Anti-inflammatory effect of *Artemisiae annuae* herba in lipopolysaccharide-stimulated RAW 264.7 Cells

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ABSTRACT

Background: *Artemisiae annuae* herba (AAH) has been traditionally used as a drug for the treatment of malaria, heat stroke, bacterial infection, and fever in East-Asia. Although AAH has been used for the treatment of inflammation-related symptoms, the underlying mechanism of antiinflammatory activity of AAH is still unknown. **Objective:** We investigated whether AAH have an inhibitory effect on the production of pro-inflammatory mediators in lipopolysaccharide-stimulated RAW 264.7 macrophage cells. **Materials and Methods:** The investigation was forced on the inhibitory effect of AAH on the production of tumor necrosis factor (TNF)-α, interleukin (IL)-6, nitric oxide (NO), and inducible NO synthase (iNOS) in macrophages. Furthermore, we examined the effect of AAH on the activation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) pathways. **Results:** We found that AAH suppresses NO production and TNF-α, IL-6, and iNOS gene expression. Moreover, AAH inhibited the nuclear translocation of p65 and IκBα degradation in NF-κB pathway and decreased the extracellular signal-regulated kinase, p38, c-Jun NH_2-terminal kinase phosphorylation in MAPK signaling pathway. **Conclusions:** Consequently, these results indicate that AAH contains antiinflammatory activity and this effect is derived from the repression on the activation of NF-κB and MAPKs pathways. We first demonstrated that antiinflammatory effect of AAH and its underlying mechanism in macrophage cells.

Key words: *Artemisiae annua*e herba, inducible nitric oxide synthase, inflammatory cytokine, mitogen-activated protein kinases, nuclear factor-kappa B

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INTRODUCTION

*Artemisiae annua*e herba (AAH) is an aerial part of the *A. annua* or *Artemisia apiacea*, which belongs to the Compositae family. AAH is a traditional herbal drug used for the treatment of nausea, headache, and vomiting due to malaria and heat stroke since ancient times in Korea, China, and Japan. Previous studies have demonstrated that AAH has an ability against uncomplicated falciparum malaria in adult.[1] Other reports have shown that the effect of AAH on intestinal microbiota, clostridium perfringens infection,[2] and *in vitro* trematocidal effects.[3] Furthermore, several reports have demonstrated sedative effects of AAH in mice[4] and against *Rhipicephalus*.[5] In addition, recent studies have shown that the effect of AAH against monogenean parasites[6] and its immunosuppressive effect.[7]

Inflammation is a complex physiological and immune process to against foreign challenge such as pathogens and cell or tissue injury.[8] In immune-stimulation condition, macrophages play a central role and counteract external stimuli including lipopolysaccharide (LPS). LPS derived from the outer membrane of Gram-negative bacteria activates macrophages to overexpress inflammatory mediators.[9] These inflammatory mediators such as nitric oxide (NO), prostaglandin (PG) E_2, tumor necrosis factor (TNF)-α, and interleukin (IL)-6 are essential for host survival and repair of tissue injury.[10] Among them, NO synthesized from inducible NO synthase (iNOS) regulates the expression of pro-inflammatory mediators and TNF-α and IL-6 are known as important mediators involved in the process of various inflammatory diseases.[11]

Nuclear factor kappa B (NF)-κB and mitogen-activated protein kinase (MAPK) are typical inflammatory signaling pathways in macrophages. NF-κB composed of p65/p50 subunit, is a transcription factor that modulates the expression of inflammatory cytokine and their genes.[12] In normal cells, p65 of NF-κB is sequestered by IκBα in the cytoplasm. Upon stimulation by various inflammatory stimuli including LPS, IκB kinase pathway is activated to phosphorylate IκBα. Phosphorylated IκBα is dissociated

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from p65/IκBα complex and free NF-κB translocates into nucleus.[13] The NF-κB translocated into the nucleus binds to the promoter and regulates the expression of these inflammatory mediator genes including iNOS, cyclooxygenase (COX-2), TNF-α and IL-6.[12,14] In this point, many drugs for the treatment of various inflammatory diseases focus to suppress NF-κB activation.[15]

