Regular Article

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Secoiridoid Glycosides from the Twigs of *Ligustrum obtusifolium* Possess Anti-inflammatory and Neuroprotective Effects

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Two new secoiridoid glycosides, obtusifolisides A and B (1, 2), together with 7 known secoiridoid glycosides (3–9) were isolated from the twigs of *Ligustrum obtusifolium*. The chemical structures of new compounds were determined by a spectroscopic data analysis, including one and two dimensional (1D-, 2D)-NMR, High resolution-MS, and experiments involving chemical reactions. The isolated secoiridoid glycosides were evaluated for their anti-inflammatory effects in lipopolysaccharide (LPS)-stimulated BV-2 murine microglia cells. Compounds 2, 5, 6, 8, and 9 significantly reduced the production of nitric oxide (NO), with IC_{50} values of 5.45, 11.17, 14.62, 15.45, and 14.96 μM, respectively. None of the compounds were toxic to the cells. Additionally, we evaluated the neuroprotective effects of compounds 1–9 on nerve growth factor (NGF) induction in a C6 rat glioma cell line. Compounds 2 and 6 upregulated NGF secretion to 155.56±7.16%, and 139.35±11.65%, respectively, without significant cell toxicity.

Key words *Ligustrum obtusifolium*; secoiridoid glycoside; anti-inflammation; nerve growth factor (NGF) regulation

Glial cells are major defence cells present in the central nervous system (CNS). Microglia and astrocytes are major defending glia cells. In normal condition microglia gets activated and tries to protect the neuron from hazardous conditions or specially pathogens and CNS injury *etc*. However, under pathological conditions, microglia cells are over-activated and produce a variety of proinflammatory mediators, including nitric oxide (NO). Lipopolysaccharide (LPS) is being used as a neuroinflammation inducer for *in vitro* experiments.[1)] LPS induced neuroinflammation is also responsible for the induction of neuronal apoptosis characterized by neurodegeneration. LPS is a toll like receptor 4 (TLR4) agonist, that can initiate the TLR4 mediated inflammatory cascades during inflammation as well as neuroinflammation. LPS-TLR4 mediated neuroinflammation induce the expression of inducible nitric oxide synthase (iNOS) protein which is responsible to increase the production of nitric oxide.[2)] NO is a major biomarker against inflammatory conditions, so we did the screening of the compound to inhibit the nitrite production that proves the anti-inflammatory activity of our compounds. Additionally, nerve growth factor (NGF) is an important neurotrophin that helps neuron to survive and differentiate during brain development. It is also believed that, NGF helps to improve neurodegeneration and shows its neuroprotective role in the developing brain. It protects neuron against toxin induced neuronal death and cholinergic cell loss. Hence in this study we screened our compounds to increase NGF production through astrocyte. In this way inhibition of NO production and increased NGF production are responsible for anti-inflammatory and neuroprotective effects.[3)]

Ligustrum obtusifolium, known as Ibota privet, is a shrub that is widely distributed in East Asia.[4)] *L. obtusifolium* has been used as a Korean traditional medicine, and it has been known that fruits have a cardiac and roborant effect, and the trunks have been used for the treatment of diabetes, and hypertension. Additionally, leaves of this plant have been used for oropharyngeal inflammation, rheumatic condition and diuretic conditions in the traditional medicine of southern Europe.[5)] Taken together we hypothesized that, the compounds present in this plant may play important role in the treatment of neuroinflammation as well as neurodegenerative conditions. Previous phytochemical investigations on *L. obtusifolium* reported the isolation of secoiridoid glycosides, phenolic glycosides, and lignans.[6–8)] In particular, several secoiridoid glycosides isolated from *L. obtusifolium* were associated with lysine decreasing activity and anti-hyperglycemic activities.[9,10)] However, only a few phytochemical studies on *L. obtusifolium* have been reported. As a part of our continuing search for bioactive constituents from Korean medicinal plants, we investigated methanol (MeOH) extracts of the twigs of *L. obtusifolium*. The EtOAc-soluble fraction was separated using repeated silica gel and reversed phase column chromatography followed by preparative HPLC, to yield nine secoiridoid glycosides (**1**–**9**), including two new secoiridoid glycosides derivatives, named obtusifolisides A and B (**1**, **2**). The structural elucidation of the new compounds was based on MS, ^{1}H- and ^{13}C-NMR, and two dimensional (2D)-NMR analysis (^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond connectivity (HMBC), and nuclear Overhauser effect spectroscopy (NOESY)) as well as acid hydrolysis. Herein, we report the isolation and structural determination of the isolates (**1**–**9**) and their biological activities.

