Anti-inflammatory components isolated from *Atractylodes macrocephala* in LPS-induced RAW264.7 macrophages and BV2 microglial cells

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**Abstract**

The phytochemical investigation on the methanol extract of the rhizomes of *Atractylodes macrocephala* resulted in the discovery of one new compound 9α-hydroxyatractylenolide (1) and 21 known compounds including atractylon (2), 3β-acetoxyatractylon (3), atracylenolide I (4), atracylenolide II (5), 8-epiasterolid (6), atracylenolide III (7), atracylenolide VII (8), 8-epiastrelactenolide III (9), eudesm-4(15)-ene-7α,11-diol (10), linoleic acid (11), myristic acid (12), 3-O-caffeoyl-1-methyquinic acid (13), (2E,8E,10E)-tetradecatetriene-4,6-diyne-1,14-diol (14), 14-aceroxy-12-sene-cioxyoctadeca-2E,8Z,10E-trien-4,6-dyn-1-ol (15), isoscopoletin (16), caffeic acid (17), protocatechic acid (18), 3-O-caffeoylquinic acid (19), 4-O-caffeoylquinic acid (20), 1,5-di-O-cafeoylquinic acid (21), and nicotinic acid (22).

Their structures were identified using nuclear magnetic resonance (NMR) and mass spectroscopy, and by comparison with previously published data. Compounds 4, 5, 6, 8, and 10–22 significantly inhibited lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW264.7 macrophages, and compounds 4, 5, 6, 16, and 17 showed those responses in BV2 microglial cells. Especially, compound 6 showed the second-best effect, and inhibited the LPS-induced production of prostaglandin E2 (PGE2), the protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, and the production of cytokines including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in both cells. These inhibitory effects were mediated by the inactivation of nuclear factor kappa B (NF-κB) signaling pathway.

**Keywords:** Atractylodes macrocephala, Anti-inflammatory effect, Macrophages, Microglia, Nuclear factor kappa B

**Introduction**

Inflammation is associated with the activation of macrophages or monocytes that are responsible for the innate and adaptive immune responses of the human body. Following various stimuli, these immune cells release a series of pro-inflammatory mediators including nitric oxide (NO), prostaglandin E2 (PGE2), cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in both cells. These inhibitory effects were mediated by the inactivation of nuclear factor kappa B (NF-κB) signaling pathway.

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and neurodegenerative diseases [3–5]. Macrophages are the most dominant and widely distributed immune cell types throughout the body, and microglia are considered resident macrophages in the central nervous system (CNS) [6]. Macrophages and microglia are important sources of pro-inflammatory mediators through the activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) due to lipopolysaccharide (LPS) stimulation [7, 8]. LPS is the most abundant lipidic component of the gram-negative bacterial cell wall [9]. Since macrophages and microglia stimulated by LPS produce excessive pro-inflammatory effects, which belong to the Compositae family, is a perennial herb, widely distributed in East Asia [12]. Traditionally, this plant has been used for the treatment of an abnormal function of the digestive system including malfunction of the spleen, anorexia, abdominal distension, diarrhea, dizziness, and palpitation due to the retention of phlegm and fluid, edema, spontaneous sweating, threatened abortion, oedema, excessive perspiration, and an abnormal fetal movement [13, 14]. In several recent studies, various components of A. macrocephala were isolated including essential oils, sesquiterpenoids, polysaccharides, amino acids, vitamins, and resins [15], and their physiological activity including anti-inflammatory [16], neuroprotective [17], anti-cancer [18], anti-oxidant [19], and immunological enhancement effects [20] were evaluated. In this investigation, we described the isolation and structural identification of 22 compounds from the rhizomes of A. macrocephala together with the evaluation of their anti-inflammatory effects using RAW264.7 macrophage and BV2 microglial cell lines.

Materials and methods

Plant materials

The rhizomes of A. macrocephala were harvested in Andong (Gyeongbuk) in December 2018. The plant material was identified by Prof. Youn-Chul Kim, College of Pharmacy, Wonkwang University, Iksan, Korea. A specimen of A. macrocephala (No. WSY-2019-003) has been deposited at the College of Pharmacy, Wonkwang University, Korea.

Extraction and isolation

The dried rhizomes of A. macrocephala (2.0 kg) were cut and extracted using MeOH three times for 3 h at 80 °C. The resultant MeOH extract (100.9 g) was suspended in water (1.0 L × 3) and then sequentially partitioned using equal volumes of n-hexane, dichloromethane, ethyl acetate, and n-butanol. Each fraction was evaporated in vacuo to yield the residues of n-hexane (8.9 g), CH₂Cl₂ (3.7 g), EtOAc (1.5 g), n-BuOH (3.9 g), and water (72.3 g) extracts, respectively.

