15</sup> Acid hydrolysis of 1 yielded glucose and apiose detected by co-TLC comparison with authentic samples. A large coupling constant ( $J=7.5\text{Hz}$ ) for the anomeric proton ( $\delta_{\text{H}}$  4.83) of the glucose in the  $^1\text{H}$ -NMR spectrum suggested a  $\beta$ -configuration in glucose, and the apiose unit was determined to have a  $\beta$ -configuration due to a coupling constant ( $J=1.5\text{Hz}$ ) of H-1''' and the chemical shift of its anomeric carbon ( $\delta_{\text{C}}$  109.9) in the  $^{13}\text{C}$ -NMR spectrum.<sup>15</sup> The glucose C-2'' signal appeared at  $\delta_{\text{C}}$  73.8, while that of C-6'' appeared at  $\delta_{\text{C}}$  68.0, suggesting that the interglycosidic linkage is apiosyl-(1 $\rightarrow$ 6)-glucose,<sup>15</sup> which was also confirmed by the HMBC correlation between H-1''' ( $\delta_{\text{H}}$  5.00) and C-6'' ( $\delta_{\text{C}}$  68.0). The glycosidation position was determined as C-4' by the HMBC correlation between the glucosyl anomeric proton H-1'' ( $\delta_{\text{H}}$  4.83) and C-4' ( $\delta_{\text{C}}$  145.6) of A ring (Fig. 2). Furthermore,

the D-glucose and D-apiose were identified by gas chromatography (GC) analysis of their chiral derivatives in the acidic hydrolysate.<sup>16,17</sup> Thus, compound **1** was characterized as orobol 4'-O-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside.

The known compounds were identified as pratensein-7-O-β-D-glucoside (**2**),<sup>18</sup> orobol 7-O-β-D-glucoside (**3**),<sup>18</sup> orobol 4'-O-β-glucopyranoside (**4**),<sup>11</sup> kelampayoside A (**5**),<sup>19</sup> osmanthuside H (**6**),<sup>20</sup> salidroside (**7**),<sup>21</sup> dihydroconiferin (**8**),<sup>22</sup> isotachioside (**9**),<sup>23</sup> tachioside (**10**),<sup>23</sup> koaburside (**11**),<sup>24</sup> 2-methoxyhydroquinone (**12**),<sup>25</sup> scopoletin (**13**),<sup>26</sup> scopolin (**14**),<sup>27</sup> fraxin (**15**),<sup>28</sup> *n*-butyl β-D-glucopyranoside (**16**),<sup>29</sup> and adenosine (**17**)<sup>30</sup> by comparing the spectroscopic data with previously reported values.

The isolated compounds (**1–17**) were examined for their anti-neuroinflammatory activities by measuring the NO levels produced in LPS-activated BV-2 cells, a microglial cell line. In this study, twelve compounds had an IC<sub>50</sub> of less than 200 μM. Among them, compounds **1–3**, **6–7**, **13** and **15** showed significant inhibitory effects on NO production. These compounds had no effect on cell viability in LPS-treated BV-2 at their respective IC<sub>50</sub> values (data not shown). The new isoflavonoid glycoside, compound **1** showed the highest activity with an IC<sub>50</sub> value of 23.42 μM (Table 2).

Some previous studies reported anti-inflammatory activities of flavonoid glycosides.<sup>31–33</sup> Isoflavonoid glycosides (**1–3**) isolated from *T. amurensis* also exhibited inhibitory activities on inflammation in activated microglial cells. However, orobol 4'-O-β-glucopyranoside (**4**) does not have influence NO production in LPS-treated BV-2, unlike orobol 4'-O-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (**1**), the most active compound. These results suggested that the addition of β-D-apiofuranosyl group to glycosylation in C-4' hydroxyl group might play a role on NO inhibition in BV-2. In this study, compounds **6** and **7** also significantly inhibited NO production in LPS-stimulated murine microglial cell line. The inhibitory effect of salidroside (**7**) on NO production was also reported in murine macrophage RAW264.7.<sup>34</sup> Both osmanthuside H (**6**) and salidroside (**7**) are glucosides of tyrosol. The inhibitory effect of tyrosol on NO synthesis is already known.<sup>35,36</sup> Therefore, it is possible that the tyrosol skeleton structure may be important to anti-neuroinflammatory efficacy. According to previous studies, scopoletin (**13**) and fraxin (**15**) can also suppress NO production by inhibiting inducible nitric oxide