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Results and Discussion

The MeOH extract from the twigs of *L. obtusifolium* was suspended in distilled water and then successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH in order to identify the bioactive constituents responsible for anti-cancer and anti-inflammatory activities. Each fraction was evaluated for cytotoxicity against human cancer cell lines and inhibition of NO production in LPS-stimulated BV-2 cells. The EtOAc-soluble fraction was showed inhibition NO production in LPS-stimulated BV-2 microglial cells. Therefore, we separated the EtOAc-soluble fraction using repeated silica gel and reversed phase column chromatography followed by preparative HPLC, to afford two new secoiridoid glycosides derivatives (**1**, **2**), together with seven known compounds (**3**–**9**) (Fig. 14S).

Compound **1** was obtained as a yellowish gum. The molecular formula of **1** was determined to be $C_{37}H_{46}O_{17}$ by positive mode high-resolution (HR)-FAB-MS data at *m/z* 785.2630 $[M+Na]^+$ (Calcd for $C_{37}H_{46}O_{17}Na$, 785.2633). The ^{1}H-NMR spectrum (Table 1) of **1** exhibited the presence of two 1,3,4-trisubstituted aromatic ring moieties [δ_H 7.13 (1H, d, *J*=2.0 Hz, H-2″), 6.89 (1H, dd, *J*=8.0, 2.0 Hz, H-6″), and 6.78 (1H, d, *J*=8.0 Hz, H-5″)/6.95 (1H, d, *J*=2.0 Hz, H-2‴), 6.79 (1H, d, *J*=8.0 Hz, H-5‴), and 6.75 (1H, dd, *J*=8.0, 2.0 Hz, H-6‴)], one oxygenated methine [δ_H 4.67 (1H, d, *J*=8.0 Hz, H-7″)], two oxygenated methylenes [δ_H 4.34 (1H, dd, *J*=11.6, 6.0 Hz, H-9″a), and 4.21 (1H, dd, *J*=11.6, 6.0 Hz, H-9″b)/3.85 (1H, overlap, H-9‴a), and 3.64 (1H, overlap, H-9‴b)], one methylene [δ_H 2.93 (1H, d, *J*=13.9 Hz, H-7‴a), and 2.86 (1H, d, *J*=13.9 Hz, H-7‴b)], and two methoxyl groups [δ_H 3.88 (6H, s, 3″,3‴-OCH_3)]. Through this data, it was confirmed that there was lignan, (−)-olivil, in the chemical structure of **1**.[11] In addition, ^{1}H-NMR data of **1** displayed signals as two olefinic protons [δ_H 7.55 (1H, s, H-3), and 6.12 (1H, q, *J*=6.7 Hz, H-8)], one hemiacetalic proton [δ_H 5.96 (1H, br s, H-1)], one methine [δ_H 4.01 (1H, dd, *J*=9.0, 5.0 Hz, H-5)], one methylene [δ_H 2.67 (1H, dd, *J*=14.0, 5.0 Hz, H-6*β*), and 2.45 (1H, dd, *J*=14.0, 9.0 Hz, H-6*α*)], one methyl [δ_H 1.69 (3H, dd, *J*=7.0, 1.5 Hz, H-10)], one methyl ester group [δ_H 3.71 (3H, s, 11-OCH_3)], and one glucopyranosyl unit [δ_H 4.87 (1H, d, *J*=7.8 Hz, H-1′), 3.87 (1H, overlap, H-6′a), 3.65 (1H, overlap, H-6′b), 3.41 (1H, t, *J*=9.0 Hz, H-5′), 3.32 (1H, m, H-3′), 3.31 (1H, m, H-2′), and 3.30 (1H, m, H-4′)], suggesting that the structure of **1** to have a secoiridoid glycoside moiety.[12] The ^{13}C-NMR spectrum revealed 38 carbon signals including eighteen in a lignan unit, three methoxy signals, six in a glucopyranosyl unit, and the remaining eleven in secoiridoid moiety. These spectroscopic data suggested that the structure of **1** was connected to a (−)-olivil and an oleoside 11-methyl ester. The (−)-olivil located at C-7 based on the HMBC correlation from H-9″ [δ_H 4.34 (1H, dd, *J*=11.6, 6.0 Hz), and 4.21 (1H, dd, *J*=11.6, 6.0 Hz)] with C-7 (δ_C 173.1). Also, the location of the glucose unit was determined to be at C-1 by analysis of the HMBC data showing correlation from H-1 to C-1′. The planar structure of **1** was established on the basis of the consideration and analysis of ^{1}H–^{1}H COSY, HMQC, and HMBC. Alkaline methanolysis of **1** afforded (−)-olivil (**1a**) and oleoside-7,11-dimethyl ester (**1b**=**4**). The **1a** was identified by comparing their ^{1}H-NMR data and specific optical rotation with reported literature.[11] Acid hydrolysis of **4** (**1b**) afforded D-glucopyranose, which was identified by co-TLC with the authentic sample and specific optical rotation. The relative configuration of the lignan moiety in **1** was established by analysis of the coupling constant. The large coupling constant (*J*=8.0 Hz) of H-7″ and H-8″ observed in the ^{1}H-NMR spectrum of **1**, established that H-7″ and H-8″ are in the opposite orientation. The circular dichroism (CD) spectrum of **1** showed the negative Cotton effect at 246 and 282 nm, suggesting that the absolute configuration of **1** was determined to be 7″*R*,8″*S*, and 8‴*S*.[13] Also, the relative configuration of secoiridoid part was assigned by NOESY spectrum, which showed correlation between H-1 and CH_2-6, suggesting that H-5 was on the *β* configuration, and H-1 and