Mitogen-activated protein kinases consist of three kinds of kinases such as extracellular signal-regulated kinase (ERK) 1/2, p38, c-Jun NH_2-terminal kinase (JNK) and play an important role in conveying external inflammatory signal into the cytoplasm and nucleus.[16] The cellular signals came from MAPKs lead to the activation of inflammatory mediators.[17] Further, phosphorylated MAPKs are involved in NF-κB activation and lead to expression of iNOS.[18]

In this study, we assessed the inhibitory effects of AAH on LPS-induced inflammation. Furthermore, we first demonstrated the underlying mechanism of anti-inflammatory effect of AAH in RAW 264.7 macrophage cells.

MATERIALS AND METHODS

Materials and reagents

Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), antibiotics, LPS, bovine serum albumin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT), various primary antibodies, secondary antibodies, enzyme-linked immunosorbent assay (ELISA) kits and RNA extraction kits were purchased from Hyclone (Logan, UT, USA), Sigma (St. Louis, MO, USA), Cell Signaling Technology (Boston, MA, USA), BD Biosciences (San Jose, CA, USA) and iNtRON Biotech (Daejeon, Korea). The oligonucleotide primers for reverse transcription-polymerase chain reaction (RT-PCR) were synthesized by Bioneer Corp. (Daejeon, Korea). The standard chemicals of chlorogenic acid, caffeic acid, vitexin, quercetin, and artemisinin were obtained from Sigma (St. Louis, MO, USA). High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from J. T. Baker (Austin, TX, USA). Trifluroacetic acid (TFA) was purchased from Sigma (St. Louis, MO, USA). The twice distilled water was filtered by a pump (Division of Mixllipore, Waters, Milford, MA, USA) and filter (FH-0.2 μm, Waters, Milford, MA, USA).

Extraction and preparation of *Artemisiae annuae* herba

A*rtemisiae annua*e herba was obtained from Yeongcheon Oriental Herbal Market (Yeongcheon, Korea). All voucher specimens were deposited in the herbal bank of KM-Based Herbal Drug Development Group, Korea Institute of Oriental Medicine. To prepare AAH water extract, the dried aerial parts of AAH were placed in distilled water and then extracted by heating for 3 h at 115°C (Gyeongseo Extractor Cosmos-600, Inchon, Korea). Following extraction, the solution was filtered out using standard testing sieves (150 μm) (Retsch, Haan, Germany), freeze-dried, and maintained in desiccators at 4°C prior to use.

Cell culture and drug treatment

The murine macrophage RAW 264.7 cells were obtained from Korea Cell Line Bank (Seoul, Korea) and grown in complete RPMI 1640 medium. The cells were then incubated in humidified 5% CO_2 atmosphere at 37°C. To stimulate cells, LPS (200 ng/mL) was added in the presence or absence of AAH (10, 100 and 500 μg/mL) for the indicated periods.

3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenylthiazolium bromide assay for cell viability

Cytotoxicity of AAH was analyzed using an MTT assay. AAH was added to each well and incubated for 48 h at 37°C with 5% CO_2. MTT assay was conducted in accordance with the method described in the previous report with some modifications.[19]

Measurement of nitric oxide production

Nitric oxide production was analyzed by measuring the nitrite in the supernatants of cultured macrophage cells. Nitrite was measured by Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2.5% phosphoric acid) reaction according to the method described in the recent study.[19]

Measurement of tumor necrosis factor-α and interleukin-6 cytokines production

The amounts of TNF-α and IL-6 cytokines in the culture medium were determined by ELISA. ELISA plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with anti-mouse TNF-α or IL-6 antibodies diluted in coating buffer (0.1 M carbonate, pH 9.5) and then washed four times with PBS containing 0.05% Tween 20. The nonspecific protein binding sites were blocked with assay diluent (PBS containing 10% FBS, pH 7.0) for at least 1 h. Immediately, each samples and standards were added to the wells. After incubation for overnight at 4°C, a working detector (biotinylated antimouse TNF-α or IL-6 monoclonal antibody and streptavidin-horseradish peroxidase reagent) was added and incubated for 1 h. Subsequently, substrate solution (tetramethylbenzidine) was added to the wells and incubated for 30 min in the dark until the reaction was stopped with stop solution (2 NH_3PO_4). The absorbance at 450 nm was read.

