FULL PAPER

Neolignans from the Fruits of *Magnolia obovata* Inhibit NO Production and Have Neuroprotective Effects

by **Kyeong-Hwa Seo**[a]), **Dae-Young Lee**[b]), **Jae-Woo Jung**[a]), **Dong-Sung Lee**[c]), **Youn-Chul Kim**[d]), **Youn-Hyung Lee**[e]), and **Nam-In Baek***[a])

[a]) Department of Oriental Medicine Biotechnology, Kyung Hee University, Yongin 17104, Republic of Korea (phone: +82-31-201-2661; fax: +82-31-204-8116; e-mail: nibaek@khu.ac.kr)
[b]) Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, RDA, Eumseong 27709, Republic of Korea
[c]) College of Pharmacy, Chosun University, Dong-gu, Gwnagju 61452, Republic of Korea
[d]) College of Pharmacy, Wonkwang University, Iksan 54538, Republic of Korea
[e]) Department of Horticultural Biotechnology, Kyung Hee University, Yongin 17104, Republic of Korea

One new sesquineolignan, obovatalignan A (**1**), and one new neolignan, obovatalignan B (**2**), were isolated from the *Magnolia obovata* fruits. Their chemical structure, including absolute configuration, was determined based on various spectroscopic methods, such as HR-EI-MS, 1D-NMR (^{1}H, ^{13}C, DEPT), 2D-NMR (gCOSY, gHSQC, gHMBC, NOESY), and CD spectroscopy. The compounds were evaluated for protective effects against glutamate-induced oxidative stress in HT22-immortalized hippocampal cells and inhibitory activity against NO production in LPS-induced RAW 264.7 cells. Compounds **1** and **2** exhibited protective effects against glutamate-induced oxidative stress with EC_{50} values of 18.1 ± 1.23 and 7.10 ± 0.78 μM, respectively, as well as inhibitory effects on NO production with IC_{50} values of > 30.0 and 8.22 ± 2.01 μM, respectively.

Keywords: HT22, Neuroprotection, NO production, Obovatalignan, RAW 264.7.

Introduction

Magnolia obovata THUNB. (Magnoliaceae) is a deciduous tree widely distributed in East Asia. Its fruits, called 'Hoo-Bahk-Ja' in Korea, have been traditionally used as a digestive aid and a tonic in oriental herbal medication [1]. Additionally, *M. obovata* exhibits anti-inflammatory [2], cytotoxic [3], and antiplatelet [4] activities. Our previous phytochemical investigation into the fruits of *M. obovata* led to the isolation of new neolignans [2]. Our ongoing search for secondary metabolites of this plant yielded two further new neolignans. The fruits of *M. obovata* were extracted with aqueous MeOH and the concentrated extract was partitioned into AcOEt, *n*-BuOH, and H_2O fractions. After repeated SiO_2 and ODS column chromatography for the AcOEt-soluble fraction, one new sesquineolignan and one new neolignan were isolated. Their chemical structures were identified based on spectroscopic data (*Fig. 1*). In addition, we evaluated the neuroprotective effects of the compounds against glutamate-induced neurotoxicity in the mouse hippocampal HT22 cell line and also examined the inhibition effect on NO production in LPS-induced RAW 264.7 macrophage cells.

