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Article

Flavonoid Glycosides from *Hosta longipes*, Their Inhibition on NO Production, and Nerve Growth Factor Inductive Effects

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Investigação fitoquímica das folhas da *Hosta longipes* identificou um novo flavonóide glicosídeo, o caempferol-3-*O*-β-D-glucopiranosil-(1→2)-[6'''-*O*-acetil-β-D-glucopiranósido]-7-*O*-β-D-glucopiranósido, e mais cinco derivados flavonóides conhecidos. As estruturas de dois compostos foram reveladas por vários métodos de RMN (^{1}H e ^{13}C RMN, ^{1}H-^{1}H COSY, HMQC HMBC) e hidrólise química. Dados de RMN de um deles são publicados pela primeira vez. As atividades biológicas de seis compostos revelaram que cinco inibiram fortemente a produção de óxido nítrico (NO), com valores de IC_{50} de 11,56-15,97 μm em células BV-2 estimuladas por lipopolissacarídeo (LPS), sem toxicidade celular. Dois compostos mostraram indução moderada da secreção no fator de crescimento do nervo (NGF) em linhagem de células C6 de glioma (124,70 ± 7,71% e 117,02 ± 3,60%, respectivamente).

An extended phytochemical investigation of the leaves of *Hosta longipes* identified the new flavonoid glycoside, kaempferol-3-*O*-β-D-glucopyranosyl-(1→2)-[6'''-*O*-acetyl-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside and five known flavonoid derivatives. The structures of two compounds were revealed by extensive NMR methods (^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HMQC and HMBC) and chemical hydrolysis. NMR data of one of them are published for the first time. Bioactivities of six compounds revealed that five strongly inhibited the production of nitric oxide (NO) with IC_{50} values of 11.56-15.97 μm in lipopolysaccharide (LPS)-stimulated BV-2 cells without cell toxicity. Two compounds showed moderate induction of secretion of nerve growth factor (NGF) in C6 glioma cells (124.70 ± 7.71% and 117.02 ± 3.60%, respectively).

Keywords: *Hosta longipes*, flavonoid glycoside, nitric oxide, nerve growth factor

Introduction

More than 8,000 flavonoids have been isolated from plant sources. Flavonoids have a variety of pharmacological effects that include anti-cancer, anti-microbial, anti-oxidant, and anti-inflammatory activities.[1,2] Neuroprotective effects of flavonoids from *Citrus* species are reportedly associated with their anti-inflammatory action, the ability to traverse the blood-brain barrier, and multiple neuroprotective mechanisms.[3] NO and NGF have important roles in neuropathological conditions. These roles include regulation of inflammatory response and recovery of tissue damage in brain injury.[4,5] Regulation of NO production or/and NGF secretion in microglia and astrocytes is a good target for the treatment of neurodegenerative disorders.

As part of our efforts to screen bioactive constituents of Korean medicinal plants with anti-neuroinflammatory activities, we found that the MeOH extract of the leaves of *Hosta longipes* (FR. et SAV.) MATSUMURA (Liliaceae) inhibited NO production in murine microglia BV-2 cells. *H. longipes* is an edible vegetable in Korea and has long been used as a traditional Korean medicine for treating cough, sputum, laryngopharyngitis, and burns.[6,7] Previous phytochemical investigations from this source led to the

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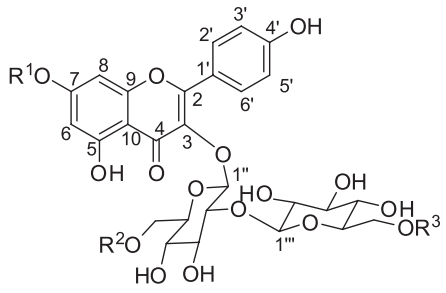
isolation of cytotoxic steroidal saponins.[8,9] Our earlier phytochemical investigation of *H. longipes* resulted in the isolation of steroidal constituents capable of inhibiting NO production.[10] Our continuing research for active constituents in MeOH extracts led to the isolation of six flavonoid glycosides (**1**-**6**) (Figure 1), including one new compound (**1**). All six compounds were tested for their inhibitory effects on NO production in a LPS-activated murine microglial cells and their effects on NGF secretion from C6 glioma cells.



