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A biphenyl derivative from the twigs of *Chaenomeles speciosa*

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ABSTRACT

In our continuing search for bioactive constituents of Korean medicinal sources, we investigated an 80% MeOH extract of the twigs of *Chaenomeles speciosa*. Column chromatographic purification of the $CHCl_3$ fraction resulted in the isolation of a new biphenyl derivative (**1**), along with four known biphenyl compounds (**2**–**5**) and six triterpenes (**6**–**11**). The chemical structure of the new compound was determined on the basis of spectroscopic analyses including 1D and 2D NMR data. Among isolates, compound **3** exhibited potent cytotoxic activities against SK-OV-3, SK-MEL-2, and XF498 cell lines (IC_{50} = 5.91, 4.22, and 6.28 μM, respectively). Also, Compounds **9** and **10** showed strong anti-neuroinflammatory activities (IC_{50} 2.38, and 6.70 μM, respectively).

1. Introduction

Microglia are a type of neuronal cell that plays an immune function in the central nervous system (CNS). However, if they are uncontrolled and activated chronically, they contribute to operating various proinflammatory factors such as nitric oxide (NO), tumor necrosis factor alpha (TNF-α) and prostaglandin E_2 (PGE_2) [1,2]. These factors cause inflammation of the CNS and eventually lead to neurological diseases such as Parkinson's disease [3]. Recently, it has been reported that neurotrophic factor can prevent LPS-induced neuroinflammation by inhibiting JNK signaling in neurons [4]. This means that neurotrophic factor may be potential targets of neuroinflammatory therapy in central nervous system (CNS) disorders, and therefore need to focus on finding new compounds that regulate the integration of neurotrophic factors. As part of our continuing search for bioactive constituents that exhibit various activities such as anti-inflammatory, anti-cancer and neuroprotective effects from Korean medicinal resources, we found that the MeOH extract from the twigs of *Chaenomeles speciosa* (Sweet) Nakai showed inhibitory effect on NO levels in lipopolysaccharide (LPS)-stimulated murine microglia BV2 cells in Preliminary screening test.

C. speciosa is a deciduous shrub belonging to the Rosaceae family and distributed in Korea, and China [5,6]. *C. speciosa* has been used in traditional medicine. The fruit of this plant has been used for the treatment of rheumatoid arthritis, hepatitis, and common cold [7,8]. Previous studies have suggested that terpenoids, flavonoids, and tannins from the fruit of *C. speciosa* associated with anti-inflammatory, antimicrobial, and antioxidant effects [8–10]. Recently, several isolated components, such as triterpenoids and lignan glycosides, from the twigs of same genus plant, *Chaenomeles sinensis,* have reported that they exhibit good cytotoxic and anti-inflammatory activity [11,12]. In a process of searching for more active substances from another *Chaenomeles* genus plants, we found that the $CHCl_3$ layer of a *C. speciosa* MeOH extract showed strong cytotoxicity against A549, SK-OV-3, SK-MEL-2, and XF498 cells and inhibitory to produce NO in lipopolysaccharide (LPS)-stimulated murine microglia BV2 cells. The chemical investigation of the extracts from the twigs of *C. speciosa* resulted in the isolation of a new biphenyl compound (**1**), along with ten known ones (**2**–**11**). The chemical structures of the isolated compounds were elucidated by extensive NMR data (^1H and ^{13}C NMR, ^1H-^1H COSY, HSQC, and HMBC), optical rotation and MS analysis. The isolated compounds (**1**–**11**) were evaluated for their cytotoxic, anti-neuroinflammatory, and NGF-potentiating activities. Herein, we report the isolation and structural elucidation of bioactive compounds from the twigs of *C. speciosa* and their biological activities.