Table 1. ^{1}H- (700 MHz) and ^{13}C- (175 MHz) NMR Data for **1** and **2** in CD_3OD (δ in ppm, 700 MHz for ^{1}H and 175 MHz for ^{13}C)[a]

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1	5.96 br s	95.2	5.96 br s	94.6
3	7.55 s	155.3	7.51 overlap	155.1
4		109.4		109.4
5	4.01 dd (9.0, 5.0)	32.1	4.09 overlap	32.6
6	2.67 dd (14.0, 5.0)	41.3	2.77 dd (15.0, 4.0)	41.3
	2.45 dd (14.0, 9.0)		2.50 dd (15.0, 9.7)	
7		173.1		173.0
8	6.12 q (6.7)	124.9	6.18 t (7.0)	124.7
9		130.7		134.4
10	1.69 dd (7.0, 1.5)	13.7	4.97 dd (13.2, 7.5)	62.2
			4.77 overlap	
11		168.7		168.5
1′	4.87 d (7.8)	100.9	4.78 d (7.6)	101.1
2′	3.31 m	75.0	3.28 overlap	74.8
3′	3.32 m	78.6	3.29 overlap	78.6
4′	3.30 m	71.6	3.27 overlap	71.6
5′	3.41 t (9.0)	78.1	3.35 m	78.1
6′	3.87 overlap	62.9	3.85 dd (12.0, 1.8)	62.8
	3.65 overlap		3.63 dd (12.0, 5.6)	
1″		134.5		130.8
2″	7.13 d (2.0)	111.7	6.61 d (2.0)	117.2
3″		149.3		146.4
4″		147.7		145.1
5″	6.78 d (8.0)	116.0	6.63 d (8.0)	116.6
6″	6.89 dd (8.0, 2.0)	121.3	6.47 dd (8.0, 2.0)	121.5
7″	4.67 d (8.0)	85.9	2.72 t (7.0)	35.4
8″	2.52 m	59.0	4.18 m	67.1
			4.10 overlap	
9″	4.34 dd (11.6, 6.0)	63.5		
	4.21 dd (11.6, 6.0)			
1‴		130.0		122.6
2‴	6.95 d (2.0)	115.5	7.48 overlap	113.8
3‴		148.7		148.9
4‴		146.4		153.1
5‴	6.79 d (8.0)	115.9	6.78 d (8.0)	116.1
6‴	6.75 dd (8.0, 2.0)	124.2	7.50 overlap	125.4
7‴	2.93 d (13.9)	40.9		168.0
	2.86 d (13.9)			
8‴		82.4		
9‴	3.85 overlap	78.0		
	3.64 overlap			
11-OCH_3	3.71 s	52.0	3.68 s	52.0
3″-OCH_3	3.88 s	56.6		
3‴-OCH_3	3.88 s	56.6	3.82 s	56.6

