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Chemical Constituents from *Buddleja officinalis* and Their Inhibitory Effects on Nitric Oxide Production

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Abstract – Bioactivity-guided fractionation of a methanolic extract of *Buddleja officinalis* led to the isolation of two monoterpenes, crocusatin M (**1**), crocusatin C (**2**), a flavonoid, acacetin (**3**), three lignans, lariciresinol (**4**), pinoresinol (**5**), and syringaresinol (**6**), and two triterpenoidal saponins, mimengoside B (**7**) and songarosaponin A (**8**). The structures of isolates were identified based on 1D-, 2D-NMR, and MS data analysis. All isolates were tested for their inhibition on LPS-induced NO production in RAW 264.7 cells. As a result, mimengoside B (**7**) and songarosaponin A (**8**) showed a mild inhibitory activity of NO production.
Keywords – *Buddleja officinalis*, Buddlejaceae, NO production inhibitor

Introduction

Buddleja officinalis Maxim. (Buddlejaceae), a shrub tree, has been widely distributed in temperate regions of America, Africa, and Asia. The flower buds of *B. officinalis* have been used in traditional Korean medicine for the treatment of stroke, headache, neurological disorders, conjunctivitis, and other ophthalmologic diseases.[1] Previous phytochemical investigations on *B. officinalis* have reported to contain several types of compounds such as terpenoids, flavonoids, iridoids, phenylethanoids, and saponins.[1-7] In the course of an investigation for the isolation of plant-derived nitric oxide (NO) production inhibitors, a MeOH extract of the flower buds of *B. officinalis* showed inhibitory effects on LPS-induced NO production. The dried flower buds of *B. officinalis* were extracted with MeOH, and the resultant extract was partitioned successively with *n*-hexane, CH_2Cl_2, and water. Using various types of column chromatography and preparative HPLC, two monoterpenes, a flavonoid, three lignans, and two triterpenoidal saponins were isolated from the CH_2Cl_2 soluble fraction. Their structures were identified based on 1D-, 2D-NMR, and MS data analysis, as well as by comparison with previously reported spectroscopic data. This paper herein deals with the isolation and structure elucidation of the isolated compounds, and their inhibitory effects on LPS-induced NO production in RAW 264.7 cells.

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Experimental

General experimental procedures – UV spectra were obtained using a JASCO UV-550. NMR spectra were recorded on a Bruker AMX-500 MHz NMR spectrometer. ESIMS was performed on a Waters Q-TOF micro mass spectrometer. Open column chromatography and MPLC were performed using a silica gel (Kiesel gel 60, 700 - 230 mesh, Merck) and a LiChroprep RP-18 (40 - 60 μM, Merck), respectively. Preparative HPLC was conducted using a Waters system (515 pump and 2996 photodiode array detector) with a YMC J'sphere ODS-H80 column (4 μm, 20 × 250 mm, i.d., flow rate 6 mL/min). Thin layer chromatography (TLC) was performed using pre-coated silica gel 60 F254 plates (0.25 mm, Merck).

Plant materials – The dried flower buds of *B. officinalis* were purchased from Kyungdong Oriental Herbal Market (Seoul, Korea) in 2009. A voucher specimen (CBNU-2009-03-BO) was authenticated by one of the authors (B. Y. H.) and deposited at the Herbarium of the College of Pharmacy, Chungbuk National University, Cheongju, Korea.

Extraction and isolation – The dried and powdered flower buds of *B. officinalis* (2.6 kg) were extracted with MeOH at room temperature, and then the solution was evaporated under reduced pressure. The residue was suspended in H_2O and partitioned successively with *n*-hexane, CH_2Cl_2, and EtOAc. The CH_2Cl_2 soluble fraction (25 g) was chromatographed on a silica gel column eluted

with a gradient solvent system consisting of *n*-hexane-CH_2Cl_2 (1:0 to 0:1) and CH_2Cl_2-MeOH (1:0 to 0:1), to yield nine subfractions (BOC1-BOC9). Fraction BOC4 was subjected to RP-18 MPLC eluted with MeOH-H_2O gradient system (20:80 to 100:0) to yield five subfractions (BOC4A-BOC4E). Subfraction BOC4B was purified by preparative HPLC (MeCN-H_2O, 10:90 to 30:70) to afford compound **1** (5 mg). Subfraction BOC4C was further purified by preparative HPLC (MeCN-H_2O, 20:80 to 40:60) to afford compounds **2** (5 mg) and **4** (3 mg). Compound **3** (7 mg) was obtained by recrystallization from the fraction BOC2 in CH_2Cl_2 and MeOH. Fraction BOC1 was subjected to RP-18 MPLC eluted with MeOH-H_2O gradient system (30:70 to 100:0) to yield four subfractions (BOC1A-BOC1D). Subfraction BOC1B was purified by preparative HPLC (MeCN-H_2O, 30:70 to 80:20) to give compounds **5** (3 mg) and **6** (3 mg). BOC8 was subjected to RP-18 MPLC eluted with MeOH-H_2O gradient system (20:80 to 100:0) to yield four subfractions (BOC8A-BOC8D). Subfraction BOC8C was further purified by preparative HPLC (MeCN-H_2O, 40:60 to 100:0) to afford compounds **7** (7 mg) and **8** (40 mg).

