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Inhibitory Constituents of LPS-induced Nitric Oxide Production from *Arctium lappa*

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Abstract – The methanolic extract from the seeds of *Arctium lappa* was found to inhibit the LPS-induced nitric oxide (NO) production in murine macrophage RAW264.7 cells. Bioassay-guided fractionation of a methylene chloride soluble fraction led to the isolation of three lignan compounds, arctiin (**1**), arctigenin (**2**), and lappaol B (**3**). Their structures were elucidated by UV, IR, MS, and NMR data, as well as by comparison with those of the literatures. Arctigenin (**2**) and lappaol B (**3**) had an iNOS inhibitory activity with IC_{50} values of 12.5 and 25.9 μM, respectively.

Keywords – *Arctium lappa*, Compositae, Lignan, Nitric oxide synthase inhibitor

Introduction

Arctium lappa L. (Compositae), a perennial herb, has been cultivated as a vegetable for a long time in Korea. The roots are widely used as a food, whereas the seeds are known as one of folk medicine as diuretics, anti-inflammatory or detoxifying agent (Jung and Shin, 1992). A number of dibenzylbutyrolactone lignans have been isolated from *Arctium* species (Han *et al.*, 1994; Ichihara *et al.*, 1976; 1977; 1978; 1979). Previous studies have shown that lignans from the fruits of *A. lappa* possesses an inhibitory effect on the binding of platelet-activating factor to rabbit platelets (Iwakami *et al.*, 1992). It has also been reported to have differentiation inducing activity against mouse myeloid leukemia cells (Umehara *et al.*, 1993; 1996).

Nitric oxide, a molecular messenger synthesized by nitric oxide synthase (NOS) from L-arginine and molecular oxygen, is involved in a number of physiological and pathological processes in mammalians (Moncada *et al.*, 1991). Three isoforms of NOS are known to exist: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). nNOS and eNOS are constitutively expressed and essential for maintaining tissue homeostasis. In contrast, iNOS is expressed in a variety of cells including macrophages, endothelial cells, and smooth muscle cells in response to pro-inflammatory stimuli such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and lipopolysaccharide (LPS), and is thought to be responsible for the overproduction of NO, which have been implicated in the tissue destruction and pathogenesis of a number of immunological and inflammatory diseases. NO is also known to be responsible for the vasodilation and hypotension observed in septic shock. In addition, NO can enhance the production of a variety of other inflammatory mediators, including TNF-α and IL-1β which participate in the macrophage-dependent inflammatory response (Anggard, 1994). Therefore, the effective suppression of NO production, via inhibition of iNOS expression and/or activity, is an attractive therapeutic strategy for the pathological conditions such as septic shock and inflammation.

A recent study demonstrated that arctigenin inhibited the production of TNF-α and NO (Cho *et al.*, 1999; 2004). Further study also demonstrated that arctigenin potently inhibited LPS-inducible iNOS expression through inhibition of NF-κB activation by suppression of I-κBα phosphorylation and nuclear translocation of p65 (Cho *et al.*, 2002).

In our search for anti-inflammatory substances from natural products, the methanol extract of the seeds of *A. lappa* inhibited the production of NO in LPS-stimulated murine macrophage RAW264.7 cells. Activity-guided fractionation of a methylene chloride soluble fraction led to the isolation of three known lignans, arctiin (**1**), arctigenin (**2**), and lappaol B (**3**). In this paper, we report the isolation and structure elucidation, as well as their inhibitory effects on NO production.

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Experimental

Plant material – The dried seeds of *A. lappa* were purchased from herbal drug store at Cheongju, Korea, in April, 2003 and identified by emeritus professor Kyong Soon Lee, a plant taxonomist at Chungbuk National University. The voucher specimens (CBNU 03021) were deposited at the Herbarium of College of Pharmacy, Chungbuk National University.

General experimental procedures – Melting points were measured on Bûchi model B-540. Optical rotations were determined on JASCO DIP-370 polarimeter at 25 °C. UV and IR spectra were obtained on a JASCO UV-550 and Perkin-Elmer model LE599 spectrometer, respectively. ^{1}H-NMR, ^{13}C-NMR, DEPT, HMQC, HMBC, and NOESY spectra were recorded on a Bruker AMX-500 MHz NMR spectrometer. ESI-MS and EI-MS were measured on Finnigan Navigator and Hewlett Packard 5989A mass spectrometer, respectively. Open column chromatography was conducted on silica gel 60 (230-400 mesh, Fisher Scientific), Sephadex LH-20 (25-100 μM, Pharmacia), and LiChroprep RP-18 (particle size 40-63 μM, Merck). Thin layer chromatography (TLC) was performed on pre-coated silica gel 60 F_{254} (0.25 mm layer thickness, Merck). For visualization of TLC plates, UV lamp and vanillin-sulfuric acid reagent were used. All other chemicals and reagents were analytical grade.

