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Chemical Constituents of *Abies koreana* Leaves with Inhibitory Activity against Nitric Oxide Production in BV2 Microglia Cells†

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Abstract – Eleven compounds were isolated from fresh leaves of *Abies koreana* (Pinaceae), and structures of these compounds were determined to be 3-hydroxy-2-methyl-4-pyrone (**1**), maltol-3-*O*-β-D-glucoside (**2**), (–)-epicatechin (**3**), naringenin 7-*O*-β-D-glucopyranoside (**4**), naringenin-7-*O*-rhamnoglucoside (**5**), kaempferol 3-*O*-β-D-glucopyranoside (**6**), (+)-isolariciresinol (**7**), secoisolariciresinol (**8**), rhododendrol (**9**), ferulic acid (**10**) and 4-(4-hydroxyphenyl)butan-2-one (rheosmin) (**11**) by comparing ^{1}H-, ^{13}C-NMR and MS spectroscopic data with reference values. Compounds **3**, **5**, **7**, **8**, **9**, **10**, **11** were isolated for the first time from *A. koreana*. Among eleven isolates, compounds **1, 7** and **11** showed inhibitory activities against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in BV-2 microglia in a concentration dependent manner.
Keywords – *Abies koreana,* Pinaceae, Nitric oxide

Introduction

Nitric oxide (NO), a gaseous paramagnetic radical, is a very important and versatile messenger in biological systems. NO is synthesized from L-arginine by inducible nitric oxide synthase (iNOS). NO is crucial for many physiological functions. Inappropriate release of this mediator has been linked to a number of pathologies. In particular, neurons and oligodendrocytes in the central nervous system (CNS) are exquisitely vulnerable to NO toxicity owing to their inability to sustain a high level of glycolysis and their lower reserves of intracellular antioxidants such as glutathione and α-tocopherol (Heales *et al*., 1999). Accordingly, agents that reduce the formation of NO may be of considerable therapeutic value (Alderton *et al*., 2001).

Abies koreana (Pinaceae) is a fir indigenous to the higher mountains of South Korea such as Jeju island. The seeds of this tree have been used for the treatment of hypertension, uterine bleeding and so on in Korean traditional medicine (Ahn, 2002). Lanostane terpenoids (Kim *et al*., 2004), secocycloartenoids (Kim *et al*., 2001), lignans (Kim *et al*., 1994) and essential oils (Baran *et al*., 2007; Jeong *et al*., 2007) have been isolated, and this plant has shown antimicrobial activity (Oh *et al.,* 2007; Bagci *et al*., 1996) and memory-enhancing effects on scopolamine-induced amnesia in mice (Kim *et al*., 2006).

The present study describes inhibitory activities of *A. koreana* and its constituents against NO production, and their effects on BV2 microglia cell survival. Cell viability was determined by an MTT assay, and inhibition of nitric oxide production in lipopolysaccharide (LPS) treated cells was evaluated by reaction with the Griess reagent.

Materials and Methods

General experimental procedures – Column chromatography (CC) was carried out using silica gel 60 (40 - 60 μm, 230 - 400 mesh, Merck, Germany), YMC-GEL ODS-A (5 - 150 μm, YMC, Japan) and Sephadex LH-20 (25 - 100 μm, Pharmacia, Sweden). Thin layer chromatography (TLC) was performed with silica gel 60 F_{254} (Art. 5715, Merck, Germany) and RP-18 F_{254S} (Art. 15685, Merck, Germany) plates. Detection was achieved by heating TLC plates after spraying them with anisaldehyde-H_2SO_4. ^{1}H. ^{13}C NMR, and 2D NMR experiments (^{1}H-^{1}H COSY, HSQC, and HMBC) were performed on a Bruker AMX 500 spectrometer (Germany). Solvent signals were used as internal standards. FT-IR spectra were measured on a JASCO FT/IR-300 spectrophotometer (Japan). High-resolution and low-resolution FAB MS were obtained on a JEOL JMS-AX505WA (Japan). The HPLC system consisted of a Gilson-321 pump (Gilson Co. Ltd., USA) and a Gilson-151 UV detector (Gilson Co. Ltd., USA).

