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ORIGINAL ARTICLE

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Antioxidative activity and anti-inflammatory effects of diarylheptanoids isolated from *Alnus hirsuta*

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Abstract Two diarylheptanoids, 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-*β*-D-xylopyranoside (I) and 1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-*β*-D-xylopyranoside (II), were isolated from the bark of *Alnus hirsuta*. Compounds I and II exhibited strong antioxidative activity against 1,1-diphenyl-2-picrylhydrazyl radicals, with IC_{50} values of 8.36 ± 0.54 and 8.67 ± 1.46 μM, respectively. In addition, we demonstrated that compounds I and II inhibited the production of nitric oxide and reactive oxygen species and the expression of proinflammatory molecules such as inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide-induced macrophages. According to our results, the two diarylheptanoids isolated from the bark of *A. hirsuta* exhibited significant antioxidative activity and anti-inflammatory effects and may be useful in the pharmaceutical industry for alleviating oxidative stress.

Key words Diarylheptanoids · *Alnus hirsuta* · Antioxidation · Anti-inflammatory

Introduction

It is commonly accepted that under oxidative stress conditions, reactive oxygen species (ROS) such as superoxide radicals (O_2^-, OOH^-) and hydroxyl radicals (·OH) play an important role in the pathogenesis of various serious diseases, including cancer, neurodegenerative disorders, atherosclerosis, cardiovascular diseases, cataracts, and inflammation.[1]

Inflammation is a physiological response of the body to a stimulus (e.g., tissue injury or infection).[2] High levels of ROS contribute to the pathophysiological mechanisms associated with various inflammatory skin disorders.[3,4] Inhibition of cyclooxygenase (COX) has been established as an important target of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin.[5] COX-2 induction is a response to proinflammatory stimuli such as bacterial lipopolysaccharide (LPS), which induces pathological conditions.[6] Nitric oxide (NO) generated by inducible nitric oxide synthase (iNOS) is involved in a variety of physiological and pathophysiological functions.[7] Improper iNOS expression plays an important role in human inflammatory diseases such as multiple sclerosis, asthma, neurodegenerative diseases, colitis, transplant rejection, and psoriasis.[8] Therefore, the induction of iNOS expression and iNOS-generated NO seem to be involved in the pathomechanisms of these diseases.[9]

Alnus hirsuta is a deciduous, broad-leaved tree that thrives in damp locations, and the bark of *Alnus* spp. has been used in traditional oriental medicine as a remedy for hemorrhage, fever, diarrhea, and alcoholism.[10] A large number of chemical compounds such as triterpenoids, flavonoids, and diarylheptanoids have been identified in *A. hirsuta*.[11–13] Diarylheptanoids isolated from the rhizome of Chinese ginger showed potent cytotoxicity against the HL-60 human promyelocytic leukemia cell line.[14] Two diarylheptanoids, isolated from the stem bark of *A. hirsuta*, were characteristic components of *Alnus* spp., and their chemical structures were elucidated on the basis of one-dimensional and two-dimensional NMR spectroscopy.

The aim of this work was to evaluate the free radical scavenging and anti-inflammatory properties of diarylheptanoids using an in vitro model. The present study determined that two diarylheptanoids isolated from the bark of *A. hirsuta* possess potent antioxidative and anti-inflammatory potential.

Materials and methods

Materials

Two diarylheptanoids, 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-*β*-D-xylopyranoside and 1,7-bis-(3,4-

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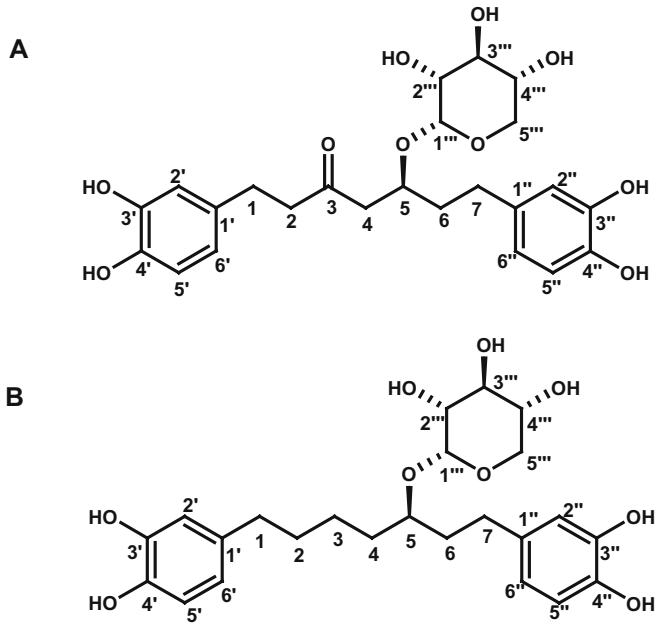


Fig. 1. Structure of compounds isolated from the bark of *Alnus hirsuta*. **A**, Compound I: 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-*β*-D-xylopyranoside (oregonin); **B**, compound II: 1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-*β*-D-xylopyranoside

dihydroxyphenyl)-heptane-5-*O*-*β*-D-xylopyranoside, were isolated from methanol extract of the bark of *A. hirsuta* (Fig. 1). Mass spectrum data were recorded on an Autospec M363 series (Micromass, Euroscience, Manchester, UK) mass spectrometer. ^{1}H and ^{13}C NMR spectra were measured using a Bruker DPX 400 (400 MHz for ^{1}H, 100 MHz for ^{13}C) spectrometer.

