Diarylheptanoids from *Alnus hirsuta* Inhibit the NF-*k*B Activation and NO and TNF-α Production

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Six diarylheptanoids (1—6) from the stem bark of *Alnus hirsuta* were investigated for their inhibitory activity against LPS-induced NF-*k*B activation and NO and TNF-α production. Among them, compounds 2, 3, and 6 displayed inhibitory activity against NF-*k*B activation and NO and TNF-α production with IC_{50} values of 9.2—9.9 μM, 18.2—19.3 μM, and 22.3—23.7 μM, respectively, in RAW264.7 cells. Three active compounds had no significant cytotoxicity in RAW264.7 cells at their effective concentrations. This is the first report of NF-*k*B-inhibitory activity of these compounds and supports the pharmacological use of *A. hirsuta*, which has been employed as a herbal medicine for the treatment of inflammatory diseases.

Key words *Alnus hirsuta*; diarylheptanoid; NF-*k*B activation; NO production; TNF-α production

Nuclear factor-*k*B (NF-*k*B) is a dimeric transcription factor that activates the expression of many genes involved in the inflammatory process, *e.g.*, the cytokines IL-1β, IL-2, and TNF-α, adhesion molecules, and enzymes such as iNOS, cyclooxygenase-II, and 5-lipoxygenase.[1)] NF-*k*B is inactive without stimulation, and it is activated by extracellular signals such as TNF-α, IL-1, lipopolysaccharide (LPS), UV light, and phorbol esters. In unstimulated cells, NF-*k*B is retained in the cytoplasm *via* interaction with its inhibitor I*k*B. In response to various proinflammatory stimuli, I*k*B is phosphorylated by I*k*B kinase complex. This leads to the ubiquitination and subsequent proteasome-mediated degradation of I*k*B, allowing NF-*k*B to enter the nucleus. NF-*k*B is highly activated at the site of inflammation of diverse diseases such as rheumatoid arthritis, atherosclerosis, asthma, inflammatory bowel disease, and *Helicobacter pylori*-associated gastritis[1)] and associated with cancer, cachexia, diabetes, euthyroid sick syndrome, and AIDS.[2)] With its apparent involvement in a variety of human diseases, NF-*k*B has been shown to be the target of several anti-inflammatory and anticancer drugs.[2)]

In our search for inhibitors of NF-*k*B activity from natural products, the MeOH extract of the stem bark of *Alnus hirsuta* showed significant inhibitory activity of NF-*k*B activation (IC_{50} value, 16.5 μg/ml). The species *A. hirsuta* belongs to the Betulaceae, and geographically distributes in Korea, Japan, Northeast China, and Russia. It is a broad-leaved deciduous tree that grows in damp places. The bark of this species has been used in Chinese and Korean traditional medicine as a remedy for fever, hemorrhage, diarrhea, and alcoholism.[3)] Previous studies on the chemical constituents of *A. hirsuta* have led to the isolation of various natural products such as tannins,[4,5)] flavonoids,[6,7)] diarylheptanoids,[8—10)] and triterpenoids.[11)] It has also been reported to exhibit a variety of bioactivities such as antioxidant activity,[12,13)] antiinflammation,[14)] cytotoxicity, and antitumor effects.[15—18)] Here we describe the isolation and effects of six diarylheptanoids (**1**—**6**) from the stem bark of *A. hirsuta* on LPS-induced NF-*k*B activation, on nitric oxide (NO) production, and on tumor necrosis factor-α (TNF-α) production in LPS-stimulated RAW264.7 cells.

MATERIALS AND METHODS

Plant Material The stem bark of *Alnus hirsuta* TURCZ. was collected at Yangu, Kangwon, Korea in Aug. 2002 and the plant was identified by one of the authors, Prof. KiHwan Bae. The voucher specimen (CNU-0304) has been deposited at the herbarium of Chungnam National University, Daejeon, Korea.