Results and Discussion

The molecular formula of compound **1** was determined to be $C_{29}H_{30}O_7$ from a molecular ion peak *m*/*z* 490.1991 (calc. for $C_{29}H_{30}O_7$, 490.1992) in the HR-EI-MS. Optical rotation showed dextrorotatory characteristics, $[\alpha]_D$ = +6.5 (*c* = 0.11). The IR spectrum showed absorption bands at 3357, 2921, 1591, and 1504 $cm^{-1,}$ suggesting the presence of OH and aromatic ring functionalities. Compound **1** showed ^{1}H- and ^{13}C-NMR spectra similar to those of magnolianin, which is a sesquineolignan composed of one obovatol and one magnolol moiety [5]. The ^{1}H-NMR spectrum (*Table 1*) showed four H–C (sp^2) signals at δ (H) 7.11 (*d*, *J* = 8.4 Hz, H–C(2″, 6″)) and 6.92 (*d*, *J* = 8.4 Hz, H–C(3″, 5″)) due to a *para*-substituted benzene ring and two *meta*-coupled H–C (sp^2) signals at δ(H) 6.64 (*d*, *J* = 2.0 Hz, H–C(2′)) and 6.48 (*d*, *J* = 2.0 Hz, H–C(6′)) due to a tetrasubstituted benzene ring. In addition, two overlapping H–C (sp^2) signals at δ(H) 5.97 – 5.92 (*m*, H–C(8′)) and 5.93 – 5.88 (*m*, H–C(8″)) and two *exo*-CH_2 (sp^2) signals δ(H) 5.07 – 5.02 (*m*, H_a–C(9′)) and 5.05 – 5.01 (*m*, H_b–C(9″)) were also observed. In the upfield region, two allyl–CH_2 signals at δ(H) 3.34 (*d*, *J* = 6.4 Hz, CH_2(7″)) and 3.24 (*d*, *J* = 6.8 Hz, CH_2(7′)) were

DOI: 10.1002/hlca.201500101

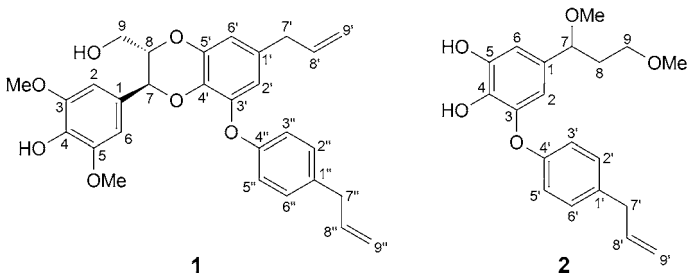

Fig. 1. Structures of compounds **1** and **2**.

Table 1. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) data of compounds **1** and **2** (in CD_3OD, δ in ppm, J in Hz)

No.	**1**		No.	**2**	
	δ(H)	δ(C)		δ(H)	δ(C)
1		127.0	1		134.3
2	6.62 (*s*)	104.2	2	6.65 (br. *s*)	109.0
3		147.3	3		144.8
4		135.4	4		134.1
5		147.3	5		144.0
6	6.62 (*s*)	104.2	6	6.38 (br. *s*)	108.5
7	4.88 (*d*, *J* = 8.4)	76.5	7	4.07 (*dd*, *J* = 6.4, 6.0)	80.2
8	3.95 (*ddd*, *J* = 8.4, 4.0, 2.4)	78.2	8a	2.00 (*dd*, *J* = 13.6, 6.4)	37.9
			8b	1.75 (*dd*, *J* = 13.6, 6.0)	
9a	3.65 (*dd*, *J* = 12.4, 2.4)	61.3	9a	3.46 (overlapped)	69.1
9b	3.43 (*dd*, *J* = 12.4, 4.0)		9b	3.35 (overlapped)	
1′		133.0	1′		135.5
2′	6.64 (*d*, *J* = 2.0)	113.6	2′	7.14 (*d*, *J* = 8.4)	129.9
3′		144.9	3′	6.93 (*d*, *J* = 8.4)	118.1
4′		133.6	4′		154.7
5′		144.5	5′	6.93 (*d*, *J* = 8.4)	118.1
6′	6.48 (*d*, *J* = 2.0)	112.7	6′	7.14 (*d*, *J* = 8.4)	129.9
7′	3.24 (*d*, *J* = 6.8)	39.5	7′	3.43 (*d*, *J* = 6.8)	39.4
8′	5.97 – 5.92 (*m*)	136.9	8′	5.97 – 5.90 (*m*)	137.2
9′	5.07 – 5.02 (*m*)	116.0	9′	5.09 – 5.04 (*m*)	115.9
1″		134.3	7-MeO	3.25 (*s*)	56.4
2″	7.11 (*d*, *J* = 8.4)	129.5	9-MeO	3.13 (*s*)	58.6
3″	6.92 (*d*, *J* = 8.4)	117.1			
4″		156.2			
5″	6.92 (*d*, *J* = 8.4)	117.1			
6″	7.11 (*d*, *J* = 8.4)	129.5			
7″	3.34 (*d*, *J* = 6.4)	39.3			
8″	5.93 – 5.88 (*m*)	137.5			
9″	5.05 – 5.01 (*m*)	115.7			
3,5-MeO	3.88 (*s*)	56.4			

