PHCOG MAG. ORIGINAL ARTICLE

Flavonoids isolated from *Lespedeza cuneata* G. Don and their inhibitory effects on nitric oxide production in lipopolysaccharide-stimulated BV-2 microglia cells

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Submitted: 01-10-2014 Revised: 07-01-2015 Published: 10-07-2015

ABSTRACT

Background: *Lespedeza cuneata* (Dum. Cours.) G. Don, a perennial legume native to Eastern Asia, has been used therapeutically in traditional Asian medicine to protect the function of liver, kidneys and lungs. However, its effect on inflammatory nitric oxide (NO) production and the active constituents have not yet been explored. **Objective:** In this study, we investigated the phytochemical constituents of *L. cuneata* and evaluated their effect on NO production using lipopolysaccharide (LPS)-stimulated BV2 cells. **Materials and Methods:** The 80% methanol extract of the aerial part of *L. cuneata* were used for the isolation of flavonoids. The isolated compounds were elucidated by various spectroscopic methods including nuclear magnetic resonance and mass spectrometry spectrometry. To evaluate the effect on inflammatory NO production, LPS-stimulated murine microglia BV-2 cells were used as a screening system. **Results:** Nine flavonoids were isolated from the aerial parts of *L. cuneata*. Among the isolated flavonoids, compounds 4, 5, 7 and 9 are reported from the genus *Lespedeza* for the first time. Moreover, compounds 1 and 6 showed significant inhibitory effects on NO production in LPS-stimulated BV2 cells without cell toxicity. **Conclusion:** In this study, nine flavonoids were isolated from *L. cuneata*. Among the compounds, only 1 and 6, which have free hydroxyl groups at both C3 and C7 showed significant inhibitory activity on NO production in LPS-stimulated BV2 cells. These results suggested *L.* cuneata and its flavonoid constituents as possible candidate for the treatment of various inflammatory diseases.

Key words: BV2 microglia, flavonoids, *Lespedeza cuneata*, nitric oxide

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.160466

Quick Response Code:

INTRODUCTION

Plants belonging to the genus *Lespedeza* (Leguminosae) are widely distributed both in Eastern North America and in Eastern Asia, and about 40 species have been reported.[1] It is recognized for its tolerance of drought and acidity and for its ability to grow in shallow soils of low fertility. *Lespedeza cuneata* is now being considered as an energy crop for increasing the sustainability of agriculture and energy production in the United States.[2] In the pharmaceutical field, the aerial parts of this plant have been used to protect the function of liver, kidneys and lungs in traditional Asian medicine.[3] *L. cuneata* is known to contain flavonoids, pinitol, tannins and β-sitosterol.[4] Phytochemical studies have revealed that flavonoid compounds including *C*-glycosyl flavones (e.g. isoorientin, isovitexin, vicenin II, lucenin II, desmodin and homoadonivernith), *O*-glycosyl flavonols (e.g. avicularin, juglanin, trifolin, hyperin and hirsutrin) and aglycones (e.g. quercetin and kaempferol) were isolated from extracts of the aerial parts of *L. cuneata*.[5,6]

Flavonoids are naturally occurring polyphenolic compounds, which contain two benzene rings linked together with a heterocyclic pyran or pyrone ring. Flavonoids are normal constituents of the human diet and are known for a variety of biological activities.[7] Recently, there has been much interest in the neuroprotective effects of flavonoids, which have been shown to be effective in protecting against both age-related cognitive and motor decline *in vivo*.[8-10] This potential may reside in a number of physiological functions,

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including their antioxidant properties,[11] the interactions with intracellular signaling pathways, the regulation of cell survival/apoptotic genes and mitochondrial function.[12,13] These signaling cascades are also critical for the control of inflammatory processes, helping the activation of microglia in response to cytokines and the induction of inducible nitric oxide synthase and nitric oxide (NO) production.[14-16] Consequently, flavonoids have been suggested as the novel therapeutic agents for the reduction of the deleterious effects of inflammation and as the potential preventive drugs for degenerative disease development.[17]

Nitric oxide is a signaling molecule that plays a key role in the pathogenesis of inflammation.[18] They regulate inflammatory response and recovery from tissue damage.[19,20] Therefore, regulation of NO production in microglia could be a good target for the treatment of degenerative disorders.

In this study, we tried to identify NO production inhibitory constituents of *L. cuneata* employing lipopolysaccharide (LPS)-stimulated BV2 cells as a screening system. Since the structures of flavonols 1–8 were similar to each other, the effects due to hydroxylated and the position of the sugar moiety against NO production inhibitory activity was investigated.