General Experimental Procedures Optical rotations were measured by JASCO DIP-370 digital polarimeter. IR spectra were taken on a JASCO Report-100 spectrometer (KBr pellet). FAB-MS were obtained using a JEOL JMS-DX 300 spectrometer. ^{1}H-NMR (300 MHz), ^{13}C-NMR (75 MHz), and DEPT data were obtained on a Varian Unity NMR spectrometer. High-performance liquid chromatography (HPLC) was carried out on analytical and preparative scales using YMC ODS-H80 (YMC Co., Japan) [150×4.6 mm i.d., S-4 μm (analytical); 150×20 mm i.d., S-4 μm (preparative)]. Silica gel 60 (40—63 μm and 60—200 μm, Merck, Darmstadt, Germany) was used for column chromatography. TLC was carried out on precoated TLC sheets (silica gel 60 F_{254}, Merck), and the spots were detected by spraying with anisaldehyde–H_2SO_4 then heating on a hot plate. Parthenolide was purchased from Calbiochem (La Jolla, CA, U.S.A.) and Sigma-Aldrich Co. and used as positive standard.

Cell Line and Cell Culture RAW264.7 cells were maintained in DMEM medium. Fetal bovine serum, media, and supplement materials for cell culture were purchased from GIBCO-BRL (Gaithersberg, MD, U.S.A.). The medium was supplemented with penicillin (100 units/ml), streptomycin (100 units/ml) (Invitrogen), and 10% heat-inactivated fetal bovine serum (Hyclone). Cells were cultured at 37 °C in a 5% CO_2 incubator.

NF-*k*B Activity Assay The NF-*k*B inhibitory activity assay was carried out according to the established protocol.[19)] RAW264.7 cells transfected with a plasmid containing eight copies of *k*B elements linked to the secreted alkaline phosphatase (SEAP) gene were seeded in a 96-well plate at a density of 5×10^4 cells/well. After 3 h incubation at 37 °C, the cells were treated with various concentrations of test compounds and LPS (1 μg/ml) for 24 h. Then, 100 μl of each culture supernatant was transferred to a new 96-well plate and

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heated at 65 °C for 5 min. An additional 100 μl of 2×SEAP assay buffer (2 M diethanolamine, 1 mM $MgCl_2$, 20 mM L-homoarginine) was added to each well and incubated at 37 °C for 10 min. The reaction was initiated by addition of 20 μl of 120 mM *p*-nitrophenyl phosphate dissolved in 1×SEAP assay buffer and incubated at 37 °C. The absorbance of the reaction mixture was measured at 405 nm by microplate reader (Molecular Devices Co., Menlo Park, CA, U.S.A.).

Determination of NO Production Determination of NO production was carried out according to the established protocol.[19] RAW264.7 cells were transferred in 96-well plates at a density of 1×10^5 cells/well. After 3-h incubation, the cells were stimulated with LPS (1 μg/ml) for 24 h in the absence or presence of the compounds tested. As a parameter of NO synthesis, nitrite concentration was measured in the supernatant of RAW264.7 cells by the Griess reaction. Briefly, 100 μl of cell culture supernatant was reacted with 100 μl of Griess reagent [1 : 1 mixture of 0.1% *N*-(1-naphthyl)ethylenediamine in H_2O and 1% sulfanilamide in 5% phosphoric acid] in a 96-well plate, and absorbance at 570 nm was read by microplate reader. The nitrite concentration in the supernatants was calculated by comparison with a sodium nitrite standard curve.