observed. Taken together, the above data suggested that compound **1** included one molecule of obovatol. Two H–C (sp^2) signals at δ(H) 6.62 (*s*, H–C(2, 6)) due to a symmetrically tetrasubstituted benzene ring, two CH–O signals at δ(H) 4.88 (*d*, *J* = 8.4 Hz, H–C(7)) and 3.95 (*ddd*, *J* = 8.4, 4.0, 2.4 Hz, H–C(8)), a CH_2–O signal at δ(H) 3.65 (*dd*, *J* = 12.4, 2.4 Hz, H_a–C(9)) and 3.43 (*dd*, *J* = 12.4, 4.0 Hz, H_b–C(9b)), and two MeO signals at δ (H) 3.88 (*s*, MeO–C(3,5)) were observed. The deshielded doublet corresponding to a benzylic CH–O signal at δ(H) 4.88 (H–C(7)) and the doublet of CH–O signal at δ(H) 3.95 (H–C(8)) indicated the linkage of two phenylpropanoid units *via* a 1,4-dioxane ring system [6][7]. Taken together, the above data suggested that **1** was a benzodioxane-type sesquineolignan composed of an obovatol and a syringyl glycerol unit. The ^{13}C-NMR spectrum (*Table 1*) showed 29 C-atom signals including those of MeO signals δ(C) 56.4 (MeO–C(3, 5)), confirming **1** as a sesquineolignan. In the downfield region, seven O–C (sp^2) signals at δ(C) 156.2 (C(4″)), 147.3 (C(3, 5)), 144.9 (C (3′)), 144.5 (C(5′)), 135.4 (C(4)), and 133.6 (C(4′)), three C (sp^2) signals at δ(C) 134.3 (C(1″)), 133.0 (C(1′)), and

127.0 (C(1)), 10 H–C (sp^2) signals at δ(C) 137.5 (C(8″)), 136.9 (C(8′)), 129.5 (C(2″, 6″)), 117.1 (C(3″, 5″)), 113.6 (C(2′)), 112.7 (C(6′)), and 104.2 (C(2, 6)), and two *exo*-CH_2 (sp^2) signals at δ(C) 116.0 (C(9′)) and 115.7 (C(9″)) were observed. Two CH–O signals at δ(C) 78.2 (C(8)) and 76.5 (C(7)), one CH_2–O signal at δ(C) 61.3 (C(9)), and two allyl–CH_2 signals at δ(C) 39.5 (C(7′)), 39.3 (C(7″)) were also observed. Consequently, the above data were in agreement with ^{1}H-NMR data for the presence of three C_6C_3 units linked through ether O atoms. In addition, the gCOSY spectrum showed the correlation of δ(H) H–C(7)/H–C(8) and H–C(8)/H–C(9). In the gHMBC spectrum, the correlation peaks between H–C(2, 6)/C(1), C(2), C(3), C(4), C(5), C(6), and C(7), and between H–C(7)/C(1), C(2), C(6), and C(8) confirmed the presence of a syringyl glycerol unit. The H-atom signals of two MeO groups showed a nuclear *Overhauser* effect (NOE) with H–C(2) and H–C(6), which also showed NOE with H–C(8) and H–C(7), respectively, indicating the MeO groups to be placed at C(3) and C(5) (*Fig. 2*). The *threo*-configuration at C(7) and C(8) of the dioxane ring was deduced from the large coupling constant (J = 8.4 Hz). Based on the positive CD *Cotton* effect at 238 nm, **1** was assigned the absolute configuration 7*S*,8*S* [8]. Hence, compound **1** was determined to be (2*S*,3*S*)-2,3-dihydro-3-(4-hydroxy-3,5-dimethoxyphenyl)-7-(2-propen-1-yl)-5-[4-(2-propen-1-yl)phenoxy]-1,4-benzodioxin-2-methanol, and was named obovatalignan A (**1**).