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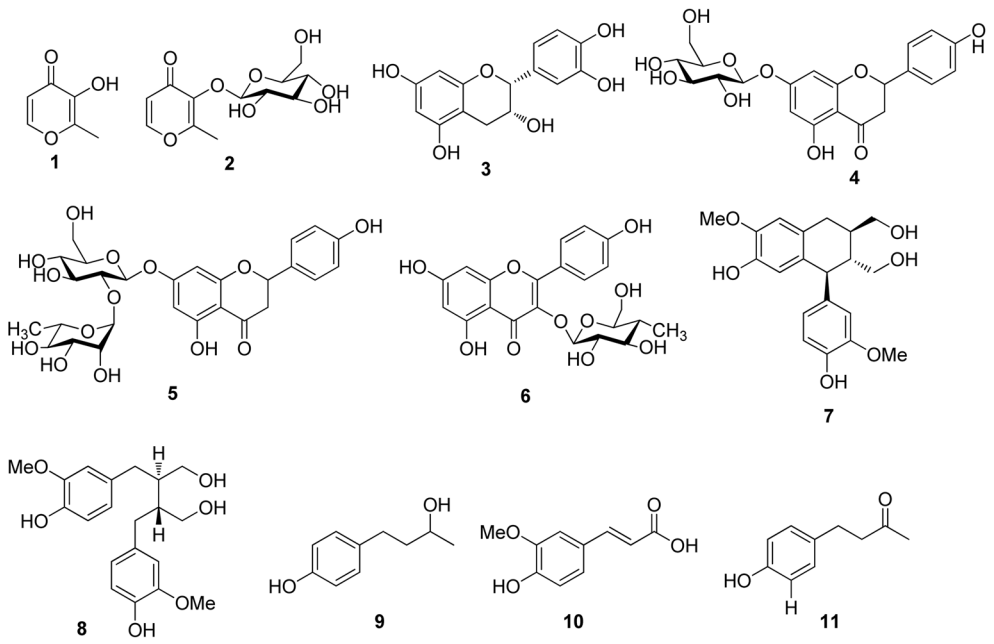

Fig. 1. Chemical structures isolated from *Abies koreana*.

An YMC hydrosphere C_{18} column (250 mm × 10 mm i.d.; 5 mm) was used for isolation, and chromatograms were monitored at 210 nm. Recycling preparative HPLC was performed with an L-6050 pump (JAI, Co. Ltd., Japan), 3702B UV detector and 50 RI detector, and a JAIGEL-ODS-AP-50 C18 column (500 mm × 50 mm i.d.; 5 μm). HPLC grade solvents (Fisher Scientific Korea Ltd., Korea) were used in the CH_3CN-H_2O mobile phase system.

Plant materials – Leaves of *Abies koreana* E.H. Wilson (Pinaceae) were collected at the Medicinal Herb Garden (Goyang-si, Gyeonggi-do), College of Pharmacy, Seoul National University in March 2008. A voucher specimen (SNUPH-100) has been deposited at the herbarium of the Medicinal Herb Garden, College of Pharmacy, Seoul National University.

Extraction and isolation – The leaves of *A. koreana* (10 kg) were extracted with 80% MeOH (3 × 10 L) at room temperature. The methanolic extract was concentrated *in vacuo* to give a crude extract (989.9 g) The methanol-soluble extract was then suspended in H_2O and partitioned successively with *n*-hexane, ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH). The EtOAc-soluble fraction was evaporated to dryness *in vacuo* and yielded 186.3 g. It was subjected to HP-20 column chromatography with a gradient elution of H_2O-MeOH to give six fractions (E1~6). Fraction E3 was further separated by a RP-C_{18} column (H_2O : MeOH = 80 : 20 → MeOH) to give ten subfractions (E3-1~10). Fraction E3-2 was purified with an RP-C_{18} prep. HPLC (MeCN : H_2O = 15 : 85) to afford compound **2** (maltol-3-*O*-β-D-glucoside, 54.6 mg). Fraction E3-3 was separated over a RP-C_{18} column (MeCN : H_2O = 15 : 85) to afford compound **5** (naringenin-7-*O*-rhamno-glucoside, 14.9 mg) and compound **3** ((−)-epicatechin, 3.1 mg). Fraction E4 was further separated over a silica gel CC ($CHCl_3$: MeOH = 50 : 1 → MeOH) to give thirteen subfractions (E4-1~13). Compound **1** (3-hydroxy-2-methyl-4-pyrone, 2.9 mg) was obtained from Fraction E4-1 by recrystallization. Fraction E4-3 was separated over a RP-C_{18} column (H_2O : MeOH = 20 : 80 → MeOH) and rechromatographed on an RP C_{18} column with 70% H_2O, which afforded compound **9** (rhododendrol, 9.8 mg). Fractions E4-5 and E4-10 were separated over a RP-C_{18} column with 70% H_2O, which afforded compounds **7** (Isolariciresinol, 57.4 mg), **8** (secoisolariciresinol, 97.9 mg), **4** (naringenin 7-*O*-β-D-glucopyranoside, 2.5 mg), and **6** (kaempferol 3-*O*-β-D-glucopyranoside, 3.0 mg), repectively. Fraction E5 was further separated over a silica gel CC ($CHCl_3$: MeOH = 50 : 1 → MeOH) to give twelve subfractions (E5-1~12). Fraction E5-4 was separated over a RP-C_{18} column (H_2O : MeOH = 20 : 80 → MeOH) and rechromatographed on an RP C_{18} column with 75% H_2O, which afforded compounds **10** (ferulic acid, 8.9 mg) and **11** (4-(4-hydroxyphenyl)butan-2-one, 37 mg) (Fig. 1).