The n-hexane soluble fraction (8.5 g) was subjected to column chromatography (CC) using a silica gel column and eluted with an n-hexane/EtOAc (100:0 → 1:1) gradient system. The fractions were combined based on their thin layer chromatography (TLC) pattern to yield subfractions, which were designated H1–H7. Fraction H1 (3.8 g) was purified by Sephadex LH-20 CC (n-hexane, 100:0) to yield six subfractions (H11–H16). Subfraction H11 was purified by silica gel CC (n-hexane/EtOAc, 100:1 → 10:1, and by ODS CC (MeOH/H₂O, 1.5:1 → 2:1) to yield 1 (1.5 mg). In addition, subfraction H13 was purified by silica gel CC (n-hexane/EtOAc, 30:1 → 10:1), and by ODS CC (MeOH/H₂O, 1:1 → 3:1) to yield 9 (12.0 mg) and 5 (90.2 mg). Subfraction H15 was purified by silica gel CC (n-hexane/EtOAc, 20:1 → 5:1), and by ODS CC (MeOH/H₂O, 1:1 → 2:1) to yield 6 (2.6 mg). Fraction H2 (1.1 g) was subjected to silica gel CC eluting with an n-hexane/EtOAc (50:1 → 10:1) gradient system to yield three subfractions (H21–H23). Subfraction H21 was then purified by repeated ODS CC (MeOH/H₂O, 2.5:1 → 3.5:1) to yield 3 (13.2 mg), and 8 (50.6 mg). Fraction H3 (1.2 g) was subjected to silica gel CC eluting with a n-hexane/EtOAc (15:1 → 5:1) gradient system to yield six subfractions (H31–H36). Subfraction H31 was purified by silica gel CC (n-hexane/EtOAc, 25:1 → 15:1), and by ODS CC (MeOH/H₂O, 1.5:1 → 3.5:1) to yield 4 (55.6 mg) and 7 (165.6 mg). Moreover, subfraction H34 was then purified by ODS CC (MeOH/H₂O, 4.5:1 → 6:1) to yield 11 (73.2 mg), and 12 (23.0 mg). Fraction H4 (435.2 mg) was subjected to silica gel CC eluting with a n-hexane/EtOAc (8:1 → 4:1) gradient system to achieve five subfractions (H41–H45). Subfraction H42 was purified by ODS CC (MeOH/H₂O, 1.5:1 → 3:1) to yield 10 (13.2 mg).

The CH₂Cl₂ soluble fraction (3.5 g) was subjected to CC using a Sephadex LH 20 column and eluted with CH₂Cl₂/MeOH (1:1 and 100% MeOH) system. The fractions were combined based on their TLC pattern to yield subfractions, which were designated D1–D4. Fraction D2 (596.8 mg) was subjected to silica gel CC eluting with n-hexane/EtOAc (5:1 → 1:1) gradient system to yield eight subfractions (D21–D28). Subfraction D25 was purified by ODS CC (MeOH/H₂O, 1:1 → 2:1), followed by preparative high-performance liquid chromatography (HPLC) eluted with MeOH/H₂O (60:40) to give 14 (12.4 mg). Fraction D3 (464.6 g), containing 15, and 16 was purified by silica gel CC n-hexane/EtOAc (4:1 → 1:1), followed by medium pressure liquid chromatography

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The EtOAc soluble fraction (1.1 g) was subjected to CC using a silica gel column and eluted with CHCl₃/MeOH/H₂O (20:1:1) gradient system. The fractions were combined based on their TLC pattern to yield subfractions, which were designated E1–E10. Fractions were combined based on their TLC pattern to give 15 (33.2 mg) and 16 (11.2 mg).

The EtOAc soluble fraction (1.1 g) was subjected to CC using a silica gel column and eluted with CHCl₃/MeOH/H₂O (20:1:1) gradient system. The fractions were combined based on their TLC pattern to yield subfractions, which were designated E1–E10. Fraction E3 (135.7 mg) was subjected to ODS CC eluting with MeOH/H₂O (3:1) to give six subfractions (E31–E36). Subfraction E33 was purified by silica gel CC (CHCl₃/MeOH, 9:1) to give 17 (32.2 mg).

Fraction E5 (359.2 mg) was subjected to silica gel CC eluting with CHCl₃/MeOH/H₂O (4:1:1) gradient system to give eight subfractions (E51–E58). Subfraction E53 was purified by MPLC (MeOH/H₂O, 1:2→1:1) to give 18 (6.0 mg). Fraction E8 (204.5 mg) was subjected to silica gel CC eluting with CHCl₃/MeOH/H₂O (3:1:0→1:1:0.1) gradient system to give six subfractions (E81–E86). Subfraction E85 was purified by ODS CC (MeOH/H₂O, 1:2→1:1), followed by preparative HPLC eluting with MeOH/CH₃OH (20:80→60:40) gradient system, to give 19 (10.6 mg), 20 (2.6 mg), and 21 (9.8 mg). Besides, subfraction E87 was purified by ODS CC (MeOH/H₂O, 1:1.5) to give 22 (14.5 mg).