The molecular formula of compound **2** was determined to be $C_{20}H_{24}O_5$ from a molecular ion peak *m*/*z* 344.1623 (calcd. for $C_{20}H_{24}O_5$, 344.1624) in the HR-EI-MS. The IR spectrum showed absorption bands at 3357, 2928, 1600, and 1504 cm^{-1}, suggesting the presence of hydroxyl and aromatic ring functionalities. The ^{1}H-NMR spectrum (*Table 1*) showed four H–C (sp^2) signals at δ(H) 7.14 (*d*, J = 8.4 Hz, H–C(2′, 6′)), 6.93 (*d*, J = 8.4 Hz, H–C(3′, 5′)) due to a *para*-substituted benzene ring and two *meta*-coupled H–C (sp^2) signals at δ(H) 6.65 (br. *s*, H–C(2)), 6.38 (br. *s*, H–C(6)) due to an unsymmetrically tetrasubstituted benzene ring, one overlapping H–C (sp^2) signal at δ(H) 5.97 – 5.90 (*m*, H–C(8′)), and one *exo*-CH_2 (sp^2) signal at δ(H) 5.09 – 5.04 (*m*, H_a–C(9′) and H_b–C(9′)). In addition, one CH–O signal at δ(H) 4.07 (*dd*, J = 6.4, 6.0 Hz, H–C(7)), one CH_2-O signal at δ(H) 3.46 (overlapped, H_a–C(9)), 3.35 (overlapped, H_b–C(9)), and two MeO signals at δ(H) 3.25 (*s*, MeO–C(7)), 3.13 (*s*, MeO–C(9)) were also observed. In the upfield region, one allyl–CH_2 signal at δ(H) 3.43 (*d*, J = 6.8 Hz, CH_2(7′)) and one CH_2 signal at δ(H) 2.00 (*dd*, J = 13.6, 6.4 Hz, H_a–C(8)), 1.75 (*dd*, J = 13.6, 6.0 Hz, H_b–C(8)) were observed. These findings suggested that **2** contained two propylbenzene units. The ^{13}C-NMR spectrum (*Table 1*) showed 20 C-atom signals including two MeO signals δ(C) 58.6 (*s*, (MeO–C(9)), 56.4 (*s*, MeO–C(7)) confirming **2** as a neolignan. In the downfield region, four O–C (sp^2) signals at δ(C) 154.7 (C(4′)), 144.8 (C(3)), 144.0 (C(5)), and 134.1 (C(4)), two C (sp^2) signals at δ(C) 135.5 (C(1′)) and 134.3 (C(1)), seven H–C (sp^2) signals at δ(C) 137.2 (C(8′)), 129.9 (C(2′, 6′)), 118.1 (C(3′, 5′)), 109.0 (C(2)), and 108.5 (C(6)), and one exo-CH_2 (sp^2) signal at δ(C) 115.9 (C(9′)) were observed. One CH–O signal at δ(C) 80.2 (C(7)), one CH_2–O signal at δ(C) 69.1 (C(9)), one allyl–CH_2 signal at δ(C) 39.4 (C(7′)), and one CH_2 signal at δ(C) 37.9 (C(8)) were also observed. Consequently, the above data were in agreement with ^{1}H-NMR data for the presence of two C_6C_3 units linked through an ether O atom. In addition, the gCOSY spectrum of **2** showed a correlation between H–C(7)/H–C(8) and H–C(8)/H–C(9). In the gHMBC spectrum, the correlation peaks between H–C(7)/C(2), C(6) and between two MeO groups/C(7) and C(9) indicated the presence of a CH(MeO)–CH_2–CH_2OMe unit. Compound **2** was determined to be 5-(1,3-dimethoxypropyl)-3-[4-(2-propen-1-yl)phenoxy]-1,2-benzenediol, and was named obovatalignan B (**2**).