3-hydroxy-2-methyl-4-pyrone, **Maltol (1)** – White amorphous powder; EIMS *m/z* : 126 $[M]^+$; UV λ_{max} (MeOH) nm : 214.5, 276; IR ν_{max} (KBr): 3260, 1635, 1612, 1605, 1260, 700 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) : δ 8.8 (1H, s, OH), 8.0 (1H, d, *J* = 5.5 Hz, H-5), 6.3

Table 1. The effect of compounds isolated from the leaves of *A. koreana* on LPS-induced NO- production in BV-2 microglia

	1 μM		10 μM		100 μM	
	NO (%)	Viability (%)	NO (%)	Viability (%)	NO (%)	Viability (%)
1	80.15 ± 3.29*	91.18 ± 0.96	73.30 ± 1.79*	86.94 ± 0.88	68.68 ± 3.30*	82.12 ± 3.77
2	98.72 ± 2.14	84.75 ± 2.26	99.57 ± 0.95	77.63 ± 1.88	88.21 ± 1.59*	78.97 ± 5.94
3	96.17 ± 4.64	75.53 ± 1.92	92.83 ± 0.60	70.18 ± 1.24	90.82 ± 4.82	71.53 ± 0.22
4	97.34 ± 1.30	78.78 ± 2.56	101.35 ± 2.67	84.29 ± 5.26	102.27 ± 4.78	77.34 ± 1.39
5	99.21 ± 5.15	104.91 ± 2.45	85.19 ± 5.89	99.27 ± 0.36	96.11 ± 0.95	94.75 ± 2.35
6	107.64 ± 1.02	85.36 ± 3.74	95.35 ± 0.79	80.35 ± 2.54	21.52 ± 0.83**	81.25 ± 0.92
7	85.71 ± 1.90*	101.66 ± 0.92	79.78 ± 1.33*	86.79 ± 2.34	77.57 ± 1.37*	66.50 ± 5.25
8	99.65 ± 2.97	88.32 ± 0.48	99.83 ± 1.72	76.55 ± 0.23	93.18 ± 0.14	71.96 ± 1.01
9	95.35 ± 0.20	73.29 ± 0.51	93.38 ± 1.05	68.93 ± 2.62	86.67 ± 1.74	71.52 ± 1.53
10	96.18 ± 1.85	98.65 ± 0.76	99.96 ± 5.44	89.57 ± 1.78	94.03 ± 0.94	87.59 ± 1.37
11	85.85 ± 1.18*	78.65 ± 1.42	83.69 ± 2.02*	77.38 ± 0.70	69.49 ± 7.35	71.78 ± 2.41

BV-2 microglia were washed with DMEM and incubated with test compounds for 1 hr. The cultures were then stimulated by 100 ng/ml of LPS for 24 hrs. After incubation, NO production was measured by the Griess reaction and sodium nitrite was used as a standard. NO production (NP) of the control and the LPS-treated cultures were 5.63 ± 0.53 and 72.92 ± 1.90 μM, respectively. Relative production (%) was calculated as (NP of sample treated – NP of control) / (NP of LPS treated – NP of control) × 100. Mean value is significantly different (*$p < 0.05$, **$p < 0.01$) from the value of the LPS-treated.