Compound I: light-brown amorphous powder

Negative FAB-MS: *m*/*z* 477[M-H]$^{-}$

^{1}H-NMR (400 MHz, CD_3OD) *δ*: 1.71 (1H, m, H-6), 2.45–2.77 (8H, m, H-1, 2, 4 and 7), 3.13 (1H, dd, *J* = 8.9, 7.5 Hz, xyl-2), 3.18 (1H, m, xyl-5ax), 3.32 (1H, m, xyl-3), 3.52 (1H, m, xyl-4), 3.86 (1H, dd, *J* = 11.4, 5.3 Hz, xyl-5eq), 4.07 (1H, m, H-5), 4.21 (1H, d, *J* = 7.6 Hz, xyl-1), 6.47 (2H, d, *J* = 8.0 Hz, H-6′ and 6″), 6.62–6.68 (4H, m, H-2′, 2″, 5′ and 5″).

^{13}C-NMR (100 MHz, CD_3OD) *δ*: 212.8 (C-3), 146.5 (C-3″), 146.4 (C-3″), 144.8 (C-4″), 144.5 (C-4′), 135.7 (C-1″), 134.6 (C-1′), 121.4 (C-6″), 121.3 (C-6′), 117.2 (C-5″), 117.2 (C-5′), 117.0 (C-2″), 116.8 (C-2′), 104.7 (xyl-1), 78.3 (xyl-3), 76.9 (C-5), 75.6 (xyl-2), 71.7 (xyl-4), 67.4 (xyl-5), 49.0 (C-4), 46.8 (C-2), 38.9 (C-6), 32.2 (C-7), 30.5 (C-1).

Compound II: dark-brown amorphous powder

ESI-MS: *m*/*z* 464[M]$^{+}$, 332[M-xylose]$^{+}$

^{1}H-NMR (400 MHz, CD_3OD) *δ*: 1.67–1.79 (4H, m, H-2, 3), 2.41–2.78 (8H, m, H-1, 6, 4 and 7), 2.79 (1H, m, H-5), 3.16 (2H, m, xyl-5ax and xyl-2), 3.49 (1H, m, xyl-3), 3.85 (1H, m, xyl-4), 4.08 (1H, dd, *J* = 5.4, 11.3 Hz, xyl-5eq), 4.21 (1H, d, *J* = 7.6 Hz, xyl-1), 6.47 (2H, dd, *J* = 8, 2.3 Hz, H-6′ and 6″), 6.61 (2H, d, *J* = 2.3 Hz, H-2′ and 2″), 6.65 (2H, d, *J* = 8 Hz, H-5′ and 5″).

^{13}C-NMR (100 MHz, CD_3OD) *δ*: 146.6 (C-3′), 144.7 (C-3″), 143.0 (C-4″), 142.7 (C-4′), 138.1 (C-1″), 132.7 (C-1′), 115.3 (C-5″), 115.2 (C-5′), 119.4 (C-6″), 119.3 (C-6′), 115.1 (C-2″), 114.9 (C-2′), 102.9 (xyl-1), 76.5 (xyl-3), 75.0 (C-5), 73.7 (xyl-2), 69.9 (xyl-4), 65.6 (xyl-5), 48.1 (C-4), 45.0 (C-2), 37.2 (C-6), 30.4 (C-7), 28.7 (C-1), 24.2 (C-3).

1,1-Diphenyl-2-picrylhydrazyl free radical scavenging assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity was assessed as described in Yin et al.[15] with some modification. Briefly, 100 µl of DPPH solution (0.2 mM in methanol) was added to a 96-well plate containing 100 µl of sample at various concentrations (1, 5, 10, 20, 50, 100, or 200 µM). The mixture was then immediately shaken and kept at room temperature in the dark; the decrease in absorbance at 515 nm was measured using a multiplate spectrophotometer (ELx800TM, BioTek, USA) after 25 min. All experiments were performed in triplicate. DPPH radical scavenging activity was calculated as follows:

$$\text{Scavenging effect (\%)} = (1 - (A_i - A_j)/A_0) \times 100$$

where A_0 is the A_{515} of DPPH without a sample (control), A_i is the A_{515} of a sample and DPPH, and A_j is the A_{515} of a sample without DPPH (blank). The effective concentration required for 50% plaque reduction (IC_{50}) was determined from a curve relating plaque number to the concentration of the sample.

Cell culture maintenance

The murine macrophage RAW 264.7 cell line was obtained from the Korea Cell Bank; the cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated at 37°C in an air/carbon dioxide (95:5) atmosphere.

Assay for cell viability

RAW 264.7 cells were seeded into 96-well plates at a density of 1×10^4 cells/well and exposed to medium in the presence of compounds with various concentrations (1, 5, 10, 20, 50, or 100 µM) for 24 h. After removing the supernatant of each well, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) [5 mg/ml in phosphate buffered saline (PBS)] was added to each well at the time of incubation. After 4 h of incubation, the supernatant was discarded and 200 µl of dimethyl sulfoxide (DMSO) was added to each well to terminate the reaction. The absorbance was measured at 550 nm using an enzyme-linked immunosorbent assay multiplate spectrophotometer (ELx800TM).[16] The mean value of optical density (OD) of three wells was used for calculating the viability (percentage of control). All experiments were performed in triplicate.

Inhibition of NO production by LPS-stimulated RAW 264.7 cells

A total of 1×10^4 RAW 264.7 cells were seeded per well in a 96-well plate for 12 h. Cells were preincubated with com-

pound solutions (1–100 μM, final concentration) for 30 min and then treated with 100 ng/ml LPS. After incubation for 24 h, 100 μl supernatant was transferred to a new 96-well plate, 100 μl Griess reagent was added to each well, and it was incubated for 15 min.[17] Absorbance was measured at 550 nm by using a multiplate spectrophotometer (ELx800TM).