Chemicals and reagents
Tissue culture reagents RPMI1640, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL Co. (Grand Island, NY, USA). LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide sodium (MTT) was obtained from Glentham Life Sciences (Corsham, UK). The anti-inducible nitric oxide synthase (iNOS) primary antibodies were purchased from Cayman (Ann Arbor, MI, USA), anti-cyclooxygenase (COX)-2, anti-inhibitor kappa B (IκB)-α, anti-p-IκB-α, anti-p-p65, anti-p65, anti-p-extracellular signal-regulated kinase (ERK), anti-ERK, anti-p-c-Jun N-terminal kinase (JNK), anti-JNK, anti-p-p38, and anti-p38 from Cell Signaling (Danvers, MA), and anti-β-actin from Santa Cruz Biotechnology (Dallas, TX, USA), respectively. Anti-mouse, anti-goat, and anti-rabbit secondary antibodies were purchased from Merck Millipore (Darmstadt, Germany).

Cell culture
The immortalized murine RAW264.7 macrophages and BV2 microglial cells were maintained at 5 × 10⁵ cells/mL in 100 mm dishes in RPMI1640 medium supplemented with 10% (v/v) heat-inactivated FBS, penicillin G (100 units/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM), and cultured at 37 °C in a humidified atmosphere containing 5% CO₂.

Cell viability assay
An MTT assay was conducted to determine cell viability. RAW264.7 and BV2 cells were cultured in a 96-well plate at a density 1 × 10⁵ cell/mL, and the cultured cells were treated with the test compounds for 24 h. Subsequently, 50 μL of an MTT solution were added to each well at a final concentration of 0.5 mg/mL, and the cells were incubated for 3 h in a humidified incubator at 37 °C. After removing the supernatant, the formazan formed was dissolved in 150 μL of dimethyl sulfoxide (DMSO), and mixed for 15 min. The optical density of the DMSO solution was measured at 540 nm wavelength using a microplate reader (Bio-Rad, Hercules, CA, USA). The optical density of the formazan formed in the control (untreated group) cells was considered to represent 100% viability, and the viability of cells in other groups was expressed as a percentage of obtained viable cells relative to the control. This assay was independently conducted three times.

Determination of nitrite
Nitrite is an indicator of NO production; its concentration in the culture medium was measured using the Griess reaction. RAW264.7 and BV2 cells were cultured in a 24-well plate at a density of 2 × 10⁵ cell/mL for 12 h. Cells were treated with test compounds for 3 h, and then stimulated with LPS (1 μg/mL) for 24 h. Each culture medium (100 μL) was mixed with the same volume of Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at room temperature. The absorbance was measured spectrophotometrically at 540 nm wavelength using an enzyme-linked immunosorbent assay (ELISA) plate reader. The nitrite concentration in the culture medium was determined from a standard curve of sodium nitrite.

PGE₂ assay
The culture media were collected to determine the PGE₂ level present in each sample using the appropriate ELISA kit from ENZO Life Sciences (Farmingdale, NY, USA). Three independent assays were performed according to the manufacturer’s instructions.

IL-1β, IL-6, and TNF-α assays
The levels of IL-1β, IL-6, and TNF-α present in each sample were determined using a commercially appropriate kit from R&D Systems (Minneapolis, MN). Three independent assays were performed according to the manufacturer’s instructions.
Western blot analysis

RAW264.7 and BV2 cells were cultured in a 6-well plate at a density of $2 \times 10^5$ cell/mL for 12 h. Then cells were treated with the test compounds for 3 h, followed by stimulation with LPS (1 μg/mL) for 24 h. Radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, MA, USA) was used to prepare lysate. The concentration of protein was measured using Bradford protein assay (Bio-Rad Laboratories, CA, USA) and normalized to ensure that equal amounts were loaded. Subsequently, 30 μg of protein from each sample were resolved using 6%, 8%, and 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were electrophoretically transferred onto Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% skim milk and sequentially incubated with particular primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies, followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis

The data are expressed as mean ± standard deviation (SD) of at least three independent experiments. To compare three or more groups, a one-way analysis of variance (ANOVA) was used, followed by Tukey’s multiple comparison tests. Statistical analysis was performed using GraphPad Prism software, version 3.03 (GraphPad Software Inc., San Diego, CA, USA).