(1H, d, J = 5.5 Hz, H-6), 2.2 (3H, s, CH_3); ^{13}C-NMR (125 MHz, DMSO- d_6) : δ 172.5 (C-4), 154.5 (C-3), 149.2 (C-6), 142.9 (C-2), 113.5 (C-5), 13.9 (2-CH_3).

maltol-3-*O*-β-D-glucoside (2) – Dark brownish oil; FABMS *m/z* : 289 $[M + H]^+$; UV λ_{max} (MeOH) nm : 276, 402; IR ν_{max} (KBr): 3350, 1650, 1620, 1260, 890, 705 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) : δ 8.1 (1H, d, J = 5.6 Hz, H-6), 6.4 (1H, d, J = 5.6 Hz, H-5), 4.7 (1H, d, J = 7.7 Hz, H-1'), 3.6 (1H, dd, J = 12.1, 2.2 Hz, H_a-6'), 3.4 (1H, dd, J = 11.8, 5.0 Hz, H_b-6'), 2.4 (3H, s, CH_3); ^{13}C-NMR (125 MHz, DMSO- d_6) : δ 174.3 (C-4), 161.4 (C-2), 155.8 (C-6), 141.9 (C-3), 116.3 (C-5), 103.7 (C-1'), 77.3 (C-5'), 76.3 (C-3'), 73.9 (C-2'), 69.6 (C-4'), 60.9 (C-6'), 15.3 (C-7).

(–)-epicatechin (3) – Brownish syrup; EI MS *m/z* : 290 $[M]^+$; UV λ_{max} (MeOH) nm : 279, 307; IR ν_{max} (KBr): 3306 cm^{-1}; ^{1}H-NMR (400 MHz, CD_3 OD) : δ 6.8 (1H, d, J = 1.6 Hz, H-2), 6.8 (1H, d, J = 8.1 Hz, H-5'), 6.7 (1H, dd, J = 1.8, 8.1 Hz, H-6'), 5.9 (1H, d, J = 2.1 Hz, H-6), 5.8 (1H, d, J = 2.1 Hz, H-8), 4.6 (1H, d, J = 7.6 Hz, H-2), 4.0 (1H, m, H-3), 2.8 (1H, J = 5.4, 16.1 Hz, H_a-4), 2.5 (1H, J = 8.1, 16.1 Hz, H_b-4)); ^{13}C-NMR (125 MHz, CD_3 OD) : δ 157.9 (C-5), 157.6 (C-7), 156.9 (C-9), 146.3 (C-3'), 146.2 (C-4'), 132.3 (C-1'), 120.1 (C-6'), 116.1 (C-2'), 115.3 (C-5'), 100.9 (C-10), 96.3 (C-6), 95.6 (C-8), 82.9 (C-2), 68.9 (C-3), 28.6 (C-4).

Naringenin 7-*O*-β-D-glucopyranoside (4) – Yellowish oil; FAB MS *m/z* : 435 $[M + H]^+$; $[\alpha]^{20}_D$: –66.6 (*c* 0.3, MeOH); UV λ_{max} (MeOH) nm : 228, 285, 330; IR ν_{max} (KBr): 3300, 1615, 1570, 1520, 1050 cm^{-1}; ^{1}H-NMR (300 MHz, CD_3 OD) : δ 7.3 (2H, d, J = 8.6 Hz, H-2', 6'), 6.8 (2H, d, J = 8.6 Hz, H-3', 5'), 6.2 (1H, d, J = 2.4 Hz, H-8), 6.2 (1H, d, J = 2.4 Hz, H-6), 5.4 (1H, dd, J = 2.8, 12.9 Hz, H-2), 5.0 (1H, d, J = 7.3 Hz, H-1"), 3.3~3.9 (5H, m, H-2", 3", 4" 5", 6"), 3.2 (1H, dd, J = 12.9, 17.2 Hz, H_b-3), 2.7 (1H, dd, J = 3.1, 17.2, H_a-3). ^{13}C-NMR (125 MHz, CD_3 OD) : δ 198.6 (C-4), 167.0 (C-7), 165.0 (C-5), 164.6 (C-9), 159.1 (C-4'), 130.9 (C-1'), 129.1 (C-2'), 129.1 (C-6'), 116.3 (C-3'), 116.3 (C-5'), 104.9 (C-10), 101.3 (C-1"), 98.0 (C-6), 96.9 (C-8), 80.7 (C-2), 78.3 (C-5"), 77.8 (C-3"), 74.7 (C-2"), 71.2 (C-4"), 62.4 (C-6"), 44.2 (C-3).