	R^1	R^2	R^3
1	β-D-Glc	CH_3CO	H
2	β-D-Glc	H	(*E*)-feruloyl
3	H	H	H
4	H	α-L-Rha	H
5	β-D-Glc	H	H
6	β-D-Glc	α-L-Rha	H

Figure 1. Structures of compounds **1**-**6** isolated from *H. longipes*.

Experimental

General procedures

Optical rotations were measured on a Jasco P-1020 polarimeter in MeOH (Jasco, Easton, MD). IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany). UV spectra were recorded using a Schimadzu UV-1601 UV-Visible spectrophotometer (Shimadzu, Tokyo, Japan). HR-FAB and ESI mass spectra were obtained on a JEOL JMS700 mass spectrometer (JEOL, Peabody, MA). NMR spectra, including COSY, HMQC, and HMBC experiments were recorded on a Varian UNITY INOVA 500 and 900 NMR spectrometer with chemical shifts given in ppm (Varian, Palo Alto, CA). Preparative high performance liquid chromatography (HPLC) was conducted using a Gilson 306 pump (Gilson, Middleton, WI) with Shodex refractive index detector (Shodex, New York, NY). Silica gel 60 and RP-C_{18} silica gel (230-400 mesh, Merck, Darmstadt, Germany) were used for column chromatography. The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Spots were detected by thin layer chromatography (TLC) under UV light or by heating after spraying with 10% H_2SO_4 in C_2H_5OH (v/v).

Plant materials

Leaves of *H. longipes* were collected in Taebaek City, Korea, in June 2010. The plant was identified by one of the authors (K. R. Lee). A voucher specimen (SKKU-NPL 1103) of the plant has been deposited at the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and isolation

Leaves of *H. longipes* (2.5 kg) were extracted with 80% MeOH at room temperature and filtered. The filtrate was evaporated under reduced pressure to give a MeOH extract (190 g), which was suspended in water (800 mL) and solvent-partitioned to give *n*-hexane (3 g), $CHCl_3$ (14 g), EtOAc (3 g), and BuOH (24 g) layers. The BuOH soluble layer (24 g) was separated over a RP-C_{18} silica gel column (600 g) with a MeOH-H_2O gradient (1:1, 3:1, 5:1, and 1:0, v/v) to give six fractions (Fractions A to F). Fraction A (10 g) was chromatographed on a Diaion HP-20 column (Sigma, St. Louis, MO) eluting with a gradient solvent system of 100% H_2O and 100% MeOH, yielding subfractions A1 and A2. Subfraction A2 (2.3 g) was separated over a silica gel column (40 g) with $CHCl_3$-MeOH-H_2O (2:1:0.2, v/v) to afford nine subfractions (A21-A29). Subfraction A26 (350 mg) was separated on a RP-C_{18} silica gel column (10 g) with 45% MeOH to give five subfractions (A261-A265). Subfraction A262 (50 mg) was purified by RP-C_{18} preparative HPLC with MeOH-H_2O (2:1, v/v) at a flow rate of 2.0 mL/min (Econosil RP-18 10 μm column; 250 × 10 mm; 10 μm particle size; Shodex refractive index detector) to give **1** (3 mg, *Rt* = 13.0 min) and **6** (3 mg, *Rt* = 15.2 min). Compounds **2** (50 mg, MeOH-H_2O, 1:1.5, v/v, *Rt* = 9.2 min) and **4** (40 mg, MeOH-H_2O, 1:1.5, v/v, *Rt* = 11.6 min) were isolated from subfractions A264 and A265, respectively, through HPLC purification. Subfraction A28 (420 mg) was separated over a Sephadex LH-20 (150 g) with MeOH-H_2O (1:1, v/v) to give **5** (200 mg). Fraction B (2.5 g) was separated over a Sephadex LH-20 (150 g) with MeOH-H_2O (4:1, v/v), chromatographed over a silica gel column (30 g) with $CHCl_3$-MeOH-H_2O (2:1:0.2, v/v), and further purified with a silica gel prep. HPLC with $CHCl_3$-MeOH-H_2O (2:1:0.2, v/v) at a flow rate of 2.0 mL min^{-1} (Apollo Silica column; 250 mm × 10 mm i.d., 5 μm, Alltech; Shodex refractive index detector) to yield **3** (6 mg, *Rt* = 13.6 min).