MATERIALS AND METHODS

General procedures

All organic solvents, such as hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), methanol (MeOH) and n-butanol (n-BuOH) used for extraction and column chromatography were of analytical grade and purchased from Duksan Chemical (Anseong, Korea). 1H nuclear magnetic resonance (^{1}H NMR) (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on an Agilent 400-MR NMR spectrometer (Agilent Technologies, Santa Clara, CA) and TMS was used as an internal standard. Data processing was carried out with the MestReNova 6.0.2 program (Mestrelab research SI, www.mestrelab.com, 2009). HRESIMS spectra were obtained using an Agilent 6550 iFunnel Q-TOF liquid chromatography/mass spectrometry (LC/MS) system (Agilent Technologies, Santa Clara, CA). Preparative high-performance liquid chromatography (HPLC) was carried out using an Agilent 1260 HPLC system. Column chromatography was performed on silica gel (Kieselgel 60, 70–230 mesh and 230–400 mesh, Merck, Darmstadt, Germany) and YMC RP-18 resins (Fuji Silysia Chemical, Aichi, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL. Co. Glutamate and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carbboxylic acid (trolox), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and scopolamine were purchased from Sigma (USA).

Plant materials

The aerial part of *L. cuneata* were collected at Yongdu Mountain, Jecheon, Chungcheongbuk province, South Korea in October 2011, and authenticated by Dr. Jong Hee Park, professor of Pusan National University. A voucher specimen (YIPS-LC-140815) was deposited at the Herbarium of Pharmacy, Yonsei Institute of Pharmaceutical Sciences, Yonsei University, Incheon, Korea.

Extraction and isolation

The dried and powdered aerial part of *L. cuneata* (1.8 kg) were extracted with 80% MeOH four times for 3 days at room temperature. After removal of the solvent under reduced pressure *in vacuo*, the MeOH extract (196.74 g, yield: 10.9%) was suspended in H_2O and then partitioned successively with hexane (16.15 g), $CHCl_3$ (11.52 g) EtOAc (10.40 g) and *n*-BuOH (62.9 g) fraction. The *n*-BuOH fraction was chromatographed on a Diaion HP-20P column eluting with H_2O containing increasing concentrations of MeOH (0, 40, 60, 80 and 100%) to obtain five sub-fractions Fr. A (23.72 g), Fr. B (12.55 g), Fr. C (2.12 g) Fr. D (0.32 g) and Fr. E (0.03 g). The Fr. B was chromatographed on a LH-20 column eluting with 80% MeOH to give five smaller fractions, Fr. B1 (0.8 g), Fr. B2 (0.9 g), Fr. B3 (9.24 g) Fr. B4 (0.32 g) and Fr. B5 (0.03 g). The Fr. B3 fraction was chromatographed on HPLC using J'sphere ODS H-80 (250 mm × 20 mm, 4 μm, 8 nm) column eluting with 15% aqueous acetonitrile at a flow rate of 3 mL/min to yield 2 (5.6 mg), 3 (0.7 mg), 4 (4.7 mg), 5 (3.6 mg), 7 (0.6 mg) and 8 (5.2 mg). The Fr. C was chromatographed on LH-20 column eluting with 80% MeOH to yield 1 (6.4 mg), 6 (8.1 mg) and 9 (2.0 mg). Isolated compounds were elucidated by electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS) and several NMR techniques including 1D and 2D NMR spectroscopic methods and by comparison of their data with those reported previously in the related literatures [Table 1].

Structure elucidation of isolated compounds

Kaempferol (1) - Yellow amorphous powder; ^{1}H-NMR (400 MHz, CD_3OD) δ_H: 6.17 (1H, d, J = 1.4 Hz, H-6), 6.38 (1H, s, H-8), 8.07 (1H, d, J = 8.7 Hz, H-2), 6.89 (1H, d, J = 8.7 Hz, H-3), 6.89 (1H, d, J = 8.7 Hz, H-5), 8.07 (1H, d, J = 8.7 Hz, H-6); ^{13}C-NMR (100 MHz, CD_3OD) δ_C: 148.0 (C-2), 137.1 (C-3), 177.4 (C-4), 104.5 (C-4a), 162.5 (C-5), 99.2 (C-6), 165.6 (C-7), 94.4 (C-8), 160.5 (C-8a), 123.7 (C-1), 130.7 (C-2), 116.3 (C-3), 158.2 (C-4), 116.2 (C-5), 130.7 (C-6).