Naringenin-7-*O*- rhamnoglucoside (5) – Whitish amorphous powder; FAB MS *m/z* : 379$[M - H]^+$; UV λ_{max} (MeOH) nm : 282, 326; IR ν_{max} (KBr): 1645, 1298 cm^{-1}; ^{1}H-NMR (500 MHz, CD_3 OD) : δ 7.3 (2H, d, J = 8.5 Hz, H-2' and H-6'), 6.8 (2H, d, J = 8.5 Hz, H-3' and H-5'), 6.2 (1H, d, J = 2.1 Hz, H-8), 6.1 (1H, d, J = 2.1 Hz, H-6), 5.4 (1H, dd, J = 12.9, 2.5 Hz, H-2), 5.2 (1H, d, J = 1.3 Hz, H-1'"), 5.1 (1H, d, J = 7.7 Hz, H-1"), 3.1 (1H, m, H_a-3), 2.7 (1H, dd, J = 17.1, 2.8 Hz, H_b-3), 1.3 (3H, d, J = 6.2 Hz, H-6'"); ^{13}C-NMR (125 MHz, CD_3 OD) : δ 199.3 (C-4), 167.3 (C-7), 165.7 (C-5), 165.4 (C-9), 159.9 (C-4'), 131.5 (C-1'), 130.0 (C-2', 6'), 117.1 (C-3', 5'), 105.7 (C-10), 103.4 (C-1'"), 100.1 (C-1"), 98.7 (C-6), 97.6 (C-8), 81.4 (C-2), 80.0 (C-2"), 79.7 (C-5"), 78.9 (C-3"), 74.7 (C-4'"), 73.0 (C-2'", 3'"), 72.0 (C-4"), 70.8 (C-5'"), 63.1 (C-6"), 44.7 (C-3), 19.0 (C-6'").

Kaempferol 3-*O*-β-D-glucopyranoside (6) – Yellowish oil; FAB MS *m/z* : 449 $[M + H]^+$; UV λ_{max} (MeOH) nm : 267, 300, 353; IR ν_{max} (KBr): 3420, 1680, 1620, 1590,

1510 cm^{-1}; ^{1}H-NMR (600 MHz, CD_3OD) : δ 8.1 (2H, d, *J* = 9.0 Hz, H-2' and H-6'), 6.9 (2H, d, *J* = 9.0 Hz, H-3' and H-5'), 6.4 (1H, d, *J* = 1.8 Hz, H-8), 6.2 (1H, d, *J* = 1.8 Hz, H-6), 5.3 (1H, d, *J* = 7.8 Hz, H-1''), 3.2~3.9 (5H, m, H-2'', 3'', 4'', 5'', 6''); ^{13}C-NMR (150 MHz, CD_3OD) : δ 179.7 (C-4), 166.1 (C-7), 163.2 (C-5), 161.7 (C-4'), 159.2 (C-9), 158.6 (C-2), 137.3 (C-3), 132.4 (C-2'), 132.4 (C-6'), 122.9 (C-1'), 116.2 (C-3'), 116.2 (C-5'), 104.3 (C-10), 100.0 (C-1''), 94.9 (C-8), 78.6 (C-3''), 78.2 (C-5''), 75.9 (C-2''), 71.5 (C-4''), 62.8 (C-6'').