Determination of TNF-α Production TNF-α production was quantitated by Opt-ELATM assay kit according to the manufacturer's instructions (Pharmingen, San Diego, U.S.A.). Briefly, RAW264.7 cells were seeded in 96-well plates at a density of 1×10^4 cells/well, pretreated with different concentrations of compounds 1 h, then stimulated with LPS (1 μg/ml for 18 h). The wells of the immuno-plate were incubated overnight at 4 °C with 100 μl anti-mouse TNF-α monoclonal antibody. Before performing the next assay steps, the coated plates were washed with washing buffer (PBS with 0.05% Tween 20) and blocked by 200 μl assay diluent (PBS with 10% FBS, pH 7.0) for 1 h. Recombinant mouse TNF-α was diluted and used as standard. All reagents used in this assay were incubated for 2 h. Assay plates were washed then exposed to 100 μl working detector (Detection Antibody-Biotinylated mouse TNF-α monoclonal antibody and Enzyme Reagent-Avidin-horseradish peroxidase conjugate) for 1 h. Then, the plates were re-washed and 100 μl substrate solution [hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine (TMB)] was added at room temperature in dark conditions. After 30 min, the reaction was terminated by adding 50 μl stop solution (2 N H_2SO_4), with the absorbance recorded at 450 nm by microplate reader.

Extraction and Isolation The stem bark of *A. hirsuta* (8 kg) was extracted with methanol (MeOH) three times under reflux for 24 h and filtered and concentrated to give MeOH extract (626 g). The MeOH extract was suspended in H_2O then respectively extracted with hexane, ethyl acetate (EtOAc), and *n*-butanol (BuOH). The resulting fractions were concentrated *in vacuo* to give a hexane-soluble fraction (65 g), EtOAc-soluble fraction (121 g), and BuOH-soluble fraction (92 g). The active fraction EtOAc-soluble fraction (IC_{50} value, 14.2 μg/ml, 121 g) was chromatographed by silica gel column chromatography eluting with a gradient of CH_2Cl_2–MeOH (80 : 1→1 : 1) to afford eight fractions (Fr. 1—Fr. 8). Compounds **1** (3.2 mg) and **2** (2.2 mg) were purified from the active fraction Fr. 4 (IC_{50} value, 10.4 μg/ml) by preparative HPLC with elution of 30% acetonitrile (MeCN) to H_2O. Another active fraction, Fr. 5 (IC_{50} value, 11.3 μg/ml), was subjected to silica gel column chromatography with a gradient of CH_2Cl_2–MeOH (40 : 1→1 : 1) to afford four subfractions (Fr. 51—Fr. 54). Compounds **3** (2.8 mg), **4** (3.6 mg) were purified from the subfraction Fr. 52 by preparative HPLC with elution of 25% MeCN to H_2O, compound **5** (32.2 mg) and compound **6** (52.1 mg) were purified from subfraction Fr. 54 by HPLC with elution of 23% MeCN to H_2O. These compounds were demonstrated pure as evidenced by NMR and HPLC analysis (purity >95%).

1-(4-Hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-4-hepten-3-one (**1**): Colorless oil, IR ν_{max} (KBr) cm^{-1}: 3380, 2933, 1685, 1652, 1515. FAB-MS *m/z*: 326 $[M]^+$. ^{1}H-NMR (300 MHz, $CDCl_3$) and ^{13}C-NMR (75 MHz, $CDCl_3$) spectra and physical constants were identical to those in a previous report.[20]

2-Oxatrycyclo[13.2.2.13,7]eicosa-3,5,7(20),15,17,18-hexaen-10-16-diol (**2**): White amorphous powder, $[\alpha]_D^{25}$ −74.2° (*c*=0.2, $CHCl_3$), IR ν_{max} (KBr) cm^{-1}: 3380, 2933, 1652, 1515. FAB-MS *m/z*: 358 $[M]^+$. ^{1}H-NMR (300 MHz, $CDCl_3$) and ^{13}C-NMR (75 MHz, $CDCl_3$) spectra and physical constants were identical to those in a previous report.[21]

2-Oxatrycyclo[13.2.2.13,7]eicosa-3,5,7(20),15,17,18-hexaen-10-one] (**3**): White amorphous powder, IR ν_{max} (KBr) cm^{-1}: 3380, 2933, 1720, 1652, 1515. FAB-MS *m/z*: 342 $[M]^+$. ^{1}H-NMR (300 MHz, $CDCl_3$) and ^{13}C-NMR (75 MHz, $CDCl_3$) spectra and physical constants were identical to those in a previous report.[22]

