

RESEARCH ARTICLE

Constituents of *Limonia acidissima* inhibit LPS-induced nitric oxide production in BV-2 microglia

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Abstract

The ethyl acetate (EtOAc) soluble fraction of the 85% ethanol (EtOH) extract of the dried bark of *Limonia acidissima* potently inhibited nitric oxide (NO) production in lipopolysaccharide (LPS) activated BV-2 cells, a microglial cell line. Bioassay-guided column chromatography separation afforded a new stereoisomer of neolignan, (7'E)-(7R,8S)-4-hydroxy-3,5'-dimethoxy-4',7'-epoxy-8,3'-neolig-7'-en-9,9'-diyl diacetate (**1**), together with two known lignans, (+)-yangambin (**2**) and (+)-syringaresinol (**3**), three known triterpenoids, hederatriol (**4**), basic acid methyl ester (**5**), and 3β-hydroxyolean-12-en-11-one (**6**), and four known fatty acid derivatives, cascarillic acid (**7**), (+)-α-dimorphecolic acid (**8**), 8(R)-hydroxylinoleic acid (**9**), and (6Z,9Z,12Z)-pentadecatrienoic acid (**10**). The structure of the new compound **1** was elucidated by detailed analysis of spectroscopic data and circular dichroism (CD) spectroscopy. Compounds **1**, **3-6**, and **8-10** isolated from *L. acidissima* significantly reduced NO production in LPS-stimulated BV-2 microglia cells.

Keywords: *Limonia acidissima*; neoligna; BV-2 cells; nitric oxide

Introduction

Microglia are the primary immune cells in the central nervous system and mediate inflammation-mediated neurotoxicity [1]. Under normal conditions microglia act as immune surveillance; however, activated microglia secrete excessive pro-inflammatory substances such as nitric oxide (NO), tumour necrosis factor-α, interleukin-1β, and prostaglandins [2,3]. The NO produced by inducible nitric oxide synthase is a primary pro-inflammatory mediator and plays an important role in neuroinflammatory diseases [4].

Thus, in the course of our continuing search for biologically active compounds from natural medicinal sources, we investigated the inhibitory constituents from dried bark of *Limonia acidissima* L. on NO production in lipopolysaccharide (LPS)-stimulated microglia cells, as the EtOAc-soluble fraction of the 85% EtOH extract of this herb inhibited NO production.

L. acidissima, or wood apple, belongs to the family of Rutaceae. Various parts of this plant have medicinal properties; the fruit pulp has been used for a remedy for insect bites [5] and thanaka, a root paste made from its pulp, is used as a facial cosmetic to remove small spots and lesions [6]. Various constituents, including coumarins, steroids, triterpenoids, benzoquinones, and tyramine derivatives have been isolated from different parts of this plant [7–12]. We also reported that benzamide derivatives and coumarins from *L. acidissima* inhibit NO production [13,14].

In the present study, a bioassay-guided column chromatographic separation of *L. acidissima* led to the isolation of a new stereoisomer of neolignan (**1**), (7'E)-(7R,8S)-4-hydroxy-3,5'-dimethoxy-4',7'-epoxy-8,3'-neolig-7'-en-9,9'-diyl diacetate, together with nine known compounds (**2-10**). Furthermore, the ability of all ten isolated compounds (**1-10**) to inhibit NO production was evaluated in LPS-activated BV-2 cells, a microglial cell line.

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Materials and methods

General experimental procedures

Optical rotations were measured on a Jasco P-1020 polarimeter (Jasco, Easton, MD, USA) in MeOH. Circular dichroism (CD) spectra were measured on a JASCO J-715 spectropolarimeter (Bruker, Karlsruhe, Germany). IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer (Shimadzu, Tokyo, Japan). UV spectra were recorded using a Shimadzu UV-1601 UV-Visible spectrophotometer (JEOL, Peabody, MA, USA). Fast atom bombardment (FAB) and high resolution fast atom bombardment (HRFAB) mass spectra were obtained on a JEOL JMS700 mass spectrometer (JEOL, Peabody, MA, USA). Nuclear magnetic resonance (NMR) spectra, including ^1H - ^1H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correlation (HMBC), and nuclear Overhauser effect spectroscopy (NOESY) experiments, were recorded on a Varian UNITY INOVA 500 NMR spectrometer (Varian, Palo Alto, CA, USA) operating at 500 MHz (^1H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). Preparative HPLC was conducted using a Gilson 306 pump (Gilson, Middleton, WI, USA) with a Shodex refractive index detector (Shodex, New York, NY, USA) and an Apollo Silica 5 μ column (250 \times 10 mm; Alltech, Nicholasville, KY, USA). Silica gel 60 (Merck, Darmstadt, Germany; 70-230 mesh and 230-400 mesh) was used for column chromatography. The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Spots were detected on thin layer chromatography (TLC) under UV light or by heating after spraying with 10% H_2SO_4 in $\text{C}_2\text{H}_5\text{OH}$ (v/v).