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2. Experimental

2.1. General experimental procedures

Optical rotations were measured on a Jasco P-1020 polarimeter (Jasco, Easton, MD, USA). Infrared (IR) spectra were recorded on a Bruker IFS-66/S Fourier-transform IR spectrometer (Bruker, Karlsruhe, Germany). Ultraviolet (UV) spectra were recorded with a Shimadzu UV-1601 UV–Visible spectrophotometer (Shimadzu, Tokyo, Japan). HRFABMS was conducted on a Waters SYNAPT G2 (Milford, MA, USA). NMR spectra were recorded on a Bruker AVANCE III 700 NMR spectrometer at 700 MHz (^{1}H) and 175 MHz (^{13}C). The preparative high performance liquid chromatography (HPLC) system had a Gilson 306 pump (Middleton, WI, USA) with a Shodex Refractive Index Detector (New York, NY, USA). Column chromatography was performed with silica gel 60 (70–230 and 230–400 mesh; Merck, Darmstadt, Germany) and RP-C_{18} silica gel (Merck, 230–400 mesh). LPLC was performed over a LiChroprep Lobar-A RP-C_{18} column (Merck, 240 mm × 10 mm i.d.) equipped with a FMI QSY-0 pump. Merck precoated silica gel F_{254} plates and reversed-phase (RP)-18 F_{254s} plates (Merck) were used for thin-layer chromatography (TLC). 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 *C. speciosa* were collected from Suwon, Korea, in June 2014. Samples of plant material were identified by one of the authors (K.R. Lee). A voucher specimen (SKKU-NPL-1403) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

2.3. Extraction and isolation

The twigs of *C. speciosa* (7 kg) were extracted with 80% MeOH (3 × 4 L every 3 days) under reflux, and filtered. The methanol extract (398 g) was suspended in distilled water (2.4 L) and then successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, yielding 28, 25, 19, and 81 g of residue, respectively. Each layer was tested for cytotoxicity against A549, SK-OV-3, SK-MEL-2, and XF498 cells using a SRB assay. The $CHCl_3$-soluble fraction showed the most significant cytotoxic activity against the tested tumor cell lines. In our screening test, the active $CHCl_3$-soluble fraction also strongly inhibited NO production in LPS-stimulated BV-2 microglial cells. The $CHCl_3$ fraction (20 g) was separated over a silica gel (230–400 mesh, 100 g) column with a solvent system of $CHCl_3$/MeOH (20:1 → 1:1, gradient system) to obtain eight fractions (A-H). Fraction B (1.4 g) was chromatographed on a RP-C_{18} silica gel column with 80% aqueous MeOH to give six subfractions (A1–A6). Fraction A1 (48 mg) was purified by semi-preparative HPLC (2 mL/min, 50% aqueous CH_3OH) to yield compounds **3** (2 mg), **4** (3 mg), and **5** (2 mg). Fraction B (4.2 g) was chromatographed on a RP-C_{18} silica gel column with 80% aqueous MeOH to give nine subfractions (B1–B9). Fraction B1 (270 mg) was separated by a Lobar-A RP-C_{18} column with 50% aqueous MeOH and further purified by semi-preparative HPLC (2 mL/min, 35% aqueous CH_3CN) to yield compound **1** (5 mg). Fraction B2 (120 mg) was separated by a Lobar-A RP-C_{18} column with 70% aqueous MeOH and further purified by semi-preparative HPLC (2 mL/min, 35% aqueous CH_3CN) to yield compound **2** (6 mg). Fraction B6 (137 mg) was purified by semi-preparative HPLC (2 mL/min, 90% aqueous CH_3OH) to yield compound **8** (3 mg). Fraction B8 (540 mg) was purified by semi-preparative HPLC (2 mL/min, 90% aqueous CH_3OH) to yield compounds **7** (3 mg), **9** (3 mg), and **11** (3 mg). Fraction C (1.0 g) was chromatographed on a RP-C_{18} silica gel column with 80% aqueous MeOH to give 5 subfractions (C1–C5). Fraction C4 (90 mg) was purified by semi-preparative HPLC (2 mL/min, 85% aqueous CH_3OH) to yield compound **6** (3 mg).

2.3.1. Chaenomin B (*1*)

Yellowish gum; $[\alpha]_D^{25}$ − 3.1 (*c* 0.30, $CHCl_3$); IR (KBr): ν_{max} = 3400, 2944, 2830, 1746, 1690, 1647, 1488, 1362, 1249, 1032, 758 cm^{-1}; UV (MeOH) λ_{max} (log ε) 205 (1.3), 262 (0.3), 288 (0.2) nm ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR data, see Table 1; HR-FAB-MS (negative mode): *m/z* 453.1543 [M − H − H_2O]$^-$ (calcd. for $C_{25}H_{25}O_8$, 453.1549).