Compound **1**

Yellow gum; $[\alpha]_D^{25}$ –6.5° (*c* 0.5, MeOH); IR (KBr) ν_{max}/cm^{-1} 3729, 3395, 2931, 1656, 1531, 1518, 1240, 1058, 669; UV (MeOH) λ_{max}/nm (log ε) 261 (4.1), 339 (4.5); ^{1}H and ^{13}C NMR (see Table 1); HR-ESI-MS (positive mode) *m/z* 837.2086 $[M + Na]^+$ (calcd for $C_{35}H_{42}NaO_{22}$, 837.2065).

Compound **2**

Yellow gum; $[\alpha]_D^{25}$ –74.0° (*c* 0.5, MeOH); IR (KBr) ν_{max}/cm^{-1} 3729, 3395, 2931, 1656, 1531, 1518, 1240, 1058, 669; UV (MeOH) λ_{max}/nm (log ε) 268 (4.2), 328 (4.0); ^{1}H and ^{13}C NMR (see Table 1); HR-FAB-MS (positive mode) *m/z* 949.2612 $[M + H]^+$ (calcd for $C_{43}H_{49}O_{24}$, 949.2614).

Alkaline hydrolysis of **1** and **2**

A solution of compound **1** (2.0 mg) in 0.1 N KOH (3 mL) was stirred at room temperature for 24 h. The reaction mixture was neutralized with Dowex HCR W2 (H^+ form) and the resin was removed by filtration. A portion of the reaction product was partitioned between $CHCl_3$/H_2O (1 mL each) and kaempferol-3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside-7-*O*-β-D-glucopyranoside (**5**, 1.1 mg) was obtained from the H_2O layer. Through a similar procedure, **5** (1.0 mg) and (*E*)-ferulic acid (0.5 mg) were obtained from H_2O and $CHCl_3$ layer, respectively, from **2** (2.0 mg).

Compound **5**

Yellow gum; ^{1}H NMR (500 MHz, CD_3OD) δ 8.07 (d, 2H, *J* 9.0 Hz), 6.90 (d, 2H, *J* 9.0 Hz), 6.77 (d, 1H, *J* 2.0 Hz), 6.48 (br s, 1H), 5.48 (d, 1H, *J* 7.5 Hz), 5.06 (d, 1H, *J* 7.0 Hz), 4.76 (d, 1H, *J* 7.5 Hz); FAB-MS *m/z* 773.2 $[M + H]^+$.

Acid hydrolysis of **5** and sugar determination

Compound **5** (1.0 mg) was refluxed with 1 mL of 1 N HCl for 4 h at 100 °C. The hydrolysate was extracted with EtOAc and the extract was evaporated *in vacuo* to yield the aglycone kaempferol (0.5 mg) as a yellow gum. The H_2O layer was neutralized by passage through an Amberlite IRA-67 column (Sigma, St. Louis, MO) and was repeatedly evaporated to give D-glucose identified by co-TLC ($CHCl_3$:MeOH:H_2O = 2:1:0.2, R_f value: 0.2) with an authentic sample and optical rotation {$[\alpha]_D^{25}$ +61.0 (c = 0.10, H_2O)}.

Kaempferol

Yellow gum; ^{1}H NMR (500 MHz, CD_3OD) δ 8.08 (d, 2H, *J* 8.5 Hz), 6.90 (d, 2H, *J* 8.5 Hz), 6.39 (br s, 1H), 6.18 (br s, 1H).

Measurement of NO production and cell viability in LPS-activated BV-2 cells

BV-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin. To measure NO production, BV-2 cells were dispensed in wells of a 96-well plate (3×10^4 cells/well). After 24 h, the cells were pretreated with compounds for 30 min and stimulated with 100 ng/mL LPS for 24 h. Nitrite, a soluble oxidation product of NO, was measured in the culture media using the Griess reaction. The supernatant was harvested and mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using an Emax microplate reader (molecular devices). Sodium nitrite was used as a standard to calculate the nitrite concentration. Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. N^G-Monomethyl-L-arginine (L-NMMA), a well-known NO synthase inhibitor, was tested as a positive control.