Table 1: Identification of flavonoids from *L. Cuneata*

t_R (min)[a]	UV λmax (nm)	Molecular formula	MS fragments (positive, m/z)	Identification	References
28.24	258, 325sh, 370	$C_{15}H_{10}O_6$	287.0535 $[M+H]^+$	kaempferol	[23]
16.66	215, 223, 268, 307	$C_{21}H_{20}O_{10}$	433.1110 $[M+H]^+$, 455.0927 $[M+Na]^+$	afzelin	[24]
17.99	268, 351	$C_{27}H_{30}O_{15}$	595.1638 $[M+H]^+$, 617.1455 $[M+Na]^+$	nicotiflorin	[25]
14.29	203, 257, 355	$C_{27}H_{30}O_{15}$	595.1635 $[M+H]^+$, 617.1450 $[M+Na]^+$	kaempferol-3-*O*-glu-7-*O*-rha	[26]
17.79	268, 334	$C_{27}H_{30}O_{14}$	579.1674 $[M+H]^+$, 601.1486 $[M+Na]^+$	rhoifolin	[27]
18.90	259, 308sh, 376	$C_{15}H_{10}O_7$	303.0485 $[M+H]^+$	quercetin	[28]
17.41	258, 263sh, 360	$C_{21}H_{20}O_{12}$	465.1017 $[M+H]^+$	isoquercitrin	[29]
16.38	257, 264sh, 358	$C_{27}H_{30}O_{16}$	611.1596 $[M+H]^+$	rutin	[25]
23.59	234sh, 249, 261sh, 305sh	$C_{15}H_{10}O_4$	255.0585 $[M+H]^+$	daidzein	[30]

[a]The retention times refer to those obtained in the LC-UV-MS set up

Afzelin (2) - Yellow amorphous powder; ^{1}H-NMR (400 MHz, DMSO-d_6) δ_H: 0.78 (3H, d, J = 5.1 Hz, C-6”), 5.29 (1H, s, C-1”), 6.22 (1H, d, J = 1.6 Hz, H-6), 6.42 (1H, d, J = 1.6 Hz, H-8), 6.92 (2H, d, J = 7.3 Hz, H-3, 5), 7.76 (2H, d, J = 7.3 Hz, H-2, 6), 12.62 (1H, s, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ_C: 17.5 (C-6”), 70.1 (C-2”), 70.3 (C-3”), 70.7 (C-5”), 71.2 (C-4”), 93.8 (C-8), 98.8 (C-6), 101.8 (C-1”), 104.2 (C-10), 115.5 (C-3’,5’), 120.6 (C-1’), 130.7 (C-2’,6’) 134.2 (C-3), 156.6 (C-9), 157.3 (C-2), 160.0 (C-4’), 161.3 (C-5), 164.4 (C-7), 177.8 (C-4).

Nicotiflorin (3) - Yellow amorphous powder; ^{1}H-NMR (400 MHz, CD_3OD) δ_H: 6.20 (1H, d, J = 2.0 Hz, H-6), 6.39 (1H, d, J = 2.0 Hz, H-8), 8.06 (2H, dd, J = 2.0, 6.8 Hz, H-2’, 6’), 6.89 (2H, dd, J = 2.0, 6.8 Hz, H-3’, 5’), 5.13 (1H, d, J = 7.6 Hz, H-1”), 3.26-3.48 (5H, m, H-2”~5” and H-6b”), 3.81 (1H, d, J = 10.0 Hz, H-6a”), 4.52 (1H, d, J = 1.6 Hz, H-1’”), 3.64 (1H, dd, J = 1.6, 3.2 Hz, H-2’”), 3.53 (1H, dd, J = 3.8, 9.4 Hz, H-3’”), 3.26-3.48 (2H, m, H-4’”, 5’”), 1.13 (3H, d, J = 6.0 Hz, H-6’”); ^{13}C-NMR (100 MHz, CD_3OD) δ_C: 158.67 (C-2), 135.69 (C-3), 179.54 (C-4), 163.11 (C-5), 100.11 (C-6), 166.13 (C-7), 95.07 (C-8), 159.55 (C-9), 105.81 (C-10), 122.89 (C-1’), 132.53 (C-2’), 116.28 (C-3’), 161.63 (C-4’), 116.28 (C-5’), 132.53 (C-6’), 104.79 (C-1”), 75.92 (C-2”), 78.28 (C-3”), 71.58 (C-4”), 77.34 (C-5”), 68.72 (C-6”), 102.57 (C-1’”), 72.23 (C-2’”), 72.44 (C-3’”), 74.05 (C-4’”), 69.88 (C-5’”), 18.07 (H-6’”).