2.4. Cytotoxicity assessment

The cytotoxicity of the compounds against cultured human tumor cell lines was evaluated by the SRB method. The assays were performed at the Korea Research Institute of Chemical Technology. Each tumor cell line was seeded in standard 96-well flat-bottomed microplates and incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. The attached cells were incubated with serially diluted samples. Following 48 h of continuous exposure to the compounds, the culture medium was removed, 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 then solubilized with 10 mM unbuffered Tris base solution (pH 10.5). Absorbance was measured spectrophotometrically at 520 nm using a microtiter plate reader. Etoposide (≥98%; Sigma Chemical Co., St. Louis, MO, USA) was used as a positive control. Etoposide showed IC_{50} values against A549 (non-small cell lung adenocarcinoma), SK-OV-3 (malignant ovarian ascites), SK-MEL-2 (skin melanoma), and XF498 (human CNS solid tumor) of 0.92, 1.75, 0.19, and 0.23 μM, respectively.

Table 1
^{1}H and ^{13}C NMR data of **1** in $CDCl_3$. (δ in ppm, 700 MHz for ^{1}H and 175 MHz for ^{13}C).[a]

Position	**1**	
	δ_H	δ_C
1		131.1
2	6.74 (d, 2.0)	111.3
3		144.2
4		132.1
5		148.5
6	6.64 (d, 2.0)	106.4
1′		123.4
2′		157.6
3′	6.46 (d, 2.0)	104.9
4′		160.5
5′	6.47 (dd, 8.0, 2.0)	99.2
6′	7.16 (d, 8.0)	131.2
1″		128.4
2″	6.88 (d, 1.8)	109.9
3″		147.1
4″		146.6
5″	6.88 (d, 8.0)	114.8
6″	6.90 (dd, 8.0, 1.8)	121.1
7″	4.92 (d, 8.1)	76.4
8″	3.98 m	78.5
9″	3.82 m, 3.51 m	61.7
5-OCH_3	3.84 s	56.3
2′-OCH_3	3.73 s	55.8
4′-OCH_3	3.77 s	55.6
3″-OCH_3	3.85 s	56.4

[a] *J* values are in parentheses and reported in Hz; the assignments were based on ^{1}H-^{1}H COSY, HSQC, and HMBC experiments.

2.5. Measurement of nitric oxide production and cell viability

BV-2 microglia cells were stimulated with 100 ng/mL LPS in the presence and absence of samples for 24 h. Nitrite in the culture media, a soluble oxidation product of NO, was measured by the Griess reaction. 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). Absorbance at 540 nm was measured after 10 min using a microplate reader (Emax, Molecular Device, Sunnyvale, CA, USA). Graded sodium nitrite solutions were used as standards to calculate nitrite concentrations. Cell viability was measured using an MTT colorimetric assay. The NO synthase inhibitor N^G-monomethyl-l-arginine (Sigma) was used as a positive control.

2.6. NGF and cell viability assays

C6 glioma cells were used to measure NGF release. C6 cells were purchased from the Korean Cell Line Bank and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified incubator with 5% CO_2. C6 cells were seeded into 24-well plates (1×10^5 cells/well) to measure NGF content in the medium and to assess cell viability. After 24 h, the cells were treated with DMEM containing 2% FBS and 1% penicillin-streptomycin with 20 μM of each sample for one day. The media supernatant was used for NGF ELISA (R&D Systems, Minneapolis, MN, USA). Cell viability was assessed using an MTT assay.

3. Results and discussion

3.1. Bioactivity-guided isolation of isolated compounds

The 80% MeOH extract from the twigs of *C. speciosa* revealed significant cytotoxic and anti-inflammatory activities. The 80% MeOH extract was fractionated to yield *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH soluble fractions, and then each fraction was evaluated for cytotoxicity against tumor cell lines using a sulforhodamine B (SRB) assay. It was found that the $CHCl_3$-soluble fraction showed cytotoxic activity against the tumor cell lines, with IC_{50} values ranging from 18.01 to 25.97 μg/mL. while the *n*-hexane, EtOAc, and *n*-BuOH soluble fraction exhibited weak cytotoxic activities. Therefore, we investigated the most active $CHCl_3$-soluble fraction and isolated a new biphenyl derivative (**1**), together with ten known compounds (**2**–**11**) (Fig. 1). This paper reports the isolation and structural elucidation of compounds (**1**–**11**) and their antitumor, anti-neuroinflammator, and NGF release activities.