NGF and cell viability assays

C6 glioma cells were used to measure NGF release into the medium.[11] C6 cells were purchased from the Korean Cell Line Bank and maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin 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% penicillin-streptomycin with 20 μM of each sample for one day. Media supernatant was used for the NGF assay using an ELISA development kit (R&D Systems). Cell viability was assessed by the MTT assay.

Results and Discussion

Leaves of *H. longipes* (2.5 kg) were extracted with 80% MeOH and the extract was partitioned with *n*-hexane, $CHCl_3$, EtOAc, and BuOH. The BuOH layer (24 g) was successively chromatographed over silica gel, Sephadex LH-20, and preparative HPLC to give one new flavonoid glycoside (**1**), together with five known flavonol glycosides (**2**-**6**). The known compounds, kaempferol 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside (**3**),[12] kaempferol 3-*O*-β-D-glucopyranosyl-(1→2)-[α-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (**4**),[13] kaempferol 3-*O*-β-D-[β-D-glucopyranosyl-(1→2)-

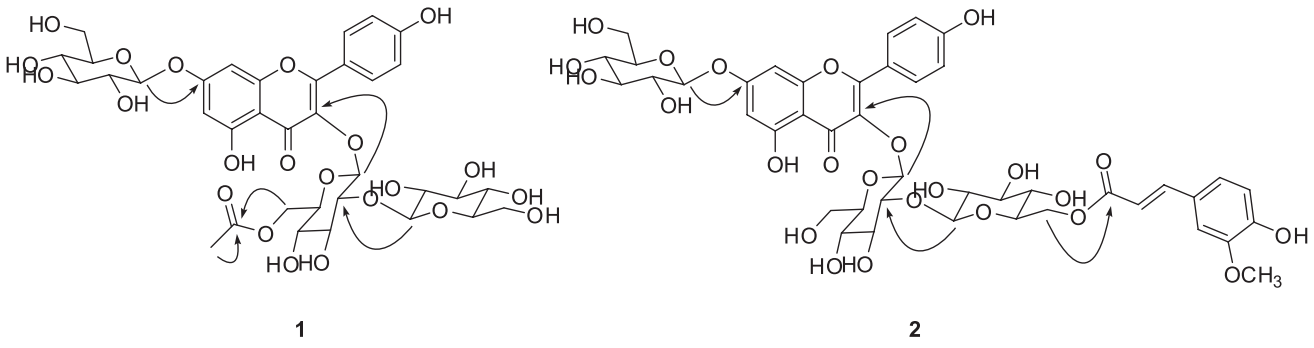


Figure 2. Key HMBC correlations (H→C) of compounds **1** and **2**.

glucopyranoside]-7-*O*-β-D-glucopyranoside (**5**),[14] and kaempferol 3-*O*-(2^G-glucosylrutinoside)-7-*O*-glucoside (**6**)[15] were identified in comparison with previously published data. Although compound **2** (kaempferol-3-*O*-[6-(*E*)-feruloyl]-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside-7-*O*-β-D-glucopyranoside) was previously reported from *Brassica rapa* L. Ssp. c*hinensis* L. (Hanelt.) by LC-MS/MS,[16] NMR spectral data have not been reported. By extensive NMR studies (^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HMQC, and HMBC), full NMR data were assigned for the first time.

