Original Article

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An ethanol extract of *Aster yomena* (Kitam.) Honda inhibits lipopolysaccharide-induced inflammatory responses in murine RAW 264.7 macrophages

Hye-Joo Kang[1,2,§], Ji-Suk Jeong[3,§], No-Jin Park[4], Geun-Bae Go[4], Sung Ok Kim[5], Cheol Park[6], Byung Woo Kim[7], Su-Hyun Hong[1,*], Yung Hyun Choi[1,2,*]

[1] *Department of Biochemistry, Dongeui University College of Korean Medicine, Busan, Korea;*
[2] *DAnti-Aging Research Center & Blue-Bio Industry RIC, Dongeui University, Busan, Korea;*
[3] *Gurye Wild Flower Institute, Gurye, Korea;*
[4] *Gurye-gun Agricultural Center, Gurye, Korea;*
[5] *Department of Food Science & Biotechnology, College of Engineering, Kyungsung University, Busan, Korea;*
[6] *Department of Molecular Biology, College of Natural Sciences & Human Ecology, Dongeui University, Busan, Korea;*
[7] *Department of Life Science and Biotechnology, College of Natural Sciences & Human Ecology, Dongeui University, Busan, Korea.*

Summary ***Aster yomena* (Kitam.) Honda has been widely used as a traditional herbal medicine for centuries to treat cough, asthma, insect bites, etc. Recent reports indicate that *A. yomena* possesses a wide spectrum of pharmacological activities; however, few experiments have described its anti-inflammatory properties. The present study examined the anti-inflammatory effects of an ethanol extract of *A. yomena* leaves (EEAY) on lipopolysaccharide (LPS)-stimulated murine RAW 264.7 macrophages. Treatment with EEAY significantly reduced the secretion of pro-inflammatory molecules, such as nitric oxide and interleukin-1β, in LPS-stimulated RAW 264.7 cells, without incurring any significant cytotoxicity. These protective effects were accompanied by a marked reduction in the expression of regulatory genes at the transcription level. Treatment with EEAY also inhibited the DNA-binding activity of nuclear factor-κB (NF-κB) by suppression of nuclear translocation of NF-κB and by degradation of the inhibitor of NF-κB; these effects were associated with suppression of the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase signaling pathways. The EEAY treatment also potently suppressed LPS-induced toll like receptor (TLR) 4 expression and attenuated the binding of LPS to the macrophage cell surface. In addition, EEAY treatment markedly inhibited LPS-induced accumulation of intracellular reactive oxygen species in RAW 264.7 macrophages. Therefore, the inhibitory effects of EEAY on LPS-stimulated inflammatory responses in RAW 264.7 macrophages were apparently associated with suppression of the TLR-mediated NF-κB signaling pathway. More work is needed to fully understand the critical role and clinical usefulness of EEAY treatment, but the findings of the present study provide some insights into the potential of EEAY as a therapeutic agent for treatment of inflammatory disorders.**

Keywords: *Aster yomena* (Kitam.) Honda, macrophages, anti-inflammation, NF-κB TLR4

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[§]These authors contributed equally to this works.
**Address correspondence to:*
Drs. Yung Hyun Choi and Su-Hyun Hong, Department of Biochemistry, College of Korean Medicine, Dongeui University, Busan 47227, Korea.
E-mail: choiyh@deu.ac.kr (Choi YH) and hongsh@deu.ac.kr (Hong SH)

1. Introduction

Inflammation is one of the first protective responses of the body and involves activation of immune system processes. The inflammatory response is a highly regulated self-limiting process for identifying and destroying invading pathogens and restoring normal tissue structure and function (*1,2*). However, in many

diseases, an excessive inflammatory response is a very common and important basic pathologic process (*2,3*). Inflammatory stimulants, such as the lipopolysaccharide (LPS) endotoxins of Gram-negative bacteria, activate macrophages through Toll-like receptor 4 (TLR4), a member of the TLR family. These macrophages, together with neutrophils and dendritic cells, act as the first cellular mediators of the inflammatory process (*4-6*).

TLR4 activation by LPS triggers activation of several intracellular signaling pathways, including phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs). This further activation ultimately allows nuclear translocation of nuclear factor κB (NF-κB), a dimer of p65 and p50 subunits, which, in turn, promotes secretion of pro-inflammatory mediators, including nitric oxide (NO), and pro-inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) (*7-9*). Overproduction of these pro-inflammatory molecules eventually leads to deleterious consequences associated with pathogenesis of many inflammatory diseases (*9,10*).

Another important component of inflammation is oxidative stress, which reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify these reactive intermediates (*11,12*). Overproduction of ROS by activated macrophages is an important contributor to the manifestation of inflammation (*13,14*), and ROS are also involved in the production of inflammatory cytokines in LPS-stimulated macrophages (*15*). Suppression of production of inflammatory factors by blocking macrophage activation can be viewed as a valuable therapeutic approach for relieving progression of inflammatory disorders (*16,17*). Development of anti-inflammatory agents that can prevent activation of LPS signaling and its downstream molecules, is therefore a necessary prerequisite for prevention and treatment of various inflammatory diseases.