Compounds **1** and **2** were evaluated for their protective effects against glutamate-induced cell death in HT-22 cells (*Table 2*). *Trolox*® (50 μM) was used as a positive control. Compounds **1** and **2** showed considerable cell viability (91.5, 88.5, 91.8 and 95.3, 95.1, 94.3%; data not shown) in the range of concentration from 10, 20 and 40 μM. The EC_{50} values of compounds **1** and **2** were 18.1 ± 1.23 and 7.10 ± 0.78 μM, respectively, which were higher levels of protection activity than seen with the positive control (EC_{50} 79.3 ± 0.78 μM). Additionally, compounds **1** and **2** were evaluated for an inhibitory effect on NO production in LPS-induced RAW 264.7 cells (Table *2*). Compounds **1** and **2** showed considerable cell viability (93.4, 94.4 and 90.5, 85.3%; data not shown) in the range of concentration from 15 and 30 μM. The IC_{50} values of compounds **1** and **2** were > 30.0 and

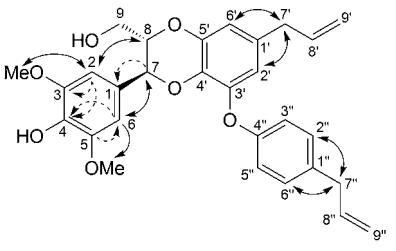

Fig. 2. Key gHMBC (⇢) and NOESY correlations (↔) in compound **1**.

Table 2. Neuroprotective effects of Compounds **1** and **2** from the fruits of *Magnolia obovata* on cell death in glutamate-induced HT22 cells and inhibitory effects on NO production in LPS-induced RAW 264.7 cells

Compound	HT22 (EC_{50}) [μM]	RAW 264.7 (IC_{50}) [μM]
Trolox	79.3 ± 0.78	–
Butein	–	7.70 ± 2.55
1	18.1 ± 1.23	> 30.0
2	7.10 ± 0.78	8.22 ± 2.01

8.22 ± 2.01 μM, respectively. Butein was used as a positive control. Compound **1** showed a lower degree of inhibition than was seen with butein (IC_{50} 7.70 ± 2.55), but compound **2** showed a level of NO inhibition that was similar to that of the positive control. In conclusion, compounds **1** and **2** showed significant protective activities against glutamate-induced oxidative injury in HT22 cells and compound **2** showed inhibitory activity on NO production in LPS-induced RAW 264.7 cells. Therefore, these results demonstrate the potential of these compounds and plant extracts as neuroprotective and anti-inflammatory agents. Further studies are needed to determine the mechanism of activity for each of these compounds.

This work was supported by the Next Generation BioGreen 21 (Project No. PJ01122302) Project from *Rural Development Administration, Korea*.