Western blot analysis and preparation of cytosolic, nuclear extracts for nuclear factor-kappa B and IκBα

Protein expression was evaluated via Western blot analysis according to standard procedures. The cells were lysized using PRO-PREP lysis buffer (iNtRON, Sungnam, Korea)

and concentration of protein was determined by Bradford's method (Bio-Rad Laboratories, Hercules, CA, USA). Western blot analysis was performed according to the method described in the previous study.[19] Cytosolic and nuclear fractions were isolated using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo scientific, Waltham, MA, USA) according to the manufacturer's instruction. The fractions were stored in the deep freezer at − 80°C before use.

Reverse transcription-polymerase chain reaction

Total cellular RNA was isolated using easy-BLUE™ RNA extraction kit (iNtRON Biotech, Daejeon, Korea) in accordance with the manufacturer's recommendations. And, total RNA was converted to cDNA using RevoScript™ RT PreMix (iNtRON, Daejeon, Korea). Then, cDNA was amplified by polymerase chain reaction using specific primers [Table 1]. The following PCR conditions were applied: iNOS, TNF-α, IL-6 and β-actin, 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C (iNOS), 55°C (TNF-α), 58°C (IL-6) and 58°C (β-actin) for 30 s, and extension at 72°C for 30 s.[19,20]

High-performance liquid chromatography analysis

The AAH sample extract was injected into a Dionex Ultimate 3000 HPLC system (Thromo scientific, Waltham, MA, USA). The chromatographic columns used in this experiment are commercially available; one was obtained from RS-tech (0.46 × 25 cm, 5 μm, C_{18}, Daejeon, Korea). The injection volume was 10 μL, and the flow rate of the mobile phase was 1.0 mL/min delivered by a Dionex Ultimate 3000 Pump. The wavelengths of the UV detector were fixed at 210, 254, 280 and 320 nm. Data were analyzed using Chromeleon 7 software. The composition of the mobile phases was reservoir A (Water/TFA = 99.9/0.1 vol%) and reservoir B (Acetonitrile 100 vol%). The run time was 60 min and the solvent program was the linear gradient method, as follows; A: B 0-50 (90:10-30:70), 50-60 (30:70).

Preparation of standard solutions and samples

Samples were prepared by dissolving 2 mg of the standard chemicals chlorogenic aced, caffeic acid, vitexin, quercetin, and artemisinin in 10 ml of methanol and adjusting the concentration to 200 ppm. To prepare analytical samples, after the lyophilization of the extract, the AAH was filtered out using standard testing sieves (150 μm, Retsch, Haan, Germany). As a result, whitish AAH powder was obtained and stored at 4°C before use. The 50 mg of AAH powder was dissolved in 100% water at the concentration of 50 mg/mL. Each sample was filtered with a 0.2 μm polyvinylidene fluoride membrane filter before HPLC analysis.

Statistical analysis

The results are expressed as means ± standard deviation values for the number of experiments. Statistical significance was determined through comparison of each treated group with the control and determined through one-way ANOVA test. Each experiment was repeated at least 3 times to yield comparable results. $P < 0.05$ and $P < 0.01$ were considered to be significant.

RESULTS

Inhibition of nitric oxide and its synthesizing enzyme inducible nitric oxide synthase by *artemisiae annuae* herba treatment in lipopolysaccharide-stimulated RAW 264.7 macrophages

We evaluated the cytotoxicity of AAH using MTT assay to determine the optimal concentration that would be effective for anti-inflammation with minimum toxicity. As shown in Figure 1a, AAH did not affect cell viability up to 500 μg/mL, indicating AAH is not toxic to cells. NO, synthesized by iNOS is considered as an important inflammatory parameter. Therefore, we investigated whether AAH inhibits NO production and iNOS expression by LPS stimulation. We compared the inhibitory activity of AAH with that of dexamethasone, which is used to treat many inflammatory and autoimmune conditions, such as rheumatism and allergodermia. As shown in Figure 1b, NO production was repressed by AAH treatment in a dose-dependent manner. At high concentration (500 μg/mL), NO generation was reduced up to 73%, when compared with LPS alone. Furthermore, AAH showed dose-dependent inhibitory effect on iNOS expression at both protein and mRNA levels [Figure 1c].