synthase expression in LPS-stimulated RAW264.7.<sup>28,37</sup> However, anti-neuroinflammatory effects of these compounds in microglial cells have not been reported yet. Our study is the first to show the anti-neuroinflammatory properties of these compounds. Excess production of NO by activated microglia induces neuronal cell death,<sup>3,4</sup> which consequently leads to various neurodegeneration in the CNS. Among isolates (**1–17**) from the active EtOAc-soluble fraction of *T. amurensis*, compounds **1–3**, **6–7**, **13** and **15** were determined to be the active ingredients responsible for anti-neuroinflammatory property of the EtOAc-soluble fraction. Particularly, compounds **2**, **3** and **15** (relatively high-yield isolates) are possible to be main contributors to the activity. The present study suggests that the anti-neuroinflammatory compounds isolated from *T. amurensis* have beneficial therapeutic potential against neurodegenerative diseases.

Next, the antiproliferative activities of the isolates **1–17** were additionally evaluated by determining their inhibitory effects on four human tumor cell lines, namely A549 (non-small cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma) using the SRB bioassay.<sup>38</sup> The results (Table 3) showed that only compounds **1–4**, which are isoflavonoid glycosides among the isolates, showed moderate antiproliferative activities against SK-MEL-2 cell line with IC<sub>50</sub> values ranging from 12.31 to 19.67 μM. In particular, compound **3** showed antiproliferative activities against all the tumor cell lines, A549, SK-OV-3, SK-MEL-2, and HCT-15 with IC<sub>50</sub> values of 8.41, 22.56, 13.93, and 28.28 μM, respectively (Table 3).

Table 3. Antiproliferative Activities of Compounds **1–4** from *T. amurensis*

Compounds	IC <sub>50</sub> (μM) <sup>a)</sup>			
	A549	SK-OV-3	SK-MEL-2	HCT-15
<b>1</b>	>30.0	>30.0	19.67	>30.0
<b>2</b>	>30.0	>30.0	18.24	>30.0
<b>3</b>	8.41	22.56	13.93	28.28
<b>4</b>	>30.0	>30.0	12.31	>30.0
Doxorubicin <sup>b)</sup>	0.001	0.003	0.002	0.081

a) IC<sub>50</sub> value of compounds against each cancer cell line defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*; Values are means of triplicate determinations. b) Doxorubicin as a positive control.

Table 2. Inhibitory Effects on NO Production of Fractions and Compounds **1–17** in LPS-Activated BV-2 Cells

Compounds	IC <sub>50</sub> (μg/mL or μM) <sup>a)</sup>	Compounds	IC <sub>50</sub> (μM) <sup>a)</sup>
Crude MeOH extract	37.81	<b>8</b>	158.49
<i>n</i> -Hexane fraction	>200	<b>9</b>	50.29
CHCl <sub>3</sub> fraction	84.92	<b>10</b>	116.66
EtOAc fraction	31.03	<b>11</b>	>200
<i>n</i> -BuOH fraction	172.38	<b>12</b>	58.87
<b>1</b>	23.42	<b>13</b>	37.69
<b>2</b>	32.23	<b>14</b>	148.92
<b>3</b>	31.85	<b>15</b>	30.02
<b>4</b>	>200	<b>16</b>	>200
<b>5</b>	>200	<b>17</b>	>200
<b>6</b>	25.99	NMMA <sup>b)</sup>	14.77
<b>7</b>	35.64		

a) IC<sub>50</sub> value of extract and fractions was defined as the concentration (μg/mL) that caused 50% inhibition of NO production in LPS-activated BV-2 cells, while that of compounds was defined as the concentration (μM). b) NMMA (*N*<sup>G</sup>-monomethyl L-arginine, nitric oxide synthase inhibitor) as a positive control.

All the other compounds were inactive ( $IC_{50} > 30 \mu M$ ) in all the cell lines.