Intracellular ROS inhibition activity

A total of 1×10^4 RAW 264.7 cells were plated per well in a 96-well plate for 12 h. Cells were pretreated with various concentrations of compound I or II for 30 min before being stimulated with LPS (100 ng/ml, final concentration) for 24 h. After incubation, the supernatant of each well was removed and cells were washed with preheated PBS at 37°C; then 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, final concentration 100 μM) was added and the cells were incubated for 30 min. DCFH-DA was removed from each well and 100 μl cold PBS was added; fluorescence intensity (485 nm/535 nm, ex/em) was measured using a fluorescence spectrophotometer (Victor 3, PerkinElmer, New York, USA).[18]

Flow cytometric analysis

RAW 264.7 cells (2×10^5 cells/ml) were incubated in a 6-well plate for measurement of intracellular ROS content using a fluorescence-activated cell sorting machine (FACS, Becton Dickinson, CA, USA). Cells were pretreated with compounds I or II at various concentrations (10, 20, 50, or 100 μM, final concentration) for 30 min before treatment with LPS (100 ng/ml, final concentration) and incubated for 24 h. After incubation, the supernatant medium of each well was removed and washed with preheated PBS at 37°C; DCFH-DA (final concentration 100 μM) was added and incubated for 30 min. DCFH-DA was removed from the each well and washed with cold PBS twice, and then the fluorescence intensity was measured.[19]

Detection of ROS formation with laser scanning confocal microscopy

LPS-induced RAW 264.7 cells were prepared with a view to imaging using a confocal laser microscope. The cells were treated with various concentrations of compound I or II 30 min before stimulated with 100 ng/ml LPS (final concentration) for 24 h. After incubation, the supernatant medium of each well was removed, cells were washed with preheated PBS at 37°C, and then 100 μM DCFH-DA was added and incubated for 30 min.[20] DCFH-DA was removed from the sample, washed with cold PBS twice, and then the fluorescence intensity was measured by using a laser scanning confocal microscope (Zeiss Axiovert 135 microscope, laser excitation 488 nm, emission long-pass LP515-nm filter set).

RNA preparation and reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was used to analyze gene expression in RAW 264.7 cells after stimulation with LPS in the presence of different concentrations of compound I or II for 24 h. Total RNA was isolated with Trizol reagent in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from the total RNA (2 μg) containing oligo (dT) primers and Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen). The primer sequences for iNOS, β-actin, and COX-2 were as follows: iNOS sense 5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′, antisense 5′-GGC TGT CAG AGC CTC GTG GCT TTG G-3′; β-actin sense 5′-TCA CCC TGA AGT ACC CCA TC-3′, antisense 5′-CCA TCT CTT GCT GCA AGT CC-3′; COX-2 sense 5′-CAC TAC ATC CTG ACC CAC TT-3′, antisense 5′-ATG CTC CTG CTT GAG TAT GT-3′. The PCR reaction involved an initial 5 min of denaturation at 94°C followed by 25 cycles of 94°C for 30 s, 55°–60°C for 30 s, and 72°C for 1 min with a final 7 min of extension. Aliquots of individual PCR products were separated on 1% agarose gel, stained with ethidium bromide, and imaged.[21]

Statistical analysis

Means were calculated from three replications of each experiment. Data were analyzed employing SPSS v13.0 (Statistical Package for the Social Sciences, Chicago, IL, USA). Duncan's multiple range test was used to determine the significance of differences ($P < 0.01$ was taken as significant).

Results and discussion

Identification of compounds I and II and their DPPH free radical scavenging activity

Compound I, a light-brown amorphous powder, has a molecular formula of $C_{24}H_{30}O_{10}$ according to FAB-MS ($[M\text{-}H]^-$ at $m/z = 477$). The ^{1}H-NMR (CD_3OD, 400 MHz) spectrum of compound I showed multiples at δ1.71–2.77 corresponding to 10H that were attributed to five methylene groups and two pairs of 1,3,4-trisubstituted aromatic rings: 6.47 (2H, dd, $J = 8$, 2.3 Hz, H-6′ and 6″), 6.61 (2H, d, $J = 2.3$ Hz, H-2′ and 2″), 6.65 (2H, d, $J = 8$ Hz, H-5′ and 5″). The ^{1}H-NMR spectrum also showed an anomeric proton signal at δ4.21 (1H, d, $J = 7.6$ Hz). These spectral data indicated that compound I was a bis-(3,4-dihydroxyphenyl) heptane glycoside. The signals at δ3.13 (1H, m, $J = 8.9$, 7.5 Hz, xyl-2), 3.18 (1H, m, $J = 10.9$ Hz, xyl-5ax), 3.32 (1H, m, xyl-3), 3.52 (1H, m, xyl-4), 3.86 (1H, dd, $J = 5.3$, 11.4 Hz, xyl-5eq), and 4.21 (1H, d, $J = 7.6$ Hz, xyl-1) indicated the presence of xylopyranosyl groups. The assignment of the sugar as a xylopyranosyl was supported by the signals for

oxygenated carbons at δ104.7 (xyl-1), 78.3 (xyl-3), 76.9 (C-5), 75.6 (xyl-2), 71.7 (xyl-4), and 67.4 (xyl-5). Also, the structure of compound I was elucidated to be (5*S*)-1,7-bis(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-β-D-xylopyranoside (oregonin), by comparing its spectroscopic data with previously reported data.[22,23]

Compound II, a dark-brown amorphous powder, has a molecular formula of $C_{24}H_{32}O_9$ according to ESI-MS ($[M]^+$ at $m/z = 464$). The spectroscopic data of compound II is very similar to that of compound I. Comparing the ^{1}H-NMR spectrum of compound II with that of compound I, compound II has one more methylene at δ1.67–1.79 than compound I does. ^{13}C-NMR showed the presence of a methylene at δ24.2 (C-3) instead of 212.8 (C-3) in compound I. Thus, the structure of compound II was identified as (5*R*)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-β-D-xylopyranoside by comparison with previously reported data.[24]

Free radicals are the primary cause of oxidative damage of biological molecules in the human body, and they are associated with coronary heart disease, aging, cancer, and dementia.[25] DPPH, a stable free radical, decrease significantly after exposure to proton radical scavengers. Antioxidants transfer either an electron or a hydrogen atom to DPPH, thus neutralizing its free radical character. DPPH has been used extensively as a free radical to evaluate reducing substances in foods and biological systems.[26]

