Research Article

Ethyl Acetate Extract of Artemisia anomala S. Moore Displays Potent Anti-Inflammatory Effect

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Artemisia anomala S. Moore has been widely used in China to treat inflammatory diseases for hundreds of years. However, mechanisms associated with its anti-inflammatory effect are not clear. In this study, we prepared ethyl acetate, petroleum ether, n-BuOH, and aqueous extracts from ethanol extract of Artemisia anomala S. Moore. Comparing anti-inflammatory effects of these extracts, we found that ethyl acetate extract of this herb (EAFA) exhibited the strongest inhibitory effect on nitric oxide (NO) production in LPS/IFN-γ-stimulated RAW264.7 cells. EAFA suppressed the production of NO in a time- and dose-dependent manner without eliciting cytotoxicity to RAW264.7 cells. To understand the molecular mechanism underlying EAFA’s anti-inflammatory effect, we showed that EAFA increased total cellular anti-oxidant capacity while reducing the amount of inducible nitric oxide synthase (iNOS) in stimulated RAW264.7 cells. EAFA also suppressed the expression of IL-1β and IL-6, whereas it elevates the level of heme oxygenase-1. These EAFA-induced events were apparently associated with NF-κB and MAPK signaling pathways because the DNA binding activity of p50/p65 was impaired and the activities of both ERK and JNK were decreased in EFA-treated cells comparing to untreated cells. Our findings suggest that EAFA exerts its anti-inflammatory effect by inhibiting the expression of iNOS.

1. Introduction

Artemisia anomala S. Moore (Nan-Liu-Ji-Nu) is a perennial herbaceous plant categorized to Artemisia genus Compositae family. Many species of Artemisia have been used as medicinal materials. In fact, Artemisia anomala S. Moore has been used for centuries to treat fever, empyrosis, inflammation, and dissipated liver function caused by hepatitis in China. For example, Artemisia oil can potently inhibit the growth of bacteria, yeasts, dermatophytes, and Aspergillus niger and has thus been extensively used as an anti-inflammatory agent [1]. The most well-known medicine from Artemisia genus is probably artemisinin and its derivatives that have the rapidest action against malaria among all antimalaria drugs. The regimen containing at least one artemisinin derivative (artemisinin-combination therapies) is the standard protocol to treat P. falciparum malaria worldwide [2]. The therapeutic effect of Artemisia anomala S. Moore is likely to be linked to its ability to counteract against inflammation, oxidation [3], and viral infection [4]. Recent studies show that dimeric guaianolides and sesquiterpenoids extracted from the aerial part of Artemisia anomala can suppress cyclooxygenase 2- (COX2-) associated effects [5]. Commonly used prostaglandin-like fatty acid derivatives anomalone A-D were actually isolated from Artemisia anomala [6]. Although sufficient evidences have demonstrated Artemisia anomala S. Moore as an effective anti-inflammatory agent, systematically evaluating its anti-inflammatory effects with inflammatory parameters has yet been performed.

Acute inflammatory response represents an initial protective mechanism in the body. In contrast, excessive and chronic inflammation results in severe damages of cells and tissues. Emerging evidences support the hypothesis that chronic inflammation plays a critical role in various pathological conditions, including hypertension, atherosclerosis, stroke, metabolic diseases, cancer, autoimmune disorders, and neurodegenerative diseases [7–10]. Nitric oxide (NO) is a free radical that is synthesized from L-arginine by nitric oxide synthase (NOS). There are three types of NOS: two constitutive NOS, eNOS and nNOS, and one
inducible NOS (iNOS). Constitutive NOSs generate nanomolar concentration of NO and are known to mediate various physiological functions. Contrarily, iNOS produces NO at the level of micromolar that often results in pathological consequences such as chronic inflammation. Inflammatory stimuli can induce iNOS expression through distinct signaling pathways. Proinflammatory cytokines released from inflammation-stimulated cells, for example, macrophages, can further upregulate iNOS expression and augment inflammatory responses [11, 12]. The expression of proinflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), is often regulated through the NF-κB and MAPK signaling pathways [13, 14]. Endogenous anti-inflammatory response is also involved in inducible heme oxygenase-1 (HO-1). Because of the ability of HO-1 to attenuate iNOS expression [15, 16], HO-1 is thought to play a protective role during inflammation [17, 18].

