Article

Atraric Acid Exhibits Anti-Inflammatory Effect in Lipopolysaccharide-Stimulated RAW264.7cells and Mouse Models

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Abstract: As symbionts of fungi and algae, lichens produce a variety of secondary products which pharmacological activities. This study aimed to investigate the anti-inflammatory activities of *Heterodermia hypoleuca* and its main compound, atraric acid. The results confirmed that atraric acid could regulating induced pro-inflammatory cytokine, nitric oxide, induced nitric oxide synthase and cyclooxygenase-2 expression by lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Means while, atraric acid down-regulated expression of phosphorylated IκB, ERK and nuclear factor kappa B (NFκB) signaling pathway to exhibit anti-inflammatory effects in LPS-stimulated RAW264.7 cells. Based on these results, the anti-inflammatory effect of atraric acid during LPS-induced endotoxin shock in a mouse model was confirmed. In the atraric acid treated-group, cytokine production was decreased in the peritoneum and serum, and each organ damaged by LPS-stimulation was recovered. These results show that atraric acid has an anti-inflammatory effect and its molecular mechanism may be involved in the inactivation of the ERK/NFκB signaling pathway, demonstrating its value as a potential therapeutic for inflammatory diseases.

Keywords: Anti-inflammation; Endotoxin shock; Atraric acid; Lichen; *Heterodermia hypoleuca*

1. Introduction

Inflammation is an inherent immune mechanism that occurs as a results of pathogen invasion in the body [1, 2]. During an inflammatory response, macrophages release pro-inflammatory mediators, such as nitric oxide (NO), induced nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), as well as pro-inflammatory cytokines and chemokines [3,4]. Nuclear factor kappa B (NFκB) is inactivated by binding to IκB in the cytoplasm and activated by phosphorylation and IκB degradation. The activation of NFκB can be regulated by a mitogen-activated protein kinase (MAPK) and an extracellular regulatory kinase [5-7]. However, abnormally high inflammatory response leads to severe tissue damage and endotoxin shock, and chronic inflammatory processes cause a series of diseases such as neurodegeneration, cardiovascular diseases, and cancer [3]. Endotoxin shock is a severe inflammatory reaction caused by Gram-negative bacteria induced infection of the bloodstream. Lipopolysaccharide (LPS), an endotoxin on the surface of

Gram-negative organisms, stimulates the release of pro-inflammatory cytokines and NO, increasing vasodilation and vascular permeability. This leads to impaired cardiovascular function, pulmonary dysfunction, acute renal impairment, septic shock and death. Many studies have demonstrated that the inhibition of NFκB and MAPKs activation is an important strategy for the treatment of inflammatory diseases [8-11].

Lichens are a symbiont of algae and fungi, can grow in cold and harsh environments, and are abundantly found in the surroundings [3,12]. In addition, lichens have been traditionally used as herbal tea and food ingredients in China and Japan. Their capacity to produce and accumulate secondary metabolites gives rise to their wide chemical diversity. Many metabolites, including atraric acid, atranorin, usnic acid and olivetoric acid, have been linked to different biological activities such as antibacterial, antioxidant and antiproliferation effects [13-15].

Atraric acid is able to inhibit the growth of androgen receptor-expressing human prostate cancer, and it is therefore considered an androgen receptor-specific antagonist [16]. It shows good antioxidant and antimicrobial properties in vitro and inhibits carrageenan-induced edema and wound healing activity *in vivo* [17]. In this study, atraric acid was isolated from *Heterodermia hypoleuca* (HH) and we determined its anti-inflammatory effect in LPS-stimulated RAW264.7 cells and on LPS-induced endotoxin shock in mice models.

2. Results

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.