a) *J* values are in parentheses and reported in Hz; The assignments were based on ^{1}H–^{1}H COSY, HMQC and HMBC experiments.

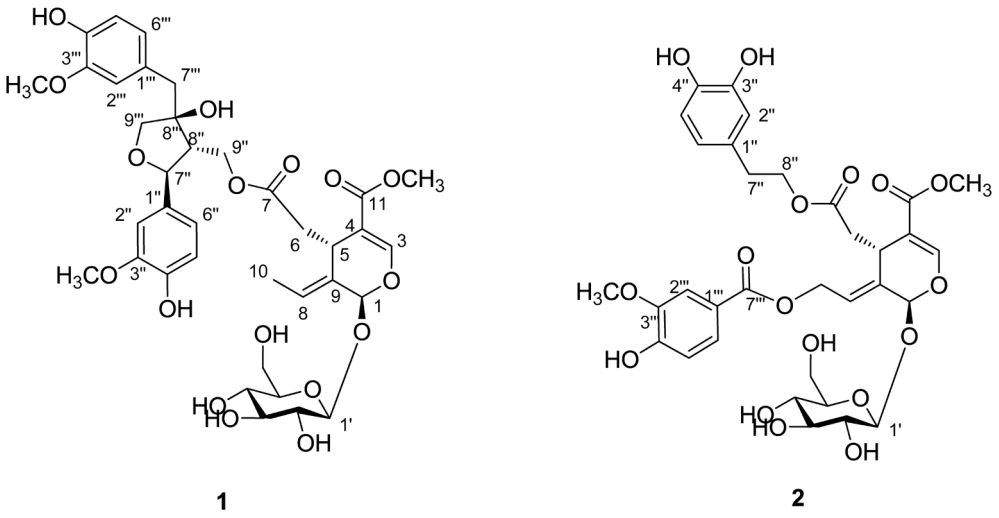

Fig. 1. Chemical Structures of Compounds **1** and **2**

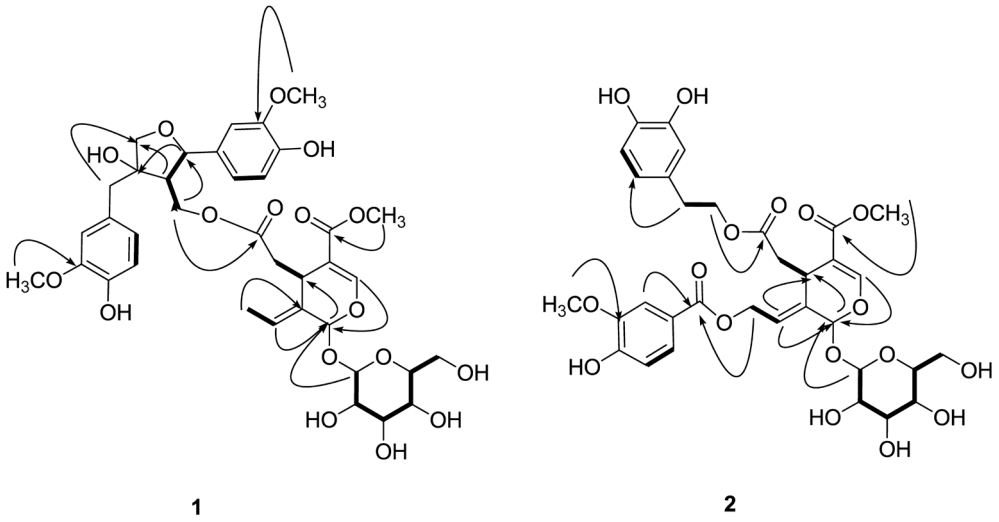

Fig. 2. Key ^{1}H–^{1}H COSY (▬) and HMBC (↷) Correlations of **1** and **2**

CH_2-6 were on the α configuration. Thus, the structure of **1** was established as shown in Fig. 1, and named obtusifoliside A.

Compound **2** was obtained as a yellowish gum. The molecular formula of **2** was established as $C_{33}H_{38}O_{17}$ using HR-electrospray ionization (ESI)-MS, which showed a negative ion $[M-H]^-$ *m/z* 705.2024 (Calcd for $C_{33}H_{37}O_{17,}$ 705.2031). The ^{1}H- and ^{13}C-NMR spectra were similar to those of **5**,[14] except for the presence of vanillic acid [δ_H 7.50 (1H, overlap, H-6‴), 7.48 (1H, overlap, H-2‴), 6.78 (1H, d, J=8.0 Hz, H-5‴), and 3.82 (3H, s, 3‴-OCH_3); δ_C 168.0 (C-7‴), 153.1 (C-4‴), 148.9 (C-3‴), 125.4 (C-6‴), 122.6 (C-1‴), 116.1 (C-5‴), 113.8 (C-2‴), and 56.6 (3‴-CH_3)] and a deshielded signal of C-10 [**2**: δ_C 62.2; **5**: δ_C 59.1]. The HMBC correlation from H-10 with C-7″ showed that the vanillic acid unit was located at C-10. The glucopyranosyl moiety of **2** was confirmed to be located at C-1 by HMBC correlation between H-1′ and C-1. This gross structure was confirmed by analysis of the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra (Fig. 2). The coupling constant of the anomeric proton (J=7.6 Hz) indicated the presence of a β-glucopyranosyl unit.