Kaempferol 3-*O*-glucosyl-7-*O*-rhamnoside (4). Yellow amorphous powder; ^{1}H NMR (400 MHz, CD_3OD) δ_H: 8.08 (2H, d, J = 8.7 Hz, H-2’, 6’), 6.91 (2H, d, J = 8.7 Hz, H-3’, 5’), 6.23 (1H, d, J = 1.5 Hz, H-6), 6.41 (1H, d, J = 1.5 Hz, H-8), 5.14 (1H, d, J = 7.2 Hz, H-1”), 3.24-3.98 (6H, H-2”-H-6”), 4.53 (1H, d, J = 5.8 Hz, H-1’”), 3.24-3.98 (4H, H-2’”-H-5’”), 1.14 (3H, d, J = 6.0 Hz, H-6’”); ^{13}C NMR (100 MHz, CD_3OD) δ_C: 156.6 (C-2), 133.5 (C-3), 177.5 (C-4), 161.3 (C-5), 99.8 (C-6), 164.2 (C-7), 93.8 (C-8), 156.9 (C-9), 104.2 (C-10), 121.1 (C-1’), 130.9 (C-2’, C-6’), 115.2 (C-3’, C-5’), 159.9 (C-4’), 101.6 (C-1”), 74.4 (C-2”), 76.7 (C-3”), 70.9 (C-4”), 76.0 (C-5”), 67.1 (C-6”), 100.8 (C-1’”), 70.5 (C-2’”), 70.2 (C-3’”), 72.1 (C-4’”), 68.3 (C-5’”), 17.7 (C-6’”).

Rhoifolin (5) - White amorphous powder; ^{1}H NMR (400 MHz, CD_3OD) δ_H: 1.32 (3H, d, J = 6.0 Hz, 6’”–CH3), 5.18 (1H, d, J = 7.5 Hz, H-100), 5.28 (1H, d, J = 1.8 Hz, H-1’”), 6.44 (1H, d, J = 2.1 Hz, H-6), 6.64 (1H, s, H-3), 6.76 (1H, d, J = 2.1 Hz, H-8), 6.91 (1H, d, J = 9.0 Hz, H-30, 50), 7.86 (1H, dd, J = 9.0 Hz, H-2’, 6’); ^{13}C NMR (100 MHz, CD_3OD) δ_C: 164.4 (C-2), 104.1 (C-3), 184.0 (C-4), 159.0 (C-5), 99.8 (C-6), 166.7 (C-7), 95.9 (C-8), 162.9 (C-9), 107.1 (C-10), 123.0 (C-1’), 129.6 (C-2’), 117.1 (C-3’), 162.9 (C-4’), 117.1 (C-3’), 129.6 (C-6’), 99.8 (C-1”), 79.1 (C-2”), 78.3 (C-3”), 72.2 (C-4”), 79.1 (C-5”), 62.4 (C-6”), 101.0 (C-1’”), 71.4 (C-2’”), 72.2 (C-3’”), 74.0 (C-4’”), 70.0 (C-5’”), 18.3 (C-6’”).

Quercetin (6) - Yellow amorphous powder; ^{1}H-NMR (400 MHz, DMSO-d_6) δ_H: 6.17 (s, 1H, H-6), 6.404 (s, 1H, H-8), 7.54 (d, 1H, H-2’), 6.88 (d, 1H, H-3’), 7.66 (s, 1H, H-6’). ^{13}C-NMR (100 MHz, DMSO-d_6) δ_C: 147.20 (C-2), 136.14 (C-3), 176.24 (C-4), 161.18 (C-5), 98.59 (C-6), 164.35 (C-7), 93.74 (C-8), 156.60 (C-9), 103.40 (C-10), 122.41 (C-1’), 115.42 (C-2’), 145.47 (C-3’), 148.10 (C-4’), 115.94 (C-5’), 120.40 (C-6’).