## Experimental

**General Experimental Procedures** Optical rotations were measured on a Jasco P-1020 polarimeter (Jasco, Easton, MD, U.S.A.). IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany). ESI and HR-ESI mass spectra were recorded on a SI-2/LCQ DecaXP Liquid chromatography (LC)-mass spectrometer (Thermo Scientific, West Palm Beach, FL, U.S.A.). NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer (Varian, Palo Alto, CA, U.S.A.) operating at 500 MHz ( $^1H$ ) and 125 MHz ( $^{13}C$ ), with chemical shifts given in ppm ( $\delta$ ). Preparative HPLC used a Gilson 306 pump (Gilson, Middleton, WI, U.S.A.) with a Shodex refractive index detector (Shodex, New York, NY, U.S.A.). Low-pressure liquid chromatography (LPLC) was carried out over a LiChroprep Lobar-A Si 60 column (240 mm  $\times$  10 mm i.d.; Merck, Darmstadt, Germany) with a FMI QSY-0 pump (Teledyne Isco, Lincoln, NE, U.S.A.). Column chromatography was performed with a silica gel 60 (Merck, 230–400 mesh). The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Merck precoated silica gel F<sub>254</sub> plates and reversed-phase (RP)-18F<sub>254s</sub> plates (Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC). Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde–sulfuric acid.

**Plant Materials** The trunk of *T. amurensis* was collected from Hwacheon-Myun, Hongcheon city, Gangwon-do, Korea, in March 2010. Samples of plant material were identified by one of the authors (K. R. Lee). A voucher specimen (SKKU 2010–03) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

**Extraction and Isolation** The air-dried *T. amurensis* trunks (4 kg) were extracted twice with 80% aqueous MeOH (2  $\times$  4 h) under reflux. The extract was filtered and then concentrated under vacuum to afford a crude MeOH extract (360 g). The extract was then partitioned with *n*-hexane,  $CHCl_3$ , EtOAc, and *n*-BuOH to yield 11.8, 38.5, 10.2, and 72.5 g of residues, respectively. Each fraction was evaluated for its anti-neuroinflammatory effect in an activated murine microglial cell line. Among the four fractions, the EtOAc-soluble fraction showed the most significant activity (Table 2). Thus, the EtOAc-soluble fraction was separated with silica gel column chromatography using a gradient of MeOH to  $CHCl_3$  from 10 to 50% and this yielded 9 fractions (PNM1–9). Fraction PNM7 was subjected to Sephadex LH-20 with 100% MeOH to give 8 fractions (PNM71–78). Fraction PNM76 and PNM77 was consolidated and subjected to fractionation with silica gel column chromatography ( $CHCl_3$ –MeOH– $H_2O$ , 9:3:0.5) to yield 4 subfractions (PNM771–774). Subfraction PNM772 was further purified by semi-preparative reverse-phase HPLC (flow rate; 2 mL/min, 55% MeOH) to give compounds **1** (6 mg) and **4** (3 mg). Subfraction PNM773 was further purified by semi-preparative reverse-phase HPLC (flow rate; 2 mL/min, 35% MeOH) to give compounds **2** (12 mg), **3** (10 mg), and **17** (5 mg). Fraction PNM73 was also purified by semi-preparative reverse-phase HPLC (flow rate; 2 mL/min, 45% MeOH) to yield compounds **5** (9 mg) and **6** (9 mg). Fraction PNM72 was further purified by semi-preparative reverse-phase HPLC

(flow rate; 2 mL/min, 50% MeOH) to yield compound **8** (4 mg). Fraction PNM5 was separated with Sephadex LH-20 with 100% MeOH to yield 7 fractions (PNM51–57). Fraction PNM51 was separated by the purification of semi-preparative reverse-phase HPLC using 50% MeOH to furnish compound **16** (4 mg). Fraction PNM52 was applied to C<sub>18</sub> Waters Sep-Pak Vac 6 cc with 40% MeOH and purified by preparative reverse-phase HPLC using 38% MeOH to yield compounds **7** (4 mg) and **11** (4 mg). Fraction PNM53 was separated by chromatography on LiChroprep Lobar-A RP-18 column with 40% MeOH to give five subfractions (PNM531–535). From those subfractions, subtraction PNM532 was further purified by semi-preparative reverse-phase HPLC (flow rate; 2 mL/min, 25% MeOH) to yield compounds **9** (7 mg) and **10** (6 mg). Subfraction PNM533 was further purified by semi-preparative reverse-phase HPLC (flow rate; 2 mL/min, 40% MeOH) to obtain compounds **14** (12 mg) and **15** (19 mg). Fraction PNM1 and PNM2 were consolidated and separated by RP-C<sub>18</sub> silica gel column chromatography with MeOH to  $H_2O$  gradient increasing from 80 to 100% to give three fractions (PNM21–23). Fraction PNM21 was subjected to fractionation with C<sub>18</sub> Waters Sep-Pak Vac 6 cc with 100% MeOH to yield subfractions PNM211 and PNM212. Subfraction PNM211 was purified by preparative reverse-phase HPLC with a solvent system of 45% MeOH (flow rate; 2 mL/min) to give compounds **12** (4 mg) and **13** (4 mg).