Results

Compounds isolated from *A. macrocephala*

Twenty-two compounds were isolated from the rhizomes of *A. macrocephala* using various combined chromatographic methods. The nuclear magnetic resonance (NMR) and mass spectroscopy (MS) data of the isolated compounds were analyzed and compared with those reported in the literature to elucidate the structures of the isolated compounds. Compound 1 was obtained as a white solid, $[\alpha]_{D}^{23} + 232.4$ (c 0.07, MeOH). Its molecular formula of $C_{15}H_{20}O_{3}$ was determined based on the analysis of HR-ESI-MS data showing m/z peak at 249.1496 [M + H]$^+$ (calculated for $C_{15}H_{21}O_{3}$: 249.1412) (Fig. 1),
and the analysis of $^1$H NMR and $^{13}$C NMR data (Table 1, Fig. 2, Additional file 1: Fig. S1). The $^1$H-NMR spectrum of 1 (in CDCl$_3$) showed the following signals: two tertiary methyl groups at δ 0.89 (3H, s) and 1.83 (3H, t, J = 1.6 Hz), which were typical of CH$_3$-14 and CH$_3$-13 of eudesmanolides; together with one terminal double bond at δ 4.60 (1H, d, J = 1.6 Hz) and 4.88 (1H, d, J = 1.6 Hz) for H-15. The $^1$H-NMR patterns of 1 were similar to those of the known compound 5 (attractylenolide II) [21], as stated in the Supplementary data. However, the $^1$H-NMR spectrum of 1 was distinct from that of 5 because there was one more proton signal at δ 3.88 (1H, d, J = 3.6 Hz). Moreover, in $^{13}$C-NMR spectrum, there was an additional carbon signal at δ 75.4. Taking the molecular formula into account, it was suggested that hydroxylation had taken place on one of the secondary carbons. Compared with 5, H-8 signals of 1 notably shifted to lower fields, from δ 4.80 (1H, t, J = 6.4 Hz) to δ 4.98 (1H, d, J = 2.0 Hz). Therefore, it was supposed that a hydroxylation took place at C-9, resulting in increased chemical shift values of H-8 for an inductive effect. In the heteronuclear multiple bond correlation (HMBC) spectrum, the long-range correlations from the carbon signal at δ 75.4 (C-9), to H-8 signals confirmed the hydroxyl group located at C-9 (Table 1, Additional file 1: Fig. S3, S5). The coupling constants and the cross peak between H-9 and the signal at δ 4.98 (1H, d, J = 2.0 Hz) and δ 0.89 (3H, s), assigned to H-8 and CH$_3$-14, respectively, also CH$_3$-14 correlated with H-2β, in NOESY spectrum confirmed that the H-9 was axial and β-oriented (Additional file 1: Fig. S4, S6). Finally, the chemical structure of 1 is formed by adding an extra oxygen in comparison with 5, and 1 was established as 9α-hydroxyatractylenolide, namely 9α-hydroxyeudesma-4(15),7(11)-dien-8α,12-olide. When compound 1 was separated and purified by ODS CC, it was found that its $R_f$ value was very close to that of compound 7 by TLC analysis. It was worth noting that 1 has no fluorescence under 254 nm ultraviolet light, while 7 shows dark brown fluorescence. After heating and color development spraying with 10% sulfuric acid, 1 showed blue-purple and blue fluorescence, however, 7 showed dark brown and yellow-green fluorescence at 254 and 365 nm, respectively. There was a significant difference between the two compounds. This difference is related to the position of OH. The lone pair electron on the 8-position OH of 7 formed a p-π conjugation effect with a double bond and carbonyl, enhancing the fluorescence, while the OH in 1 did not have the above effect at the 9-position. As far as we know, its spectroscopic data had not been reported before. In addition, the other compounds were identified by comparing with previously published data as presented in Fig. 3.

Table 1  NMR data for compound 1 in CDCl$_3$

| No | δC (ppm) | δH (J, Hz) | HMBC |
|----|---------|------------|------|
| 1  | 34.6    | 1.33 (1H, m) 2.06 (1H, dd, J = 13.6, 4.4 Hz) | C$_2$, C$_{10}$, C$_{14}$ |
| 2  | 22.3    | 1.60 (2H, m) |      |
| 3  | 36.3    | 1.96 (1H, m) 2.35 (1H, m) | C$_2$, C$_4$, C$_{15}$ C$_4$ |
| 4  | 148.9   |            |      |
| 5  | 42.2    | 2.38 (1H, m) | C$_{10}$ |
| 6  | 25.6    | 2.31 (1H, m) 2.67 (1H, dq, J = 12.8, 2.8 Hz) | C$_4$, C$_8$, C$_{10}$ |
| 7  | 159.1   |            |      |
| 8  | 79.9    | 4.98 (1H, d, J = 2.0 Hz) | C$_8$, C$_9$, C$_9$ |
| 9  | 75.4    | 3.88 (1H, d, J = 3.6 Hz) | C$_9$, C$_9$, C$_9$ |
| 10 | 41.0    |            |      |
| 11 | 121.9   |            |      |
| 12 | 175.1   |            |      |
| 13 | 8.4     | 1.83 (3H, t, J = 1.6 Hz) | C$_2$, C$_{11}$, C$_{12}$ |
| 14 | 15.7    | 0.89 (3H, s) | C$_1$, C$_3$, C$_8$, C$_{10}$ |
| 15 | 107.3   | 4.88 (1H, d, J = 1.6 Hz) 4.60 (1H, d, J = 1.6 Hz) | C$_2$, C$_3$ |

Fig. 2  The key HMBC (A) and NOESY (B) correlations (arrow) of compound 1
Comparing with previously published data, the other compounds were identified as atractylosine (2) [22], 3β-acetoxyatractylon (3) [22], atractylenolide I (4) [23], atractylenolide II (5) [21], 8-epiasterolid (6) [24], atractylenolide III (7) [14], atractylenolide VII (8) [25], 8-epitractylenolide III (9) [14], eudesm-4(15)-ene-7α,11-diol (10) [26], linoleic acid (11) [27], myristic acid (12) [27], 3-O-caffeoyl-1-methyquinic acid (13) [28], (2E,8E,10E)-tetradecatriene-4,6-diyne-1,14-diol (14) [29], 14-aceroxy-12-senecioyloctadeca-2E,8Z,10E-trien-4,6-diyn-1-ol (15) [30], isoscopoletin (16) [31], caffeic acid (17) [32], protocatechic acid (18) [33], 3-O-caffeoylquinic acid (19) [28], 4-O-caffeoylquinic acid (20) [34], 1,5-di-O-caffeoylquinic acid (21) [35], and nicotinic acid (22) [36] (Fig. 3).