Extraction and isolation – The dried seeds of *A. lappa* (3 kg) were extracted with MeOH (3×5 L) at room temperature. After filtration and evaporation of the solvent, the resulting extract was diluted with H_2O and extracted with CH_2Cl_2 and BuOH (each 3×3 L). The CH_2Cl_2 soluble fraction (150 g), exhibiting inhibitory effect on the production of NO, was subjected to column chromatography on silica gel (7.5×25 cm) eluted with CH_2Cl_2-MeOH gradient system to obtain five fractions (AF1-AF5). Repeated chromatotraphy of the fraction AF1 on silica gel column (CH_2Cl_2-Acetone, 20 : 1) and RP-18 flash column (20% aq. MeOH) afforded arctigenin (**2**, 486 mg) and lappaol B (**3**. 17 mg). Fraction AF3 was further applied to RP-18 flash column (50 % aq. MeOH) to give arctiin (**1**, 23 g).

Arctiin (1) – white amorphous powder; mp 115-118 °C; $[\alpha]_D^{25}$ –47.5°C (c, 0.2 in MeOH); UV λ_{max} MeOH nm (log ε): 230 (3.8), 280 (3.3); IR ν_{max} cm^{-1}: 3400 (OH), 1760 (γ-lactone), 1600 (aromatic C = C); ESI-MS *m/z*: 557 $[M+Na]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.89 (1H, d, *J* = 8.3 Hz, H-5), 6.72 (1H, d, *J* = 8.2 Hz, H-5'), 6.61 (1H, br s, H-2), 6.56 (1H, br d, *J* = 8.3 Hz, H-6), 6.53 (1H, br d, *J* = 8.2 Hz, H-6'), 6.47 (1H, br s, H-2'), 4.93 (1H, d, *J* = 6.0 Hz, glc-1), 4.09 (2H, m, H-9'), 2.90 (2H, m, H-7), 2.83 (2H, m, H-7'), 2.67 (2H, m, H-8, 8'); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 179.3 (C-9), 150.1 (C-4), 149.7 (C-3), 148.6 (C-3'), 145.7 (C-4'), 133.8 (C-1), 131.0 (C-1'), 122.5 (C-6), 121.3 (C-6'), 117.9 (C-5), 113.9 (C-5'), 112.6 (C-2), 112.1 (C-2'), 102.6 (glc-1), 76.8 (glc-3), 76.6 (glc-5), 73.9 (glc-2), 71.9 (C-9'), 70.1 (glc-4), 62.2 (glc-6), 56.6 (OCH_3×3), 47.1 (C-8), 41.7 (C-8'), 38.7 (C-7'), 35.0 (C-7).

Arctigenin (2) – white amorphous powder; mp 96-99 °C; $[\alpha]_D^{25}$ –16.5°C (c, 0.2 in MeOH); UV λ_{max} MeOH nm (log ε): 230 (5.0), 280 (4.6); IR ν_{max} cm^{-1}: 3400 (OH), 1750 (γ-lactone), 1600 (aromatic C = C); EI-MS *m/z*: 372 $[M]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.83 (1H, d, *J* = 7.8 Hz, H-5), 6.75 (1H, d, *J* = 8.4 Hz, H-5'), 6.64 (1H, d, *J* = 1.8 Hz, H-2), 6.61 (1H, dd, *J* = 7.8, 1.8 Hz, H-6), 6.55 (1H, dd, *J* = 8.4, 1.8 Hz, H-6'), 6.46 (1H, d, *J* = 1.8 Hz, H-2'), 4.14 (1H, dd, *J* = 9.0, 4.0 Hz, H-9'), 3.89 (1H, dd, *J* = 9.0, 4.0 Hz, H-9'), 3.86 (3H, s, OCH_3), 3.82 (6H, s, OCH_3), 2.95 (1H, dd, *J* = 14.1, 5.1 Hz, H-7), 2.90 (1H, dd, *J* = 14.1, 6.9 Hz, H-7), 2.64 (2H, m, H-7'), 2.56 (2H, m, H-8, 8'); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 178.7 (C-9), 148.9 (C-3'), 147.7 (C-4'), 146.6 (C-3), 144.3 (C-4), 130.3 (C-1), 129.4 (C-1'), 122.0 (C-6), 120.5 (C-6'), 114.0 (C-5), 111.6 (C-2), 111.4 (C-5'), 111.1 (C-2'), 71.2 (C-9'), 55.8 (OCH_3×3), 46.5 (C-8), 40.8 (C-8'), 38.1 (C-7'), 34.4 (C-7).