Orobol 4'-O- $\beta$ -D-apiofuranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside (**1**): Yellowish gum;  $[\alpha]_D^{25} + 20.6$  ( $c = 0.30$ , MeOH); UV (MeOH)  $\lambda_{max}$  (log  $\epsilon$ ) 288 (1.2), 258 (1.9), 218 (3.8) nm; IR (KBr)  $\nu_{max}$  3354, 2946, 2832, 1656, 1508, 1450, 1365, 1177, 1055, 998  $cm^{-1}$ ;  $^1H$ - (500 MHz) and  $^{13}C$ - (125 MHz) NMR data, see Table 1; ESI-MS (positive-ion mode)  $m/z$ : 603 [M+Na]<sup>+</sup>. HR-ESI-MS (positive-ion mode)  $m/z$ : 603.1323 [M+Na]<sup>+</sup> (calcd for C<sub>26</sub>H<sub>28</sub>O<sub>15</sub>Na, 603.1326).

**Acid Hydrolysis of 1 and Sugar Analysis** Compound **1** (2 mg) was hydrolyzed by 1 N HCl (dioxane– $H_2O$ , 1:1, 5 mL) under reflux conditions for 3 h. After cooling, the reaction mixture was diluted with  $H_2O$  and extracted with  $CHCl_3$ . A sample of the aqueous layer was neutralized by passage through an Amberlite IRA-67 column and repeatedly evaporated under reduced pressure to give the sugar fraction. The sugars in the fraction were analyzed by silica gel TLC by comparison with authentic samples. The solvent system was  $CHCl_3$ –MeOH– $H_2O$  (8:5:1). Spots were visualized by spraying with 95% EtOH– $H_2SO_4$ –anisaldehyde (9:0.5:0.5), then heated at 120°C for 3 min. The  $R_f$  of glucose and apiose were 0.30 and 0.45, respectively for sugars of **1**. For GC analysis, each sugar fraction was dissolved in anhydrous pyridine (100  $\mu$ L), and 0.1 M L-cysteine methyl ester hydrochloride in anhydrous pyridine (200  $\mu$ L) was added.<sup>16,17</sup> The mixture was stirred at 60°C for 1 h. Then 150  $\mu$ L of HMDS/TMCS (hexamethyldisilazane–trimethylchlorosilane–pyridine, 3:1:10) was added, and the mixture was stirred at 60°C for another 30 min. The precipitate was centrifuged off, and the supernatant was concentrated under an N<sub>2</sub> stream. The residue was partitioned between *n*-hexane and  $H_2O$  (0.1 mL each), and the hexane layer (1  $\mu$ L) was analyzed by GC experiment. D-Glucose and D-apiose were detected by co-injection of the hydrolysate with standard silylated samples (D-glucose: 11.38 min; L-glucose: 12.62 min; D-apiose: 5.08; L-apiose: 5.65). The retention times of sugars obtained by acid hydrolysis were D-glucose

(11.41 min) and D-apiose (5.05 min) for **1**. The standards of sugars, D-glucose, L-glucose, and D-apiose were obtained from Sigma-Aldrich, U.S.A, and L-apiose was from Santa Cruz Biotechnology, Inc., U.S.A.