[15] Acid hydrolysis of **2** afforded the sugar unit, which was identified by co-TLC with the authentic sample and specific optical rotation. The relative configuration of **2** was assumed to be the same as that of **1** based on the NOESY correlation between H-1/CH_2-6. Thus, the structure of **2** was established as shown in Fig. 1 and named obtusifoliside B.

The known compounds were identified as oleoside-11-methylester (**3**),[16] oleoside-7,11-dimethylester (**4**),[17] 10-hydroxyoleuropein (**5**),[14] oleuropein (**6**),[16] ligstroside (**7**),[18] (2″*R*)-2″-methoxyoleuropein (**8**),[19] neonuezhenide (**9**)[14] by comparison of their spectroscopic and physical data with previously reported values.

As LPS activated microglia induced NO is a major biomarker of inflammatory conditions. Compounds that can inhibit the NO production poses an anti-inflammatory activity against LPS induced neuroinflammation.[2] The secoiridoid glycoside is known to be associated with anti-inflammatory activity.[20,21] Therefore, compounds **1**–**9** were tested for production of NO levels in LPS-activated BV-2. As shown in Table 2, among the isolates, compounds **2**, **5**, **6**, **8**, and **9** significantly reduced the production of NO, with IC_{50} values of 5.45, 11.17, 14.62,

Table 2. Inhibitory Effects on NO Production of Compounds **1–9** in LPS-Activated BV-2 Cells

Compounds	IC_{50}[a] (μM)	Cell viability[b] (%)
1	33.85	131.07±7.93
2	5.45	123.96±7.24
3	38.67	131.83±8.60
4	38.89	152.58±4.57
5	11.17	125.64±4.07
6	14.62	123.16±0.08
7	61.25	127.97±2.88
8	15.45	127.29±6.99
9	14.96	145.93±7.42
L-NMMA[c]	18.47	120.23±4.562

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±S.D. *c*) L-NMMA as positive control.