Artemisiae annuae herba represses inflammatory cytokine expression in macrophages

Next, we researched the effect of AAH on LPS-induced inflammatory cytokine expression including TNF-α and IL-6. The expression of cytokines and their mRNA genes were analyzed by ELISA and RT-PCR, respectively. As

Table 1: Primers used for RT-PCR analysis

Target gene	Primer sequence
iNOS	F: 5'-AGCCCAACAATACAAATGACCCTA-3'
	R: 5'-TTCCTGTTGTTTCTATTTCCTTTGT-3'
TNF-α	F: 5'-AGCACAGAAAGCATGATCCG-3'
	R: 5'-GTTTGCTACGACGTGGGCTA-3'
IL-6	F: 5'-CATGTTCTCTGGGAAATCGTGG-3'
	R: 5'-AACGCACTAGGTTTGCCGAGTA-3'
β-actin	F: 5'-ATGAAGATCCTGACCGAGCGT-3'
	R: 5'-AACGCAGCTCAGTAACAGTCCG-3'

F: Forward; R: Reverse; RT-PCR: Reverse transcription-polymerase chain reaction; iNOS: Inducible nitric oxide synthase; TNF: Tumor necrosis factor; IL: Interleukin

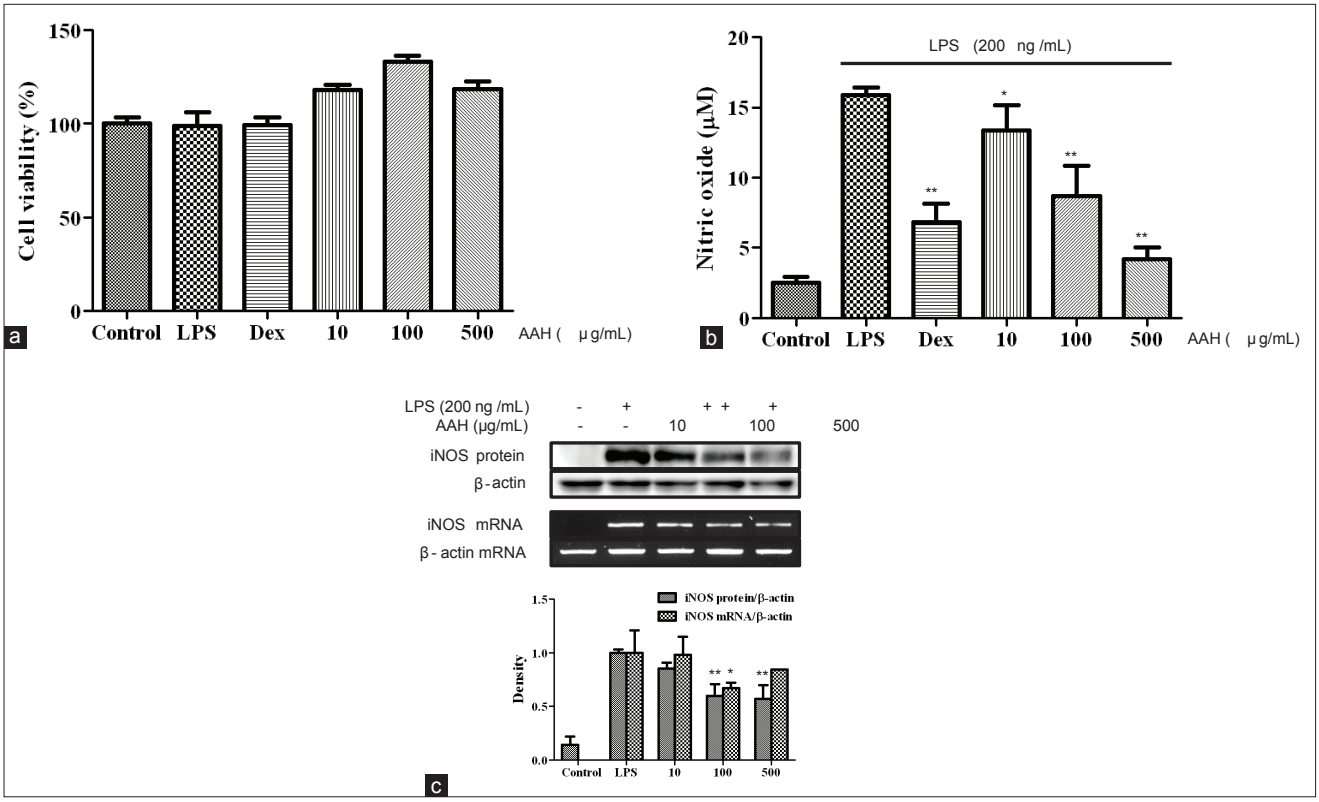

Figure 1: The effect of *Artemisiae annuae* herba on (a) cell viability, (b) nitric oxide (NO) secretion and (c) inducible NO synthase expression in RAW 264.7 cells

shown in Figure 2, TNF-α expression was a little decreased by high concentration of AAH, both cytokine and mRNA levels. However, IL-6 expression was significantly inhibited by AAH in a dose-dependent fashion and showed statistical significance [Figure 3].

Inhibitory effect of *Artemisiae annuae* herba on lipopolysaccharide-stimulated nuclear factor-kappa B nuclear translocation and IκBα degradation

The activation of NF-κB signaling pathway influences the expression of inflammatory mediators including iNOS and cytokines. Accordingly, we investigated the effect of AAH on NF-κB activation to elucidate the correlation between the suppressive effect of AAH on inflammatory parameters and inhibition of