Rhoiptelol B (**4**): White amorphous powder, $[\alpha]_D^{25}$ +97.0° (*c*=0.3, MeOH), IR ν_{max} (KBr) cm^{-1}: 3380, 2933, 1652, 1515. FAB-MS *m/z*: 383 $[M+Na]^+$. ^{1}H-NMR (300 MHz, $CDCl_3$) and ^{13}C-NMR (75 MHz, $CDCl_3$) spectra and physical constants were identical to those in a previous report.[23]

Hirsutanone (**5**): Brown amorphous powder, IR ν_{max} (KBr) cm^{-1}: 3365, 1690, 1606, 1520, 1519. FAB-MS *m/z*: 351 $[M+Na]^+$. ^{1}H-NMR (300 MHz, $CDCl_3$) and ^{13}C-NMR (75 MHz, $CDCl_3$) spectra and physical constants were identical to those in a previous report.[24]

Hirsutanonol (**6**): Brown amorphous powder, $[\alpha]_D^{25}$ −15.5° (*c*=1.0, acetone), IR ν_{max} (KBr) cm^{-1}: 3390, 1690, 1605, 1520. FAB-MS *m/z*: 369 $[M+Na]^+$. ^{1}H-NMR (300 MHz, $CDCl_3$) and ^{13}C-NMR (75 MHz, $CDCl_3$) spectra and physical constants were identical to those in a previous report.[25]

RESULTS AND DISCUSSION

The methanolic extract of the dried stem bark of *A. hirsuta* showed strong inhibition upon NF-*k*B activation (IC_{50} value, 16.5 μg/ml) in LPS-stimulated murine macrophage RAW264.7 cells. Solvent partition of the methanolic extract resulted in the localization of the active components in EtOAc-soluble fraction. Further bioactivity-guided fractionation of combined EtOAc-soluble fraction using the NF-*k*B reporter gene assay, afforded six known diarylheptanoids (**1**—**6**) isolated by repeated column chromatography and preparative HPLC. As shown in Fig. 1, the structures of these compounds were identified by comparing physicochemical and spectroscopic data with previously reported results as 1-(4-hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-4-hepten-3-one (**1**),[20] 2-oxatrycyclo[13.2.2.13,7]eicosa-3,5,7(20),15,17,18-hexaen-10-16-diol (**2**),[21] 2-oxatrycyclo-

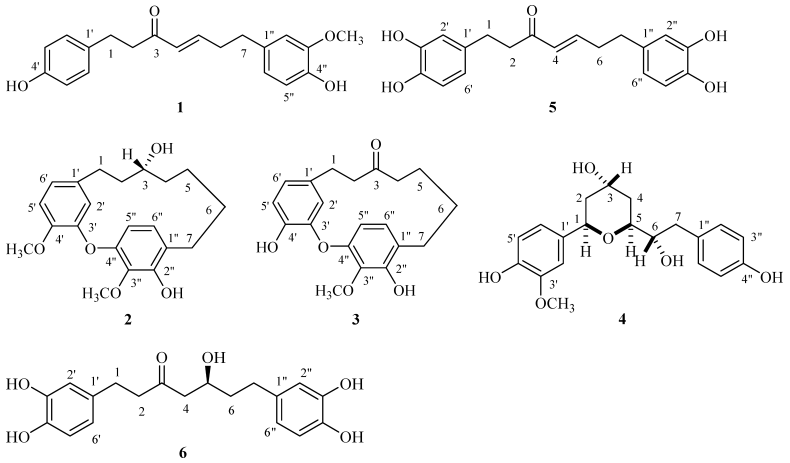

Fig. 1. Chemical Structures of Diarylheptanoids Isolated from *Alnus hirsuta*

Table 1. IC_{50} Values (μM)[a] of Compounds **1**—**6** in NF-*k*B Activation and NO and TNF-α Production

Compound	NF-*k*B activation	NO production	TNF-α production
1	>100	>100	>100
2	9.2±0.08	18.2±0.09	22.3±0.25
3	9.4±0.09	18.5±0.12	22.9±0.28
4	>100	>100	>100
5	>100	>100	>100
6	9.9±0.07	19.3±0.13	23.7±0.32
PTN[b]	3.4±0.08	2.4±0.11	2.6±0.11

a) Data are mean±S.D. from three separate experiments. *b*) Positive control. PTN, parthenolide.