Table 3. Effects of Compounds **1–9** on NGF Secretion and Cell Viability in C6 Cells[a]

Compounds	NGF secretion	Cell viability[b]
1	90.64±14.58	119.23±5.30
2	155.56±7.16	101.24±4.27
3	65.32±10.44	109.70±0.69
4	111.73±8.85	100.79±12.85
5	86.02±7.78	102.00±4.80
6	139.35±11.65	109.83±0.71
7	53.54±14.00	72.23±38.19
8	114.39±14.65	107.39±2.49
9	79.30±9.29	100.40±4.06
6-Shogaol[c]	164.25±4.56	106.53±5.34

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±S.D. 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±S.D. *c*) 6-Shogaol as positive control.

15.45, and 14.96 μM, respectively, without cell toxicity. The activity of these compounds exhibited more potent effects than a positive control, N^G-monomethyl-L-arginine (L-NMMA), which inhibited NO production with an IC_{50} value of 18.47 μM. Interestingly, the secoiridoid glycosides which contained the 3,4-dihydroxylphenylethyl alcohol moiety at C-7 showed remarkably better anti-inflammatory effects compared to structures without this moiety. Although the structures of **2** and **5** are very similar, except for the presence of vanillic acid at C-10, compound **2** was more active than compound **5**. These data suggested that vanillic acid at C-10 may be important for the inhibition of NO production in BV2 cells.

NGF is a main important neurotrophins responsible for the neuroprotective effect against neuroinflammation and other pathological condition.[22] The neuroprotective activity of compounds **1–9** was also tested by measuring their induction potentials on NGF secretion in C6 cells. As shown in Table 3, compounds **2** and **6** had moderate effects on NGF release with stimulation levels of 155.56±7.16%, and 139.35±11.65%, respectively (without cell toxicity at a concentration of 20 μM).

NO inhibitory activity, as well as NGF production, increase the anti-inflammatory and neuroprotective effect of compounds from *L. obtusifolium*. This study showed that secoiridoid glycosides are the main constituents of the twigs of *L. obtusifolium*, and two new secoiridoid derivatives, obtusifolisides A and B (**1**, **2**), were isolated from this plant. Moreover, we investigated anti-inflammatory, and neuroprotective activities of isolated compounds from *L. obtusifolium*. As a result, the new compound **2** showed better effects than other isolates in the inhibition of NO production in LPS-stimulated BV-2 cells and NGF secretion in C6 cells. This study could be useful for the development of novel anti-inflammatory and neuroprotective agents.

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). UV spectra were recorded with a Shimadzu UV-1601 UV-Visible spectrophotometer (Shimadzu, Kyoto, Japan). ESI and HR-ESI mass spectra were recorded on an Applied Biosystems Mariner time-of-flight (TOF) mass spectrometer with an electrospray interface. FAB and HR-FAB mass spectra were obtained on a JEOL JMS700 mass spectrometer (JEOL, Peabody, MA, U.S.A.). NMR spectra, including ^1H–^1H COSY, HMQC, HMBC, and NOSEY experiments, were recorded on a Bruker AVANCEIII 700 NMR spectrometer (Bruker, U.S.A.) operating at 700 MHz (^1H) and 175 MHz (^{13}C), with chemical shifts given in ppm (δ). 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.). Silica gel 60 (Merck, 70–230 mesh and 230–400 mesh) and RP-C_{18} silica gel (Merck, 230–400 mesh) were used for column chromatography. The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia Co., Ltd.). Merck precoated silica gel F_{254} plates and RP-18 F_{254s} plates (Merck, Darmstadt, Germany) were used for TLC. Spots were detected on TLC under UV light or by heating after spraying with 10% H_2SO_4 in C_2H_5OH (v/v).