3.2. Structure elucidation of isolated compounds

Compound **1** was obtained as a yellowish gum. The molecular formula was determined to be $C_{25}H_{28}O_9$ from the molecular peak $[M - H - H_2O]^-$ at *m/z* 453.1543 (calcd. for $C_{25}H_{25}O_8$, 453.1549) in the negative-ion HRFABMS (Fig. S1, Sup. material). The IR spectrum of **1** showed absorption bands at 3400, 2944, 1453 and 1249 cm^{-1}, which were indicative of a hydroxyl group, phenyl group and ether groups, respectively. The ^1H NMR spectrum of **1** (Table 1) indicated the presence of a 1,2,4-trisubstituted aromatic ring δ_H 7.16 (1H, d, *J* = 8.0 Hz), 6.47 (1H, dd, *J* = 8.0, 2.0 Hz), and 6.46 (1H, d, *J* = 2.0 Hz)], a 1,3,4-trisubstituted aromatic ring [δ_H 6.90 (1H, dd, *J* = 8.0, 1.8 Hz), 6.88 (1H, d, *J* = 8.0 Hz) and 6.88 (1H, d, *J* = 1.8 Hz)], an asymmetric 1,3,4,5-tetrasubstituted aromatic ring [δ_H 6.74 (1H, d, *J* = 2.0 Hz) and 6.64 (1H, d, *J* = 2.0 Hz)], two oxygenated methines [δ_H 4.92 (1H, d, *J* = 8.1 Hz, H-7″) and 3.98 (1H, m, H-8″)], an oxygenated methylene [δ_H 3.82 (1H, m, H-9″a) and 3.51 (1H, m, H-9″b)], and four methoxy groups [δ_H 3.85 (3H, s), 3.84 (3H, s), 3.77 (3H, s), and 3.73 (3H, s)] (Fig. S2, Sup. material). The ^{13}C NMR spectrum of **1** showed 25 carbon signals including 18 aromatic carbons, three oxygenated carbons, and four methoxy

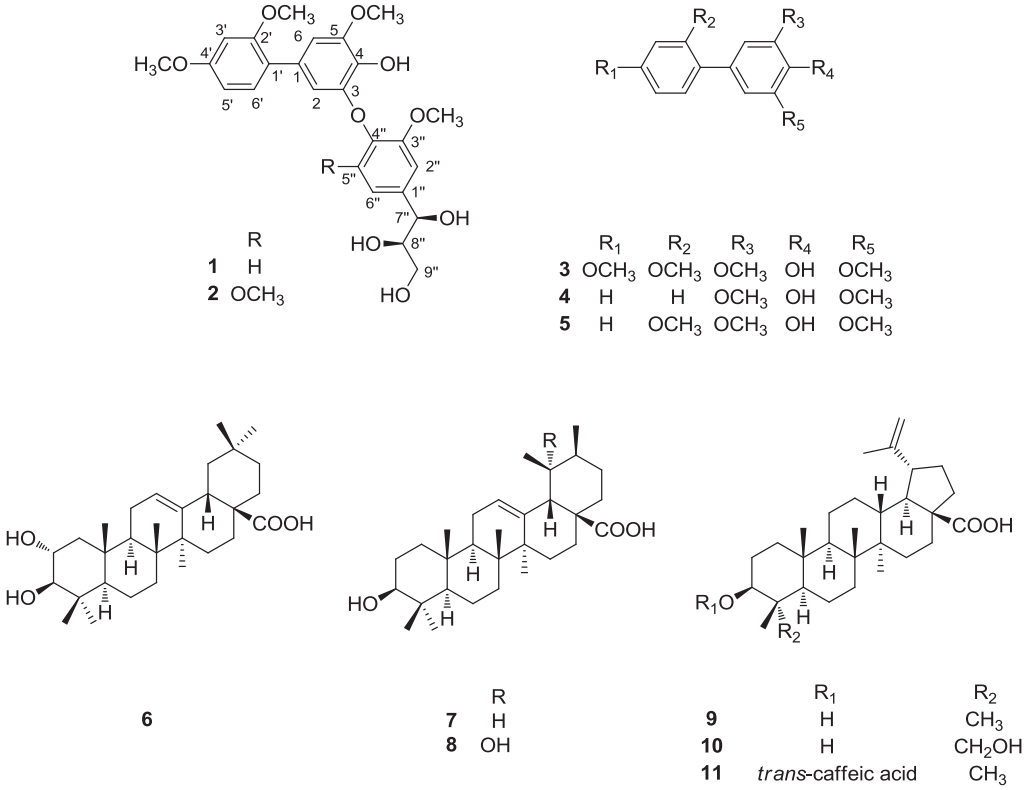

Fig. 1. Chemical structures of compounds **1**–**11**.