Plant materials

The dried bark of *L. acidissima* was imported from Yangon, Union of Myanmar, in October 2006, and identified by Dr W. Bae, researcher of the R & D Institute, Miwon Commercial (Ansan, Korea) and a voucher specimen (SKKU 2006-10) was deposited at the R & D Institute, Miwon Commercial, Co., Ltd, Ansan, Korea.

Extraction and isolation

The dried bark (3 kg) of *L. acidissima* was extracted with 85% EtOH three times at 85°C. The resulting ethanol extract (250 g) was suspended in distilled water (7.2 L) and then partitioned with EtOAc, yielding an EtOAc soluble extract (50 g). The EtOAc-soluble fraction (50 g) was separated over a silica gel column (800 g, 10 \times 80 cm), eluted with a gradient of *n*-hexane-EtOAc (10:1, 5:1, 1:1, and 0:1, v/v) to afford 12 fractions (fractions A to L). The active fraction H (810 mg) was separated further over a silica gel column (80 g, 3 \times 40 cm) using CHCl_3 -MeOH (17:1, v/v) to give three sub-fractions (fractions H1 to H3). Fraction H2 (450 mg) was separated over a Sephadex LH-20 column (Pharmacia, 150 g, 3 \times 80 cm), using a solvent system of CH_2Cl_2 -MeOH (1:1, v/v) to yield three sub-fractions (fractions H21 to H23). Fraction H22 (70 mg) was separated by preparative HPLC, over 30 min at a

flow rate of 2 mL/min (Apollo Silica 5 μ column; 250 \times 10 mm; 5 μ particle size; Shodex refractive index detector; *n*-hexane-EtOAc, 1:1, v/v) to obtain compound **1** (8 mg, retention times (*Rt*)=17.5). Compounds **5** (5 mg, *n*-hexane-EtOAc, 1:1, v/v), **6** (18 mg, *n*-hexane-EtOAc, 1:1, v/v), and **7** (5 mg, *n*-hexane-EtOAc, 1:1, v/v) were isolated from fraction H22, and compound **8** (5 mg, MeOH- H_2O , 4:1, v/v) was isolated from fraction H3 through HPLC purification. The active fraction I (760 mg) was separated over a silica gel column (80 g, 3 \times 40 cm) using CHCl_3 -MeOH (15:1, v/v) to give three subfractions (fractions I1 to I3). Compound **2** (8 mg, MeOH- H_2O , 1:1, v/v) was isolated from fraction I2 and compounds **4** (7 mg, MeOH- H_2O , 1:0, v/v), **9** (4 mg, MeOH- H_2O , 19:1, v/v), and **10** (3 mg, MeOH- H_2O , 19:1, v/v) were isolated from fraction I3 through HPLC purification. Compound **3** (10 mg) was isolated from subfraction of active fraction J (2.7 g) by HPLC purification using MeOH- H_2O (9:11, v/v).

Compound (1): Amorphous gum (8 mg); $[\alpha]_D^{25}$ -60.5° (C=0.4, MeOH); IR (KBr) ν_{max} 3729, 3395, 2931, 1656, 1531, 1518, 1240, 1058, 669 cm^{-1} ; UV (MeOH) λ_{max} 278 (4.1), 303 (3.5) 345 (3.0) nm; CD (MeOH) $[\theta]_{234}^{25}$ +5300, $[\theta]_{265}^{25}$ +3200, $[\theta]_{287}^{25}$ - 5700; ^1H and ^{13}C NMR (see Table 1). HRFABMS (positive mode) *m/z* 443.1701 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{24}\text{H}_{27}\text{O}_8$, 443.1706).