### 2.1. HH exhibited anti-inflammatory effect

The anti-inflammatory activity of HH in LPS-stimulated RAW264.7cells was investigated. Prior to the experiment, cell counting kit-8 (CCK-8) was used to evaluate the cell viability of HH extracts. The survival of the cells was not affected by HH extracts (Figure 1a). As shown in Figure 1, HH inhibited the production of NO stimulated by LPS in a concentration dependent manner (Figure 1b). In addition, the levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and granulocyte-macrophage colony stimulating factor (GM-CSF) released in LPS-stimulated RAW 264.7 macrophages were significantly reduced by 20 %, 30 %, 58 % and 45 %, respectively, at 30 μg/mL of HH (Figure 1c-f).
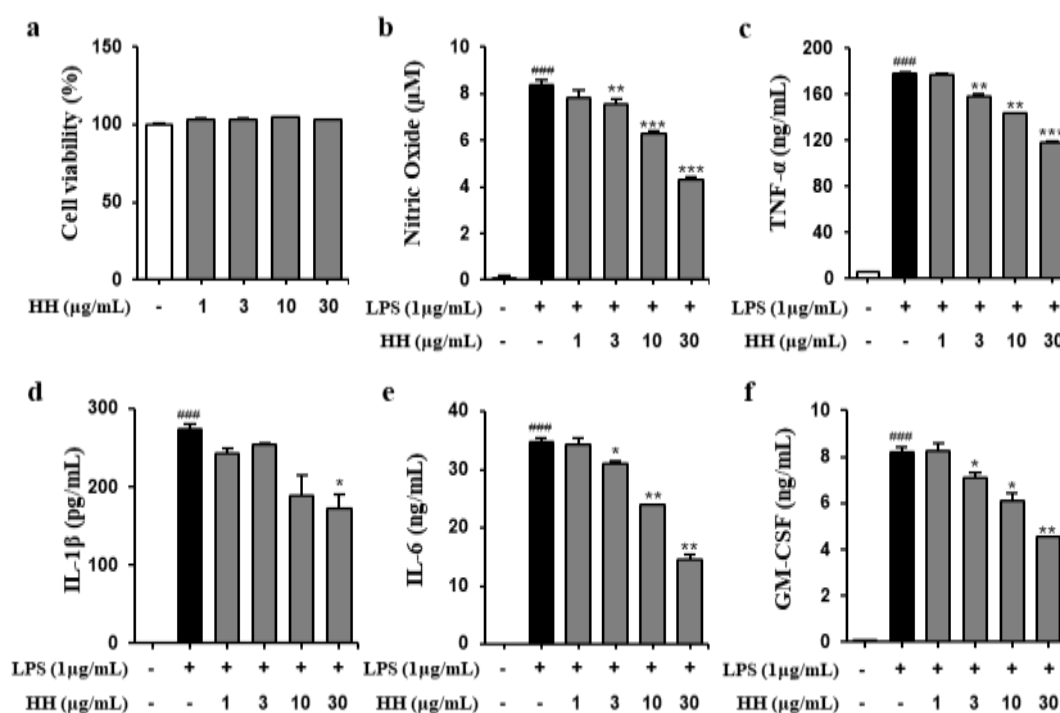
Figure 1. The screening of an anti-inflammatory activity of the methanol extract of HH. (a) The cells were treated with different concentrations of HH for 24 h, and the viability of the treated cells was determined by cell counting kit-8 assay. The cells were preincubated with 1 μg/mL LPS for 1h and then treated with 1~30 μg/mL HH for 24 h. (b) The concentration of nitric oxide in the cultured medium was measured by using the Griess reaction. The release of TNF-α (c), IL-1β (d), IL-6 (e) and GM-CSF (f) was determined by ELISA. Data are presented as mean ± SD from three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *versus* the LPS group).

2. 2. Identification of atraric acid

Atraric acid isolated from HH was identified by comparing the spectroscopic data and optical rotations with previously reported data. Atraric acid: 1H NMR (methanol-d4, 400 MHz); 6.25 (1H, s, H-6), 3.83 (3H, s, H-10), 2.31 (3H, s, H-8), 1.89 (3H, s, H-9), 13C NMR (methanol-d4, 100 MHz); 172.5 (C-1), 162.1 (C-3), 160.1 (C-5), 140.0 (C-7), 111.1 (C-6), 108.4 (C-4), 104.4 (C-2), 52.4 (C-10), 23.9 (C-8), 8.1 (C-9) ; ESI-MS showed an [M+H]+ ion at m/z = 197.0 (M+H)+, 219.0 (M+Na)+ (13C NMR chemical shifts were determined using 2D NMR spectroscopic data and confirmed by comparing them with previously reported data) (Figure 2) [18-20].
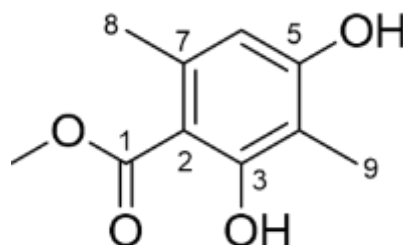

Figure 2. Chemical structure of atraric acid.