NF-κB pathway. Figure 4a shows AAH significantly repressed the translocation of p65 into nucleus, dose-dependently. Consistent with that, the phosphorylation and degradation of IκBα were inhibited by AAH treatment [Figure 4b]. These results suggest that AAH suppresses NF-κB pathway activation via the inhibition of p65 translocation and IκBα degradation.

Artemisiae annuae herba inhibits lipopolysaccharide-induced phosphorylation of mitogen-activated protein kinases in RAW 264.7 cells

Because MAPK activation is closely related with NF-κB stimulation, we further researched whether AAH inhibits MAPKs activation by phosphorylation. We checked the effect of AAH on the phosphorylation of MAPKs including ERK 1/2, p38 and JNK. As shown in Figure 5a and b, AAH exerted a little inhibitory effect on ERK 1/2 and p38 activation at high concentration (500 μg/mL). However, AAH strongly suppressed the level of JNK phosphorylation in a dose-dependent manner [Figure 5c]. AAH did not affect the levels of all nonphosphorylated MAPKs.

High-performance liquid chromatography analysis of *Artemisiae annuae* herba

Figure 6 shows the details of a HPLC system which analyzes the extracts and Figure 7 demonstrated the structure of compounds. The retention time (R_t) of chlorogenic acid (R_t: 9.96 min), caffeic acid (R_t: 11.84 min), vitexin (R_t: 15.65 min) quercetin (R_t: 24.93 min) and artemisinin (R_t: 44.83 min) were reported by this work.

DISCUSSION

*Artemisiae annua*e herba is usually used for the treatment of malaria and heat stroke in traditional oriental medicine. In modern, it has been reported that AAH contains various biological activities including the inhibitory effects against microorganism and parasite. However,