The objective of this study is to determine the most effective fraction of Artemisia anomala S. Moore that can inhibit iNOS-induced NO production. To identify such fraction, we prepared ethyl acetate, petroleum ether, n-BuOH, and aqueous extracts from ethanol extract of Artemisia anomala S. Moore. With the aid of the well-established murine macrophage RAW264.7 cell inflammation model, we found that ethyl acetate extraction of Artemisia anomala S. Moore (EAFA) exhibited the strongest inhibitory effect on LPS/IFNγ-induced NO production and proinflammatory cytokine expression. Since NF-κB and MAPK activities were significantly reduced in EAFA-treated cells, we suggest that EAFA exerts its inhibitory action by interfering with both NF-κB and MAPK signaling pathways.

2. Materials and Methods

2.1. Reagents. Murine recombinant IFNγ, NF-κB p50/p65 EZ-TFA transcription factor assay system, and mouse IL-6 ELISA kit were purchased from Millipore (MA, USA); lipopolysaccharide (LPS, Escherichia coli O111:B4), dimethyl sulfoxide, N-(1-naphthyl)-ethylenediamine dihydrochloride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT), L-N6-(1-iminoethyl)lysine hydrochloride (L-NIL), and Trolox were obtained from Sigma (St. Louis, MO). TRIzol Reagent was obtained from Invitrogen (Carlsbad, CA). Mouse IL-1βELISA was obtained from Abcam (Cambridge, MA). Oligo-ΔT was obtained from Shanghai Invitrogen (Shanghai, China). Nuclear Extraction Kit was obtained from Biyuntian (Shanghai, China). Antibodies used in this study include murine iNOS monoclonal antibody from BD Transduction Laboratories (Lexington, KY); murine β-actin and HO-1 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); phosphor-p38, p38, phosphor-JNK, JNK, and phosphor-ERK1/2 antibodies from Cell Signaling Technology (Danvers, MA).

2.2. Herb Extraction and Fractionation. Artemisia anomala S. Moore was purchased from Yang-He Tang Co. (Zhangjiang High-Tech Park, Shanghai, China) and confirmed by Shanghai Institute for Food and Drug Control (SIFDC). The dried plants were first extracted with 70% ethanol at 80°C for three times (200 g raw material/1 L/60 min each time) and the obtained ethanol extract was then suspended in water followed by the constitutive partition with petroleum ether, ethyl acetate, n-butanol, and water. After evaporation of these partitioned solutions, five extract fractions were generated: ethanol (yield 6.73%), petroleum ether (0.17%), ethyl acetate (0.25%), n-butanol (0.33%), and aqueous fractions (1.64%). Each fraction was dissolved in DMSO and stored at −20°C until use.

2.3. Cell Culture. RAW264.7 cells were originally obtained from the American Tissue Culture Collection. Cells were maintained in RPMI 1640 medium supplemented with 10% FBS at 37°C in a humidified 5% CO₂ atmosphere.

2.4. Measurement of NO Production. RAW264.7 cells were plated in a 96-well plate (5 × 10⁵ cells/well) for overnight and then serum-starved for 10 h followed by the addition of 10 U/mL IFNγ and 100 ng/mL LPS for 24 h in the presence or absence of different Artemisia anomala S. Moore fractions with finally concentration at 10, 100, 200 μg/mL and used L-NIL (50 μM) as positive drug control for primary screening. After obtaining the strongest fraction, the posttreat, pretreat, and simultaneous-treat of this fraction and stimulation would undergo process for secondary screening. To analyze NO production, 100 μL of supernatant was incubated with equal volume of Griess solution at room temperature for 10 min and absorbance was then read at 540 nm. Since NO content was reflected by the amount of nitrite, a calibration curve was generated using sodium nitrite. The amount of nitrite in the supernatants was calculated based on the calibration curve. The percentage inhibition of NO production is evaluated using the formula \( \frac{1 - \text{[nitrite amount of vehicle]}}{\text{[nitrite amount of fraction treated]}} \times 100 \).