Crocusatin M (1) – Colorless oil; UV (MeOH) λ_{max} 244.5 nm; ^{1}H NMR (CD_3OD, 500 MHz): δ 5.92 (1H, t, *J* = 1.5 Hz, H-4), 3.77 (1H, d, *J* = 11.5 Hz, H-7a), 3.64 (1H, d, *J* = 11.5 Hz, H-7b), 2.92 (1H, d, *J* = 17.5 Hz, H-2α), 2.08 (1H, d, *J* = 17.5 Hz, H-2β), 2.02 (3H, d, *J* = 1.5 Hz, CH_3-10), 1.11 (3H, s, CH_3-9), 1.01 (3H, s, CH_3-8); ^{13}C NMR (CD_3OD, 125 MHz): δ 200.5 (C-3), 166.3 (C-5), 126.9 (C-4), 77.5 (C-6), 64.5 (C-7), 49.6 (C-2), 39.9 (C-1), 23.9 (C-8), 22.7 (C-9), 18.9 (C-10); ESIMS *m/z* 185 $[M+H]^+$; HRESIMS *m/z* 185.1177 $[M+H]^+$ (calcd 185.1178).

Crocusatin C (2) – Colorless oil; UV (MeOH) λ_{max} 238.1 nm; ^{1}H NMR (CD_3OD, 500 MHz): δ 5.84 (1H, s, H-4), 3.81 (1H, dd, *J* = 12.0, 3.6 Hz, H-7a), 3.75 (1H, dd, *J* = 12.0, 3.6 Hz, H-7b), 2.61 (1H, d, *J* = 16.8 Hz, H-2α), 1.88 (1H, d, *J* = 16.8 Hz, H-2β), 1.97 (3H, d, *J* = 0.8 Hz, CH_3-10), 1.93 (1H, t, *J* = 3.6 Hz, H-6), 1.05 (3H, s, CH_3-9), 0.93 (3H, s, CH_3-8); ^{13}C NMR (CD_3OD, 125 MHz): δ 201.5 (C-3), 164.3 (C-5), 126.2 (C-4), 60.3 (C-7), 53.6 (C-6), 47.8 (C-2), 34.9 (C-1), 28.1 (C-8), 25.9 (C-9), 12.8 (C-10); ESIMS *m/z* 191 $[M+Na]^+$.

Acacetin (3) – Yellow needle; UV (MeOH) λ_{max} 217.5, 269.3, 327.5 nm; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 12.9 (1H, s, 5-OH), 8.04 (2H, d, *J* = 8.0 Hz, H-2', 6'), 7.11 (2H, d, *J* = 8.0 Hz, H-3', 5'), 6.88 (1H, s, H-3), 6.51 (1H, d, *J* = 2.0 Hz, H-6), 6.20 (1H, d, *J* = 2.0 Hz, H-8), 3.88 (3H, s, 4'-OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 182.3 (C-4), 164.8 (C-7), 163.8 (C-2), 162.8 (C-9), 161.9 (C-4'), 157.8 (C-5), 128.8 (C-2', 6'), 123.3 (C-1'), 115.1 (C-3', 5'), 104.2 (C-10), 104.0 (C-3), 99.4 (C-6), 94.5 (C-8), 56.0 (4'-OCH_3); ESIMS *m/z* 283 $[M-H]^-$.