2.3. Atraric acid inhibited the production of NO and pro-inflammatory cytokines

We investigated the production of NO and pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells to determine the anti-inflammatory activity of atraric acid isolated from HH. As shown in Figure 3, we confirmed that atraric acid did not affect the viability of RAW264.7 cells. Therefore, treatment in all experiments used dose of atraric acid up to 300 μM. To investigate the potential anti-inflammatory effects of atraric acid, LPS-stimulated RAW264.7 cells were treated with atraric acid, the levels of NO and pro-inflammatory cytokines were determined. LPS stimulation significantly increased the production of NO in RAW264.7 cells. However, LPS-stimulated NO production was significantly reduced by atraric acid in a dose-dependent manner (300 μM, 69 %, Figure 4a). The pro-inflammatory cytokines in the culture media were measured by ELISA kit. With 300 μM atraric acid, the levels of IL-1β, IL-6 and GM-CSF produced by LPS were 36.3 %, 93.3 % and 80.9 %, respectively. Therefore, atraric acid (10~300 μM) exhibited an anti-inflammatory effect by regulating the levels of inflammatory factors produced by LPS-stimulated RAW264.7 cells.
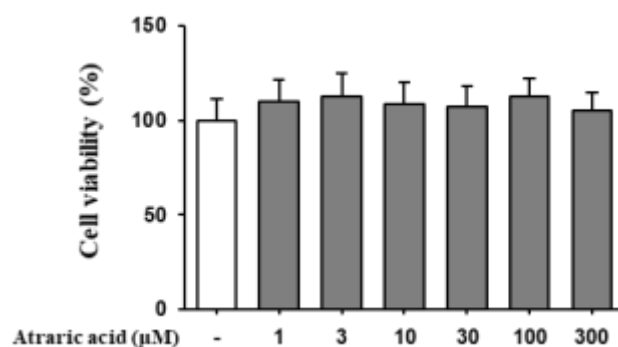
Figure 3. Effect of atraric acid on the viability of RAW264.7 cells. The cells were treated with 1~300 μM atraric acid for 24 h. Cell viability was determined using cell counting kit-8. Data are presented as mean ± SD from three independent experiments.
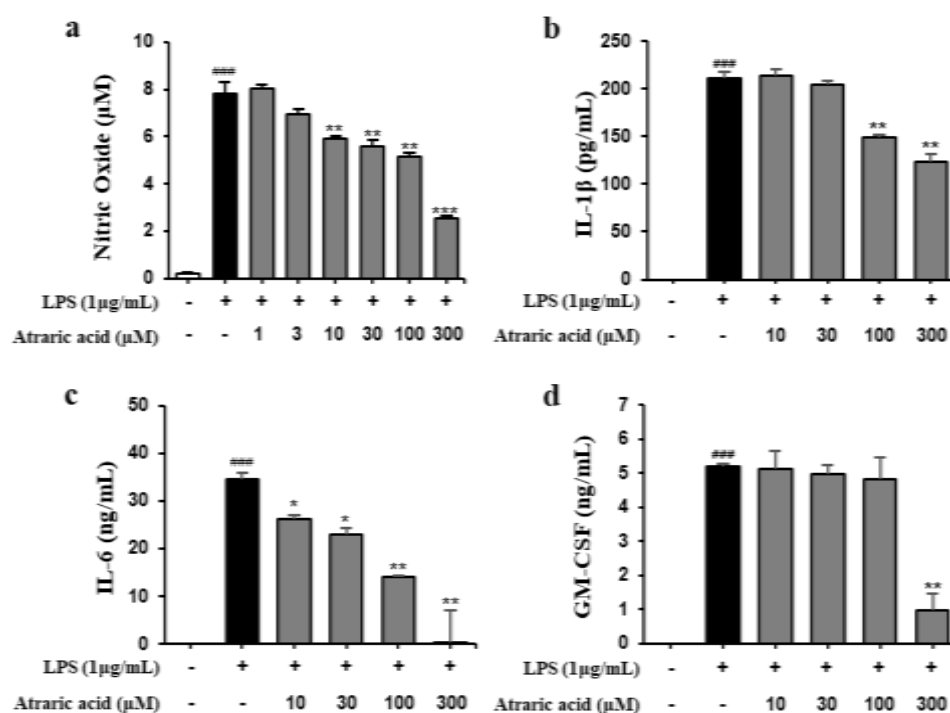
Figure 4. Effect of atraric acid on the levels of NO and pro-inflammatory cytokines produced by LPS-stimulated RAW2647 cells. The cells pre-treated with 1 μg/mL LPS for 1h and subsequently treated with 1~300 μM atraric acid for 24 h. (a) The concentration of nitric oxide in the cultured medium was measured as an indicator of NO production by using Griess reaction. The release of IL-1β (b), IL-6 (c) and GM-CSF (d) was determined by ELISA. Data are presented as mean ± SD from three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *versus* the LPS group).