2.5. Assay for Cell Viability. Cell viability was assessed by MTT assay. Briefly, after using the 100 μL supernatants to do Griess reaction, the rest cells were incubated with 10 μL MTT (5 mg/mL in phosphate-buffered saline, pH = 7.4) for 4 h at 37°C followed by adding 50 μL 0.01 mol/L HCl buffer containing 10% SDS and 10% Isopropanol. Absorbance was measured at 540 and 630 nm in a microplate reader. The absorbance of control (untreated) cells was considered as 100% of viability.

2.6. Measurement of Total Antioxidant Capacity. Total antioxidant activity was measured by modified FRAP assay as previously described [19]. Briefly, RAW264.7 cells (2 × 10⁶ cells/30 mm dish) were pretreated with varying concentration of EAFA (50, 100, 200 μg/mL) for 1 h followed by co-stimulation of 100 ng/mL LPS and 10 U/mL IFN-γ for 6 h. Cells were harvested, whereas the supernatants were collected. FRAP reagent was prepared by mixing 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mmol/L HCl solution and 20 mmol/L FeCl₃.
in a 10:1:1 ratio and 245 μL of freshly prepared FRAP solution was added to each well of a 96-well plate that contained 5 μL of supernatant. After 10 min incubation at room temperature, absorbance was measured at 593 nm with the aid of a microplate reader. A standard curve was prepared with various concentrations of Trolox (0.03125 to 2 mmol/L). The potency of total antioxidant capacity for each sample was determined by comparing the antioxidant capacity of 1 mM Trolox.

2.7. Detection of IL-1β and IL-6 in Supernatant. Inhibitory effects of EAFA on the cytokine IL-6 and IL-1β production from LPS plus IFN-γ treated RAW264.7 cells were detected by sandwich ELISA. The procedure was carried out under the instructions from respective kit. After preincubation of 1 h with different dosage of EAFA and stimulation with LPS plus IFN-γ on RAW264.7 cells for 24 h, supernatants were harvested and assayed for IL-1β and IL-6. Results of three independent experiments were used for statistical analysis.

2.8. RNA Isolation and Quantitative RT-PCR. Total RNA was isolated using TRIzol Reagent according to manufacturer's instruction. Quantitative RT-PCR (qRT-PCR) was performed with Takara SYBR kit using the primers sets in Table 1 as previously described [20]. The 2^ΔΔCT method was utilized to analyze the fold increase.

2.9. Nuclear Extract Preparation and NF-κB DNA Binding Assay. Nuclear extracts were prepared using Nuclear Extraction Kit according to the manufacturer’s instruction. DNA binding activity of NF-κB in nuclear extracts was assessed using NF-κB p50/p65 EZ-TFA transcription factor assay kit which detects the amount of NF-κB in the nucleus.

2.10. Statistical Analysis. Student’s test was used to analyze the difference between treated and untreated groups. Comparisons between multiple groups were performed with one-way ANOVA test. P < 0.05 was considered statistically significant.

3. Results

3.1. EAFA Displays the Strongest Inhibitory Effect on LPS/IFNγ-Induced NO Production. The ethanol extract of Artemisia anomala S. Moore has been shown to exhibit inhibition of NO production in our previous screening [21]. We extracted ethanol extract of Artemisia anomala S. Moore further with petroleum ether, ethyl acetate, n-butanol, and water, and each of these obtained extracts was tested for its ability to inhibit NO production in RAW264.7 cells costimulated with LPS and IFNγ. Griess reaction assay showed that IC_{50} of original ethanol extract (EAA) was 31.07 μg/mL (Figure 1). IC_{50} of petroleum ether fraction (PEFA), n-butanol fraction (BFA), and aqueous fraction (AFA) from the EEA was 21.73, 39.10, and 49.25 μg/mL, respectively (Figure 1), which were similar to that of the original EEA. In contrast, IC_{50} of ethyl acetate fraction (EAFA) was 15.85 μg/mL (Figure 1), representing twice stronger inhibitory effect over the original EEA.