Although we tried to evaluate the bioactivity of the isolated compounds, the amounts of compounds 1, 3 and 20 were too small to determine their bioactivities. In addition, compound 2 was obtained in a sufficient amount, but further NMR analysis indicated changes in the compound. Thus, the remaining 18 compounds were used to examine their bioactivity.
Inhibitory effect of compounds isolated from *A. macrocephala* on LPS-induced NO production in RAW264.7 and BV2 cells

First, we evaluated whether the compounds exerted a cytotoxic effect on RAW264.7 and BV2 cells. Cells were treated with 5, 10, 20, 40, and 80 μM of selected compounds for 24 h, and cell viability was measured by MTT assay. The compounds showed no cytotoxicity for any of the tested concentrations (data not shown). Therefore, the three highest concentrations (20, 40, and 80 μM) were used in the NO assay. Cells were pre-treated with 20, 40, and 80 μM of compounds for 3 h, and then stimulated with LPS (1 μg/mL) for 24 h. Regarding RAW264.7 cells, most compounds inhibited the LPS-induced NO production, except for compounds 7 and 9, with IC₅₀ values in the 26.8–78.1 μM range. In BV2 cells, all the tested compounds suppressed NO production in a concentration-dependent manner. Compounds 4, 5, 6, 16, and 17, showed an inhibitory effect higher than 50% within the used concentration range, with IC₅₀ values of 26.0 ± 0.23, 46.8 ± 1.12, 37.4 ± 4.03, 58.5 ± 2.23, and 72.3 ± 3.35 μM, respectively (Table 2).

### Table 2 Inhibitory effects of the test compounds on LPS-induced NO production in RAW264.7 and BV2 cells

| Compounds | IC₅₀ (μM)  |
|-----------|------------|
|           | RAW264.7   | BV2         |
| Butein    | 5.40±0.50  | 4.41±0.45  |
| 4         | 26.8±3.86  | 26.0±0.23  |
| 5         | 60.5±16.8  | 46.8±1.12  |
| 6         | 39.1±3.22  | 37.4±4.03  |
| 7         | 1.7% at 80 μM | 20.3% at 80 μM |
| 8         | 50.8±7.38  | 35.9% at 80 μM |
| 9         | 9.2% at 80 μM | 27.3% at 80 μM |
| 10        | 64.3±12.1  | 24.2% at 80 μM |
| 11        | 41.2±4.97  | 47.7% at 80 μM |
| 12        | 36.7±4.11  | 35.9% at 80 μM |
| 13        | 59.2±14.9  | 25.0% at 80 μM |
| 14        | 28.4±6.55  | 29.7% at 80 μM |
| 15        | 51.3±7.28  | 38.9% at 80 μM |
| 16        | 29.8±2.52  | 58.5±2.23  |
| 17        | 34.8±2.54  | 72.3±3.35  |
| 18        | 44.5±4.34  | 41.7% at 80 μM |
| 19        | 28.8±3.09  | 45.8% at 80 μM |
| 21        | 78.1±23.5  | 36.7% at 80 μM |
| 22        | 28.0±2.94  | 47.2% at 80 μM |

Inhibitory effect of 8-epiasterolid on LPS-induced PGE₂ production in RAW264.7 and BV2 cells

According to the above result, atracylenolide I (4) showed the lowest IC₅₀ value in both RAW264.7 and BV2 cells. However, the anti-inflammatory and anti-neuroinflammatory effects of this compound was already reported [16, 17]. Therefore, we selected 8-epiasterolid (6), which was the second most effective, and examined its further anti-inflammatory activity. RAW264.7 and BV2 cells were pre-treated with 8-epiasterolid for 3 h, followed by stimulation with LPS (1 μg/mL) for 24 h to examine whether it affected PGE₂ production. It was found that pre-treatment with 8-epiasterolid significantly inhibited the LPS-induced PGE₂ production in both RAW264.7 and BV2 cells (Fig. 4).

![Fig. 4](Fig_4.png)
Inhibitory effect of 8-epiasterolid on LPS-induced production of pro-inflammatory cytokines in RAW264.7 and BV2 cells

Based on the above results, we examined whether 8-epiasterolid suppressed the production of pro-inflammatory cytokines in LPS-induced RAW264.7 and BV2 cells. Both cells were pre-treated with 8-epiasterolid for 3 h, and then stimulated with LPS (1 μg/mL) for 24 h. At a concentration of 80 μM, 8-epiasterolid significantly suppressed the LPS-induced production of IL-1β, IL-6, and TNF-α in both RAW264.7 and BV2 cells (Fig. 5).