Experimental Part

General

Fetal bovine serum (FBS) was obtained from *Hyclone Laboratories* (Logan, UT, USA), while L-glutamate, *Trolox*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and DMSO were purchased from *Sigma Chemical Co* (St. Louis, MO, USA). *RPMI* medium *1640*, *Dulbecco*'s modified *Eagle*'s medium (DMEM), and penicillin and streptomycin were purchased from *GIBCO* (*Invitrogen*, Paisley, UK). Column chromatography (CC): SiO_2 (*Kiesel gel 60*, 230 – 400 mesh, *Merck* Darmstadt, Germany) and *ODS* (*LiChroprep RP-18*, 40 – 63 μm, *Merck*) resins. TLC Analysis: *Kiesel gel 60* F_{254} and *RP-18* F_{254}*s* (*Merck*) plates; the compounds were detected using a UV lamp *Spectroline Model ENF-240 C/F* (*Spectronics Corporation*, Westbury, NY, USA) and the spots were visualized by spraying with 10% aq. H_2SO_4 soln. followed by heating. CD Spectra: *Applied Photophysics Chirascan* (Surrey, UK) with a circular dichroism detector. Optical rotations: *JASCO P-1010* digital polarimeter (*JASCO*, Tokyo, Japan). IR Spectra: *PerkinElmer Spectrum One FT-IR* spectrometer model 599B (*PerkinElmer*, Waltham, MA, USA). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra: *Varian Unity Inova AS-400 FT-NMR* spectrometer (*Varian*, Palo Alto, CA, USA); chemical shifts are given in δ (ppm) based on tetramethylsilane (TMS) as an internal standard. HR-EI-MS was conducted on a *JEOL JMSAX-700* (Tokyo, Japan).

Plant Materials

The fruits of *M. obovata* were collected at Kyung Hee University, Yongin, Korea in May 2010 and identified by Prof. *Seung-Woo Lee*, Department of Horticultural Biotechnology, Kyung Hee University, Yongin, Republic of Korea. A voucher specimen (KHU-NPCL-201009) has been deposited with the Laboratory of Natural Products Chemistry, Kyung Hee University, Yongin, Republic of Korea.

Extraction and Isolation

The dried fruits of *M. obovata* (11 kg) were chopped and extracted in 80% MeOH (40 l × 4) at r.t. for 24 h, filtrated and concentrated under reduced pressure. The MeOH extract (740 g) was suspended in H_2O (3.5 l) and then extracted with AcOEt (3.5 l × 4) followed by concentration to give the AcOEt-soluble fraction (*MOE*, 238 g). Fraction *MOE* (238 g) was applied CC (SiO_2; 12 × 15 cm; *n*-hexane/AcOEt 5:1. 2:1, 1:2; 2.8 l of each, $CHCl_3$/MeOH/H_2O 6:4:1; 3 l) to yield 15 fractions, *MOE1* to *MOE15*. *Fr. MOE5* (5.3 g, elution volume/total volume (Ve/Vt) 0.195 – 0.286) was subjected to CC (SiO_2; 5.0 × 13.5 cm; $CHCl_3$/MeOH 40:1; 4.1 l), yielding seven fractions, *MOE5-1* – *MOE5-7*. *Fr. MOE5-3* (3.6 g, Ve/Vt 0.105 – 0.297) was subjected to CC (SiO_2; 5.0 × 14 cm; *n*-hexane/AcOEt 5:1; 21 l), yielding 16 fractions, *MOE5-3-1* – *MOE5-3-16*. *Fr. MOE5-3-12* (220 mg, Ve/Vt 0.214 – 0.257) was subjected to CC (ODS; 3.0 × 5.0 cm; MeOH/H_2O 3:2; 5 l), yielding 29 fractions, *MOE5-3-12-1* – *MOE5-3-12-29*) and a purified compound **1** (*MOE5-3-12-7*; 5.0 mg; Ve/Vt 0.060 – 0.070). *Fr. MOE6* (7.6 g, Ve/Vt 0.287 – 0.450) was subjected to CC (SiO_2; 6.0 × 13 cm; *n*-hexane/$CHCl_3$/MeOH 40:10:1; 21.8 l), yielding eight fractions, *MOE6-1* – *MOE6-8*). *Fr. MOE6-1* (452 mg, Ve/Vt 0.000 – 0.152) was subjected to CC (ODS; 3.5 × 5.5 cm; MeOH/H_2O 5:2, 3:1, 4:1, 6:1; 0.75 l of each), yielding 15 fractions, *MOE6-1-1* – *MOE6-1-15*) and a purified compound **2** (*MOE6-1-9*; 10.7 mg; Ve/Vt 0.257 – 0.300).