The assay was conducted in methyl alcohol, and the results are expressed as IC_{50}, which represents the antioxidant concentration necessary to decrease the initial DPPH concentration by 50%. Lower values correspond to a higher radical scavenging capacity. Compounds I and II exhibited strong antioxidative activity against the DPPH radical, with IC_{50} values of 8.36 ± 0.54 and 8.67 ± 1.46 μM, respectively (Table 1). These IC_{50} values are lower than that of gallic acid (10.45 ± 0.47 μM). Compounds I and II exhibited good activity in the DPPH free radical model system. It is reported that diarylheptanoids exhibit strong antioxidative activity due to reducing groups such as phenolic hydroxyls, hydroxyls, or alkenes.[27] The antioxidant activities of compounds I and II are due to the 3′,4′-dihydroxyphenyl groups linked at C-7 and C-1 of the heptane chain. Flavonoids, hydroxycinnamates, and related phenolic acids have been reported to function as potent antioxidants by virtue of their hydrogen-donating properties.[28]

Table 1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the diarylheptanoids isolated from *Alnus hirsuta*

Sample	IC_{50} (μM)
Compound I	8.36 ± 0.54
Compound II	8.67 ± 1.46
Gallic acid	10.45 ± 0.47

IC_{50} is the amount required to reduce the initial concentration of DPPH by 50%
Compound I is 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-β-D-xylopyranoside (oregonin) Compound II is 1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-β-D-xylopyranoside
Values represent the mean ± SD ($n = 3$)

Cell viability and nitric oxide analysis

After 24-h treatments with various concentrations of compound I or II, the MTT assay was performed to test macrophage viability of RAW 264.7 cells. As shown in Fig. 2A, compounds I and II did not exhibit obvious cytotoxicity in the range 0–100 μM. Based on these results, concentrations up to 100 μM of compounds I and II were selected for subsequent experiments.

Macrophages play a pivotal role in the host defense system; they have an inherent capacity for phagocytic, cytotoxic, and intracellular killing.[29] NO generated by macrophages after stimulation by LPS (a primary component of

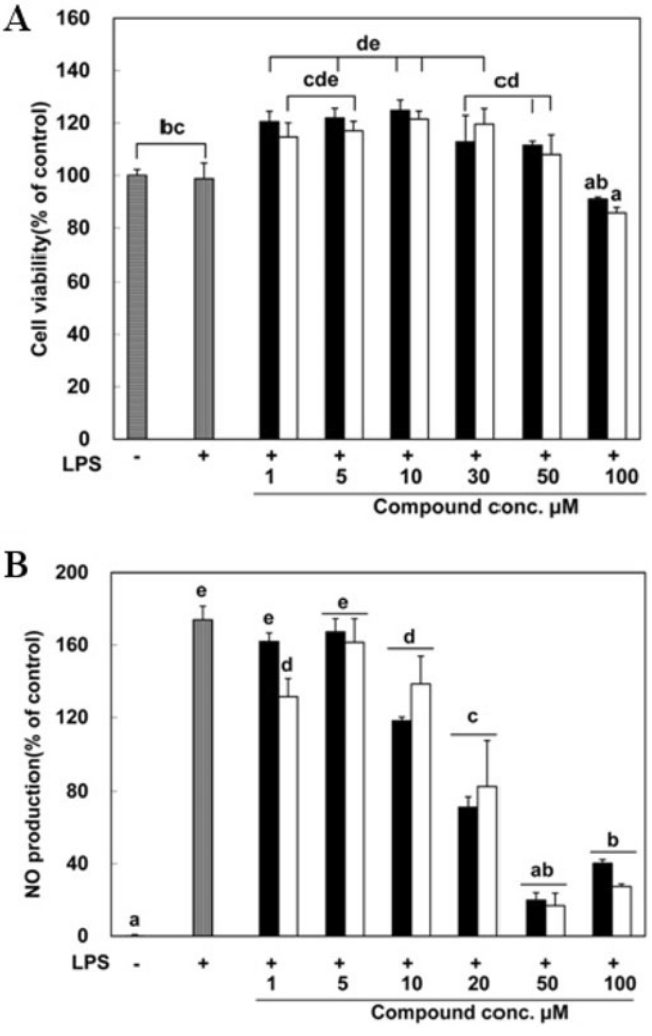


Fig. 2A,B. Effect of compounds on cell viability and NO inhibition in lipopolysaccharide (*LPS*)-induced RAW 264.7 cells. **A** RAW 264.7 cells were incubated with different concentrations of compound I or II for 24 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. **B** RAW 264.7 cells (1×10^5 cells/well) stimulated by LPS (100 ng/ml) were incubated with either compound I or II for 24 h. Supernatants were collected and the NO concentration from the supernatants was determined by Griess reagent. ▤, Control; ▥, LPS treatment; ■, compound I; □, compound II. Values are the mean ± SD ($n = 3$). Values with the same letter are not significantly different by Duncan's multiple range test ($P > 0.01$)

the Gram-negative bacteria cell wall) is a representative toxic and proinflammatory mediator and plays an important role in the various types of inflammatory processes in the animal body.[30] We examined whether compounds I and II modulate NO production in LPS-induced macrophages. The inhibitory effect of compounds I and II on LPS-induced NO production was quantitatively measured by the Griess reaction. As shown in Fig. 2B, NO production was dose-dependently suppressed by compounds I and II up to 50 μM, even though NO production slightly increased at 100 μM. These results imply that compounds I and II were effective as NO scavengers in LPS-stimulated RAW 264.7 cells. Much evidence indicates that compound I prevents the expression of iNOS and COX-2 in RAW 264.7 macrophages and BV-2 microglial cells exposed to LPS in combination with or without interferon-γ.[31,32] Kim et al.[33] reported that compounds I and II inhibited 50% of NO production in RAW 264.7 cells exposed to LPS plus interferon-γ at 16.7 and 48.6 μg/ml, respectively.