Isolariciresinol (7) – Brownish oil; FAB MS *m/z* : 361 $[M+H]^+$; $[\alpha]^{20}_D$: 68.0 (*c* 1.0, Me_2CO); UV λ_{max} (MeOH) nm : 220, 285; IR ν_{max} (KBr): 3350, 1600, 1500 cm^{-1}; ^{1}H-NMR (600 MHz, CD_3OD) : δ 6.7 (1H, d, *J* = 8.0 Hz, H-5), 6.7 (1H, d, *J* = 1.4 Hz, H-2), 6.6 (1H, s, H-2'), 6.6 (1H, dd, *J* = 1.5, 8.0 Hz, H-6), 6.2 (1H, s, H-5'), 3.8 (1H, d, *J* = 10.7 Hz, H-7), 3.8 (3H, s, 3'-OCH_3), 3.8 (3H, s, 3-OCH_3), 3.7 (1H, m, H-9'), 3.7 (3H, dd, *J* = 3.8, 10.8 Hz, H_b-9), 3.4 (1H, dd, J = 4.0, 11.2 Hz, H_a-9), 2.8 (2H, d, *J* = 7.7 Hz, H-7'), 2.0 (1H, m, H-8'), 1.8 (1H, m, H-8); ^{13}C-NMR (150 MHz, CD_3OD) : δ 149.1 (C-3), 147.3 (C-3'), 146.0 (C-4), 145.4 (C-4'), 138.7 (C-6'), 134.3 (C-1), 129.1 (C-1'), 123.3 (C-6), 117.5 (C-5'), 116.1 (C-2), 113.9 (C-5), 112.5 (C-2'), 66.1 (C-9'), 62.4 (C-9), 56.5 (OCH_3), 56.5 (OCH_3), 48.2 (C-8), 48.13 (C-7), 40.14 (C-8'), 33.7 (C-7').

Secoisolariciresinol (8) – Brownish oil; FAB MS *m/z* : 363 $[M+H]^+$; $[\alpha^{20}_D$: -16.0 (*c* 0.10, MeOH); UV λ_{max} (MeOH) nm : 226, 281; IR ν_{max} (KBr): 3425, 1516 cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD) : δ 6.7 (2H, d, *J* = 7.9 Hz, H-5 and H-5'), 6.6 (2H, d, *J* = 1.9 Hz, H-2 and H-2'), 6.5 (2H, dd, *J* = 7.9, 1.9 Hz, H-6 and H-6'), 3.7 (3H, s, OCH_3), 3.6 (4H, m, H-9 and H-9'), 2.6 (2H, dd, *J* = 13.6, 7.0 Hz, H_b-7 and H-7'), 2.5 (2H, dd, *J* = 13.6, 7.7 Hz, H_a-7 and H-7'), 1.9 (2H, m, H-2 and H-3); ^{13}C-NMR (100 MHz, CD_3OD) : δ 149.5 (C-3), 149.5 (C-3'), 146.1 (C-4), 146.1 (C-4'), 134.6 (C-1), 134.6 (C-1'), 123.4 (C-6), 123.4 (C-6'), 116.6 (C-5), 116.6 (C-5'), 113.9 (C-2), 113.9 (C-2'), 62.8 (C-9), 62.8 (C-9'), 57.0 (OCH_3), 57.0 (OCH_3), 44.8 (C-8), 44.8 (C-8'), 36.7 (C-7), 36.7 (C-7').

Rhododendrol (9) – Whitish powder; EIMS *m/z* : 166 $[M]^+$; UV λ_{max} (MeOH) nm : 214.5, 276; IR ν_{max} (KBr): 3330, 1595, 1500 cm^{-1}; ^{1}H-NMR (500 MHz, CD_3OD) : δ 7.0 (2H, d, *J* = 8.4 Hz, H-6 and H-10), 6.7 (2H, d, *J* = 8.4 Hz, H-7 and H-9), 3.8 (1H, m, H-2), 2.6 (1H, t, *J* = 7.0 Hz, H-4), 1.7 (1H, m, H-3), 1.2 (3H, d, *J* = 6.3 Hz, CH_3); ^{13}C-NMR (75 MHz, CD_3OD) : δ 157.1 (C-8), 135.2 (C-5), 131.0 (C-6), 131.0 (C-10), 116.8 (C-7), 116.8 (C-9), 68.7 (C-2), 43.2 (C-4), 33.0 (C-3), 24.3 (CH_3).

Ferulic acid (10) – Yellowish powder; EI MS *m/z* : 194 $[M]^+$; UV λ_{max} (MeOH) nm : 242, 293, 323; IR ν_{max} (KBr): 3427, 2921, 1620, 1516, 1432 cm^{-1}; ^{1}H-NMR (300 MHz, CD_3OD) : δ 7.6 (1H, d, *J* = 15.9 Hz, H-7), 7.1 (1H, d, *J* = 2.0 Hz, H-2), 7.0 (1H, dd, *J* = 8.1, 1.8 Hz, H-6), 6.9 (1H, d, *J* = 8.1 Hz, H-5), 6.3 (1H, d, *J* = 15.9 Hz, H-8), 2.9 (3H, s, OCH_3); ^{13}C-NMR (75 MHz, CD_3OD) : δ 173.3 (C-9), 153.9 (C-3), 150.5 (C-7), 149.1 (C-4), 131.5 (C-1), 125.1 (C-6), 119.1 (C-5), 117.3 (C-8), 115.0 (C-2), 58.8 (OCH_3).