Inhibitory effect of 8-epiasterolid on LPS-induced iNOS and COX-2 expression in RAW264.7 and BV2 cells

We further investigated the effect of 8-epiasterolid on the expression of iNOS and COX-2 proteins induced by LPS in both cell lines. Cells were pre-treated with 8-epiasterolid for 3 h, and then stimulated with LPS (1 μg/mL) for 24 h. The degree of expression of iNOS and COX-2 proteins in the lysates was determined using Western blot analysis. The pre-treatment with 8-epiasterolid at concentrations of 40 and 80 μM suppressed the LPS-induced iNOS and COX-2 expression in both RAW264.7 and BV2 cells (Fig. 6).

Effect of 8-epiasterolid on the LPS-induced activation of NF-κB and MAPK signaling pathways

We examined whether 8-epiasterolid suppressed the activation of NF-κB pathway in LPS-induced RAW264.7 and BV2 cells. Both cell lines were pre-treated with 8-epiasterolid for 3 h, and then stimulated with LPS (1 μg/mL) for 1 h. The pre-treatment with 8-epiasterolid inhibited the LPS-induced phosphorylation and degradation of IκB-α as well as the phosphorylation of p65 in both RAW264.7 and BV2 cells (Fig. 7).

Additionally, we investigated whether 8-epiasterolid affects the LPS-induced activation of the MAPK pathways in RAW264.7 and BV2 cells. Both cell lines were pre-treated with 8-epiasterolid for 3 h, and then stimulated with LPS (1 μg/mL) for 30 min. The phosphorylation levels of p38, ERK, and JNK remarkably increased by LPS-stimulation. However, the pre-treatment with 8-epiasterolid did not inhibit the activation of all three MAPKs (Fig. 8).

Discussion

In this investigation, we isolated 22 compounds from the rhizomes of *A. macrocephala* using various combined chromatographic methods, and evaluated their anti-inflammatory effects measuring the degree of the production of NO in LPS-induced RAW264.7 and
BV2 cells. Among them, we found that 8-epiasterolid is the second most effect compound, and it inhibited the LPS-induced production of PGE2 and pro-inflammatory cytokines, and the expression of iNOS and COX-2 proteins. In addition, we confirmed that these inhibitory effects were regulated by the inactivation of NF-κB signaling pathway by 8-epiasterolid, not MAPK pathways.

The production of NO is catalyzed by the enzymatic activity of nitric oxide synthase (NOS), which converts L-arginine to NO and L-citrulline via the intermediate N-hydroxy-L-arginine [37, 38]. NO is known to play a key role in regulating the vascular, immune, and nervous systems [39]. In inflammatory conditions, the expression of NOS increases in various immune cells including macrophages, monocytes, microglia, dendritic cells,

**Fig. 6** The effect of 8-epiasterolid on the LPS-induced expression of iNOS and COX-2 protein in RAW264.7 (A) and BV2 cells (B). Lysates were prepared from cells pre-treated with or without the indicated concentrations of 8-epiasterolid for 3 h and then with LPS (1 μg/mL) for 24 h. The level of expression of iNOS and COX-2 proteins was determined by Western blot analysis. Representative blots from three independent experiments are shown.

**Fig. 7** The effect of 8-epiasterolid on the LPS-induced activation of NF-κB pathway in RAW264.7 (A, C) and BV2 cells (B, D). Lysates were prepared from cells pre-treated with or without the indicated concentrations of 8-epiasterolid for 3 h and then with LPS (1 μg/mL) for 1 h. The level of expression of proteins was determined by Western blot analysis. Representative blots from three independent experiments are shown.
eosinophils, and neutrophils, resulting in the release of large amounts of NO [38]. Subsequently, the overproduction of NO leads to development of inflammatory disorders in the joints, gut, and lungs [38], neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease, multiple sclerosis [39], and cancer [40]. As inhibiting NO production could have a therapeutic effect on inflammatory diseases, we investigated whether the compounds isolated from *A. macrocephala* might inhibit the LPS-induced NO production in RAW264.7 macrophages and BV2 microglial cells. Our result showed that atractylenolide I (compound 4) has the lowest IC50 value among the tested compounds, indicating that it has the highest inhibitory effect. Previous studies have reported that atractylenolide I has anti-inflammatory effects in RAW264.7 macrophages by inhibiting the activation of NF-κB and MAPK signaling pathways mediated through the cluster of differentiation 14 (CD14)/toll-like receptor 4 (TLR4) pathways [16]. This compound also showed neuroprotective effects through the suppression of NF-κB pathway and induction of heme oxygenase (HO)-1 protein in BV2 microglia cells and in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced C57BL6/J models, suggesting that atractylenolide I might be effective in treating Parkinson’s disease [17]. 8-Epiasterolid was the second most effective compound for NO production in both RAW264.7 and BV2 cells; additional experiments were conducted and showed that this compound possesses anti-inflammatory properties (Table 2).