Obovatoside A (= **4-{(2*S*,3*S*)-3-(Hydroxymethyl)-6-(prop-2-en-1-yl)-8-[4-(prop-2-en-1-yl)phenoxy]-2,3-dihydro-1,4-benzodioxin-2-yl}-2,6-dimethoxyphenol**; **1**). Amorphous powder. TLC (*RP-18* F_{254}*s*; MeOH/H_2O (7:1): R_f 0.52). $[\alpha]_D^{18}$ = +6.5 (*c* = 0.11, MeOH). UV (MeOH): 312 (2.30), 282 (2.38), 273 (2.38), 215 (2.40). CD (MeOH) 238 (0.67). IR (CaF_2): 3357, 2921, 1591, 1504. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-EI-MS: 490.1991 (M^+, $C_{29}H_{30}O_7^+$; calc. 490.1992).

Obovatoside B (= **5-(1,3-Dimethoxypropyl)-3-[4-(prop-2-en-1-yl)phenoxy]benzene-1,2-diol**; **2**). Amorphous powder. TLC (*RP-18* F_{254}*s*; acetone/MeOH/H_2O 2:13:1: R_f 0.66). $[\alpha]_D^{18}$ = +10.6 (*c* = 0.26, MeOH). UV (MeOH): 273 (1.97), 226 (2.37). IR (CaF_2): 3357, 2928, 1600, 1504. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-EI-MS: 344.1623 (M^+, $C_{20}H_{24}O_5^+$; calc. 344.1624).

Cell Culture

Mouse hippocampal HT22 cells, a subclone of the HT4 hippocampal cell line, were obtained from Prof. *Inhee Mook* (Seoul National University, Seoul, Korea). The

cells were maintained at 5×10^4 cells/ml in DMEM medium supplemented with 10% heat-inactivated FBS, penicillin G (100 units/ml), streptomycin (100 mg/ml), and L-glutamine (5 mM), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

Cytoprotective Activity Assay

Cytoprotective assessment was performed by seeding HT22 cells in 96-well plates at a density of 10^5 cells/ml. After 24 h, cells were pretreated with either compound or positive control Trolox for 4 h. The cells were exposed to glutamate (5 mM) for 12 h. Individual compounds were tested at concentrations of 10, 20 and 40 μM, and each experiment was performed in triplicate. The purity of the used compounds **1** and **2** for assay was higher than 99%. Cell viability was evaluated using the MTT assay [9]. Briefly, cells were incubated with MTT (0.5 mg/ml) for 4 h at 37 °C, the medium was discarded, acidic ^{i}PrOH (0.04N HCl) was added, and after incubating for 30 min, absorbance was measured at 590 nm using a microplate reader (*Bio-Rad Laboratories*, Richmond, CA, USA). Half maximal effective concentration (EC_{50}) refers to the concentration where 50% of the maximal effect is observed. Data are also expressed as the percentage of protection relative to vehicle-treated control cultures, as follows: 100 × [optical density (OD) of glutamate and sample-treated culture – OD of glutamate-treated cultures]/(OD of control cultures – OD of glutamate-treated cultures).