2. 4. Atraric acid inhibited LPS-induced expression of iNOS and COX-2

iNOS and COX-2 are pro-inflammatory proteins that play important roles in the syntheses of NO and PEG-2, respectively [21]. Based on the strong anti-inflammatory effect display by 100, 300 μM atraric acid, we chose this concentration range to determine if the inhibition of inflammation-related factors by atraric acid was associated with the inhibition of iNOS and COX-2 expression [9]. Our results confirmed that the expression of iNOS and COX-2 proteins increased after treating LPS-stimulated RAW264.7 cells with atraric acid for 18 h (Figure 5).
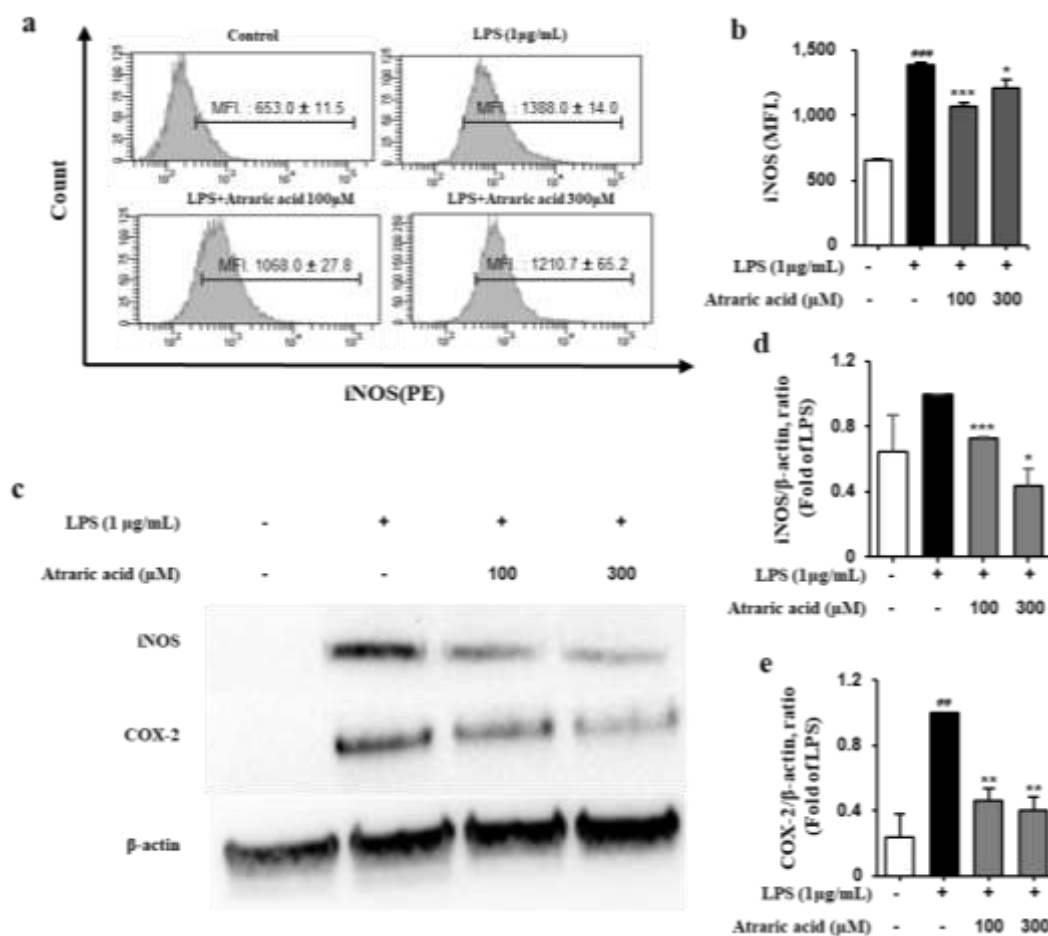
Figure 5. Effect of atraric acid on iNOS and COX-2 expression in LPS-stimulated RAW2647 cells. The cells were pre-treated with 1 µg/mL LPS for 1 h and subsequently treated with 100, 300 µM atraric acid for 18 h. (a) The expression of iNOS was analyzed by flow cytometry using specific antibodies and iso type controls. (c) Western blot analysis was performed to investigate the effect of atraric acid on the expression of pro-inflammatory proteins. The ratios of iNOS (d) and COX-2 (e) to β-actin after atraric acid treatment were determined, respectively. Data are presented as mean ± SD from two independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *versus* the LPS group).

### 2. 5. Atraric acid suppressed LPS-stimulated phosphorylation of the NFκB signaling pathway

NFκB signaling pathway is very important in inflammation. NFκB pathway exists in an inactive state with IκB; however, during an inflammatory reaction, IκB is phosphorylated and degrades due to signal transduction, thereby phosphorylating NFκB. In addition to this, MAPK phosphorylation regulates the activation of NFκB and subsequent expression of inflammatory mediators and pro-inflammatory cytokines [10,22]. To confirm the effect of atraric acid on the signal pathway, LPS-stimulated RAW264.7 cells were treated with atraric acid to analyze the protein expression of the inflammatory mediators via western blot. As shown in Figure 6, we confirmed that the expression of p-IκB and p-ERK in LPS-stimulated RAW264.7 cells was reduced by atraric acid treatment, and the degraded IκB was also upregulated. Thus, the phosphorylation of NFκB was downregulated with atraric acid treatment (Figure 6e). Atraric acid suppressed the phosphorylation of ERK, IκB and NFκB and inhibited the production of inflammatory factors, confirming the potential value of atraric acid as an anti-inflammatory agent.
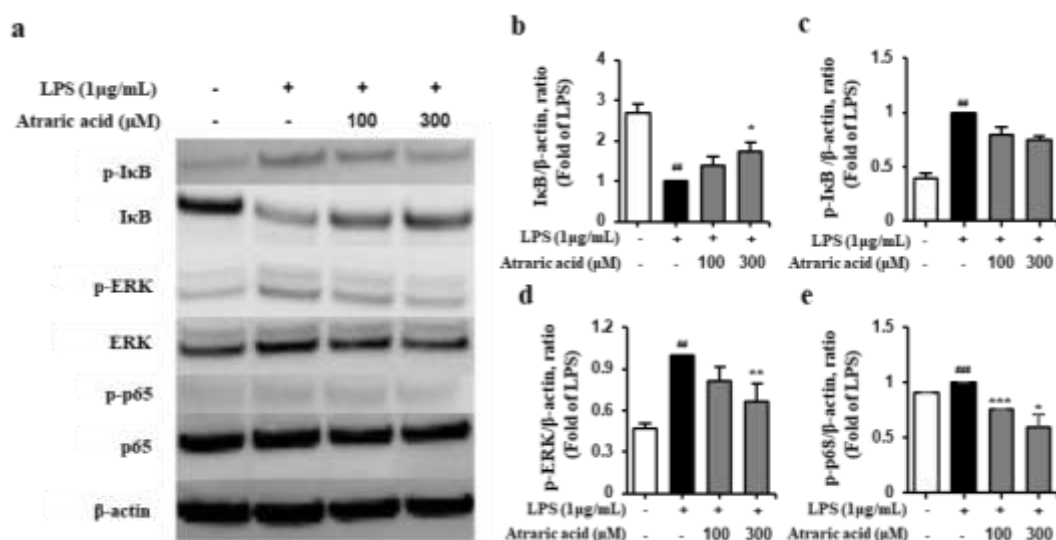
Figure 6. Effects of atraric acid on the activation of NFκB signaling. RAW264.7 cells was pre-incubated with 1 μg/mL LPS for 30 min and then treated with 100, 300 μM atraric acid for 4 h. (a) The inhibitory effect of atraric acid against the phosphorylation of IκB, ERK, and NκB was detected by western blot. The ratios of degradation of IκB (d), p-IκB (c), p-ERK (d) and p-NFκB (e) to β-actin after atraric acid treatment were determined, respectively. Data are presented as mean ± SD from two independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *versus* the LPS group).