As mentioned in “Introduction” section, macrophages are the most dominant immune cell types throughout the body, and microglia play as the first and main form of active immune defense in CNS as the resident macrophage cells in nerve system. Both cells exhibit the characteristics of macrophages, but microglial cells are specialized ones that are only found in the nerve system (brain and spinal cord) [41]. In addition, Luigina Guasti et al. investigated the in vitro effects of Apixaban (commercial product named Eliquis®) on cell proliferation, mortality, cell migration, gene expression and matrix metalloproteinase (MMP) in 5 different cancer cell lines including OVCAR3 (ovarian cancer), MDA MB 231 (breast cancer), CaCO-2 (colon cancer), LNCaP (prostate cancer), and U937 (histiocytic lymphoma). They reported that Apixaban increased the expression of tumor suppression gene p16 in all cell lines, but reduced proliferation in only 3 cancer cell lines (OVCAR3, CaCO-2, and LNCaP) [42]. These results suggest that the same compound might exhibit different effects in different cell lines. Therefore, the effects of tested compound isolated from *A. macrocephala* applied to different cells (RAW264.7 macrophages and BV2 microglial cells) could be different.

Similar to NO, prostaglandins play an important role in the inflammatory process. Arachidonic acid (AA) in the body is converted into prostaglandin H2 (PGH2) by the action of cyclooxygenase (COX)-1 and 2 enzymes [43]. Then, PGH2 acts as a substrate of specific isomerase

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**Fig. 8** The effect of 8-epiasterolid on the LPS-induced activation of MAPK cascades in RAW264.7 (A–C) and BV2 cells (D–F). Lysates were prepared from cells pre-treated with or without the indicated concentrations of compound 6 for 3 h and then with LPS (1 μg/mL) for 30 min. The expression level of proteins was determined by Western blot analysis. Representative blots from three independent experiments are shown.
and synthase enzymes to produce various prostanoids including PGE$_2$, PGI$_2$, PGD$_2$, PGF$_{2\alpha}$ and thromboxane A$_2$ (TXA$_2$) [44]. PGE$_2$ reacts with four types of receptors (EP1–EP4), each with its distinct signal-transduction properties, and exerts diverse physiological functions [45]. Our results showed that the pre-treatment with 8-epiasterolid significantly inhibited the LPS-induced production of PGE$_2$ in both RAW264.7 and BV2 cells (Fig. 4).

Cytokines are small secreted proteins produced by every cell and regulate the immune responses [46]. They consist of six major families including ILs, chemokines, interferons, TNF, growth factors of hematopoiesis and transforming growth factor-β (TGF-β) members [47]. In particular, the pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α are produced from activated macrophages or microglia, and are associated with the up-regulation of inflammatory responses [46]. Compound 4 (attractylenolide I), which is one of the isolated compounds, has been reported that this compound inhibited the production of TNF-α and IL-6 in LPS-stimulated RAW264.7 cell model [16]. Another investigation demonstrated that compound 4 inhibited the production of TNF-α, IL-6, and IL-1β in LPS-induced BV2 microglial cell model, and that of TNF-α in MPTP-induced mice model [17]. Accordingly, we investigated the inhibitory effect of 8-epiasterolid on the LPS-induced production of pro-inflammatory cytokines in RAW264.7 and BV2 cells. Our results showed that the pre-treatment with 8-epiasterolid markedly suppressed the production of IL-1β, IL-6, and TNF-α in both cells (Fig. 5).

NO is synthesized from the L-arginine via the enzymatic activity of NOS. There are three different isoforms of NOS including neuronal nNOS (NOS1), inducible iNOS (NOS2), and endothelial eNOS (NOS3) [48]. Each enzyme has its own physiological characteristics. nNOS is expressed in neurons, and has been reported to mediate the long-term regulation of synaptic transmission [49]. eNOS is released mainly by endothelial cells; as it promotes blood vessel expansion and controls blood pressure, the functional anomalies in eNOS lead to the development of cardiovascular diseases [49]. eNOS is also known to control cancer-related phenomena such as angiogenesis, apoptosis, invasion, and metastasis [50]. NOSs and eNOS are constitutive forms that continuously secrete low concentrations of NO, and thus maintain several physiological functions. However, since iNOS is inducible, its production is increased by bacterial LPS, cytokines, chemokines, and other stimuli such as stress [49, 51].

As mentioned above, COX is involved in the generation of PGE$_2$. There are two COX isoforms: COX-1 and COX-2. The former is a constitutive type that is expressed in most tissues and is associated with conducting normal physiological functions [52, 53]. On the other hand, COX-2 is an inducible form and is up-regulated through various inflammatory stimuli of cytokines, growth factors, tumor promoters, and bacterial LPS, which then increase the amount of PGE$_2$ produced [54]. Although both COX-1 and COX-2 are related to the production of PGE$_2$, the continuous suppression of COX-1 activity could lead to side effects such as gastrointestinal toxicity or mild bleeding diathesis [55]. Therefore, the selective inhibition of COX-2 is necessary to block inflammatory responses. In previous results, 8-epiasterolid inhibited the production of NO and PGE$_2$ induced by LPS. Accordingly, the pre-treatment with 8-epiasterolid also repressed the LPS-induced expression of iNOS and COX-2, which are enzymatic proteins associated with the production of NO and PGE$_2$, respectively, in both RAW264.7 and BV2 cells (Fig. 6).