Plant Material The twigs of *L. obtusifolium* was collected on Suwon, Gyeonggi-do, Korea, in June, 2014. Samples of plant material were identified by one of the authors (K. R. Lee). A voucher specimen (SKKU-NPL-1401) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and Isolation The twigs of *L. obtusifolium* (7.0 kg) were extracted with 80% aqueous MeOH three times (3×4 h) under reflux, and filtered. The filtrate was evaporated under vacuum to obtain a MeOH extract (510 g), which we suspended in distilled water (2.4 L) and then successively partitioned with *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH, yielding 20, 40, 50, and 190 g of residue, respectively. The EtOAc-soluble fraction (20 g) was separated by silica gel (230–400 mesh, 300 g) column and eluted with $CHCl_3$–MeOH (5 : 1→1 : 1, gradient system) to yield eight fractions (A–H). Fraction B (410 mg) was chromatographed on a RP-C_{18} silica gell column (230–400 mesh, 50 g) using a solvent system of MeOH–H_2O (40 : 60) and was purified by preparative reversed-phase HPLC using a 250×10 mm i.d., 10 μm, Phenomenex Luna RP-18 column with a solvent system of MeCN–H_2O (28 : 72, flow rate; 2 mL/min) as the eluant to yield **9** (50 mg). Fraction C (9.4 g)

was separated by silica gel column using a solvent system of $CHCl_3$–MeOH (10 : 1→1 : 1) to give six sub fractions (C1–C6). Fraction C4 (1.1 g) was applied to a sephadex LH-20 column using a solvent system of MeOH–H_2O (4 : 1) to yield five fractions (C41–C45). Fraction C42 (330 mg) was purified by preparative reversed-phase HPLC using a 250×10 mm i.d., 10 μm, Phenomenex Luna RP-18 column with a solvent system of MeCN–H_2O (1 : 4, flow rate; 2 mL/min) as the eluant to afford **4** (7 mg). Fraction C5 (4.8 g) was separated by RP-C_{18} silica gell column with a solvent system of MeCN–H_2O (1 : 3) to yield six sub fraction (C51–C56). Fraction C55 (170 mg) was chromatographed on a sephadex LH-20 column with 100% MeOH and purified by preparative reversed-phase HPLC using a 250×10 mm i.d., 10 μm, Phenomenex Luna RP-18 column with a solvent system of MeCN–H_2O (1 : 4, flow rate; 2 mL/min) as the eluant to afford **1** (6 mg). Fraction E36 (2.0 g) was separated by RP-C_{18} silica gell column with a solvent system of MeCN–H_2O (1 : 3) to yield seven sub fractions (C61–C67). Fraction C61 (30 mg) was purified by preparative reversed-phase HPLC using a 250×10 mm i.d., 10 μm, Phenomenex Luna RP-18 column with a solvent system of MeCN–H_2O (1 : 9, flow rate; 2 mL/min) as the eluant to afford **3** (6 mg). Fraction C63 (250 mg) was chromatographed on a silica gell column using ($CHCl_3$–MeOH, 7 : 1→1 : 1) and purified by preparative reversed-phase HPLC using a 250×10 mm i.d., 10 μm, Phenomenex Luna RP-18 column with a solvent system of MeCN–H_2O (1 : 4, flow rate; 2 mL/min) as the eluant to yield **7** (2 mg). Fraction E364 (260 mg) was chromatographed on a silica gel column using a solvent system of $CHCl_3$–MeOH (7 : 1→1 : 1) and purified by preparative reversed-phase HPLC using a 250×10 mm i.d., 10 μm, Phenomenex Luna RP-18 column with a solvent system of MeCN–H_2O (3 : 7, flow rate; 2 mL/min) as the eluant to yield **6** (38 mg) and **8** (23 mg). Fraction C67 (140 mg) was chromatographed on a sephadex LH-20 column with 100% MeOH and purified by preparative reversed-phase HPLC using a 250×10 mm i.d., 10 μm, Phenomenex Luna RP-18 column with a solvent system of MeCN–H_2O (3 : 7, flow rate; 2 mL/min) as the eluant to afford **2** (6 mg). Fraction E was separated by sephadex LH-20 column with a solvent system of MeOH–H_2O (8 : 2) to yield four sub fractions (E1–E4). Fraction E3 was chromatographed on RP-C_{18} silica gell column using a solvent system of MeOH–H_2O (1 : 3) and purified by preparative reversed-phase HPLC using a 250×10 mm i.d., 10 μm, Phenomenex Luna RP-18 column with a solvent system of MeCN–H_2O (3 : 7, flow rate; 2 mL/min) as the eluant to yield compound **5** (8 mg).