In a parallel experiment, we investigated the effect of EAFA on NO production in unstimulated RAW264.7 cells. Contrary to its ability to dose-dependently inhibit NO production in LPS/IFNγ-stimulated RAW264.7 cells (Figure 2(a)), EAFA displayed little effect on NO production in unstimulated cells (Figure 2(a)). MTT assays further showed that EAFA promoted viability of LPS/IFNγ-stimulated RAW246.7 cells in dose-dependent manner while it exhibited effect on the viability of unstimulated cells (Figure 2(b)). These results suggest that EAFA selectively inhibits NO production. Since EAFA promotes cell viability in LPS/IFNγ-stimulated RAW264.7 cells (Figure 2(b)), these results also indicate that the inhibitory effect of EAFA on NO production in LPS/IFNγ-stimulated cells is not caused by LPS/IFNγ-induced cellular toxicity.
Figure 2: Effect of EAFA on NO production (a) and cell viability (b) in LPS/IFN-γ-stimulated and unstimulated RAW264.7 cells. RAW264.7 cells were treated with EAFA (50–200 μg/mL) for 24 h with or without LPS/IFN-γ stimulation. Nitrite concentrations in the culture medium were determined by the Griess reaction. Changes in survival are represented as percentages of the control group. Bars represent the mean ± SEM. Three independent experiments were performed. **P < 0.01; *P < 0.05 versus control group; ***P < 0.01; *P < 0.05 versus model group.

Figure 3: Effect of pretreatment and posttreatment of EAFA on NO production and cell viability. Cells were plated at a density of 1 × 10^5 cells/well in a 96-well plate and allowed to attach for 2 h. EAFA was added prior to (pretreatment-12 h, pretreatment-6 h), simultaneously with (0 h) (a, b) or after the treatment of the cells with IFN-γ (10 U/mL) plus LPS (100 ng/mL) (posttreatment-6 h, posttreatment-12 h) (c, d). Nitric concentrations in the culture medium and cell viability were determined by the Griess reaction and MTT assay. The values (means ± SEM) were obtained from three independent experiments. **P < 0.01; *P < 0.05 versus control group; ***P < 0.01; *P < 0.05 versus model group.
3.2. Both Pre- and Posttreatments of EAFA Inhibit LPS/IFN-γ-Induced NO Production and Cellular Toxicity in RAW264.7 Cells. To further characterize the pharmacological action by EAFA, we pretreated RAW264.7 cells with EAFA for 6 and 12 hrs followed by LPS/IFN-γ stimulation. Griess reaction assays showed that EAFA pretreatment resulted in significantly better inhibitory effect on NO production in LPS/IFN-γ-stimulated RAW264.7 cells than adding EAFA at the time of LPS/IFN-γ stimulation (Figure 3(a)). Similarly, EAFA pretreatment promoted cell viability in a greater degree than adding EAFA simultaneously with the stimulants (Figure 3(b)). In subsequent study, RAW264.7 cells were first stimulated with LPS/IFN-γ for 6 or 12 h and then treated with EAFA. Although less inhibitory effect on NO production and cell viability was detected with EAFA posttreatment compared with EAFA pretreatment, we still observed 16.22% of reduction in NO production and 98.58% of increase in cell viability in RAW264.7 cells treated with EAFA at dosage of 200 μg/mL (Figures 3(c) and 3(d)). Taken together, these results suggest than EAFA can potentially be used both as a preventive and therapeutic agent against chronic inflammation.