ROS inhibition activity in LPS-induced RAW 264.7 cells with DCFH-DA probe

As shown in Fig. 3, the ROS level in macrophages increased more than twofold after stimulation by LPS. Pretreatment with various concentrations of compounds I and II rapidly abates the ROS in RAW 264.7 cells. Mammalian cells are constantly exposed to ROS as a result of normal metabolic processes occurring during aerobic respiration; however, excessively high levels of free radicals or ROS create oxidative stress, which leads to detrimental effects, including lipid peroxidation of cellular membranes, alteration of lipid–protein interactions, enzyme inactivation, DNA breakage, and eventually the promotion of mutations that initiate tumor progression.[34,35] Therefore, compounds I and II can act as potential ROS scavengers in an oxidative environment to balance the ROS level.

Flow cytometry analysis of ROS levels in RAW 264.7 cells

The level of ROS production in RAW 264.7 cells was determined using the fluorescence probe DCFH-DA by means of flow cytometry (Fig. 4). RAW 264.7 cells exposed to LPS

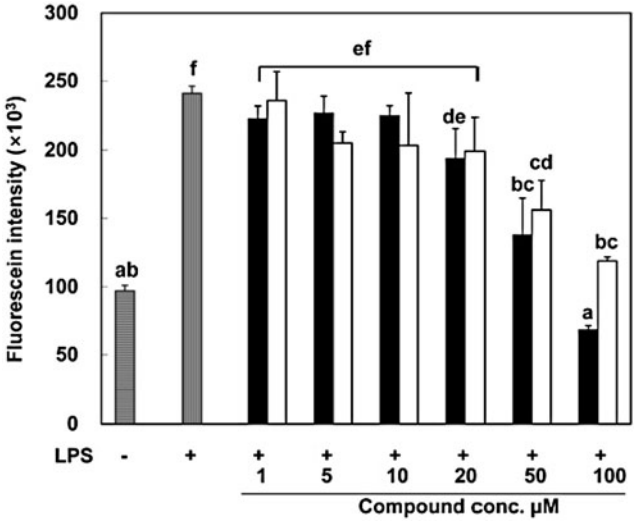
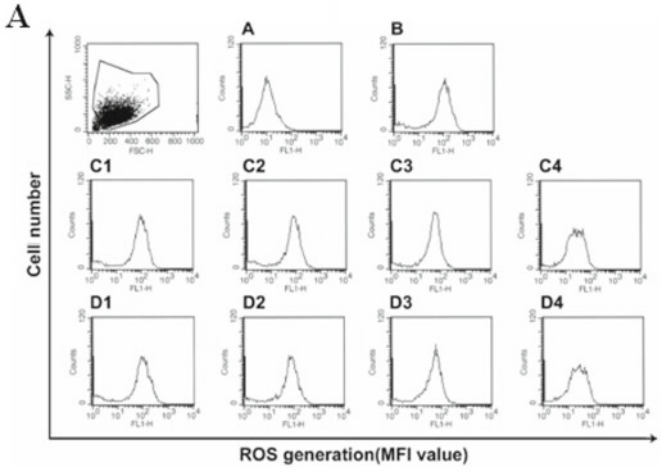


Fig. 3. Antioxidative activity of diarylheptanoids isolated from *A. hirsuta* by 2′,7′-dichlorodihydrofluorescein diacetate assay. ▤, Control; ▥, LPS treatment; ■, compound I; □, compound II. Values represent the mean ± SD ($n = 3$). Values with the same letter are not significantly different by Duncan's multiple range test ($P > 0.01$)



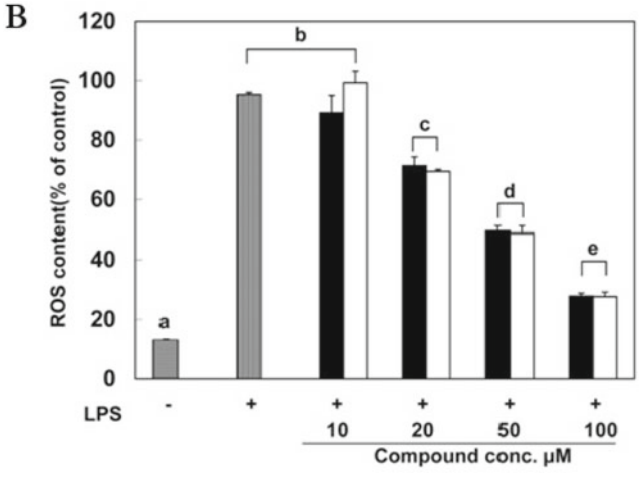


Fig. 4A,B. Suppression of LPS-induced reactive oxygen species (*ROS*) in RAW 264.7 cells in the presence of different concentrations of compound I or II. **A** RAW 264.7 cells (2×10^5 cells/ml) were stimulated by LPS (100 ng/ml) and incubated with either compound I or II for 24 h. The cells were collected and the intracellular ROS content was determined by flow cytometry. *A*, Control (no treatment); *B*, 100 ng/ml LPS treatment; *C*, 100 ng/ml LPS treatment after compound I addition; *D*, 100 ng/ml LPS treatment after compound II addition. *1–4*, Different concentrations of compounds I or II: 10, 20, 50, and 100 μM. *MFI*, mean fluorescence intensity. **B** Typical histograms showing ROS levels in LPS-induced RAW 264.7 cells with or without compound I or II. ▤, Control; ▥, LPS treatment; ■, compound I; □, compound II. Values represent the mean ± SD ($n = 3$). Values with the same letter are not significantly different by Duncan's multiple range test ($P > 0.01$)