4-(4-hydroxyphenyl)butan-2-one (11) – Brownish oil; EI MS *m/z* : 164 $[M]^+$; UV λ_{max} (MeOH) nm : 224, 278; IR ν_{max} (KBr): 3360, 3020, 2920, 2870, 1685, 1620, 1600, 1510, 1440, 1365, 1320, 1290, 1225, 1170, 1105, 1040, 960, 875, 830, 765, 730 cm^{-1}; ^{1}H-NMR (500 MHz, CD_3OD) : δ 7.0 (2H, d, *J* = 8.3, H-6 and H-10), 6.8 (2H, d, *J* = 8.3, H-7 and H-9), 2.8-2.7 (4H, m, H-3 and 4), 2.1 (3H, s, CH_3); ^{13}C-NMR (125 MHz, CD_3OD) : δ 210.0 (C-2), 154.5 (C-8), 132.6 (C-5), 129.5 (C-6, 10), 115.6 (C-7, 9), 45.6 (C-3), 30.3 (CH_3), 29.1 (C-4).

Culture of BV2 microglial cells – BV2 microglial cells were provided by Prof. Sun-yeou Kim, at Kyung Hee University (Suwon, Korea). The cell line was maintained in DMEM containing 10% FBS with penicillin (100 IU/mL) and streptomycin (10 mg/mL) at 37 °C in a humidified atmosphere of 95% air-5% CO_2.

Assay for inhibition of nitric oxide production – To remove any trace of phenol red, the cell cultures were washed and the medium was replaced with Griess medium and further incubated with test samples and LPS. After 24 hrs incubation, 100 ul aliquots of sample were mixed with 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphtylethylenediamine dihydrochloride, 2% phosphoric acid) in a 96 well plate and incubated at room temperature for 15 min. The absorbance at 550 nm was measured on a microplate reader. The concentration was determined using a nitrite standard curve (Dawson et al., 1994)

Cell viability measurements using the MTT assay – After 100 μl aliquots of sample were collected for Griess assay, MTT (0.2 mg/mL) was directly added to cultures, followed by incubation at 37 °C for 3 hrs. The supernatant was then aspirated and 100 μl of DMSO was added to dissolve the formazan. After insoluble crystals were completely dissolved, absorbance (abs) at 540 nm was measured using a microplate reader. Data were expressed as percent cell viability relative to control cultures.

$$\text{Cell viabiltity (\%)} = 100 \times \frac{\text{(Abs of LPS-treated or LPS + saple-treated)}}{\text{Abs of control}}$$

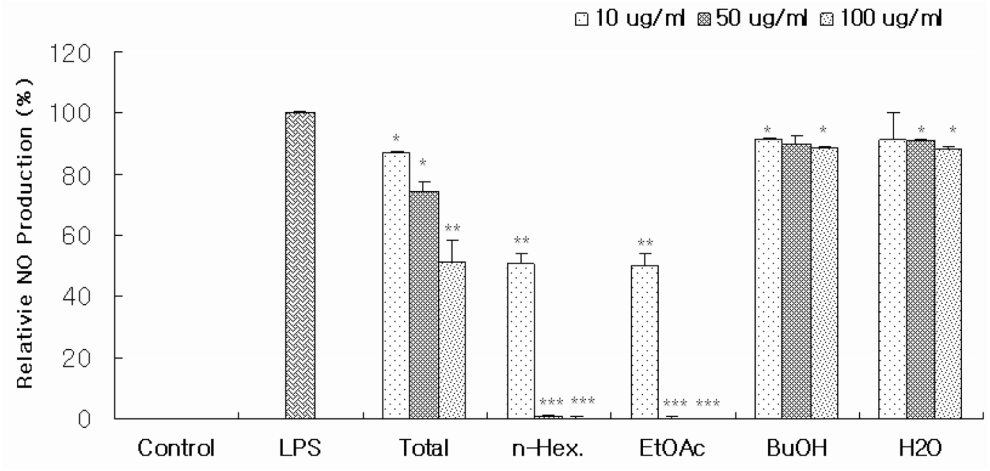

Fig. 2. Effect of each fraction from *A. koreana* leaves on LPS-induced NO production in BV-2 microglia
BV2 microglia were washed with DMEM and incubated with test fractions for 1 hr. The cultures were then stimulated by 100 ng/mL of LPS for 24 hrs. After incubation, NO production was measured by the Griess reaction and sodium nitrite was used as a standard. NO production (NP) of control and LPS-treated cultures were 1.76 ± 1.6 and 22.48 ± 1.2 μM, respectively. Relative production (%) was calculated as (NP of sample treated - NP of control)/(NP of LPS-treated - NP of control) × 100. Mean value is significantly different (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) from the value of the LPS-treated.