Compound **1** was obtained as a yellow gum, whose molecular formula was determined to be $C_{35}H_{42}O_{22}$ from its positive-ion mode HR-ESI-MS data at *m/z* 837.2086 $[M + Na]^+$ (calcd for $C_{35}H_{42}NaO_{22}$, 837.2065). The ^{1}H NMR spectrum of **1** (Table 1) showed two pairs of NMR signals at δ_H 8.05 (d, 2H, *J* 9.0 Hz, H-2' and H-6') and 6.90 (d, 2H, *J* 9.0 Hz, H-3' and H-5'), and δ_H 6.79 (d, 1H, *J* 1.8 Hz, H-8) and 6.50 (d, 1H, *J* 1.8 Hz, H-6), which were characteristic of the B ring and A ring of kaempferol derivatives, respectively. The ^{1}H and ^{13}C NMR data of **1** were very similar to those of **5**,[14] except for presence of an acetyl group resonance [δ_H 1.75 (s, 3H); δ_C 172.4 and 20.5] and up-field shift of H-6'' [δ_H 4.16 (dd, 1H, dd, *J* 11.7, 1.8 Hz) and 4.02 (dd, 1H, *J* 11.7, 5.4 Hz)] and C-6'' (δ_C 64.0) signals. The position of the acetyl group was deduced to be at C-6'' by analysis of the HMBC data showing correlation from H-6'' to C=O (Figure 2). Glucose connectivities of **1** were confirmed by HMBC correlations of H-1''/C-3, H-1'''/C-2'' and H-1''''/C-7 (Figure 2). The *J* values of anomeric protons [δ_H 5.40 (d, 1H, *J* 8.1 Hz, H-1''), 5.06 (d, 1H, *J* 7.2 Hz, H-1''''), 4.77 (d, 1H, *J* 8.1 Hz, H-1''')] of glucose indicated the β-configuration. Alkaline hydrolysis of **1** afforded kaempferol-3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside-7-*O*-β-D-glucopyranoside, which was identified to be **5** by co-TLC ($CHCl_3$:MeOH:H_2O = 1:1:0.2, R_f value: 0.4) with the similar natural compound **5**, ^{1}H NMR and MS.[14] Acid hydrolysis of **5** yielded the aglycone and D-glucose. The aglycone was confirmed as kaempferol by comparison of its ^{1}H NMR data with an authentic sample, whereas D-glucose was identified by co-TLC ($CHCl_3$:MeOH:H_2O = 2:1:0.2, R_f value: 0.2) with an authentic sample and by optical rotation {$[\alpha]_D^{25}$ +61.0 (c = 0.10, H_2O)}. The structure of **1** was thus established as kaempferol-3-*O*-β-D-glucopyranosyl-(1→2)-[6'''-*O*-acetyl-β-D-glucopyranoside]-7-*O*-β-D-glucopyranoside.

Anti-neuroinflammatory activities of the isolated compounds (**1**-**6**) were evaluated by examining NO production in LPS-activated microglia BV-2 cells (Table 2). Among the tested compounds, **1**-**5** significantly inhibited LPS-stimulated NO production with IC_{50} values of 11.56, 14.86, 13.63, 15.97, and 15.30 μM, respectively, which displayed more activity than L-NMMA, a well-known NOS inhibitor. Compound **6** showed moderate activity (29.26 μM) and all isolates (**1**-**6**) had no influence on cell viability (84.29-99.22%) at concentrations up to 20 μM. These results showed that kaempferol derivatives with 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranose unit significantly inhibited LPS-stimulated NO production, while additional substitution of β-D-glucopyranose at C-7 and α-L-rhamnopyranose at C-6'' reduced the activity.

Compounds **1**-**6** were tested using an ELISA development kit for their influence on secretion of NGF from C6 glioma cells into the medium.[17] As shown in Table 3, compounds **4** and **5** were moderate stimulants of NGF release (124.70 ± 7.71% and 117.02 ± 3.60%, respectively) without cell toxicity (97.49% and 89.21% survival, respectively) at a concentration of 20 μM.

Conclusion

From the leaves of *H. longipes* a new flavonoid glycoside (**1**) and five known flavonoid derivatives (**2**-**6**) were isolated by chromatographic methods. The structure of compounds **1** and **2** was revealed by extensive NMR methods (^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HMQC and HMBC) and chemical hydrolysis. The NMR data of compound **2** are published for the first time. Compounds **4** and **5** exhibited significant

Table 1. ^{1}H and ^{13}C NMR data for compounds **1** and **2** in CD_3OD (δ in ppm)