Lariciresinol (4) – White amorphous powder; UV (MeOH) λ_{max} 227.3, 280.0 nm; ^{1}H NMR (CD_3OD, 500 MHz): δ 6.93 (1H, br s, H-2), 6.82 (1H, br d, *J* = 8.0 Hz, H-6), 6.81 (1H, br s, H-2'), 6.79 (1H, br d, *J* = 8.0 Hz, H-5), 6.67 (1H, br d, *J* = 8.0 Hz, H-5'), 6.65 (1H, br d, *J* = 8.0 Hz, H-6'), 4.77 (1H, d, *J* = 7.0 Hz, H-7), 4.01 (1H, dd, *J* = 10.5, 7.5 Hz, Hb-9'), 3.87 (6H, s, OCH_3-3, 3'), 3.86 (1H, dd, overlapped, Hb-9), 3.75 (1H, dd, *J* = 10.5, 7.5 Hz, Ha-9'), 3.65 (1H, dd, *J* = 8.0, 6.0 Hz, Ha-9), 2.95 (1H, dd, *J* = 17.0, 6.0 Hz, Ha-7'), 2.77 (1H, m, H-8'), 2.52 (1H, dd, *J* = 17.0, 11.0 Hz, Hb-7'), 2.40 (1H, m, H-8); ^{13}C NMR (CD_3OD, 125 MHz): δ 149.0 (C-3), 147.1 (C-3', 4), 145.8 (C-4'), 135.8 (C-1), 133.5 (C-1'), 122.2 (C-6), 119.8 (C-6'), 116.2 (C-5'), 116.0 (C-5), 113.4 (C-2), 110.7 (C-2'), 84.1 (C-7), 73.5 (C-9'), 60.5 (C-9), 56.4 (OCH_3-3, 3'), 54.1 (C-8), 43.9 (C-8'), 33.7 (C-7'); ESIMS *m/z* 383 $[M+Na]^+$.

Pinoresinol (5) – White amorphous powder; UV (MeOH) λ_{max} 230.4, 280.0 nm; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.96 (2H, d, *J* = 1.5 Hz, H-2, 2'), 6.83 (2H, dd, *J* = 8.0, 1.5 Hz, H-5, 5'), 6.78 (2H, d, *J* = 8.0 Hz, H-6, 6'), 4.73 (2H, d, *J* = 4.5 Hz, H-7, 7'), 4.25 (2H, dd, *J* = 9.0, 6.5 Hz, Hb-9, 9'), 3.90 (2H, dd, overlapped, Ha-9, 9'), 3.87 (6H, s, OCH_3-3, 3'), 3.16 (2H, m, H-8, 8'); ^{13}C NMR ($CDCl_3$, 125 MHz): 147.7 (C-3, 3'), 145.9 (C-4, 4'), 132.4 (C-1, 1'), 118.7 (C-6, 6'), 114.7 (C-5, 5'), 109.6 (C-2, 2'), 86.1 (C-7, 7'), 71.1 (C-9, 9'), 55.0 (OCH_3-3, 3'), 54.0 (C-8, 8'); ESIMS *m/z* 381 $[M+Na]^+$.

Syringaresinol (6) – White amorphous powder; UV (MeOH) λ_{max} 215.1, 271.7 nm; ^{1}H NMR (CD_3OD, 500 MHz): δ 6.68 (4H, s, H-2, 2', 6, 6'), 4.74 (2H, d, *J* = 4.0 Hz, H-7, 7'), 4.29 (2H, dd, *J* = 9.0, 7.0 Hz, Hb-9, 9'), 3.91 (2H, dd, *J* = 9.0, 3.5 Hz, Ha-9, 9'), 3.87 (12H, s, OCH_3-3, 3', 5, 5'), 3.17 (2H, m, H-8, 8'); ^{13}C NMR (CD_3OD, 125 MHz): 148.0 (C-3, 3', 5, 5'), 134.8 (C-4, 4'), 131.7 (C-1, 1'), 103.1 (C-2, 2', 6, 6'), 86.2 (C-7, 7'), 71.4 (C-9, 9'), 55.4 (OCH_3-3, 3', 5, 5'), 54.1 (C-8, 8'); ESIMS *m/z* 441 $[M+Na]^+$.

Mimengoside B (7) – White amorphous powder; UV (MeOH) λ_{max} 263.0 nm; ^{1}H NMR (CD_3OD, 500 MHz): δ 5.40 (1H, d, *J* = 3.0 Hz, H-12), 4.91 (1H, overlapped, rha H-1), 4.89 (1H, overlapped, glc H-1), 4.64 (1H, d, *J* = 7.5 Hz, glc H-1), 4.50 (1H, d, *J* = 8.0 Hz, fuc H-1), 3.27 (3H, s, OCH_3), 1.30 (6H, d, *J* = 1.5 Hz, fuc CH_3-6, rha CH_3-6), 0.77, 0.92, 0.94, 1.04, 1.13, 1.29 (each 3H, s, H-24, 25, 26, 27, 29, 30); ^{13}C NMR (CD_3OD, 125 MHz), see Table 1; ESIMS *m/z* 1127 $[M+Na]^+$.