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

BV-2 microglia cells were stimulated with 100 ng/mL LPS in the presence or absence of samples for 24 h. Nitrite in

Table 1. ^1H and ^{13}C NMR data for compound **1** (δ in ppm, 500 MHz for ^1H and 125 MHz for ^{13}C , in CDCl_3).

C-atoms	^1H -NMR δ (J in Hz)	^{13}C -NMR δ
1		132.4
2	6.88 d (1.5)	108.8
3		146.9
4		144.6
5	6.86 d (8)	114.2
6	6.85 dd (1.5, 8)	119.9
7	5.47 d (7.5)	89
8	3.78 m	50.5
9	4.28 dd (7.5, 11.5)	65.4
	4.42 dd (5.5, 11.5)	
1'		130.7
2'	6.86 ^a s	110.8
3'		143.4
4'		148.5
5'		128.4
6'	6.87 ^a s	115.5
7'	6.58 d (15.5)	134.6
8'	6.16 dd (6.5, 15.5)	122
9'	4.71 d (6.5)	65.5
CH_3	3.90 s	56.1
OCH_3 '	3.87 s	56.2
OAc	2.02 s	171
		21
OAc'	2.10 s	171.1
		21.2

^aInterchangeable signals.

the culture media, a soluble oxidation product of NO, was measured by a Griess reaction. The supernatant (50 μ L) 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 a microplate reader (Emax, Molecular Device, Sunnyvale, CA, USA). Sodium nitrite was used as a standard to calculate the nitrite concentration [15]. Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [16]. *N*^G-monomethyl-L-arginine (L-NMMA, Sigma, St. Louis, MO, USA), a well-known NOS inhibitor, was tested as a positive control.

Statistical analysis

The data were analysed using Statistical Analysis System software (PRISM, GraphPad, La Jolla, CA, USA). Data are expressed as mean \pm SEM. Statistical comparisons between the different treatments were performed using one-way ANOVA with Tukey's multiple comparison post test and the data were considered to be statistically significant if the probability had a value of less than 0.05.

Results and discussion

The dried bark of *L. acidissima* was extracted with 85% EtOH and the EtOH extract was partitioned with EtOAc. The EtOAc-soluble fraction inhibited LPS-induced NO production and was subjected to silica gel open-column chromatography to afford 12 fractions (fractions A to L).

Fractions H, I, and J inhibited LPS-induced NO production in a concentration-dependent manner (Figure 1). Isolation from these three fractions afforded a new stereoisomer of neolignan (**1**), (7'*E*)-(7*R*,8*S*)-4-hydroxy-3,5'-dimethoxy-4',7-epoxy-8,3'-neolig-7'-en-9,9'-diyl diacetate, together with nine known compounds (**2-10**) (Figure 2). Two known lignans, (+)-yangambin (**2**) [17] and (+)-syringaresinol (**3**) [18], three known triterpenoids, hederatriol (**4**) [19], basic acid methyl ester (**5**) [20], and 3 β -hydroxyolean-12-en-11-one (**6**) [21], and four known fatty acid derivatives, cascarillic acid (**7**) [22], (+)- α -dimorphecolic acid (**8**) [23], 8(*R*)-hydroxylinoleic acid (**9**) [24], and (6*Z*,9*Z*,12*Z*)-pentadecatrienoic acid (**10**) [25] were identified by comparison with published data. This is the first report of these compounds in this plant, except for compound **3**.

Compound **1** was obtained as an amorphous gum with optical rotation; $[\alpha]_D^{25}$ -60.5° (C=0.4, MeOH). The molecular formula was determined to be C₂₄H₂₆O₈ by high-resolution positive fast atom bombardment ionisation mass spectroscopy (HRFABMS) at *m/z* 443.1701 [M + H]⁺ (calcd for C₂₄H₂₇O₈, 443.1706), together with the ¹H and ¹³C NMR data (Table 1). The IR spectrum exhibited the presence of hydroxyl (3395 cm⁻¹), carboxyl (1656 cm⁻¹) and aromatic (1531 and 1518 cm⁻¹) groups. The UV spectrum of **1** showed absorption maxima at 278, 303, and 345 nm in MeOH. The ¹H NMR spectrum of **1** (Table 1) displayed signals for the presence of a 3,4-disubstituted aromatic ring [δ_H 6.85 (1H, dipole-dipole (dd), *J* = 1.5, 8.0 Hz), 6.86 (1H, d, *J* = 8.0 Hz), and 6.88 (1H, d, *J* = 1.5 Hz)] and a set of *meta*-coupled signals at δ_H 6.86 (1H, s) and 6.87 (1H, s). The ¹H NMR and