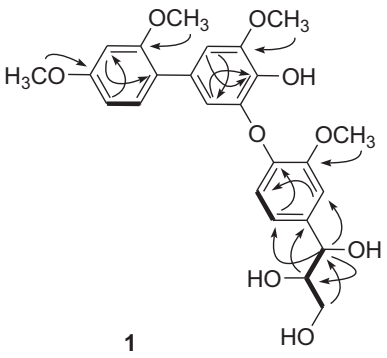

Fig. 2. Key ^{1}H-^{1}H COSY (▬) and HMBC (⟶) correlations of **1**.

carbons (Fig. S3, Sup. material). The NMR spectra of **1** were similar to those of **2** [13], except for the signals of for the absence of one methoxy group at C-5″. The gross structure of 1 was established by analysis of the ^{1}H–^{1}H COSY, HSQC (Fig. S4, Sup. material), and HMBC spectra (Fig. S5, Sup. material). The location of the four methoxy groups was determined to be C-5, C-2′, C-4′ and C-3″ through the HMBC cross-peaks between 5-OCH$_3$/C-5, 2′-OCH$_3$/C-2′, 4′-OCH$_3$/C-4′, and 3″/OCH$_3$-3″ (Fig. 2). In the arylglycerol moiety, the disposition for larger couplings in threo-form (J = 7.5–8.0 Hz) compared to their erythro-form (J = 3.5–4.5 Hz) was well confirmed [13,14], which allowed us to assign the relative configuration of 1 as 7″,8″-*threo* form. The negative optical rotation {$[\alpha]_D^{25}$ −20.1 (*c* 0.20, CHCl$_3$)} of 1 established that its configuration is 7″*R* and 8″*R* [13,15]. Thus, the structure of **1** was elucidated as shown in Fig 1, and named chaenomin B (**1**).

The known compounds were identified as chaenomin A (**2**) [13], 2′,4′-dimethoxyaucuparin (**3**) [16], aucuparin (**4**) [17], 2′-methoxyaucuparin (**5**) [18], maslinic acid (**6**) [19], dihydrotomentosolic acid (**7**) [20], ilexgenin B (**8**) [21], betulinic acid (**9**) [22], 23-hydroxybetulinic acid (**10**) [23], and pycarenic acid (**11**) [24], by comparison of their spectroscopic data with the reported data.

3.3. Antiproliferative activity

The isolated compounds **1**–**11** were evaluated for cytotoxic activities against the A549, SK-OV-3, SK-MEL-2, and XF498 cell lines using the SRB bioassay. Among them, compounds **1**–**3**, **9**, and **10** showed antiproliferative activity against the tested cells, with IC$_{50}$ values of 4.22–18.20 μM (Table 2). In particular, compound **3** exhibited the strongest cytotoxicity against SK-OV-3, SK-MEL-2, and XF498 cell lines (IC$_{50}$ = 5.91, 4.22, and 6.28 μM, respectively). The other compounds were inactive (IC$_{50}$ > 30 μM). Interestingly, the presence of a methoxy group at C-4′ in the biphenyl moiety seemed to increase cytotoxic activity against the tested cell lines based on the biological data of **3**–**5**.

Table 2
Cytotoxic activities of biphenyl compounds **1**–**11** isolated from *C. lagerania*.

Compound	IC$_{50}$ (μM)[a]			
	A549	SK-OV-3	SK-MEL-2	XF498
1	16.38	15.16	15.64	18.20
2	16.27	16.49	15.84	17.47
3	11.06	5.91	4.22	6.28
4	>30.0	>30.0	>30.0	>30.0
5	>30.0	>30.0	>30.0	>30.0
6	>30.0	>30.0	>30.0	>30.0
7	21.71	>30.0	>30.0	27.94
8	>30.0	>30.0	>30.0	>30.0
9	8.92	14.55	11.92	11.17
10	13.46	17.83	15.27	17.65
11	>30.0	>30.0	>30.0	>30.0
Etoposide[b]	0.92	1.75	0.19	0.23

[a] IC$_{50}$ value of compounds against cancer cell lines, defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*.
[b] Etoposide was used as a positive control.