Table 1. ^{13}C NMR data of compounds **7** and **8** (δ in ppm, 125 MHz, CD_3OD)[a]

Position	7	8	position	7	8
1	40.6	39.6	Fuc 1	104.5	105.3
2	26.6	27.0	2	76.7	77.3
3	84.2	86.2	3	85.5	84.9
4	44.3	44.9	4	72.2	72.9
5	49.7	49.2	5	71.2	71.8
6	17.8	17.8	6	17.7	17.4
7	33.5	31.3	Glc 1	105.0	105.7
8	42.8	41.4	2	75.2	75.3
9	54.1	56.2	3	79.2	79.8
10	37.9	36.5	4	72.1	72.8
11	76.2	127.7	5	76.6	76.9
12	122.7	127.0	6	61.6	62.2
13	150.5	138.1	Glc' 1	103.4	104.0
14	44.4	43.9	2	76.2	76.0
15	33.6	32.4	3	77.5	77.3
16	22.7	25.3	4	78.2	78.8
17	38.7	42.0	5	75.9	76.6
18	43.3	136.5	6	63.4	64.1
19	47.4	37.9	Rha 1	102.7	103.4
20	32.0	33.3	2	72.6	73.2
21	31.7	34.2	3	72.3	73.0
22	26.7	30.2	4	73.6	74.3
23	64.6	64.2	5	70.5	71.2
24	13.0	13.2	6	18.6	18.4
25	16.7	18.4			
26	18.8	19.4			
27	25.5	19.4			
28	69.6	65.1			
29	35.1	34.2			
30	23.8	24.5			
-OCH_3	53.2				

[a] The assignments were based on ^1H-^1H COSY, HMQC, and HMBC experiments.

Songarosaponin A (8) – White amorphous powder; UV (MeOH) λ_{max} 243.3, 252.8, 262.4 nm; ^1H NMR (CD_3OD, 500 MHz): δ 6.45 (1H, dd, J = 10.5, 2.5 Hz, H-11), 5.62 (1H, d, J = 10.5 Hz, H-12), 4.88 (1H, overlapped, rha H-1), 4.80 (1H, overlapped, glc H-1), 4.65 (1H, d, J = 7.0 Hz, glc H-1), 4.51 (1H, d, J = 8.0 Hz, fuc H-1), 1.29 (6H, d, J = 5.0 Hz, fuc CH_3-6, rha CH_3-6), 0.75, 0.76, 0.82, 0.97, 0.99, 1.02 (each 3H, s, H-24, 25, 26, 27, 29, 30); ^{13}C NMR (CD_3OD, 125 MHz), see Table 1; ESIMS *m/z* 1095 $[M+Na]^+$.

Measurement of LPS-induced NO production and cell viability – Griess reaction was used to determine the nitrite concentration in the medium as an indicator of NO production. Briefly, RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and penicillin (100 units/mL)-streptomycin (100 μg/mL) in an incubator at 37 °C and 5% CO_2. RAW 264.7 cells were seeded into 96 well culture plates at 2×10^6 cells/mL and treated with 1 μg/mL of LPS in the presence or absence of compounds. After incubation of 24 h, 100 μL of cell-free supernatant was mixed with 100 μL of Griess reagent containing equal volumes of 2% (w/v) sulfanilamide in 5% (w/v) phosphoric acid and 0.2% (w/v) *N*-(1-naphthyl) ethylenediamine solution to determine nitrite production. Cell viability of the remaining cells was determined by a cell counting kit (CCK) (Dojindo, Kumamoto, Japan) based colorimetric assay.

Result and Discussion

Eight known compounds **1** - **8** were isolated from the CH_2Cl_2 soluble fraction of the flower buds of *B. officinalis* by repeated chromatographic separation including silica gel, RP-18, and preparative HPLC. Their structures were identified as crocusatin M (**1**),[8] crocusatin C (**2**),[9] acacetin (**3**),[10] lariciresinol (**4**),[11] pinoresinol (**5**),[11] syringaresinol (**6**),[12] mimengoside B (**7**),[13,14] and songarosaponin A (**8**)[15] (Fig. 1) by comparing their physicochemical and spectroscopic data with those of published data.

NO, produced from L-arginine by nitric oxide synthase, is involved in various biological actions such as host-defense and regulatory molecule for homeostatic equilibrium. However, NO produced by iNOS in macrophages plays an important role in the inflammatory process, and an inhibitor of NO production may have potential therapeutic value as an anti-inflammatory agent. Therefore, compounds **1** - **8** isolated from *B. officinalis* were examined for their inhibitory effects on NO production induced by LPS in RAW 264.7 macrophage cells using the Griess reagent. Aminoguanidine was used as positive control (IC_{50} 17.5 μM) (Table 2). Cell viability was evaluated by CCK assay, indicating that all compounds did not show any significant cytotoxic activity under tested concentration (cell viability > 90%). Among the isolated compounds, compounds **7** and **8** exhibited inhibitory effects against LPS-induced NO production in a concentration-dependent manner with IC_{50} values of 22.1 and 25.6 μM, respectively.