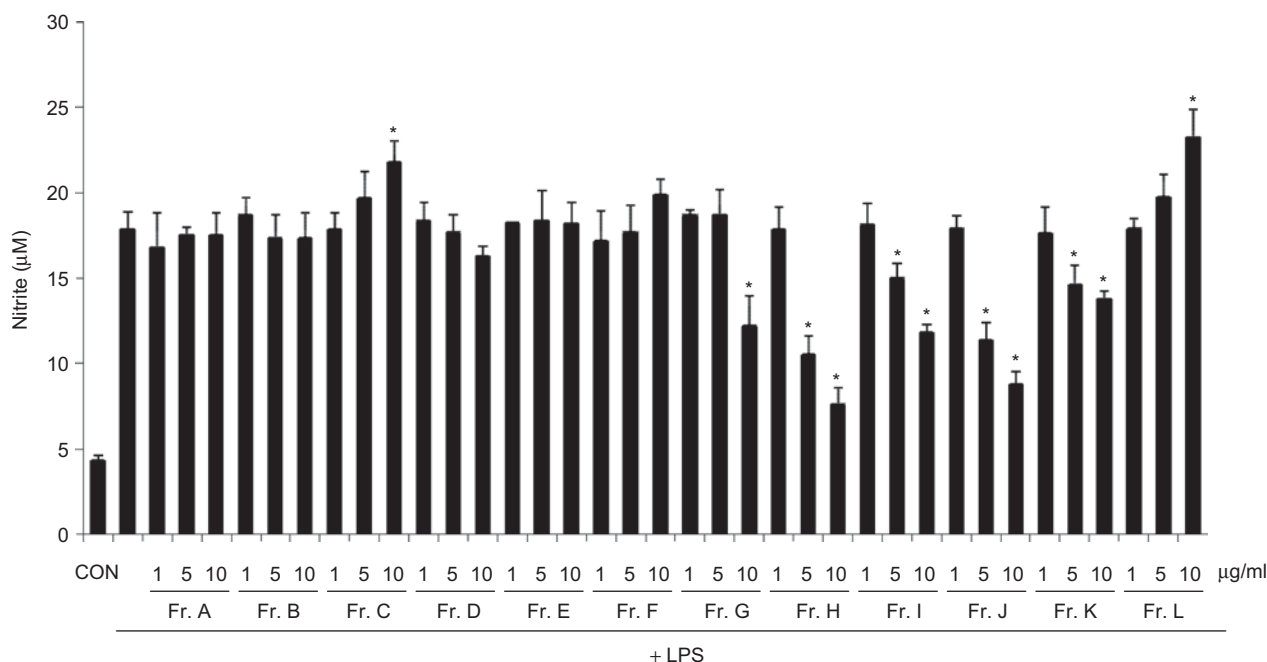


Figure 1. Effects of *Limonia acidissima* fractions (Fr. A to Fr. L) on LPS-induced NO production in BV-2 microglia cells. BV-2 cells were incubated in the absence (CON, control) or presence of LPS (100 ng/mL). Cells were pre-treated with the fractions (1, 5 and 10 μ g/mL) for 30 min, and then stimulated with LPS (100 ng/mL) for 24 h. The culture medium was then collected for a nitrite assay. Nitrite was measured using a Griess reaction and sodium nitrite was used as a standard. All data are presented as the mean \pm SEM of three independent experiments. **p* < 0.05 indicates significant difference compared to LPS alone.