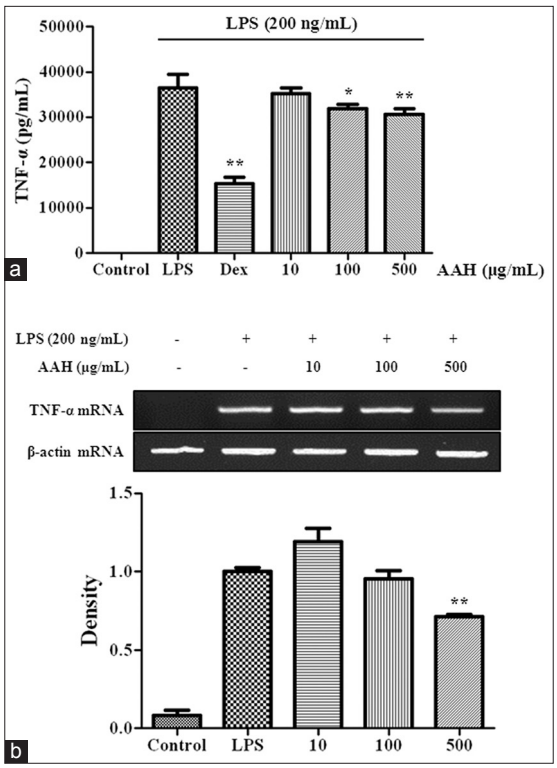

Figure 2: The effect of *Artemisiae annuae* herba on tumor necrosis factor-α (a) cytokine and (b) mRNA expression upon lipopolysaccharide stimulation

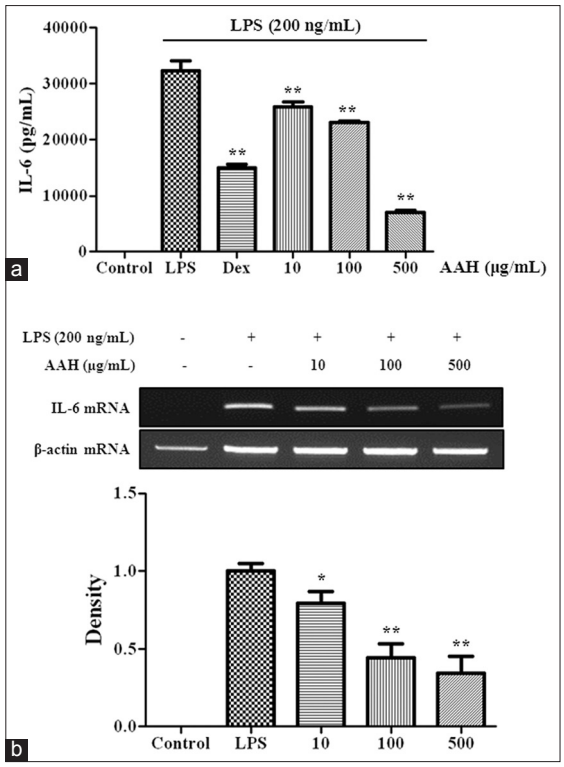

Figure 3: *Artemisiae annuae* herba suppresses interleukin-6 (a) cytokine and (b) mRNA expression

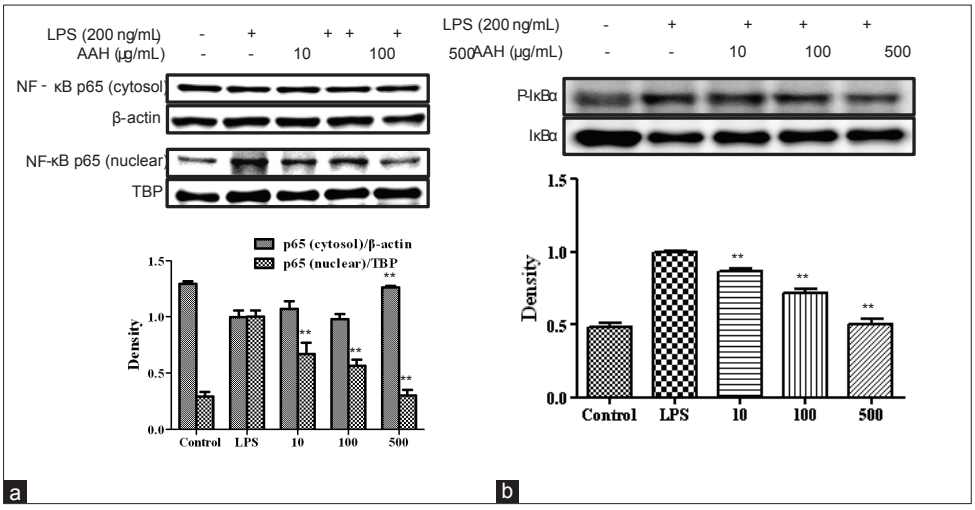

Figure 4: The extent of (a) nuclear factor-κB translocation into the nucleus and (b) degradation of IκBα into the cytosol are decreased by *Artemisiae annuae* herba treatment

the inhibitory activity of AAH on inflammation was not reported until now. In the present study, we have first demonstrated the anti-inflammatory effect of AAH using macrophage cells.