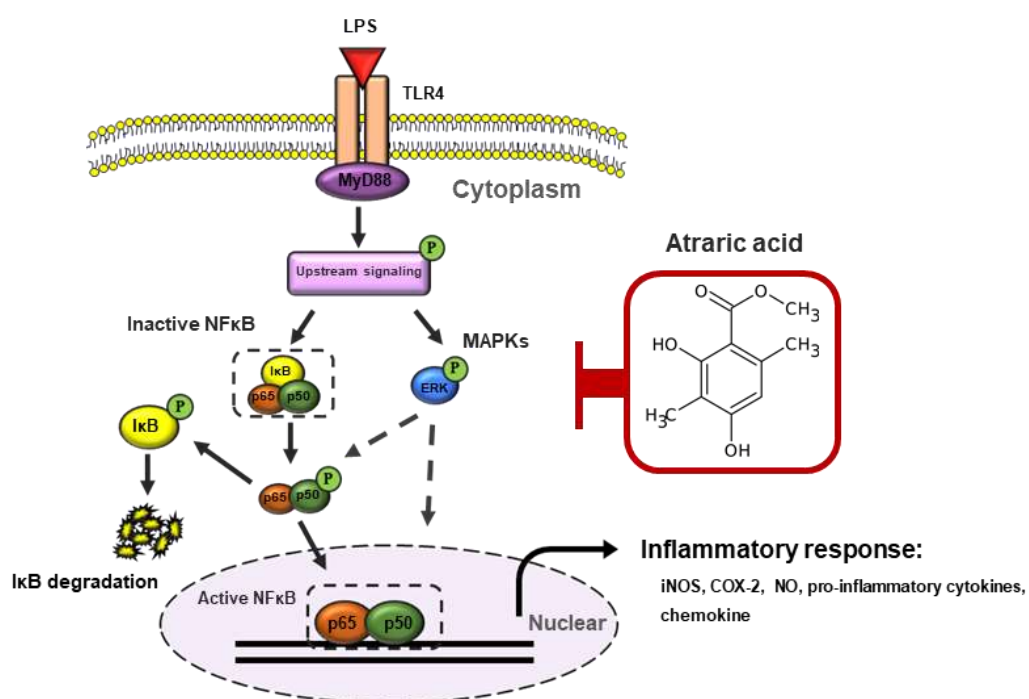## 2. 6. Atraric acid exhibited anti-inflammatory effects on LPS-induced endotoxin shock in mice