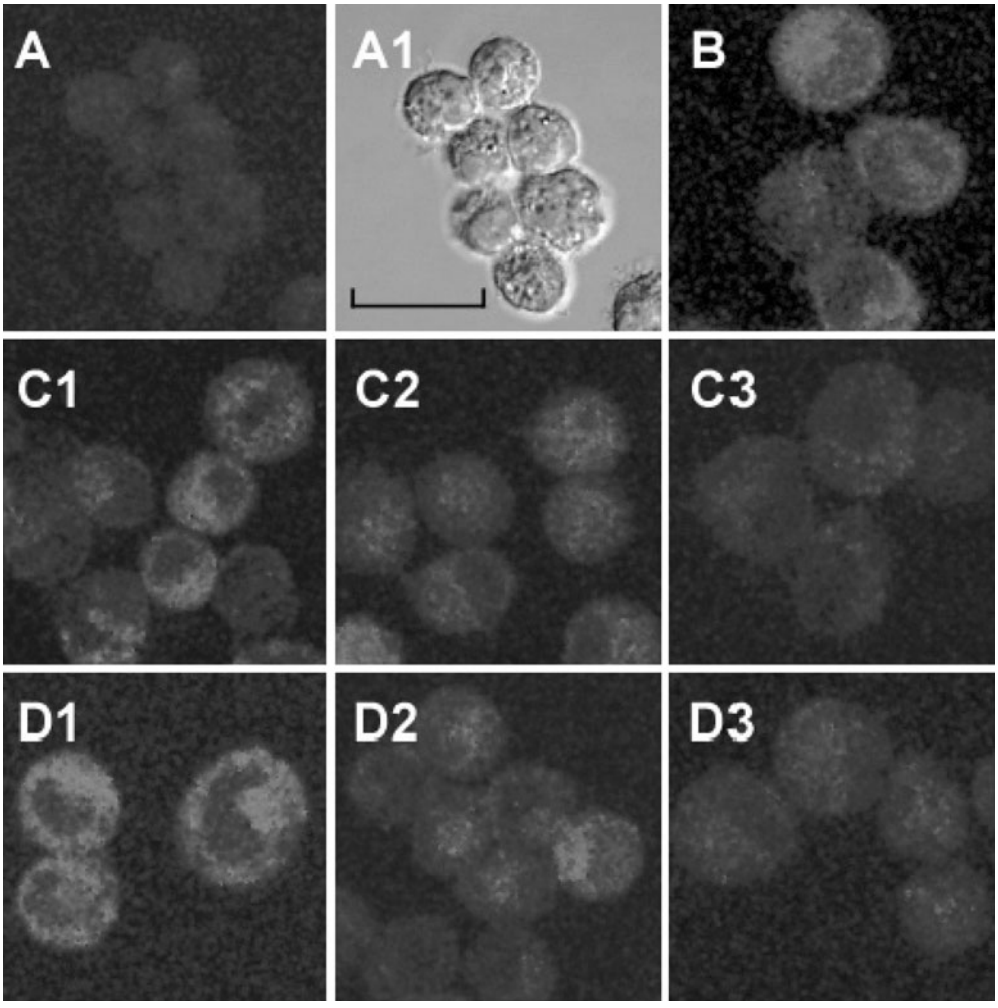


Fig. 5. ROS content of LPS-induced RAW 264.7 cells after addition of compound I or II using confocal laser microscope. *A*, Control (no treatment); *B*, 100 ng/ml LPS treatment; *C*, 100 ng/ml LPS treatment after compound I addition; *D*, 100 ng/ml LPS treatment after compound II addition. *1–3*, Different concentrations of compounds I or II: 10, 20, and 50 μM. *Bar* = 20 μm

for 24 h displayed a significant increase (greater than five-fold) in the intracellular level of ROS compared to untreated cells. Pretreatment with different concentrations of compound I or II markedly reduced the ROS levels in RAW 264.7 cells. The ROS scavenging capacity of compound I was 6.4% at 10 μM, 25.0% at 20 μM, 48.0% at 50 μM, and 70.8% at 100 μM. In comparison, the ROS scavenging capacity of compound II was 4.2% at 10 μM, 27.2% at 20 μM, 48.8% at 50 μM, and 71.0% at 100 μM. The results correlated well between flow cytometry and fluorescence spectrophotometer analysis.

Laser scanning cytometry assay

To determine the ROS scavenging ability of compounds I and II, ROS generation by RAW 264.7 cells was monitored under laser scanning confocal microscopy using fluorescence dye DCFH-DA. As shown in Fig. 5, cells not treated with LPS exhibited weaker fluorescence intensity than cells treated with LPS due to the generation of ROS in RAW 264.7 cells. Treatment with either compound I or II in conjunction with LPS reduced the fluorescence intensity, which reflects the reduction of ROS generation.

Diarylheptanoids abolished iNOS and COX-2 expression induced by LPS in RAW 264.7 cells

Among the proinflammatory enzymes, iNOS and COX-2 are believed to be involved in the pathogenesis of many chronic diseases, including multiple sclerosis, Parkinson's and Alzheimer's diseases, and cancer.[36] iNOS and COX-2 are implicated in nitric oxide upregulation and the synthesis of prostaglandins during the inflammation process. Therefore, it is recognized that downregulation of iNOS and COX-2 is an effective approach for reducing the intensity of the inflammation process.[37]

To elucidate the mechanisms of the anti-inflammatory activity of compounds I and II, semiquantitative RT-PCR was performed to determine the expression of iNOS and COX-2 mRNA. Incubation with the two compounds at several concentrations (10, 20, 50, or 100 μM, final concentration) resulted in a marked reduction of LPS-induced