**Cell Culture** BV2 (microglia from murine) was generously provided by Dr. E. Choi from Korea University (Seoul, Korea). It was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin. All tumor cell cultures were maintained using RPMI1640 cell growth medium (Gibco, Carlsbad, CA, U.S.A.), supplemented with 5% fetal bovine serum (FBS) (Gibco), 100 units/mL penicillin and 100 µg/mL streptomycin. Human tumor cell lines such as A549 (non-small cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma) were provided by the National Cancer Institute (NCI). All cells were incubated at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

**Measurement of Nitric Oxide Production and Cell Viability** BV-2 cells were plated into a 96-well plate (3×10<sup>4</sup> cells/well). After 24h, cells were pretreated with compounds **1–17** (in dimethyl sulfoxide (DMSO)) for 30 min, and then stimulated with 100 ng/mL of microglial activator LPS for another 24h.<sup>39</sup> Nitrite, a soluble oxidation product of NO, was measured in the culture media using the Griess reaction. The supernatant (50 µL) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-1-naphtylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 570 nm was measured using a microplate reader. Sodium nitrite was used as a standard to calculate the NO<sub>2</sub><sup>-</sup> concentration. Cell viability was assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. N<sup>G</sup>-monomethyl-L-arginine (L-NMMA, Sigma, St. Louis, MO, U.S.A.), a well-known nitric oxide synthase (NOS) inhibitor, was tested as a positive control.<sup>40</sup>

**Antiproliferative Effect Assessment** Antiproliferative effect of the isolated compounds against cultured human tumor cell lines was evaluated by the sulforhodamine B (SRB) method.<sup>38</sup> Each tumor cell line plated on standard 96-well flat-bottom microplates was incubated for 24h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The attached cells were then incubated with the serially diluted compounds (in DMSO). After continuous exposure to the compounds for 48h, the culture medium was removed from each well and the cells were fixed with 10% cold trichloroacetic acid at 4°C for 1h. 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 then solubilized with 10 mM unbuffered Tris base solution (pH 10.5). The absorbance was measured spectrophotometrically at 520 nm with a microtiter plate reader.

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**Conflict of Interest** The authors declare no conflict of interest.