Our results demonstrated the in vitro anti-inflammatory effect of atraric acid in LPS-stimulated RAW264.7 cells. In order to confirm if the same results would be observed in vivo, the inhibitory effect of atraric acid on the release of inflammatory cytokines from the serum and peritoneal lavage fluid was confirmed in LPS-induced endotoxin shock in mice. As shown in Figure 7, the LPS-stimulated group significantly induced high levels of cytokine production in mice, and atraric acid was able to inhibit the production of cytokines (Figure 7b-e). Endotoxin shock dose not only increase the level of cytokines in the blood, but also causes function and damage to each tissue, causing secondary disease [23,24]. Therefore, we performed hematoxylin and eosin (H&E) staining to analyze the pathological characteristics of each organ. The kidney, liver, and lung of the LPS-induced group showed bleeding and expansion of capillaries. However, atraric acid treatment showed a tendency to reduce vasodilation and bleeding, which improved the pathological properties of the organs.
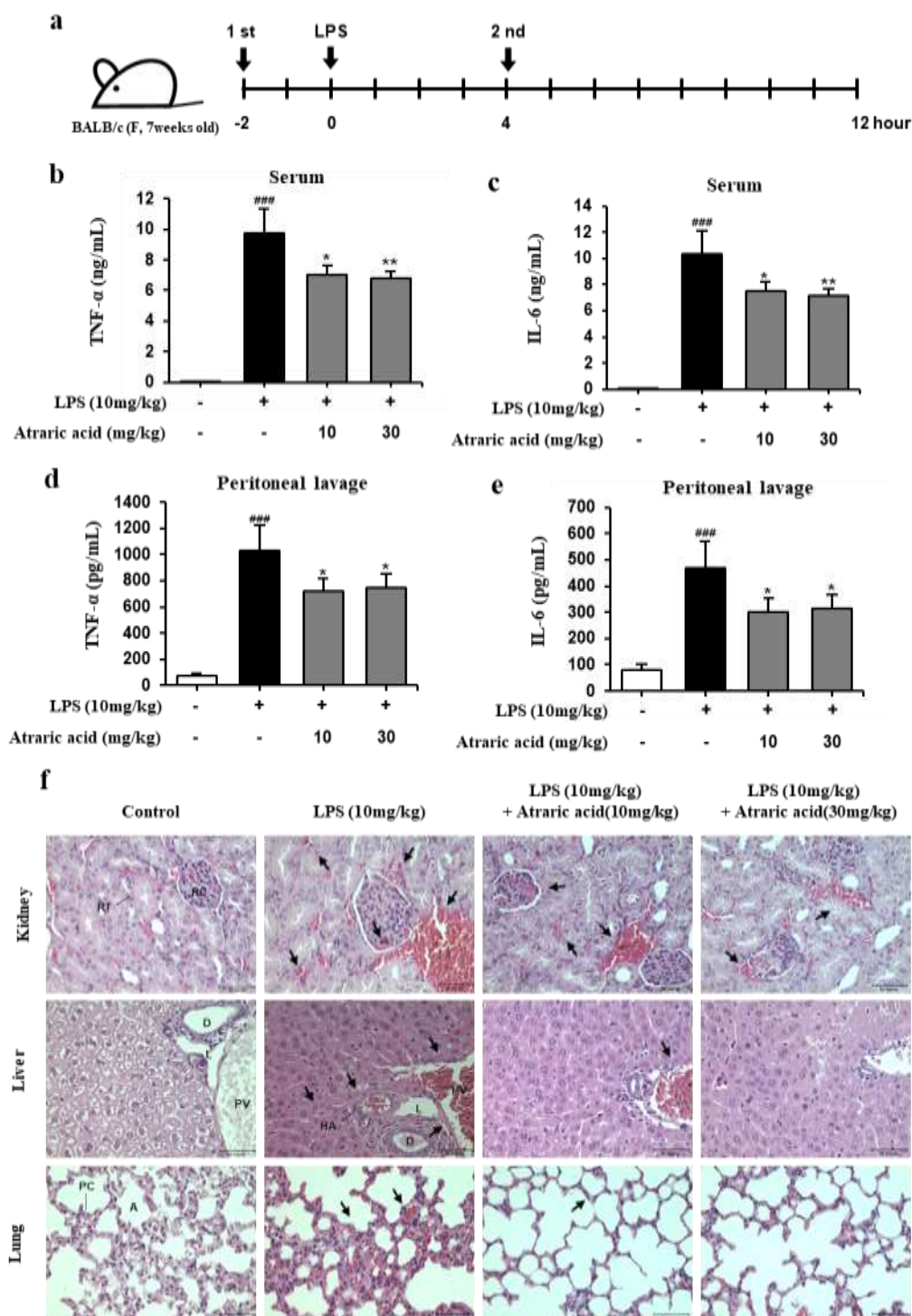
Figure 7. Effects of atraric acid on LPS-induced endotoxin shock in mice. (a) BALB/c mice (n = 5) were intraperitoneally administered (*i.p.*) LPS (10 mg/kg, *E.coli* 011:B4) and subsequently treated with atraric acid (10 mg/kg, 30 mg/kg) or control (PBS) for 4 h. The levels of pro-inflammatory cytokines released by the serum and peritoneal lavage were detected by ELISA assay (b-e). (f) Tissue samples were stained with H&E to exhibited histopathological changes (Abbreviations: RT, renal tubule; RC, renal corpuscle; D, bile duct; L, lymphatic vessels; PV, portal vein; HA, hepatic artery; PC, pulmonary capillaries; A, alveoli). The arrows showed inflammatory infiltration. Data are presented as mean ± SEM from five independent experiments (*$p <$ 0.05, **$p <$ 0.01, ***$p <$ 0.001 *versus* the LPS group).