Lappaol B (3) – Colorless oil; $[\alpha]_D^{25}$ –19.5°C (c, 0.2 in MeOH); UV λ_{max} MeOH nm (log ε): 225 (3.5), 282 (4.2); IR ν_{max} cm^{-1}: 3400 (OH), 1760 (γ-lactone), 1600 (aromatic C = C); ESI-MS *m/z*: 573 $[M+Na]^+$; ^{1}H-NMR (500 MHz $CDCl_3$) δ: 6.95 (1H, d, *J* = 1.2 Hz, H-2"), 6.91 (1H, dd, *J* = 8.4, 1.2 Hz, H-6"), 6.88 (1H, d, *J* = 8.4 Hz, H-5"), 6.77 (1H, d, *J* = 8.4 Hz, H-5'), 6.64 (1H, br s, H-2), 6.59 (1H, dd, *J* = 8.4, 1.8 Hz, H-6'), 6.54 (1H, d, *J* = 1.8 Hz, H-2'), 6.51 (1H, br s, H-6), 5.54 (1H, d, *J* = 7.2 Hz, H-7"), 4.16 (1H, dd, *J* = 8.7, 7.5 Hz, H-9'), 3.90 (1H, m, H-9'), 3.92 (2H, m, H-9"), 3.87 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 3.57 (1H, dt, *J* = 7.2, 6.6 Hz, H-8"), 2.97 (1H, dd, *J* = 14.0, 5.5 Hz, H-7), 2.92 (1H, dd, *J* = 14.0, 6.5 Hz, H-7), 2.66 (1H, m, H-7'), 2.60 (1H, m, H-8), 2.57 (1H, m, H-8'), 2.52 (1H, m, H-7'); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 178.9 (C-9), 149.3 (C-3'), 148.2 (C-4'), 147.4 (C-3), 146.9 (C-3"), 145.9 (C-4), 144.5 (C-4"), 133.2 (C-5), 131.2 (C-1"), 130.7 (C-1), 128.3 (C-1'), 120.9 (C-6'), 119.5 (C-6"), 117.3 (C-6), 114.5 (C-5"), 113.5 (C-5'), 112.2 (C-2'), 111.6 (C-2), 109.0 (C-2"), 88.2 (C-7"), 71.5 (C-9'), 64.3 (C-9"), 56.3 (OCH_3), 56.2 (OCH_3), 56.1 (OCH_3×2), 53.9 (C-8"), 46.9 (C-8), 41.5 (C-8'), 38.5 (C-7'), 34.9 (C-7).

Cell lines and cell culture – Murine macrophage Raw264.7 cells were cultured in Dulbecco's modified Essential Medium (Gibco/BRL, Gaithersburg, MD, USA) supplemented with penicillin (100 units/mL)-streptomycin (100 μg/mL) (Gibco/BRL), and 10% heat-inactivated fetal bovine serum (Gibco/BRL). Cells were incubated at 37°C, 5% CO_2 in humidified atmosphere and used for experiments between passage 5 and 20.

Measurement of no production – Raw264.7 cells were seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 2 h at 37°C. The cells were incubated for 24 h with or without 1 μg/mL of LPS (Sigma Chemical Co., St. Louis, MO, USA) in the absence or presence of compounds tested. As a parameter of NO synthesis, nitrite concentration was measured using the supernatant of RAW264.7 cells by the Griess reaction as previously described (Schmidt and Kelm, 1996). Briefly, 100 μL of cell culture supernatant were reacted with 100 μL of Griess reagent [1 : 1 mixture of 0.1 % N-(1-naphtyl) ethylenediamine in H_2O and 1 % sulfanilamide in 5 % phosphoric acid] in a 96 well plate and absorbance was read with a microplate reader (Molecular Devices Co., Menlo park, CA, USA) at 570 nm.

Determination of cell viability – Cell viability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Chemical Co., St. Louis, MO, USA]-based colorimetric assay as previously described (Scudiero *et al.*, 1988). After sampling of the supernatant for NO assay, 50 μL of fresh medium containing 0.5 mg/ml of MTT was added to each well and incubated for 2 h at 37°C. Then, the medium was removed and the violet crystals of formazan in the viable cells were dissolved in dimethyl sulfoxide. The absorbance of each well was then read at 570 nm using a microplate reader (Molecular Devices Co., Menlo park, CA, USA).

Results and Discussion

The overproduction of nitric oxide has been implicated in the pathogenesis of several diseases including rheumatoid arthritis, septic shock, and acute and chronic models of inflammation, and carcinogenesis. Therefore, regulation of NO production in tissues might be important for the treatment of inflammation and tumorigenesis. In this study, we isolated three known lignans from *Arctium lappa* as inhibitors of LPS-induced NO production in RAW264.7 cells. The structures of compounds **1** and **2** were identified as arctiin (**1**) and arctigenin (**2**), respectively, by NMR analyses and comparison with those reported in the literatures (Han *et al.*, 1994; Koubaa *et al.*, 1999; Tundis *et al.*, 2000).