Several triterpenoidal saponins were previously examined also for their inhibitory effects on NO production in RAW 264.7 cells. The inhibitory effects on LPS-induced NO production in RAW 264.7 macrophages were reported for

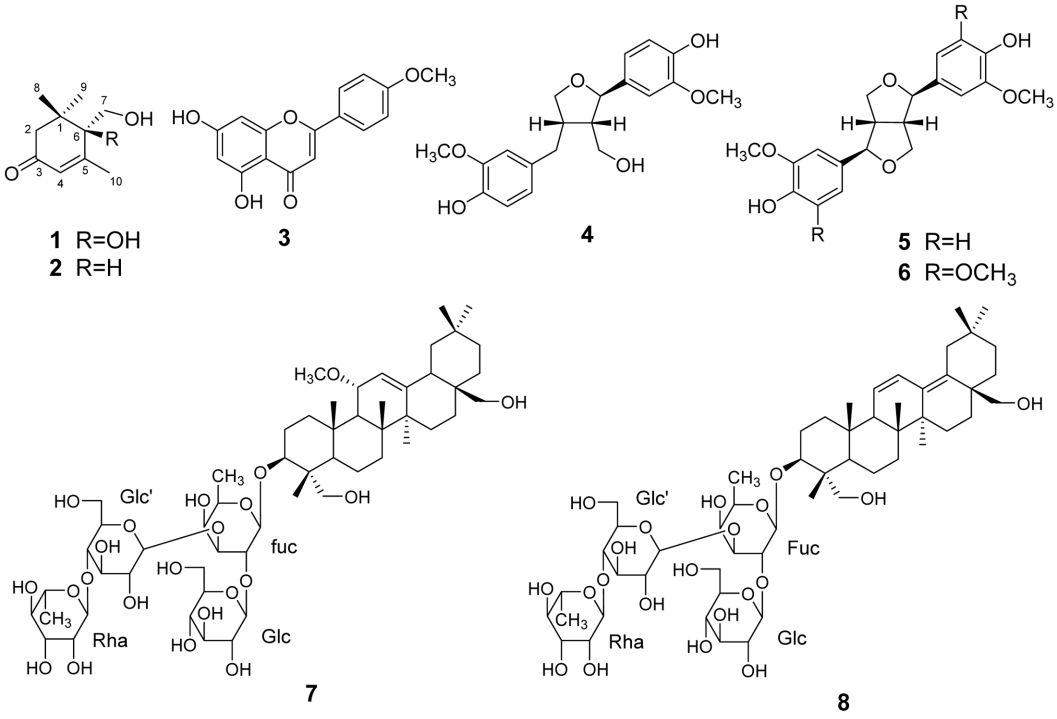

Fig. 1. Structures of **1** - **8** isolated from *B. officinalis*.

Table 2. Inhibitory effects of compounds **1** - **8** on LPS-induced NO production

compound	IC_{50} (μM) [a]
crocusatin M (**1**)	>50
crocusatin C (**2**)	>50
acacetin (**3**)	>50
lariciresinol (**4**)	>50
pinoresinol (**5**)	>50
syringaresinol (**6**)	>50
mimengoside B (**7**)	22.1 ± 0.8
songarosaponin A (**8**)	25.6 ± 1.0
aminoguanidine [b]	17.5 ± 0.7

[a] Results are expressed as means ± SD from triplicate experiments. [b] Positive control.

buddlejasaponin IV and songarosaponin D isolated from *Physospermum verticillatum*.[16] Buddlejasaponin IV isolated from *Pleurospermum kamtschatidum* showed inhibitory effects on NO, PGE_2, and TNF-α production by suppression of the expressions of iNOS, COX-2, TNF-α, IL-1β and IL-6 through blocking of NF-κB activation.[17,18] Recently, it has been reported that the water extract of *B. officinalis* inhibited LPS-induced NO release, and iNOS, IL-1β, and IL-6 expression, by the regulation of ERK 1/2 and NF-κB signaling pathways in BV-2 cells.[19]

In conclusion, our findings and previously reported data may support that triterpenoidal saponins, mimengoside B (**7**) and songarosaponin A (**8**), from *B. officinalis* could be considered as possible anti-inflammatory compounds for further investigation.

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