3. Discussion

There has been an increasing interest in the identification of natural products from plant materials and animals, as these products have been found to be harmless to humans and free of toxic and side effects [10]. Several studies have reported that natural products, a rich source of bioactive compounds, can be developed as beneficial dietary supplements and therapeutic agents [25]. The purpose of this study was determining its value as an anti-inflammatory agent by isolating secondary metabolites of the natural substance tributary and identifying new anti-inflammatory effect. LPS-stimulated macrophages have been used in vitro as established experimental models to evaluate the anti-inflammatory activity of various synthetic or naturally derived agents. LPS is a polysaccharide present in the membrane of Gram-negative bacteria, and Toll Like recptor-4 (TLR4) and MD-2 of macrophages form heterodimers that recognize common patterns in LPS molecules [26]. In LPS-stimulated macrophages, MAPKs/NFκB signaling is activated through the TLR4 pathway, leading to the overexpression of inflammatory factors [22]. The overexpression of iNOS produces high leves of NO, to activate various immunopathological pathways, and COX-2, which is an important enzyme that regulates the production of pro-inflammatory cytokines including TNF-α, IL-6, and IL-1 β [6,21]. Form the results of our in vitro experiments, we confirmed that the excessive production of NO, TNF-α, IL-6, IL-1β, and GM-CSF by LPS-stimulated macrophages was inhibited by the methanol extracts of HH (Figure 1). Although the data are not shown, we separated and purified atraric acid, using HPLC, from the methanol extract of HH (Figure 2). The isolated atraric acid inhibited the production of NO, IL-6, IL-1β and GM-CSF in LPS-stimulated RAW264.7 macrophages (Figure 4). It also reduced the protein expression of iNOS and COX-2, which are related to the activity of inflammatory factors. These data confirm that atraric acid is potential therapeutic agent against inflammatory disease. In order to confirm that the anti-inflammatory effect of atraric acid on the LPS-induced inflammatory response *in vitro* can be demonstrated *in vivo*, we determined the effect of atraric acid by endotoxin model. As shown in Figure 7a, endotoxin shock was induced by treating LPS (10mg/kg *i.p.* injection) in mice. Each concentration of atraric aicd was inoculated 2 h before and 4 h after LPS inoculation. From the results, excessive cytokines in the blood and peritoneum were released in the LPS-stimulated groups due to the systemic reactions, and there were signs of necrosis due to vasodilation and bleeding of the organs. However, in the atraric acid treatment group, the increased cytokines was controlled and histological findings showed reduced expansion of capillaries, which confirmed that atraric acid exhibited anti-inflammatory effects *in vivo*.

Figure 8. Schematic representation of the anti-inflammatory effects of atraric acid in RAW264.7 cells. Abbreviations: LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation factor 88;

MAPKs, mitogen-activated protein kinase; ERK, extracellular regulated protein kinase; NFκB, nuclear factor-κB; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; NO, nitric oxide.

4. Materials and Methods

4. 1. Plant material

Heterodermia hypoleuca (Kol.170037) was collected from the coastal rocks of southern part of Korea in 2017 and deposited at the Korean lichen and Allied Bioresource Center in the Korean Lichen Research Institute, Sunchon National University, Korea. The air-dried Lichen thalli (60 g) were extracted with 2 L methanol (MeOH) at room temperature for 48 h using sonication. The extract was then filtered and concentrated under vacuum at 40 °C using a rotary evaporator.

4. 2. Chemicals and reagents

Roswell park memorial institute-1640 medium (RPMI 1640) and fetal bovine serum (FBS) were purchased from Hyclone Laboratories (Hyclone, South Logan, UT). Cell counting kit–8 (CCK-8) was purchased from Dojindo Laboratories (Dojindo, kumamoto, Japan). DMSO, bovine serum albumin (BSA) and Lipopolysaccharides (LPS) were purchased from Sigma Aldrich (St. Louis, MO, USA). Purified rat anti-mouse (TNF-α, IL-6, IL-10, IL-1β, GM-CSF) and biotin rat anti-mouse (TNF-α, IL-6, IL-10, IL-1β and GM-CSF) were purchased from BD Biosciences (San Diego, CA, USA). HPLC grade acetonitrile, water and methanol were purchased from J.T Baker (USA), while ODS-A (40–60 mesh), Luna 5u (C_{18} 100 A 250 × 110 nm), and YMC-actus (Triat C_{18} 100 A 250 × 20 mm) were from -Merck Co. (Germany), Phenomenex Inc (USA), and YMC(Japan), respectively. The purity of atraric acic (over 90 %) that was isolated from HH in our laboratory was determined by high-performance liquid chromatography-evaporative light scattering detector.

4. 3. Fractionation and isolation

HH extract was dissolved in ethyl acetate (EtOAc) and was partitioned with water (30 % MeOH). EtOAc fraction dissolved in MeOH (50 % DMSO) was separated by preparative liquid chromatography (Prep-LC, YMC-actus Triart C_{18} 250 × 20 nm column, YMC, Japan), 15 mL/min, UV detection at 254, 365 nm using gradient elution 5% solvent B → 100 % solvent B as eluent to afford 63 fractions (1–63) (solvent A: Water + formic acid 0.5%, solvent B: Acetonitrile + formic acid 0.5 %). Atraric acid (39.5 g, Rt = 36.9 min) was obtained by reversed-phase HPLC (Luna 5u C_{18} 100A 250 × 20 nm column, Phenomenex Inc.), 1.0 mL/min, UV detections at 254, 365, 400 and 525 nm using isocratic elution, solvent B (Acetonitrile + formic acid 0.5 %): 0 ~ 60 min; as eluent 50 %.

4. 4. Instruments and data collection

All NMR spectra were recorded on a JNM-ECZS series FT 400 NMR (JEOL, Japan) spectrometer using methanol-d4 as the solvent obtained from Cambridge Isotope Laboratories (CIL), Inc. Chemical shifts were reported with reference to the respective solvent peaks. Mass spectra were recorded on an Agilent Technologies 1260 series HPLC and Electrospray ionization source (ESI) low resolution.