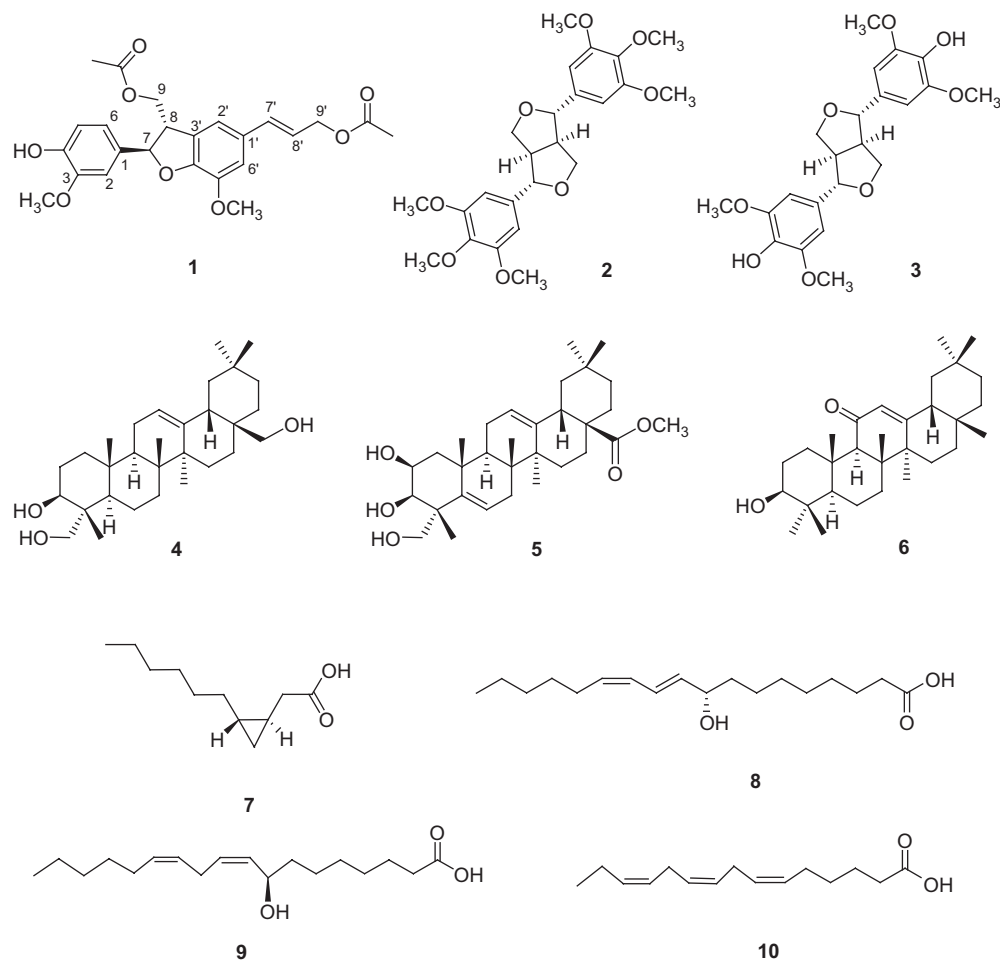


Figure 2. Structures of compounds 1-10 isolated from *L. acidissima*.

^1H - ^1H COSY spectrum also showed signals attributable to *trans* olefinic protons [δ_{H} 6.16 (1H, dd, $J=6.5, 15.5$ Hz), and 6.58 (1H, d, $J=15.5$ Hz)], two acetoxy-methylenes [δ_{H} 4.28 (1H, dd, $J=7.5, 11.5$ Hz), 4.42 (1H, dd, $J=5.5, 11.5$ Hz), and 4.71 (2H, d, $J=6.5$ Hz)], two methines [δ_{H} 3.78 (1H, m), and 5.47 (1H, d, $J=7.5$ Hz)], and two methoxy groups [δ_{H} 3.87 (3H, s), and 3.90 (3H, s)]. In addition, the signals of two additional acetyl protons at δ_{H} 2.02 (3H, s), and 2.10 (3H, s) were observed. The ^{13}C NMR spectrum also contained 18 skeletal carbon resonances. These spectral features indicated that **1** was a dihydro[*b*]benzofuran-type neolignan formed by two coniferyl alcohol acetate molecules [26,27]. Detailed analysis of COSY and HMBC correlations (Figure 3) reconfirmed the skeletal structure of **1**. The methoxy groups were determined to be at C-3 and C-3', which was confirmed by the HMBC correlations of the methoxy protons at δ_{H} 3.90 with C-3 (δ_{C} 56.1), and at δ_{H} 3.87 with C-3' (δ_{C} 56.2). The HMBC correlations of acetoxy-methylene protons at δ_{H} 4.28/4.42 (H-9) with an acetyl group (δ_{C} 171), and at δ_{H} 4.71 (H-9') with an acetyl group (δ_{C} 171.1) indicated the two acetyl groups were at C-9 and C-9'.