Isoquercitrin (7) - Yellow amorphous powder; ^{1}H-NMR (400 MHz, CD3OD) δ_H: 6.19 (1H, brs, H-6), 6.37 (1H, brs, H-8), 7.70 (1H, s, H-2’), 6.88 (1H, d, J = 8.3 Hz, H-5’), 7.59 (1H, d, J = 8.3 Hz, H-6’), 5.24 (1H, d, J = 7.5 Hz, H-1”), 3.37-3.50 (3H, m, H-2”-4”), 3.19-3.23 (1H, m, H-5”), 3.71 (1H, dd, J = 1.8, 12.0 Hz, H-6a”), 3.57 (1H, dd, J = 5.1, 12.0 Hz, H-6b”); ^{13}C-NMR (100 MHz, CD3OD) δ_C: 158.79 (C-2), 135.48 (C-3), 179.25 (C-4), 162.86 (C-5), 99.94 (C-6), 166.25 (C-7), 94.72 (C-8), 158.33 (C-9), 105.50 (C-10), 122.96 (C-1’), 115.90 (C-2’), 145.77 (C-3’), 149.72 (C-4’), 117.43 (C-5’), 123.07 (C-6’), 104.26 (C-1”), 75.69 (C-2”), 78.35 (C-3”), 71.18 (C-4”), 78.08 (C-5”), 62.53 (C-6”).

Rutin (8) - Yellow amorphous powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ_H: 1.00 (3H, d, J = 6.0 Hz, H-5'''), 3.04-3.72 (10H, m, sugar H), 4.39 (1H, s, H-1'''), 5.34 (1H, d, J = 7.5 Hz, Glc H-1), 6.20 (1H, d J = 1.2 Hz, H-6), 6.39 (1H, d, J = 1.2 Hz, H-8), 6.84 (1H, d, J = 8.5 Hz, H-5'), 7.53 (1H, d, J = 2.5 Hz, H-2'), 7.55 (1H, dd, J = 2.5, 8.5 Hz, H-6'), 12.59 (1H, s, 5-OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 156.6 (C-2), 133.3 (C-3), 177.3 (C-4), 161.2 (C-5), 98.7 (C-6), 164.2 (C-7), 93.6 (C-8), 156.4 (C-9), 104.1 (C-10), 121.6 (C-1'), 115.3 (C-2'), 144.7 (C-3'), 148.4 (C-4'), 116.3 (C-5'), 121.2 (C-6'), 101.2 (C-1''), 74.1 (C-2''), 76.5 (C-3''), 70.6 (C-4''), 75.9 (C-5''), 67.0 (C-6''), 100.7 (C-1''), 70.4 (C-2'''), 70.0(C-3'''), 71.9 (C-4'''), 68.2 (C-5'''), 17.7 (C-6''').

Daidzein (9) - White amorphous powder; ^{1}H NMR (400 MHz, DMSO-*d6*) δ_H: 6.79 (2H, d, J = 8.4 Hz, H-3',-5'), 6.84 (1H, d, J = 2.1 Hz, H-8), 6.92 (1H, dd, J = 8.8, 2.1 Hz, H-6), 7.37 (2H, d, J = 8.4 Hz, H-2', 6'), 7.95 (1H, d, J = 8.8 Hz, H-5), 8.27 (1H, s, H-2), 9.52 (1H, s, H-4'), 10.75 (1H, s, H-7); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 102.2 (C-8), 115.1 (C-3', 5'), 115.2 (C-6), 116.8 (C-4a), 122.7 (C-1'), 123.6 (C-3), 127.4 (C-5), 130.2 (C-2', 6'), 152.9 (C-2), 157.3 (C-4'), 157.6 (C-8a), 162.7 (C-7), 174.8 (C-4).

Measurements of nitric oxide in lipopolysaccharide-stimulated BV2 microglia cells

BV-2 cells were maintained in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin. To measure NO production, BV-2 cells were dispensed in wells of a 96-well plate (2 at 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 MTT assay. N^G-Monomethyl-L-arginine (L-NMMA), a well-known NO synthase inhibitor, was tested as a positive control.

Statistical analysis

Data were evaluated for statistical significance by ANOVA test using a computerized statistical package. The data were considered to be statistically significant if the $P \leq 0.05$.

RESULTS AND DISCUSSION

Nine compounds were isolated and their structures were identified as kaempferol (1), afzelin (2), nicotiflorin (3), kaempferol-3-*O*-β-glucopyranosyl-7-*O*-α-rhamnoside (4), rhoifolin (5), quercetin (6), isoquercitrin (7), rutin (8) and daidzein (9) [Figure 1]. Among them, seven flavonoids (2–5 and 7–9) were isolated from *L. cuneata* for the first time. Moreover, compounds 4, 5, 7 and 9 are reported from the genus *Lespedeza* for the first time. The present phytochemical investigation has further enriched our knowledge about the chemistry of *L. cuneata* and has identified compounds 2–5 and 7–9 could be potential chemotaxonomic markers for the species.