Obtusifoliside A (**1**)

Yellowish gum; $[\alpha]_D^{25}$ −10.4 (c=0.2, MeOH); IR (KBr) cm^{-1}: 3587, 2971, 1710, 1610, 1340, 1057; UV λ_{max} (MeOH) nm (logε): 207 (3.5), 240 (0.5), 287 (0.8); CD (MeOH) λ_{max} ($\Delta\varepsilon$): 282 (−10.54), 246 (−62.21), 232 (+14.23), 225 (−24.52); ^{1}H- and ^{13}C-NMR spectra, see Table 1; HR-FAB-MS (positive-ion mode): m/z=785.2630 $[M+Na]^+$ (Calcd for $C_{37}H_{46}NaO_{17}$, 785.2633).

Obtusifoliside B (**2**)

Yellowish gum; $[\alpha]_D^{25}$ −28.8 (c=0.50, MeOH); IR (KBr) cm^{-1}: 3360, 2943, 2829, 1649, 1471, 1402, 1032; UV (MeOH) λ_{max} (logε) nm: 202 (3.1), 248 (2.5), 288 (0.4); ^{1}H- and ^{13}C-NMR spectra, see Table 1; HR-ESI-MS (negative-ion mode): m/z=705.2024 $[M-H]^-$ (Calcd for $C_{32}H_{35}O_{17}$, 705.2031).

Alkaline Methanolysis of 1 Compound **1** (3.0 mg) was hydrolyzed with 0.5 mol/L KOH in MeOH (1 mL) at room temperature for 1 h. The mixture was subsequently eluted using an ion exchange column (Dowex® 50WX8 hydrogen form, Sigma-Aldrich) in 100% MeOH to remove KOH. The reaction mixtures of **1** were separated through semi-prep. HPLC (30% MeCN) to give **1a** (0.5 mg) and **1b** (=**4**) (0.6 mg), which were identified as (−)-olivil (**1a**) and oleoside-7,11-dimethyl ester (**1b**) by comparison comparison of ^{1}H-NMR.

Acid Hydrolysis of 1b and 2 and Sugar Analysis Each compound (**1b**, 0.6 mg; **2**, 1 mg) was refluxed with 1 N HCl (1 mL) at 90°C for 3 h. The hydrolysate was extracted with EtOAc, and the aqueous layer was neutralized using an Amberlite IRA-67 column to yield the sugar. The sugar was obtained from each H_2O layer, identified as sucrose by co-TLC comparison with an authentic sample, D-glucose [silica gel, solvent: $CHCl_3$–MeOH–H_2O (9 : 7 : 1.5), Rf value: 0.31] and its optical rotation value; $[\alpha]_D^{25}$ +85.0 (c=0.05, MeOH) from **1b** and $[\alpha]_D^{25}$ +50.0 (c=0.03, MeOH) from **2**.

Measurement of NO Production in LPS-Activated BV-2 Cells[23] The BV-2 cell line was originally developed by Dr. V. Bocchini at the University of Perugia (Perugia, Italy). BV-2 microglia cells were stimulated with 100 ng/mL of LPS in the presence or absence of samples for 24 h. 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, 0.1% N-1-napthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using a microplate reader (Emax, Molecular Device, Sunnyvale, CA, U.S.A.). N^G-monomethyl-L-arginine (L-NMMA, Sigma, St. Louis, MO, U.S.A.), a well-known nitric oxide synthase inhibitor, was tested as a positive control. Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

NGF and Cell Viability Assay[24] We used C6 glial cells to measure NGF release into the medium. C6 cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (PS) in a humidified incubator with 5% CO_2. To measure NGF content in medium and cell viability, C6 cells were seeded into 24-well plates (1×10^5 cells/well). After 24 h, the cells were treated with DMEM containing 2% FBS and 1% PS with 20 μM of each sample for one day. Media supernatant was used for the NGF assay using an ELISA development kit (R&D System, Minneapolis, MN, U.S.A.). C6 Cell viability was assessed by an MTT assay. The positive control was 6-shogaol.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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