3.4. Anti-inflammatory activity

The isolated compounds (**1**–**11**) were examined for their anti-inflammatory activities by measuring nitric oxide (NO) production in lipopolysaccharide (LPS)-activated BV-2 cells, a microglial cell line (Table 3). Among them, compounds **9** and **10** significantly reduced the production of LPS-induced NO, with IC$_{50}$ values of 2.38, and 6.70 μM, respectively, in LPS-induced BV-2 cells. Many studies reported betulinic acid derivatives, compounds **9** and **10**, to be potent anti-inflammatory agents [25]. For this reasons, one of the causes of the anti-inflammatory effects of the *C. speciosa* extract in previous studies can be expected to be that these kinds of compounds were contained in *C. speciosa.* Also, compounds **4** and **5** significantly reduced the production of LPS-induced NO, with IC$_{50}$ values of 20.04, and 22.02 μM, respectively, in LPS-induced BV-2 cells, with no cell toxicity. In particular, the inhibitory activity

Table 3
Inhibitory effect on NO production of compounds **1**–**11** in LPS-activated BV-2 cells.

Compound	IC$_{50}$[a] (μM)	Cell viability[b] (%)
1	283.33	143.74 ± 0.44
2	86.02	125.97 ± 1.01
3	272.72	127.49 ± 6.42
4	20.04	133.78 ± 3.39
5	22.02	113.16 ± 11.77
6	26.96	126.18 ± 2.13
7	19.41	127.58 ± 4.97
8	12.72	104.40 ± 3.41
9	2.38	79.04 ± 2.30
10	6.70	109.00 ± 0.87
11	67.50	123.83 ± 3.66
L-NMMA[c]	21.82	114.08 ± 2.96

[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.

Table 4
Effects of compounds **1**–**11** on NGF secretion in C6 Cells.

Compound	NGF secretion (%)[a]	Cell Viability (%)[b]
1	112.36 ± 10.89	114.20 ± 2.26
2	92.50 ± 11.65	115.08 ± 0.09
3	123.18 ± 6.37	121.42 ± 5.31
4	102.92 ± 5.08	121.58 ± 2.29
5	165.86 ± 11.22	120.29 ± 3.70
6	88.41 ± 8.11	112.95 ± 3.59
7	66.68 ± 0.29	112.74 ± 2.55
8	118.26 ± 14.25	116.99 ± 4.41
9	86.38 ± 10.16	113.48 ± 2.13
10	119.73 ± 8.68	114.66 ± 3.03
11	89.36 ± 15.65	110.95 ± 1.20
6-shogaol[c]	168.58 ± 7.16	125.80 ± 0.93

[a] C6 cells were treated with 20 μM of the compounds. After 24 h, the content of NGF secreted into C6-conditioned media was measured by ELISA. The level of secreted NGF is expressed as a percentage of the untreated control (set at 100%). Data are the mean ± SD of three independent experiments performed in triplicate.
[b] Cell viability after treatment with 20 μM of each compound was determined by the MTT assay and is expressed as a percentage (%). Results are the mean of three independent experiments, and the data are expressed as the mean ± SD.
[c] 6-shogaol was used as positive control.

of **4** was more potent than that of the positive control, L-NMMA (IC_{50} = 21.82 μM). The remaining compounds did not show any significant effects.

3.5. NGF and cell viability assays

The isolated compounds (**1**–**11**) were evaluated for their neuroprotective effects on nerve growth factor (NGF) secretion using C6 glial cells (Table 4). Among the isolates, compound **5** was a significantly potent stimulant of NGF release with stimulation levels of 165.86 ± 11.22% (positive control 6-shogaol was 168.58 ± 7.16%), without any cell toxicity at a concentration of 20 μM. On the other hand, the other compounds did not show any significant effects.

4. Conclusions

We have isolated a new biphenyl derivative, chaenomin B (**1**), along with 4 known biphenyl compounds and 6 known triterpenes from the twigs of *C. speciosa*. Moreover, we investigated the cytotoxic, anti-inflammatory, and neuroprotective activities of isolates from *C. speciosa*. As a result, compounds **3** and **9** were indicated the strongest cytotoxic activities. Of the isolated biphenyl compounds, **4** and **5** showed good anti-neuroinflammatory activity through inhibition of NO production in LPS-stimulated BV-2 cells, and triterpene type compounds except compound **11** were also active. Also, we checked the effect of NGF secretion in C6 cells, but only compound **5** had similar activity to the positive control, 6-Shogaol, and the other compounds showed no significant activities. Through this study, it is expected that *C. speciosa* and its active components could be useful for the development of novel anticancer or neuroprotective agents.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2017.04.003.

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