[13.2.2.13,7]eicosa-3,5,7(20),15,17,18-hexaen-10-one] (**3**),[22] rhoiptelol B (**4**),[23] hirsutanone (**5**),[10] hirsutanonol (**6**).[24] Among them, compounds **1**—**4** were isolated for the first time from this plant.

Six diarylheptanoids (**1**—**6**) were examined for their dose-response effect on LPS-induced NF-*k*B activation using the NF-*k*B-mediated reporter gene assay system. RAW264.7 cells transfected with a NF-*k*B-mediated reporter gene construct were stimulated with LPS in the presence of various concentrations of compounds **1**—**6** then the expression of reporter gene (SEAP) in the culture medium was measured in comprison with a known NF-*k*B inhibitor, parthenolide (Table 1).[19] Among them, compounds **2** and **3**, which have big cyclic ring-like parthenolide (IC_{50} value, 3.4 μM), showed potent inhibitory activity against reporter gene expression with IC_{50} values of 9.2 and 9.4 μM, respectively. Our results of **2** and **3** were in accordance with reported data, which showed good activity in biphenyl-type and diphenyl ether-type diarylheptanoids.[25] These results suggest that the big cyclic ring contributes to the strong inhibitory activity for NF-*k*B activation in the NF-*k*B-mediated reporter gene assay system. Compound **6**, which has a ketone and a hydroxyl group, also showed potent inhibitory activity with an IC_{50} value of 9.9 μM, whereas compounds **1** and **5**, which have only one ketone group, showed very weak activity with IC_{50} values of >100 μM. Curcumin, which has two ketone groups, has already been reported as an NF-*k*B inhibitor.[26] This result indicates that the two ketone groups or a ketone and a hydroxy group are important structure factors of diarylheptanoids in their modulation of NF-*k*B activity.

Since NF-*k*B is an important transcription factor involved in the regulation of the expression of inflammatory NF-*k*B target genes such as TNF-α, iNOS, and COX-2[27,28] and NO is produced by iNOS in macrophages and endothelial cells,[29] we determined whether compounds **1**—**6** inhibit expression of these genes in LPS-stimulated RAW264.7 cells. Compounds **1**—**6** were examined for their effects on LPS-induced production of NO and TNF-α in RAW264.7 cells (Table 1). The cells were pretreated with various concentrations of compounds for 30 min, subsequently stimulated with 1 μg/ml of LPS, then the amount of NO and TNF-α in the culture supernatants was determined. Consistent with the NF-*k*B inhibitory activities, compounds **2**, **3**, and **6** showed potent inhibitory activity on the production of NO and TNF-α with IC_{50} values of 18.2—19.3 μM, and 22.3—23.7 μM, respectively, in RAW264.7 cells. In a previous report, the big cyclic diarylheptanoids also showed inhibitory effects on NO production with IC_{50} values of 19—30 μM.[30] The posivite control, parthenolide, also significantly inhibited production of NO and TNF-α with IC_{50} values of 2.4 and 2.6 μM, respectively. Cell viability, as measured by the MTT assay, also showed that all active compounds (**2**, **3**, **6**) had no significant cytotoxicity in RAW264.7 cells at their effective concentrations for the inhibition of NF-*k*B activation and NO and TNF-α production (data not shown).

This is the first report demonstrating NF-*k*B inhibitory activity of isolated compounds and all these data support the pharmacological use of *A. hirsuta*, which has been traditionally employed as a herbal medicine for the treatment of inflammatory diseases in Asia.

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