Because NO is considered as an important parameter of inflammation and NO production is related with many inflammatory diseases,[21] we investigated the effect of AAH on LPS-induced NO production and iNOS expression, which is a synthesizing enzyme of NO. We found that AAH significantly inhibits NO production and iNOS expression both protein and mRNA levels in a dose-dependent fashion. We also checked the effect of AAH on inflammation-related cytokines, TNF-α and IL-6 production. Consistent with the inhibitory effect of AAH on NO and iNOS expressions, AAH repressed the expression of cytokines in both protein and mRNA levels. These results indicate that AAH exhibits anti-inflammatory activity through the inhibitory effect on the expression of pro-inflammatory mediators.

To investigate the effect of AAH on upstream signaling pathways, we examined whether AAH inhibits NF-κB and MAPKs activations. NF-κB is an important transcription factor involved in cellular responses to stimuli such as LPS.[22] Furthermore, NF-κB is related with cell survival and pro-inflammatory mediators production including NO, PGE_2 and cytokines.[23] For examination of the suppressive effect of AAH on NF-κB activation, we investigated the levels of nuclear translocation of p65 and degradation of IκBα. We found that AAH represses LPS-induced nuclear translocation of p65 in a dose-dependent manner through the suppression of IκBα degradation. These results suggest that the inhibitory effect of AAH on inflammatory mediators is derived from the blockade of NF-κB activation.

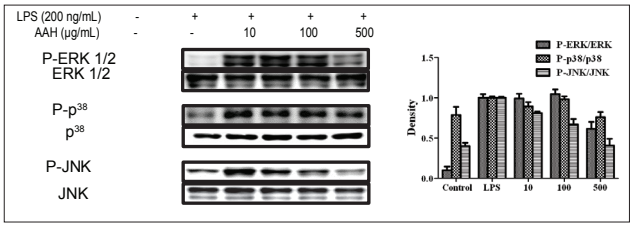

Figure 5: Inhibitory effect of *Artemisiae annuae* herba on the phosphorylation of mitogen-activated protein kinases in lipopolysaccharide-stimulated macrophages

Since ERK, p38 and JNK MAPK are known to be involved in expression of iNOS and COX-2 upon LPS stimulation in macrophages,[24] we further examined whether AAH inhibits the activation of MAPKs in LPS-stimulated RAW 264.7 cells. When the cells were pretreated with AAH, we observed AAH significantly inhibits the level of phosphorylation of JNK MAPK in a dose-dependent manner. These results suggest that antiinflammatory activity of AAH is directly related with the inhibitory effect on the activation of MAPK pathway.

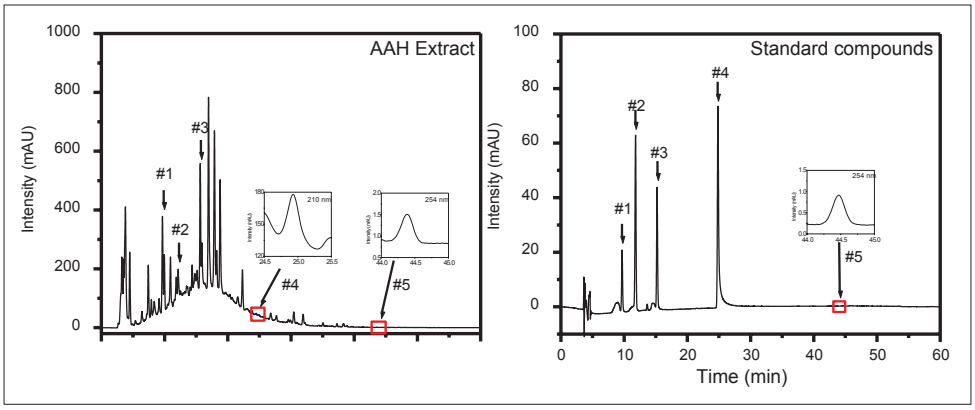

Figure 6: Analysis of five representative compounds in *Artemisiae annuae* herba with RP-high-performance liquid chromatography