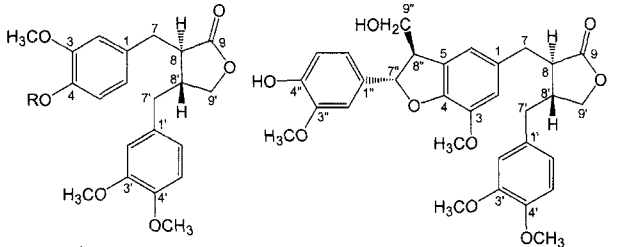

Fig. 1. Chemical structures of compounds **1-3**.

Compound **3**, the most active compound, was obtained as colorless oil. The ESI-MS spectrum of **3** gave $[M+Na]^+$ ion peak at *m/z* 573 indicating the molecular formula $C_{31}H_{34}O_9$. ^{1}H-NMR spectrum displayed signals for two sets of 1,3,4-trisubstituted benzene ring protons at δ 6.54 (1H, d, J = 1.8Hz), 6.77 (1H, d, J = 8.4 Hz), 6.59 (1H, dd, J = 8.4, 1.8 Hz), 6.95 (1H, d, J = 1.2Hz), 6.88 (1H, d, J = 8.4 Hz), and 6.91 (1H, dd, J = 8.4, 1.2 Hz), while two broad singlet signals at δ 6.64 and 6.51 indicated the protons of a 1,3,4,5-tetrasubstituted benzene ring. In addition to a dibenzylbutyrolactone system, presence of a phenylpropanoid moiety was evident from its ^{1}H-and ^{13}C-NMR data. The HMBC correlations between H-7 to C-2, C-6, C-9 and C-8', H-7' to C-8, C-2', C-6' and C-9', H-8' to C-1' and C-9', H-8 to C-1 and C-9, and H-9' to C-8, C-9 and C-7' confirmed the presence of the dibenzylbutyrolactone structure similar to that in **3**. The ^{1}H-NMR signals at δ 5.54, 3.57 and 3.90, and the ^{13}C-NMR chemical shifts at δ 88.2, 53.9 and 64.3 was characteristic of a dihydrofuran ring system and a primary alcohol group in the molecule. The HMBC correlations between H-8" to C-4, C-5, C-6, C-1" and C-9", H-7" to C-4, C-5, C-2", C-6" and C-9" confirmed that dihydrofuran system was fused with one of the two phenyl rings of the dibenzylbutyrolactone system to from the structure of lappaol B. The relative stereochemistry of **3** was assigned by direct comparison of its ^{1}H- and ^{13}C-NMR data and optical rotation value with published data (Ichihara *et al.*, 1976; Umehara *et al.*, 1993).

The effects of isolated compounds were tested on the NO production in LPS-stimulated RAW264.7 cells with respect to aminoguanidine, an iNOS inhibitor. Unstimulated Raw264.7 cells released low level of NO (2.7 ± 0.2 μM, mean ± S.D.), however, the production of NO was significantly increased up to 58.7 ± 0.3 μM for 24 h. Arctigenin (**2**) and lappaol B (**3**) inhibited LPS-induced NO production in the RAW264.7 cells dose-dependently with IC_{50} values of 12.5 and 25.9 μM, respectively. These

data are comparable to that of aminoguanidine (IC_{50} 28.0 μM). The cell viability measured by MTT assay showed that these compounds had no significant cytotoxicity to the RAW264.7 cells at their effective concentration for the inhibition of NO production (data not shown). However, arctiin (**1**), a glycoside of arctigenin (**2**), was inactive (IC_{50} : > 30 μM).

This result was in agreement with the previous data reported that arctigenin at the concentration of 0.01, 0.1, and 1 μM inhibited LPS-inducible NO production, resulting 9, 47, and 68 % inhibition, respectively (Cho *et al.*, 2002). Moreover, arctigenin slightly inhibited NO production by IFN-γ signals in a dose dependent manner at higher concentration (IC_{50} : more than 32 μM) (Cho *et al.*, 1999). The discrepancy in the inhibitory potency of arctigenin may be due to the differences in experimental conditions.

In conclusion, arctigenin (**2**) and lappaol B (**3**) isolated from *A. lappa* were identified as inhibitors of NO production in LPS-activated macrophages. Although lappaol B was previously isolated from the same plant, this is the first report on its inhibitory effect on NO production. These lignan and sesquilignan may be effective for treatment of pathological processes including inflammation and septic shock.

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