4. 5. Cell culture

RAW264.7 cells (murine macrophage cell line, KCLB 40071) were purchased from Korean Cell Line Bank (Seoul, South Korea). The cells were grown in RPMI 1640 supplemented with 10 % FBS, 100 units/mL of penicillin, 100 μg/mL of streptomycin (Invitrogen, Carlsbad, CA, USA), and 2-mercaptoethanol (50 μM) in a humidified atmosphere at 37 °C with 5 % CO_2

4. 6. Cell viability assay

Cell viability was determined by CCK-8 assay. RAW 264.7 cells (5 × 104 cell/well) were seeded in a 96well plate and incubated over-night before experimental interventions. Next the cells were treated with different concentrations of samples for 24 h. Thereafter, 10 μL of CCK-8 was added to each plated and incubated for 2 h at 37 °C with 5 % CO2. The optical density was then read at 450 nm using a microplate reader (Versa Max, molecular devices, Sunnyvale, CA). The cell viability was

evaluated by comparing the absorbance values of the sample groups with that of the control group, which was considered as 100 %.

4. 7. Measurement of NO and cytokines

The concentrations of NO, TNF-α, IL-6 IL-1β and GM-CSF were measured by Griess assay and ELISA assay. RAW 264.7 cells (5×10^5 cell/mL) were seeded in a 24well plate and incubated over-night before experimental interventions. The cells were treated with various concentrations of sample extracts in the presence of LPS (1 μg/mL) for 24 h at 37 °C with 5 % CO_2. Subsequently, the culture supernatant was assayed according to the manufacturer's instructions [9,27].

4. 8. Western blot analysis

The cell was collected and washed twice with cold phosphate-buffer saline (PBS). Total protein was extracted by using radioimmunoprecipitation assay buffer (Thermo, Rockford, IL, USA) in the presence of protease and phosphatase inhibitor cocktail (Thermo, Rockford, IL, USA). Protein concentration was determined using the BCA protein assay kit (Thermo, Rockford, IL, USA). Equal amounts of protein were separated by 4-12 % bis-tris plus gels (Thermo, Rockford, IL, USA) and transferred to nitrocellulose membranes (Thermo, Rockford, IL, USA). The membranes were incubated with blocking solution for 1 h at room temperature followed by overnight incubation at 4 °C with primary antibodies. The primary antibodies included specific antibody β-actin (1:1000, Thermo, Rockford, IL, USA), iNOS (1:500, Thermo, Rockford, IL, USA), COX-2 (1:1000, Thermo, Rockford, IL, USA), IκB (1:1000, Thermo, Rockford, IL, USA), p-IκB (1:1000, Thermo, Rockford, IL, USA), NFκB (1:1000, Thermo, Rockford, IL, USA) and p-NFκB (1:1000, Thermo, Rockford, IL, USA). The membranes were washed with TBST and incubated in horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000, Thermo, Rockford, IL, USA) for 1 h at room temperature while shaking. Next, the membranes were washed with TBST and developed with the enhanced chemilumin escence kit (Thermo, Rockford, IL, USA). The protein bands were captured and measured using a bio-imaging system (Microchemi 4.2 Chemilumineszenz-system, Neve Yamin, Israel) [28].

4. 9. Animals and experimental design

All the mice were treated in strict accordance with the guidelines issued for the care and use of laboratory animals by the Sunchon National University Institutional Animal Care and Use Committee (SCNU IACUC). All procedures were approved by the SCNU IACUC (Permit Number: SCNU IACUC-2019-15). Female BALB/c mice (7 weeks-old), weighing 17–20 g each, were purchased from orient-bio (Orientbio Inc., Seongnam, Korea). The animals were housed in a controlled environment [22 ± 2 and 50 ± 5 % (relative humidity)] in polycarbonate cages and fed a standard animal diet with water.

For the in vivo experiments, the mice were randomly divided into control group, LPS (10 mg/kg, *i.p.*) group, atraric acid treatment group (10 mg/kg, 30 mg/kg, *i.p.*) (n = 5). Before LPS injection, the mice were pretreated with atraric acid for 2 h, and 4 h after LPS injection, the mice were administered atraric acid. After 8 h, blood samples were collected and serum samples were prepared for the determination of cytokines, and the organs (Kidney, Liver, Lung, and Spleen) were extracted for tissue staining [26,29].

4. 10. Histopathological examination

Tissues were fixed in 4 % formalin and embedded in paraffin. Sections of 4 μm thickness were obtained and stained with H&E and histological changes were monitored under the microscope at × 400 magnification [10,30].

4. 11. Statistical analysis

Data are presented as mean ± standard deviation (SD) or standard error of the mean (SEM). The statistical differences between groups was analyzed by one-way SPSS version 22 (SPSS, Chicago, IL, USA) followed by Student's t test. A p value of 0.05 or less indicated statistical significance.

Abbreviations

LPS	Lipopolysaccharide
HH	*Heterodermia hypoleuca*
NFκB	Nuclear factor kappa B
NO	Nitric oxide
COX-2	Cyclooxygenase-2
TNF-α	Tumor necrosis factor-α
IL-1β	Interleukin-1β
IL-6	Interleukin-6
GM-CSF	Granulocyte-macrophage colony-stimulating factor
TLR-4	Toll-like receptor 4
CCK-8	Cell counting kit-8
i.p	Intraperitoneal

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