Position	**1**[a]		**2**[b]	
	^{1}H NMR δ (*J* in Hz)	^{13}C NMR δ	^{1}H NMR δ (*J* in Hz)	^{13}C NMR δ
2		159.9		157.6
3		135.0		135.4
4		179.8		180.0
5		162.9		162.6
6	6.50 d (1.8)	100.9	6.36 d (2.0)	100.6
7		164.9		164.4
8	6.79 d (1.8)	95.9	6.42 d (2.0)	95.8
9		158.2		158.9
10		107.6		106.4
1’		122.7		122.5
2’	8.05 d (9.0)	132.5	8.04 d (9.0)	132.8
3’	6.90 d (9.0)	116.3	6.88 d (9.0)	116.4
4’		161.9		161.7
5’	6.90 d (9.0)	116.3	6.88 d (9.0)	116.4
6’	8.05 d (9.0)	132.5	8.04 d (9.0)	132.8
1” (Glc)	5.40 d (8.1)	101.0	5.15 d (7.5)	101.1
2”	3.74 t (8.1)	82.4	3.68 m	85.1
3”	3.60 t (8.1)	77.8	3.54 m	77.7
4”	3.36 m	71.4	3.36 m	71.1
5”	3.35 m	75.6	3.11 m	78.2
6”a	4.16 dd (11.7, 1.8)	64.0	3.62 m	62.4
6”b	4.02 dd (11.7, 5.4)		3.47 m	
1”’ (Glc)	4.77 d (8.1)	104.8	4.71 d (8.0)	106.5
2”’	3.38 m	75.5	3.41 m	76.3
3”’	3.48 m	78.0	3.48 m	77.8
4”’	3.34 m	71.5	3.37 m	72.1
5”’	3.29 m	78.4	3.66 m	75.6
6”’a	3.80 dd (11.7, 1.8)	62.7	4.42 d (4.0)	64.9
6”’b	3.70 m			
1”” (Glc)	5.06 d (7.2)	101.7	5.02 d (7.5)	101.5
2””	3.49 m	74.8	3.51 m	74.9
3””	3.39 m	78.1	3.33 m	78.3
4””	3.40 m	71.3	3.42 m	71.3
5””	3.54 m	78.5	3.55 m	77.8
6””a	3.92 dd (11.7, 1.8)	62.5	3.93 dd (12.0, 2.0)	62.5
6””b	3.71 m		3.73 m	
CH_3	1.75 s	20.5		
CO		172.4		
1””” (Fer)				127.4
2”””			6.74 d (2.0)	111.0
3”””				149.0
4”””				150.3
5”””			6.63 d (8.0)	116.4
6”””			6.64 dd (8.0, 2.0)	123.9
7”””			7.28 d (16.0)	146.7
8”””			5.99 d (16.0)	115.0
9”””				168.9
OCH_3			3.73 s	56.4

[a]900 MHz (^{1}H NMR), 225MHz (^{13}C NMR); [b]500 MHz (^{1}H NMR), 125 MHz (^{13}C NMR).

Table 2. Effects of compounds **1**-**6** and L-NMMA on LPS-induced NO production in BV-2 microglia cells

Compound	IC_{50}[a] / μM	Cell viability[b] / %
1	11.56	98.12 ± 0.51
2	14.86	84.96 ± 0.65
3	13.63	99.22 ± 3.81
4	15.97	91.42 ± 5.36
5	15.30	85.12 ± 3.15
6	29.26	84.29 ± 6.07
L-NMMA[c]	16.23	101.54 ± 3.59

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

Table 3. Effects of compounds **1**-**6** on NGF secretion in C6 cells[a]

Compound	NGF secretion / %	Cell viability[b] / %
1	97.76 ± 6.76	93.07 ± 1.77
2	103.38 ± 0.27	97.18 ± 1.37
3	101.64 ± 1.18	98.06 ± 1.04
4	124.70 ± 7.71	97.49 ± 0.30
5	117.02 ± 3.60	89.21 ± 0.64
6	93.67 ± 1.02	95.26 ± 3.94
6-Sho[c]	129.54 ± 11.23	98.12 ± 3.27

[a]C6 cells were treated with 20 μM of compounds **1**-**6**. After 24 h, the content of NGF secretion in C6-conditioned media was measured by ELISA. The level of secreted NGF cells is expressed as percentage of the untreated control. The data shown represent the means ± SD of three independent experiments performed in triplicate; [b]cell viability after treatment with 20 μM of each compound was determined by MTT assay and is expressed in percentage (%). The results are averages of three independent experiments, and the data are expressed as mean ± SD; [c]6-Shogaol as positive control.

anti-neuroinflammatory activity by suppressing the release of NO in LPS-stimulated microglial cells and by inducing NGF secretion in C6 glioma cells. These results suggest that these compounds might be promising candidates for treatment of Alzheimer’s disease, Parkinson’s disease, and other neurodegenerative diseases.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as a PDF file.

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