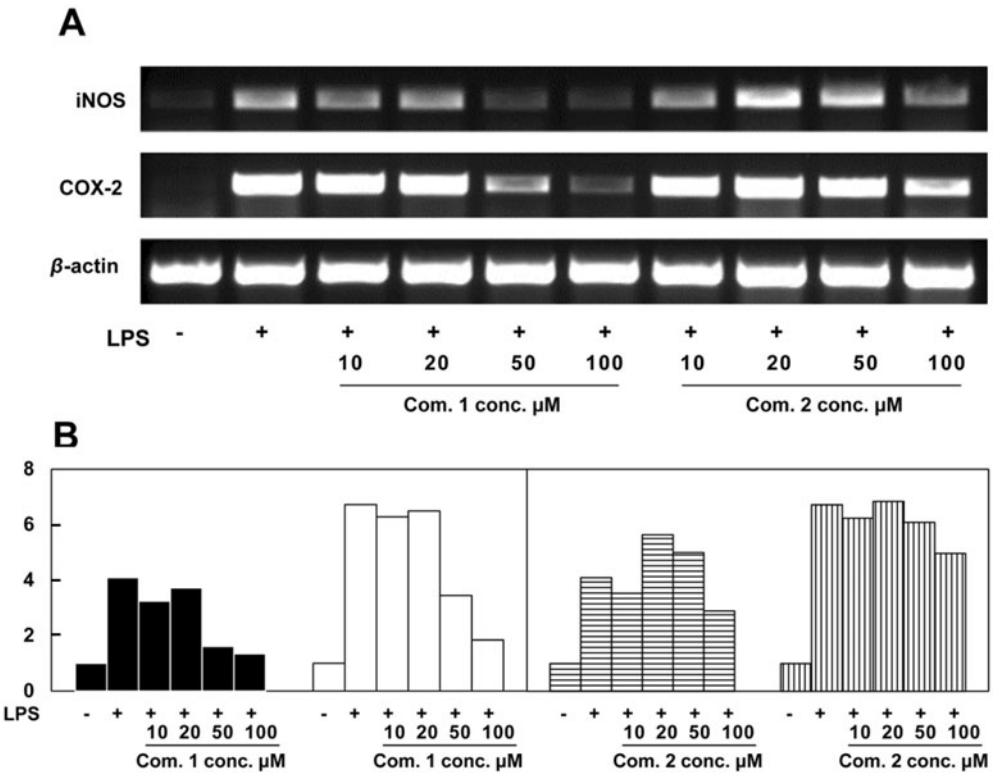


Fig. 6. RT-PCR analysis of cyclooxygenase-2 (*COX-2*) and inducible nitric oxide synthase (*iNOS*) mRNA after LPS treatment of RAW 264.7 cells with the addition of compounds I or II. *β*-actin was coamplified as an internal control. ■, iNOS expression level after compound I treatment; □, COX-2 expression level after compound I treatment; ▤, iNOS expression level after compound II treatment; ▥, COX-2 expression level after compound II treatment. **A** Typical results of agarose gel electrophoresis. **B** Band relative intensity was estimated using the image analysis software (Gel Quant, DNR bio-imaging systems, USA)

iNOS and COX-2 mRNA in RT-PCR analysis (Fig. 6). Based on image analysis results, compound I more effectively reduced the mRNA expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells than compound II did.

Conclusions

Taken together, these findings suggest that the two diarylheptanoids isolated from *A. hirsuta* have significant DPPH free radical scavenging activity. In addition, they were able to inhibit NO production, reduce ROS levels, and reduce mRNA expression levels of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. Further studies are in progress to characterize their behavior in greater detail.

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References

1. Aruoma OI (1998) Free radicals, oxidative stress, and antioxidants in human health and disease. J Am Oil Chem Soc 75:199–212
2. Korhonen R, Lahti A, Kankaanranta H, Moilanen E (2005) Nitric oxide production and signaling in inflammation. Curr Drug Targets Inflamm Allergy 4:471–479
3. Vitseva O, Varghese S, Chakrabarti S, Folts JD, Freedman JE (2005) Grape seed and skin extracts inhibit platelet function and release of reactive oxygen intermediates. J Cardiovasc Pharmacol 46:445–451
4. Hernández-Ledesma B, Hsieh CC, Lumen BO (2009) Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. Biochem Biophys Res Commun 390:803–808
5. Mouithys-Mickalad A, Deby-Dupont G, Dogne JM, Leval X, Kohnen S, Navet R, Sluse F, Hoebeke M, Pirotte B, Lamy M (2004) Effects of COX-2 inhibitors on ROS produced by *Chlamydia pneumoniae*-primed human promonocytic cells (THP-1). Biochem Biophys Res Commun 325:1122–1130
6. Fujihara M, Muroi M, Tanamoto K, Suzuki T, Azuma H, Ikeda H (2003) Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. Pharmacol Ther 100:171–194
7. Kubes P, McCafferty DM (2000) Nitric oxide and intestinal inflammation. Am J Med 109:150–158
8. Ahn KS, Noh EJ, Zhao HL, Jung SH, Kang SS, Kim YS (2005) Inhibition of inducible nitric oxide synthase and cyclooxygenase II by *Platycodon grandiflorum* saponins via suppression of nuclear factor-kappaB activation in RAW 264.7 cells. Life Sci 76:2315–2328
9. Makarov SS (2000) NF-kappaB as a therapeutic target in chronic inflammation: recent advances. Mol Med Today 6:441–448
10. Choi SE, Kim KH, Kwon JH, Kim SB, Kim HW, Lee MW (2008) Cytotoxic activities of diarylheptanoids from *Alnus japonica*. Arch Pharm Res 31:1287–1289
11. Uvarova NI, Oshitok GI, Suprunov NI, Elyakov GB (1972) Triterpenoids and other constituents from the Far Eastern species of *Alnus*. Phytochemistry 11:741–743
12. Lee MW, Jeong DW, Lee YA, Park MS, Toh SH (1999) Flavonoids from the leaves of *Alnus hirsuta*. Yakhak Hoechi 43:547–552
13. Terazawa M, Okuyama H, Miyake M, Sasaki M (1984) Phenolic compounds in living tissue of woods. IV. Hirsutoside from the green bark of keyamahannoki (*Alnus hirsuta*) [Betulaceae] and its seasonal variations in the living tissues of hannoki (*A. japonica*). Mokuzai Gakkaishi 30:587–600
14. Wei QY, Ma JP, Cai YJ, Yang L, Liu ZL (2005) Cytotoxic and apoptotic activities of diarylheptanoids and gingerol-related compounds from the rhizome of Chinese ginger. J Ethnopharmacol 102:177–184