Many flavonoids have been intensively studied on their inhibitory effects on inflammatory NO production.[21] Among the studied flavonoids, the bioactivity of various flavonoids is related to the number of hydroxyl groups on the flavonoid B-ring and the presence of the sugar moiety.[22] In the light of this, it appears that interactions might be structure-dependent, meaning that different flavonoids are likely to express different cellular outcomes. Thus, the effect of the isolated flavonoids on NO production in LPS-stimulated BV2 cells were evaluated to estimate the structure-dependency.

Nitric oxide inhibitory activities of the isolated compounds (1–9) and *L. cuneata* extract were evaluated by examining the inhibition of NO production in LPS-activated microglia BV-2 cells [Table 2]. Among the tested compounds, 1 and 6 significantly inhibited NO production with the IC_{50} values of 28.01 and 26.97 μM, respectively, which displayed more potent activity than L-NMMA, a well-known NOs inhibitor. None of the isolates (1–9) showed cytotoxicity at the concentrations up to 50 mM. The result showed that compounds with the sugar moiety (2–5 and 7–8) did not show the inhibition

Table 2: Inhibitory effect on NO production of *L. cuneata* extract and compounds 1-9 in LPS-activated BV-2 cells

Compounds	IC_{50}[a] (μM)	Cell viability[c] (%)
1	28.01	124.1±2.0
2	>500	92.5±6.4
3	>500	101.0±1.7
4	>500	98.6±1.8
5	>500	92.8±8.9
6	26.97	131.5±13.4
7	>500	112.2±7.6
8	>500	115.5±3.2
9	>500	114.4±6.9
Extract	>500	104.0±1.8
L-NMMA[c]	38.17	103.3±3.2

[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 50 μM of each extract was expressed as a percentage (%) of the LPS only treatment group. The results are averages of three independent experiments, and the data are expressed as mean±SD. [c]L-NMMA as a positive control

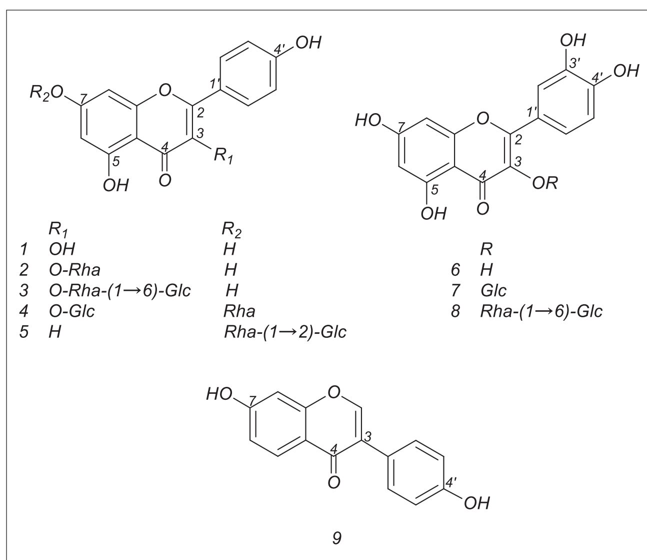

Figure 1: Chemical structures of compounds 1–9

of NO production compared to the aglycones (1 and 6). As results, we suggested that the presence of a hydroxyl group attached at C-3 on the C-ring may be the functional group responsible for the NO inhibitory properties of flavonoids.

CONCLUSION

From the aerial part of *L. cuneata*, nine flavonoids were isolated by chromatographic methods. The isolated compounds were elucidated by ESI-Q-TOF-MS and several NMR techniques. Among the isolates, compounds **1** and **6** exhibited significant NO inhibitory activities in LPS-stimulated microglial BV-2 cells. Compared to the other inactive compounds, these two active compounds have two free hydroxyl groups at both C3 and C7 positions. These results suggest the possible contribution of these hydroxyl groups to the NO inhibitory activity of flavonoids. Taken together, compounds 1 and 6 might be promising candidates for the treatment of various inflammatory diseases.

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Cite this article as: Yoo G, Park SJ, Lee TH, Yang H, Baek Y, Kim N, *et al*. Flavonoids isolated from *Lespedeza cuneata* G. Don and their inhibitory effects on nitric oxide production in lipopolysaccharide-stimulated BV - 2 microglia cells. Phcog Mag 2015;11:651-6.

Source of Support: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2011-0025129). **Conflict of Interest:** None declared.