NF-κB is one of the main transcription factors that regulates gene expression and that is involved in the production of pro-inflammatory mediators [56], development of immune cells, cell cycle, proliferation, and cell death [57]. This family consists of five structurally related members, including RelA (p65), p50, p52, RelB, and c-Rel, and these subunits form at least 12 different homo- or heterodimers [58]. There are two different signaling pathways: canonical and non-canonical pathways. The canonical pathway mainly regulates RelA (p65), p50, and c-Rel, and the non-canonical pathway predominantly activates p52 and RelB. Both pathways are important for regulating immune and inflammatory responses [56, 59]. Under basal conditions, NF-κB dimers are present in the cytoplasm with their inhibitory protein IκB. However, pro-inflammatory cytokines or LPS can induce the phosphorylation and degradation of IκB, releasing NF-κB dimers and inducing phosphorylation [60]. Then, NF-κB dimers translocate into the nucleus, bind to the κB binding site, and regulate the expression of inflammatory genes including inflammatory enzymes (iNOS, COX-2), cytokines, and adhesion molecules [61]. Previous studies demonstrated that compound 4 (attractylenolide I) inhibited the LPS-induced activation of NF-κB in RAW264.7 and BV2 cells [16, 17]. Our investigation revealed that the pre-treatment with 8-epiasterolid inhibited the phosphorylation and degradation of IκB-α, and the phosphorylation of p65 protein in both RAW264.7 and BV2 cells (Fig. 7).

MAPK cascades are groups of serine/threonine protein kinases and have an important role in the transduction of extracellular signals to various cellular responses including proliferation, stress responses, apoptosis, and immune defense [62]. In mammalian cells, MAPK cascades consist of three major types, including p38, ERK,
and JNK MAPKs, and each kinase can be activated by many different upstream MAPK kinases (MAP2K), and MAPK kinase kinases (MAP3Ks) [63]. The phosphorylation of MAPKs has been shown to be related to the enhancement of inflammatory responses through the induction of the release of pro-inflammatory mediators [62, 64]. In a previous study, compound 4 (atractylenolide I) inhibited the LPS-induced phosphorylation of p38 and ERK MAPKs, and showed anti-inflammatory activity in RAW264.7 cells [16]. Interestingly, the pre-treatment with 8-epiasterolid did not affect to the phosphorylation of p38, ERK, and JNK MAPKs. These data suggested that 8-epiasterolid regulated the inflammatory responses by inhibiting of the NF-kB signaling pathway, not MAPK pathways.

**Abbreviations**

AA: Arachidonic acid; ANOVA: Analysis of variance; CC: Column chromatography; CD: Cluster of differentiation; CD14: Cluster of differentiation 14; CDK: Cyclin-dependent kinase; COX-2: Cyclooxygenase-2; DMEM: Dulbecco’s modified Eagle’s medium; DMSO: Dimethyl sulfoxide; ECL: Enhanced chemiluminescence; ELISA: Enzyme-linked immunosorbent assay; FBS: Fetal bovine serum; HO-1: Heme oxygenase-1; HRP: Horseradish peroxidase; IL: Interleukin; iNOS: Inducible nitric oxide synthase; LPS: Lipopolysaccharide; MAPK: Mitogen-activated protein kinase; MPP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); MS: Mass spectrometry; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide sodium; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; NMR: Nuclear magnetic resonance; NO: Nitric oxide; NOS: Nitric oxide synthase; Nrf2: Nuclear factor erythroid 2-related factor 2; PGE2: Prostaglandin E2; PGE3: Prostaglandin H2; RIP-1: Radioimmunoprecipitation assay; SD: Standard deviation; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC: Thin layer chromatography; TRP4: Toll-like receptor 4; TNF: Tumor necrosis factor.

**Supplementary Information**

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**Additional file 1:** Figure S1. 1H-NMR spectrum of compound 1 in CDCl3.

**Figure S2.** 13C-NMR spectrum of compound 1 in CDCl3.

**Figure S3.** HMBC spectrum of compound 1 in CDCl3.

**Figure S4.** NOESY spectrum of compound 1.

**Figure S5.** The key HMBC correlation of compound 1.

**Figure S6.** The key NOESY correlation of compound 1.

**Authors’ contributions**

All authors contributed to the study conception and design. Dae Young Lee, Dahye Yoon, Jin Tae Jeong, and Geum-Soog Kim prepared the plant materials and funded for this investigation. Youn-Chul Kim, Ren-Bo An, and Hyuncheol Oh managed the research project, provided guidance and supervised the study. Hong-Guang Jin and Ren-Bo An contributed to the isolation of compounds, and the conduction of HPLC and NMR. Kwan-Woo Kim and Jing Li carried out the biological assays. The first draft of the manuscript was written by Hong-Guang Jin and Kwan-Woo Kim. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Competing interests**

The authors declare that they have no competing interests.

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