Macrophage RAW 264.7 Culture and Viability Assay

RAW 264.7 cells (1×10^4 cells/well) were cultured for 12 – 18 h in 96-well plates and were treated with LPS (1 μg/ml) in the presence or absence of various concentrations of compounds **1** and **2** diluted in serum-free medium for 24 h. MTT reagent was then added to the wells and incubated for 4 h. The formazan formed in the cell pellets was dissolved by adding 100 μl of DMSO, and the absorption was measured at 550 nm. The survival rates of the cells were calculated as the percentage of the viable cells according to the following formula: cell viability (%) = [OD (compound) – OD (Blank)/OD (Control) – OD (Blank)] × 100.

Measurement of Nitrite

NO was determined by measuring the amount of nitrite, a stable oxidized product. To test the inhibitory effect of the compounds on NO production, RAW 264.7 cells (1×10^4 cells/well) were cultured in 96-well cell culture plates for 12 – 18 h and then treated with 1 μg/ml LPS in a serum-free medium containing various concentrations of the compounds (15 and 30 μM) for 24 h. Subsequently, 100 μl of the cell culture supernate was mixed with 100 μl of *Griess* reagent (*Sigma–Aldrich Co*. (St. Louis, MO, USA)) in a new 96-well plate, and the absorbance was read at 550 nm with a spectrophotometer. Nitrite concentrations were determined by comparison to a sodium nitrite standard curve. Butein was used as a positive control. Half maximal inhibitory concentration (IC_{50}) refers to the concentration required for obtaining 50% inhibition. Butein (≥ 95% pure) isolated from the bark of *Rhus verniciflura* was used as a positive control [10].

Statistical Analysis

Data are expressed as the mean ± SD of at least three independent experiments. One-way analysis of variance (ANOVA) was used followed by the *Newman–Keuls post hoc* test to compare each group and treatment concentration. Statistical analysis was performed using *GraphPad Prism* software version 3.03 (*GraphPad Software, Inc.*, San Diego, CA, USA).

REFERENCES

[1] B. S. Jung, M. K. Shin, 'Hyang Yak Dae Sa Jeon', Young Lim Sa Publisher, Seoul, Korea, 2003, 3rd Edn., p. 471.
[2] K.-H. Seo, D.-Y. Lee, D.-S. Lee, J.-H. Park, R.-H. Jung, Y.-J. Jung, S. Shrestha, I.-S. Chung, G.-S. Kim, Y.-C. Kim, N.-I. Baek, *Planta Med.* **2013**, *79*, 1335.
[3] K.-H. Seo, D.-Y. Lee, R.-H. Jeong, K.-H. Yoo, I.-S. Chung, G.-S. Kim, W.-D. Seo, H.-C. Kang, E.-M. Ahn, N.-I. Baek, *J. Appl. Biol. Chem.* **2013**, *56*, 179.
[4] M. K. Pyo, Y. Y. Lee, H. S. Yun-Choi, *Arch. Pharmacal Res.* **2002**, *25*, 325.
[5] Y. Fukuyama, Y. Otoshi, K. Miyoshi, N. Hasegawa, Y. Kan, M. Kodama, *Tetrahedron Lett.* **1993**, *34*, 1051.
[6] C. Ma, H. J. Zhang, G. T. Tan, N. V. Hung, N. M. Cuong, D. D. Soejarto, H. H. S. Fong, *J. Nat. Prod.* **2006**, *69*, 346.
[7] K. H. Kim, K. W. Woo, E. Moon, S. U. Choi, S. Y. Kim, S. Z. Choi, M. W. Son, K. R. Lee, *J. Agric. Food Chem.* **2014**, *62*, 7746.
[8] K. Sawasdee, T. Chaowasku, V. Lipipun, T.-H. Dufat, S. Michel, K. Likhitwitayawuid, *Tetrahedron Lett.* **2013**, *54*, 4259.
[9] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55.
[10] D. G. Kang, A. S. Lee, Y. J. Mun, W. H. Woo, Y. C. Kim, E. J. Sohn, M. K. Moon, H. S. Lee, *Biol. Pharm. Bull.* **2004**, *27*, 366.

Received April 23, 2015
Accepted April 18, 2016