15. Yin Y, Heo SI, Roh KS, Wang MH (2009) Biological activities of fractions from methanolic extract of *Picrasma quassioides*. J Plant Biol 52:325–331
16. Wang XJ, Wei YQ, Yuan SL, Liu GJ, Lu YR, Zhang J, Wang WD (2006) Potential anticancer activity of litchi fruit pericarp extract against hepatocellular carcinoma in vitro and in vivo. Cancer Lett 239:144–150
17. Shin EM, Zhou HY, Guo LY, Kim JA, Lee SH, Merfort I, Kang SS, Kim HS, Kim SH, Kim YS (2008) Anti-inflammatory effects of glycyrol isolated from *Glycyrrhiza uralensis* in LPS-stimulated RAW 264.7 macrophages. Int Immunopharmacol 8:1524–1532
18. Sellem CH, Marsy S, Boivin A, Lemaire C, Sainsard-Chanet A (2007) A mutation in the gene encoding cytochrome *c*1 leads to a decreased ROS content and to a long-lived phenotype in the filamentous fungus *Podospora anserina*. Fungal Genet Biol 44:648–658
19. Yamada H, Arai T, Endo N, Yamashita K, Fukuda K, Sasada M, Uchiyama T (2006) LPS-induced ROS generation and changes in glutathione level and their relation to the maturation of human monocyte-derived dendritic cells. Life Sci 78:926–933
20. Gniadecki R, Christoffersen N, Wulf HC (2002) Cholesterol-rich plasma membrane domains (lipid rafts) in keratinocytes: importance in the baseline and UVA-induced generation of reactive oxygen species. J Invest Dermatol 118:582–588
21. Lee AK, Sung SH, Kim YC, Kim SG (2003) Inhibition of lipopolysaccharide- inducible nitric oxide synthase, TNF-α and COX-2 expression by sauchinone effects on I-κBα phosphorylation, C/EBP and AP-1 activation. Br J Pharmacol 139:11–20
22. Ohta S, Aoki T, Hirata T, Suga T (1984) The structures of four diarylheptanoid glycosides from the female flowers of *Alnus serrulatoides*. J Chem Soc Perkin Trans 1:1635–1642
23. Karchesy JJ, Laver ML, Barofsky DF, Barofsky E (1974) Structure of oregonin, a natural diarylheptanoid xyloside. J Chem Soc Chem Commun 16:649–650
24. Lee MW, Par MS, Jeong DW, Kim KH, Kim HH, Toh SH (2000) Diarylheptanoids from the leaves of *Alnus hirsuta* Turcz. Arch Pharm Res 23:50–53
25. Siriwardhana N, Lee KW, Jeon YJ, Kim SH, Haw JW (2003) Antioxidant activity of *Hizikia fusiformis* on reactive oxygen species scavenging and lipid peroxidation inhibition. Food Sci Tech Int 9:339–346
26. Leong LP, Shui G (2002) An investigation of antioxidant capacity of fruits in Singapore markets. Food Chem 76:69–75
27. Luo J, Feng Y, Ji X (2008) Advances in studies on naturally occurring linear diarylheptanoids. Zhong Cao Yao 39:1912–1916
28. Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB (1995) The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radic Res 22:375–383
29. al-Ramadi BK, Mustafa N, AbouHaidar M, Fernandez-Cabezudo MJ (2003) Induction of innate immunity by IL-2-expressing *Salmonella* confers protection against lethal infection. Mol Immunol 39:763–770
30. Saha K, Lajis NH, Israf DA, Hamzah AS, Khozirah S, Khamis S, Syahida A (2004) Evaluation of antioxidant and nitric oxide inhibition activities of selected Malaysian medicinal plants. J Ethnopharmacol 92:263–267
31. Lee CJ, Lee SS, Chen SC, Ho FM, Lin WW (2005) Oregonin inhibits lipopolysaccharide-induced iNOS gene transcription and upregulates HO-1 expression in macrophages and microglia. Br J Pharmacol 146:378–388
32. Lee MW, Kim NY, Park MS, Ahn KH, Toh SH, Hahn DR, Kim YC, Chung HT (2000) Diarylheptanoids with in vitro inducible nitric oxide synthesis inhibitory activity from *Alnus hirsuta*. Planta Med 66:551–553
33. Kim HJ, Yeom SH, Kim MK, Shim JG, Paek IN, Lee MW (2005) Nitric oxide and prostaglandin E2 synthesis inhibitory activities of diarylheptanoids from the barks of *Alnus japonica* steudel. Arch Pharm Res 28:177–179
34. Agarwal A, Saleh RA, Bedaiwy MA (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 79:829–843
35. Lefebvre PP**,** Malgrange B, Lallemend F, Staecker H, Moonen G, Van De Water TR (2002) Mechanisms of cell death in the injured auditory system: otoprotective strategies. Am J Audiol 7:165–170
36. Heiss E, Herhaus C, Klimo K, Bartsch H, Gerhauser C (2001) Nuclear factor κB is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. J Biol Chem 276:32008–32015
37. Pan MH, Lai CS, Dushenkov S, Ho CT (2009) Modulating of inflammatory genes by natural dietary bioactive compounds. J Agric Food Chem 57:4467–4477