One well recognized source of anti-inflammatory compounds are the botanical materials used as traditional medicines in many cultures. One of these sources is *Aster yomena* (Kitam.) Honda, an edible vegetable and perennial herb that belongs to the Asteraceae family. This plant is widely distributed in Asia and has been used in traditional medicine for the treatment of various diseases, such as cough, asthma, and insect bites (*18*). Previous studies have indicated that leaf extracts and compounds from this plant possess many pharmacological properties, including anti-microbial (*19,21*), antioxidant (*21,22*), anti-coagulant (*23*), and peroxynitrite-scavenging (*24*) activities. A recent study indicated that some of the phenolic compounds produced by *A. yomena* were potent inhibitors of IL-6 production in TNF-α stimulated osteoblast cells, a clear indication of their anti-inflammatory potential (*25*). However, to the best of our knowledge, the molecular mechanisms involved in this anti-inflammatory action have remained elusive. Therefore, the present study was conducted as part of our on-going research program that seeks out novel anti-inflammatory active substances from traditional medicinal resources. Here, we investigated an ethanol extract of *A. yomena* leaves (EEAY) for its anti-inflammatory action in LPS-stimulated RAW 264.7 macrophage cells.

2. Materials and Methods

2.1. *Preparation of the EEAY*

The dried leaves of *A. yomena* were obtained from Gurye Wild Flower Institute (Gurye, Republic of Korea) and authenticated by Professor S.H. Hong, Department of Biochemistry, Dongeui University College of Korean Medicine (Busan, Republic of Korea). The leaves (50 mg) were cut into small pieces, ground into a fine powder, and then soaked in 70% ethanol (500 mL) for 2 days. The extracted liquid was filtered twice through Whatman No. 3 filter paper to remove any insoluble materials and then concentrated using a rotary evaporator (Rikakikai Co., Ltd., Tokyo, Japan) as previously described (*26*). The concentrated extract (EEAY) was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich Co., St. Louis, MO, USA) to a final concentration of 200 mg/mL (extract stock solution) and was subsequently diluted with cell culture medium to the desired concentration prior to use.

2.2. *Cell culture*

The murine macrophage RAW 264.7 cell line was obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) and cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, WelGENE Inc., Daegu, Republic of Korea) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin (WelGENE Inc.).

2.3. *Cell viability assay*

Cell viability was determined with a colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemical Co.) assay. In brief, RAW 264.7 cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and incubated at 37°C for 24 h. Cells were treated with various concentrations of EEAY for 24 h or pretreated with EEAY for 1 h before stimulation with 100 ng/mL LPS for 24 h. After incubation, the medium was discarded, and MTT solution (5 mg/mL in phosphate-buffered saline, PBS) was added to each well and incubated for another 3 h at 37°C. The medium was removed and DMSO was added to dissolve the formazan dye. The optical density was then read at 560 nm using a

microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) to determine cell viability (*27*).

2.4. *Measurement of NO production in RAW 264.7 macrophages*

The production of NO in culture supernatants was assayed using Griess reagent (Sigma-Aldrich Chemical Co.). In brief, cells were pretreated with various concentrations of EEAY for 1 h and stimulated with LPS for 24 h. The supernatant was then collected and mixed with the same volume of Griess reagent for 10 min at room temperature in the dark. Absorbance was measured at 540 nm with a microplate reader, and NO concentrations were calculated by using a standard curve generated with known concentrations of sodium nitrite (*28*). Fresh culture medium was used as the blank in all experiments.

2.5. *Measurement of IL-1β production in RAW 264.7 macrophages*

Inhibitory efficacy of EEAY on the production of IL-1β was measured by culturing cells under the same conditions used for the NO measurement assay. Levels of IL-1β concentrations in the culture media were determined with a selective enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions (*29*).

2.6. *RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)*

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions and then reverse transcribed using an M-MLV reverse transcriptase kit (BioNEER, Daejeon, Republic of Korea) to produce cDNAs. RT-generated cDNAs encoding inducible NO synthase (iNOS) and IL-1β genes were amplified by PCR using the desired primers (BioNEER). The PCR reaction was initiated at 94°C for 2 min, followed by 31 cycles of 94°C for 30 sec, 30 sec annealing temperature, 72°C for 30 sec, and a final extension step at 72°C for 5 min. Following amplification, PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide (EtBr, Sigma-Aldrich Chemical Co.), and visualized by ultraviolet illumination. In a parallel experiment, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

2.7. *Protein extraction and Western blot analysis*

Cell pellets were collected and resuspended in extraction lysis buffer [25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol], for 30 min at 4°C. In a parallel experiment, nuclear and cytosolic proteins were separated using NE-PER nuclear and cytosolic extraction reagents (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's protocol. Protein concentration in the cell lysate was determined using a DC™ Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of protein from each sample were separated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto polyvinylidine fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were incubated overnight at 4°C with the corresponding primary antibodies purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and Cell Signaling Technology, Inc. (Boston, MA, USA). Membranes were then incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (Amersham Co., Arlington Heights, IL, USA) at room temperature for 2 h. Using an enhanced chemiluminescence (ECL, Amersham Co.) detection system, immunoreactive bands were monitored and exposed to X-ray film.