3.3. EAFA Pretreatment Prevents LPS/IFN-γ-Suppressed Antioxidant Capacity and Inhibits iNOS Expression. NO at high concentration is often considered as oxidant stress [22]. Since EAFA can effectively reduce LPS/IFN-γ-induced NO production, we hypothesized that EAFA might also possess potent antioxidant activity. To test this hypothesis, RAW264.7 cells were pretreated with varying concentrations of EAFA for 1 h followed by LPS (100 ng/mL) and IFN-γ (10 U/mL) treatment for 4 h. Total RNA was isolated and subjected to qRT-PCR. β-actin mRNA was used as an internal control for standardization. (c) RAW264.7 cells were plated at a density of 1 × 10⁶ cells in 30 mm dish for overnight. EAFA was added 1 h prior to the treatment of IFN-γ (10 U/mL) plus LPS (100 ng/mL) for 6 h. Whole cell lysates were prepared and subjected to western blotting. The data shown are representative of three independent experiments. **P < 0.01, *P < 0.05 versus control group; **P < 0.01, *P < 0.05 versus model group. (b) RAW264.7 cells (1 × 10⁶ cells/dish) were pretreated with varying concentrations of EAFA for 1 h followed by LPS (100 ng/mL) and IFN-γ (10 U/mL) treatment for 4 h. Total RNA was isolated and subjected to qRT-PCR. β-actin mRNA was used as an internal control for standardization. (c) RAW264.7 cells were plated at a density of 1 × 10⁶ cells in 30 mm dish for overnight. EAFA was added 1 h prior to the treatment of IFN-γ (10 U/mL) plus LPS (100 ng/mL) for 6 h. Whole cell lysates were prepared and subjected to western blotting. The data shown are representative of three independent experiments. **P < 0.01, *P < 0.05 versus control group; **P < 0.01, *P < 0.05 versus model group.
3.4. EAFA Blocks Inflammatory Cytokines Production and Increases HO-1 Expression in RAW246.7 Cells. LPS/IFNγ costimulation has been reported to induce the expression of a plethora of proinflammatory cytokines in macrophages [24]; we thus investigated the effect of EAFA on IL-1β and IL-6 expressions in LPS/IFNγ-stimulated RAW246.7 cells. qRT-PCR showed that LPS/IFNγ stimulation led to over 1.5-fold increase in IL-1β and 104-fold increase in IL-6 expression. Pretreatment of EAFA dose-dependently abrogated LPS/IFNγ-induced IL-1β and IL-6 expression.
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Figure 6: Effect of EAFA on NF-κB and MAPK activities in LPS/IFNγ-stimulated RAW264.7 cells. (a, b) DNA binding activity of p50 and p65 proteins in nuclear extracts was assessed using NF-κBp50/p65 EZ-TFA transcription factor assay. Absorbance was measured at 450 nm in a microplate spectrophotometer. Results were normalized to absorbance/mg protein. The data shown are representative of three independent experiments. **P < 0.01, *P < 0.05 versus control group; **P < 0.01, *P < 0.05 versus model group. (c) RAW264.7 cells were plated at a density of $1 \times 10^6$ cells/well in 30 mm dish for overnight. EAFA was added to cells for 1 h followed by 30 min stimulation of IFNγ (10 U/mL) plus LPS (100 ng/mL). Whole cell lysates were prepared and subjected to western blotting to detect phosphor-ERK, phosphor-JNK, and phosphor-p38 with the respective antibodies. Data shown are the representative of three independent experiments. **P < 0.01, *P < 0.05 versus control group; **P < 0.01, *P < 0.05 versus model group.

3.5. NF-κB and MAPK Signaling Pathways Are the Target of EAFA-Mediated Inhibition. NF-κB activity is known to be critical for the expression of iNOS [25–27]. To investigate how EAFA affected LPS/IFNγ-induced NF-κB activity, we analyzed the extent of p50 and p65 binding to NF-κB consensus sequence-containing oligonucleotides in nuclear extracts. LPS/IFNγ stimulation resulted in more than 5-fold increase in the amount of p50 and p65 bound to the NF-κB consensus sequence-containing oligonucleotides compared with unstimulated RAW246.7 cells (Figures 6(a) and 6(b)). However, EAFA inhibited LPS/IFNγ-induced NF-κB activity and, at 200 μg/mL, completely abolished this activation (Figures 6(a) and 6(b)). In addition to NF-κB, members of MAPK families have also been implicated to play an essential role in the inflammatory reaction. To determine the effect of EAFA on LPS/IFNγ-induced MAPK activation, western blots were performed to analyze the levels of phosphor-Erk, JNK, and p38 in RAW246.7 cells. LPS/IFNγ stimulation (30 min) evoked significant increases in the levels of phosphorylated Erk, JNK, and p38 in RAW246.7 cells. However, pretreatment of EAFA markedly inhibited the extent of Erk and JNK phosphorylation (Figure 6(c)). These results suggest that EAFA blocks inflammatory responses by the combination of blocking NF-κB, Erk, and JNK activation.
4. Discussion