## References

- Hanisch U. K., *Glia*, **40**, 140–155 (2002).
- Minghetti L., Levi G., *Prog. Neurobiol.*, **54**, 99–125 (1998).
- Boje K. M., Arora P. K., *Brain Res.*, **587**, 250–256 (1992).
- Takeuchi A., Isobe K. I., Miyaishi O., Sawada M., Fan Z. H., Nakashima I., Kiuchi K., *Eur. J. Neurosci.*, **10**, 1613–1620 (1998).
- Ahn D. K., "Illustrated Book of Korean Medicinal Herbs," Kyohaksa, Seoul, 2003.
- Lauriault J., "Identification Guide to the Trees of Canada," Fitzhenry and Whiteside, Ontario, 1989.
- Choi J. Y., Seo C. S., Zheng M. S., Lee C. S., Son J. K., *Arch. Pharm. Res.*, **31**, 1413–1418 (2008).
- Lee B., Weon J. B., Yun B. R., Lee J., Eom M. R., Ma C. J., *Pharmacogn. Mag.*, **10**, 195–199 (2014).
- Kim K. H., Moon E., Kim S. Y., Choi S. U., Lee K. R., *Food Chem. Toxicol.*, **50**, 3680–3686 (2012).
- Kim K. H., Moon E., Kim S. Y., Choi S. U., Lee K. R., *Bull. Korean Chem. Soc.*, **36**, 367–369 (2015).
- Kang Y. M., Lee N. H., *Bull. Korean Chem. Soc.*, **32**, 1048–1050 (2011).
- Watanabe K., Kinjo J., Nohara T., *Chem. Pharm. Bull.*, **41**, 394–396 (1993).
- Lee J. W., Lee C., Jin Q., Lee M. S., Kim Y., Hong J. T., Lee M. K., Hwang B. Y., *Arch. Pharm. Res.*, **38**, 991–997 (2015).
- Murthy M. S. R., Rao E. V., Ward R. S., *Magn. Reson. Chem.*, **24**, 225–230 (1986).
- Kim K. H., Ha S. K., Choi S. U., Kim S. Y., Lee K. R., *Chem. Pharm. Bull.*, **59**, 1425–1429 (2011).
- Bognár R., Somogyi L., Györgydeák Z., *Justus Liebigs Ann. Chem.*, **738**, 68–78 (1970).
- Hara S., Okabe H., Mihashi K., *Chem. Pharm. Bull.*, **35**, 501–506 (1987).
- Anhut S., Dietmar Zinsmeister H., Mues R., Barz W., Mackenbrock K., Köster J., Markham K. R., *Phytochemistry*, **23**, 1073–1075 (1984).
- Kanchanapoom T., Kasai R., Yamasaki K., *Phytochemistry*, **59**, 551–556 (2002).
- Lei Y., Wu L. J., Shi H. M., Tu P. F., *Helv. Chim. Acta*, **91**, 495–500 (2008).
- Chu Y. H., Chen C. J., Wu S. H., Hsieh J. F., *J. Agric. Food Chem.*, **62**, 3742–3749 (2014).
- Mazur M., Hope-Ross K., Kadla J. F., Sederoff R., Chang H. M., *J. Wood Chem. Technol.*, **27**, 1–8 (2007).
- Inoshiri S., Sasaki M., Kohda H., Otsuka H., Yamasaki K., *Phytochemistry*, **26**, 2811–2814 (1987).
- Jin H. G., Kim A. R., Ko H. J., Woo E. R., *Arch. Pharm. Res.*, **38**, 591–597 (2015).
- Ferreira J. A., Nel J. W., Vincent Brandt E., Bezuidenhout B. C. B., Ferreira D., *J. Chem. Soc., Perkin Trans. 1*, **1995**, 1049–1056 (1995).
- Cassady J. M., Ojima N., Chang C., McLaughlin J. L., *J. Nat. Prod.*, **42**, 274–278 (1979).
- Kim A. R., Ko H. J., Chowdhury M. A., Chang Y. S., Woo E. R., *Arch. Pharm. Res.*, **38**, 1059–1065 (2015).
- Kwon J. H., Kim S. B., Park K. H., Lee M. W., *Arch. Pharm. Res.*, **34**, 1459–1466 (2011).
- Antkowiak R., Antkowiak W. Z., Banczyk I., Mikolajczyk I., *Can. J. Chem.*, **81**, 118–124 (2003).
- Patching S. G., Baldwin S. A., Baldwin A. D., Young J. D., Gallagher M. P., Henderson P. J. F., Herbert R. B., *Org. Biomol. Chem.*, **3**, 462–470 (2005).
- Senthamilselvi M. M., Kesavan D., Sulochana N., *Org. Med. Chem. Lett.*, **2**, 19 (2012).
- González Mosquera D. M., Ortega Y. H., Kilonda A., Dehaen W., Pieters L., Apers S., *Phytochem. Lett.*, **4**, 231–234 (2011).
- Antunes-Ricardo M., Gutiérrez-Urbe J. A., Martínez-Vitela C.,

- Serna-Saldivar S. O., *Biomed. Res. Int.*, **2015**, 847320 (2015).
- 34) Song B., Huang G., Xiong Y., Liu J., Xu L., Wang Z., Li G., Lu J., Guan S., *J. Med. Food*, **16**, 997–1003 (2013).
- 35) De Stefano D., Maiuri M. C., Simeon V., Grassia G., Soscia A., Cinnelli M. P., Carnuccio R., *Eur. J. Pharm.*, **566**, 192–199 (2007).
- 36) Wang P., Zhu Q., Wu N., Siow Y. L., Aukema H., O K., *J. Agric. Food Chem.*, **61**, 3669–3675 (2013).
- 37) Kang T. H., Pae H. O., Jeong S. J., Yoo J. C., Choi B. M., Jun C. D., Chung H. T., Miyamoto T., Higuchi R., Kim Y. C., *Planta Med.*, **65**, 400–403 (1999).
- 38) Song J. H., Choi H. J., Song H. H., Hong E. H., Lee B. R., Oh S. R., Choi K., Yeo S. G., Lee Y. P., Cho S., Ko H. J., *J. Ginseng Res.*, **38**, 173–179 (2014).
- 39) Weinstein J. R., Swarts S., Bishop C., Hanisch U. K., Möller T., *Glia*, **56**, 16–26 (2008).
- 40) Reif D. W., McCreedy S. A., *Arch. Biochem. Biophys.*, **320**, 170–176 (1995).