Results and discussion

In search of inhibitory activity against NO production from natural sources, 80% MeOH extract of *Abies koreana* displayed moderate inhibitory activity against LPS induced NO production in BV-2 microglia in concentration dependent manner (Fig. 2). The 80% MeOH extract of *A. koreana* was partitioned with *n*-hexane, ethyl acetate, *n*-buthanol and water. The ethyl acetate soluble fraction showed potent inhibitory activity against NO production at concentrations of 50 and 100 μg/mL. The ethyl acetate soluble fraction of the 80% MeOH extract from *A. koreana* was chromatographed by repetitive column chromatography using diverse solid absorbents including silica gel, HP-20, and RP-C_{18} resin to yield eleven compounds (**1 - 11**). Isolates were identified by comparing ^{1}H-, ^{13}C-NMR and MS spectroscopic data with literature values as 3-hydroxy-2-methyl-4-pyrone (**1**, Kim *et al*., 2001), maltol-3-*O*-β-D-glucoside (**2**, Li *et al*., 2004), (−)-epicatechin (**3**, Khallouki *et al*., 2007), naringenin 7-*O*-β-D-glucopyranoside (**4,** Choi *et al*., 1990), naringenin-7-*O*-rhamnoglucoside (**5,** Akiyama *et al*., 2000), kaempferol 3-*O*-β-D-glucopyranoside (**6**, Kovganko *et al*., 2002), (+)-isolariciresinol (**7**, Baderschneider *et al*., 2001), secoisolariciresinol (**8**, Fonseca *et al*., 1978), rhododendrol (**9**, Das *et al*., 1993), ferulic acid (**10**, Anselmi *et al*., 2006) and 4-(4-hydroxyphenyl) butan-2-one (rheosmin) (**11**, Smith *et al*., 1996). Among eleven isolates, compounds **3**, **5**, and **7-11** were isolated for the first time from *Abies koreana*.

Microglial cells are known as the phagocytic immune cells of the brain, and excessive activation of these cells are associated with neurodegenerative disorders including Alzheimer's disease, Parkinson's disease and ischemia. When triggered by various stimuli, microglial cells produce proinflammatory cytokines (interleukin-1β, interleukin-6, and tumor necrosis factor-α (TNF-α), reactive oxygen species (ROS), and nitric oxide (NO), leading to neuroinflammation and neurodegeneration [Eguchi *et al*., 2008]. Therefore, it is suggested that suppression of overactivated microglial cells may mitigate the progression of neuroinflammatory and neurodegenerative diseases.

Compounds **1 - 11** were tested *in vitro* for their inhibitory effects against LPS induced nitric oxide production of microglia BV2 cells. Among the isolates, compounds **1, 7** and **11** showed inhibitory activities against LPS induced NO production in BV-2 microglia in a concentration dependent manner.

Kang *et al*. (2006) demonstrated that compound **1** isolated from four year old *Panax ginseng* inhibited NO production from nitroprusside as well as simple phenolics such as *p*-coumaric and vanillic acid. In addition, maltol levels were markedly increased by heat processing of *Panax ginseng*. Even though there was a difference in maltol concentration, the present study is consistent with previously reported data. (+)-Isolariciresinol (**7**) was reported to exert a potent *in vitro* inhibitory effect on production of a pro-inflammatory cytokine, TNF-a (Cho *et al*., 2001; Esra *et al*., 2003). It was reported that

compound **11** inhibited NO and PGE_2 production in a dose-dependent manner, and also blocked LPS-induced iNOS and COX-2 expression in RAW 264.7 cells by blocking NF-kB activation (Jeong *et al*., 2010).

Thus, the present study was able to identify constituents of *A. koreana* that inhibit NO production in BV-2 microglia cells, and the constituents which had not been reported from this plant before.

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