Inhibition of iNOS has been shown to soothe pathological conditions characterized as inflammation. For example, iNOS-knockout mice are resistant to pleurisy and lung injury caused by carrageenan [28]. Selective inhibition of iNOS improves erosive joint disease [29], prevents experimental allergic encephalomyelitis [30], and attenuates immune dysfunction following trauma [8]. In addition, expression of iNOS has also been associated with various tumor types including brain, breast, lung, pancreas, liver, colon, and prostate cancers [9]. Selective NOS-2 inhibitors L-N6-(1-iminoethyl) lysine 5-tetrazole-amide (SC-51) and aminoguanidine (AG) actually show chemopreventive effect against the incidence of azoxymethane- (AOM-) induced colon, and prostate cancers [9]. Selective NOS-2 inhibitors L-N6-(1-iminoethyl) lysine 5-tetrazole-amide (SC-51) and aminoguanidine (AG) actually show chemopreventive effect against the incidence of azoxymethane- (AOM-) induced colon, and prostate cancers [9].

Inhibition of iNOS has been shown to soothe pathologication proinflammatory cytokines in macrophages [13, 14]. EAFA can also diminish the expression and secretion of IL-1β and IL-6 (Figure 5). The expression of a number of immunity and inflammatory related genes such as iNOS, IL-1β, and IL-6 was modulated by activated NF-κB [34]. Under inflammatory conditions, inhibitory protein IκBα is promptly phosphorylated and degraded from p50 and p65 subunits binding site of NF-κB; the activated NF-κB subunits migrate to the nucleus. To investigate the possible preventive capability of EAFA on NF-κB activation, we studied p50/p65 nuclear translation by NF-κB p50/p65 EZ-TFA transcription factor assay kit. LPS/IFNγ stimulate the activation of NF-κB and induce p50/p65 movement to nucleus; EAFA repressed the amount of p50/p65 in the nucleus (Figures 6(a) and 6(b)). So EAFA displayed the interference in progress of NF-κB active heterology dimmer heading to the nucleus.

MAPKs and NF-κB signaling mechanisms have been previously linked to both iNOS and proinflammatory factor expression under inflammatory conditions. Moreover, several studies have shown that MAPKs play a critical role in the activation of NF-κB [35]. Depending on the cell system, p38, ERK, and JNK have proven to have ROS-sensitive kinase activity [36]. According to the antioxidant activity of EAFA, we investigated whether MAPK pathway was involved in attenuating inflammatory mediators express and final NO/RNS reduction. In fact, our study showed that EAFA was able to abolish LPS/IFNγ-induced activation of Erk and JNK in RAW246.7 cells (Figure 6(c)). Together, we reason that the anti-inflammatory effect of EAFA is at least partly by attenuating NF-κB and MAPKs activation.

In conclusion, our study indicates that EAFA can potently suppress inflammatory responses, and it hence warrants further identification of the effective component(s) in EAFA.

Abbreviations

EAFA: Ethyl acetate extraction of Artemisia anomala S. Moore
LPS: Lipopolysaccharide
IFN-γ: Interferon-γ
NO: Nitric oxide
iNOS: Inducible nitric oxide synthase
HO-1: Inducible haemoxygenase
IL-6: Interleukin-6
IL-1β: Interleukin-1β
NF-κB: Nuclear factor-kappa B
Erk: Extracellular signal-regulated kinase
JNK: c-Jun N-terminal kinase
MAPK: Mitogen-activated protein kinase.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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