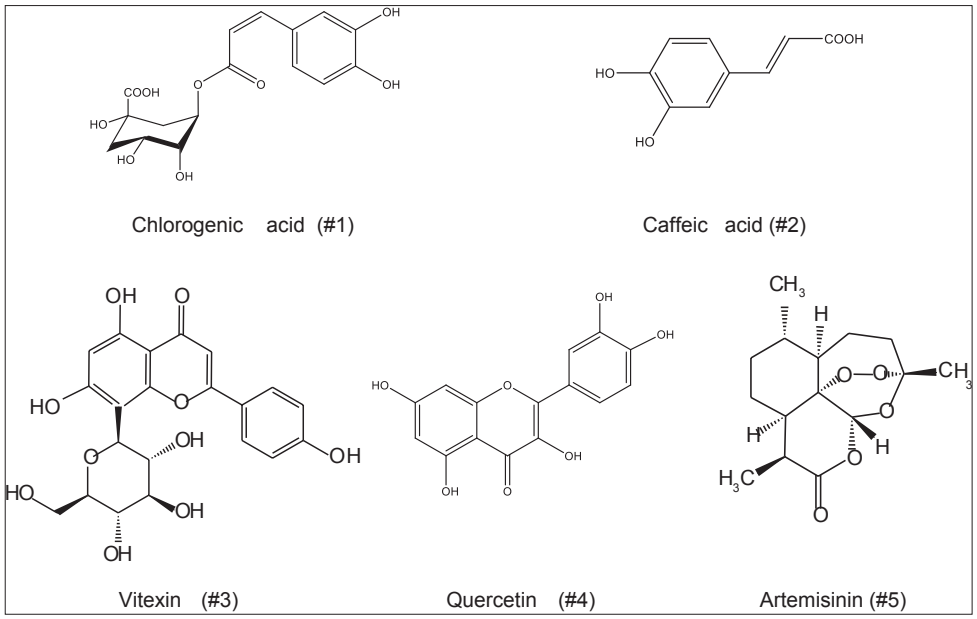

Figure 7: Chemical structures of representative compounds in *Artemisiae annuae* herba

As shown in Figure 6, we confirmed five main components (chlorogenic acid, caffeic acid, vitexin, quercetin and artemisinin) in AAH. A previous study reported that chlorogenic acid inhibits LPS-induced COX-2 expression in RAW264.7 cells through suppressing NF-κB and JNK/AP-1 activation and protects LPS-induced mice acute lung injury.[25,26] Also, it was demonstrated that quercetin inhibits NO production in LPS-stimulated peritoneal macrophages and represses LPS-induced TNF-α production in RAW 264.7 cells.[27,28] In addition, quercetin suppresses proinflammatory cytokines production through MAPK and NF-κB pathway in LPS-stimulated macrophage.[29] Another recent study has revealed that artemisinin inhibits IL-6 production in RAW 264.7 macrophage cells.[30] These facts suggest that the anti-inflammatory activity of AAH might be related with active components in AAH, including chlorogenic acid, quercetin, and artemisinin.

CONCLUSION

We demonstrated that AAH exerts strong inhibitory effect on the expression of inflammatory mediators including NO, TNF-α, IL-6 and iNOS in murine macrophage RAW 264.7 cells. Moreover, these effects were mediated by the inhibition of NF-κB activation via reduced p65 translocation, IκBα degradation and the suppression of MAPK activation through inhibited phosphorylation. These results imply that AAH could be developed as an anti-inflammatory herbal medicine. The *in vivo* studies on the antiinflammatory effect of AAH and which constituents of AAH are responsible for that are currently underway.

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