A coupling constant (7.5 Hz) between H-7 and H-8 implied the *trans*-configuration between these protons [26]. This arrangement was verified by a lack of NOESY correlations between these protons but NOESY

correlations between H-7 and H-9 and between H-8 and H-2/H-6 (Figure 3). The absolute configuration of C-7 and C-8 were confirmed as 7*R* and 8*S*, respectively, by the CD spectrum showing the positive Cotton effect at 234 nm, the positive Cotton effect at 265 nm, and the negative Cotton effect at 287 nm, and the optical rotation ($[\alpha]_{\text{D}}^{25} -60.5^\circ$) value [27,28]. For biogenesis of 8-5' neolignans, ring closure will always proceed the formation of the *trans*-form due to the more stable *trans* configuration of the C-7/C-8 bond [29]. Accordingly, compound **1** was identified as (7'*E*)-(7*R*,8*S*)-4-hydroxy-3,5'-dimethoxy-4',7'-epoxy-8,3'-neolig-7'-en-9,9'-diyl diacetate. The diastereoisomer of **1** was reported from the roots of *Lasiolaena morii* [30], which was reassigned to β form for H-7 (7*S*) and α form for H-8 (8*R*) as relative-configuration by Li et al. [29]. But the absolute stereochemistry of **1** was assigned by CD data, and **1** was the first example isolated as the 7*R*, 8*S*-form in nature.

We evaluated whether compounds (**1-10**) could inhibit NO production in LPS-activated BV-2 microglia cells (Table 2). Compounds **1**, **3-6**, and **8-10** dose-dependently inhibited LPS-stimulated NO production, but compounds **2** and **7** (up to 10 μM) were not active. Compounds **1**, **4**, **8**, **9** and **10** were more active than *N*^G-monomethyl-L-arginine (L-NMMA), a NOS inhibitor. The IC_{50} values of **1**, **4**, **8**, **9** and

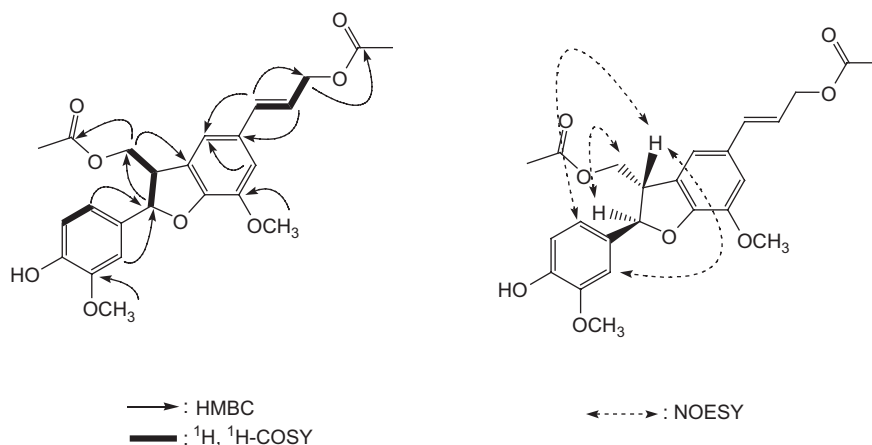


Figure 3. Key HMBC, ^1H , ^1H -COSY, and NOESY correlations of compound 1.

Table 2. Effects of compounds 1-10 and L-NMMA on LPS-induced NO production in BV-2 microglia cells.

Compounds	Inhibition (%) a 5 μM	Inhibition (%)10 μM	IC $_{50}$ (μM)
1	38.5	58.6	8.08
2	na	na	-
3	48.3	50.2	9.58
4	35.6	69.6	7.28
5	8.46	51.3	9.85
6	7.42	45.0	10.7
7	na	na	-
8	29.4	56.1	8.82
9	40.8	68.1	6.73
10	38.3	66.4	7.01
L-NMMA	29.3	51.2	9.50

a Values mean the inhibition of NO production relative to the LPS control (n=3); na, not active.

10 were 8.08, 7.28, 8.82, 6.73, and 7.01 μM , respectively. These results suggest that *Limonia acidissima* and its constituents may be good leads for the development of novel neuroprotective agents.

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Declaration of interest

The authors report no conflicts of interest.

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