2.8. *Electrophoretic mobility assay (EMSA)*

The EMSA was performed using the nuclear extract. Synthetic complementary NF-κB binding oligonucleotides (Santa Cruz Biotechnology, Inc.) were 3'-biotinylated using a biotin 3'-end DNA labeling kit (Pierce Biotechnology), according to the manufacturer's instructions, and annealed for 30 min at room temperature. Assays were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 60 min in 0.5X Tris borate/EDTA before being transferred onto a positively charged nylon membrane (Hybond™N^+) in 0.5X Tris borate/EDTA at 100 V for 30 min. The transferred DNAs were cross-linked to the membrane at 120 mJ/cm^2. Horseradish peroxidase-conjugated streptavidin was used according to the manufacturer's instructions to detect the transferred DNA.

2.9. *Immunofluorescence assay*

NF-κB p65 nuclear translocalization was detected by an immunofluorescence assay. For this study, RAW 246.7 cells were pretreated with EEAY for 1 h and then stimulated with LPS for 1 h. The cells were fixed with 3.7% paraformaldehyde in PBS for 10 min at 4°C, permeabilized with 0.4% Triton X-100 in PBS for 10 min, and blocked with 5% bovine serum albumin for 1 h. Cells were probed with anti-p65 NF-κB antibody (Santa Cruz Biotechnology, Inc.) overnight at 4°C and then incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) for 2

h at room temperature. The position of the cell nucleus was determined with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Chemical Co.) solution (1 mg/mL) for 15 min. After washing with PBS, fluorescence was visualized using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Cells were also stimulated with Alexa Fluor 488-conjugated LPS (100 ng/mL, AF-LPS; Invitrogen Life Technologies) for 30 min in the presence or absence of EEAY to assay for LPS/TLR4 complex formation. Cells were fixed, stained with anti-TLR4 antibody for 90 min at 4˚C, and then incubated with secondary antibodies conjugated with Alexa Fluor 594 (Invitrogen Life Technologies) for 1 h. Stained cells were observed under a fluorescence microscope.

2.10. *Measurement of ROS generation in RAW 264.7 macrophages*

The ROS levels in RAW 264.7 cells were measured by washing the cells twice with PBS and then lysing them with 1% Triton X-100 in PBS for 10 min at 37°C. Cells were then stained with 10 μM 2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Eugene, OR, USA) for 20 min at room temperature in the dark. Green fluorescence emitted by DCF was recorded at 515 nm using a flow cytometer (Becton Dickinson, San Jose, CA, USA) (*30*). Images showing the generation of intracellular ROS were also obtained using a fluorescence microscope.

2.11. *Statistical analysis*

All data are presented as mean ± standard deviation (SD). Significant differences among groups were determined using the unpaired Student's *t*-test. A value of $p < 0.05$ was accepted as an indication of statistical significance. All the figures shown here reflect data obtained from at least three independent experiments.

3. Results

3.1. *Cytotoxic effects of EEAY and LPS on RAW 264.7 macrophages*

The possibility of cytotoxicity caused by EEAY treatment was excluded by treating RAW 264.7 cells with various concentrations of EEAY for 24 h. The MTT assay showed no cytotoxicity at concentrations up to 500 μg/mL EEAY in the presence or absence of 100 ng/mL LPS (Figure 1). We therefore selected 300 μg/mL EEAY as the maximum concentration for further experiments using RAW 264.7 cells.

3.2. *EEAY inhibits LPS-induced NO and IL-1β production in RAW 264.7 macrophages*

Inhibitory properties of EEAY were tested on the LPS-induced production of NO and IL-1β as a representative pro-inflammatory mediator and cytokine, respectively (*8,9*), in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of EEAY for 1 h and then stimulated with 100 ng/mL LPS for another 24 h. The levels of NO and IL-1β in the culture medium were determined by the Griess reaction assay and ELISA, respectively. As shown in Figure 2A and B, stimulation

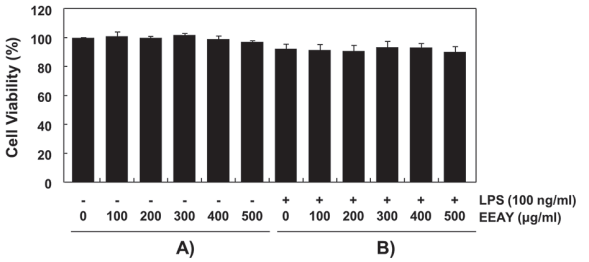

Figure 1. Effect of an ethanol extract of *Aster yomena* leaves (EEAY) on the cell viability of RAW 264.7 macrophages. Cells were treated with various concentrations of EEAY for 24 h **(A)** or pretreated with the indicated concentrations of EEAY for 1 h prior to lipopolysaccharide (LPS) (100 ng/mL) treatment for 24 h **(B)**. Cell viability was assessed with a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay, and the results are expressed as the percentage of surviving cells over control cells (no addition of EEAY and LPS).

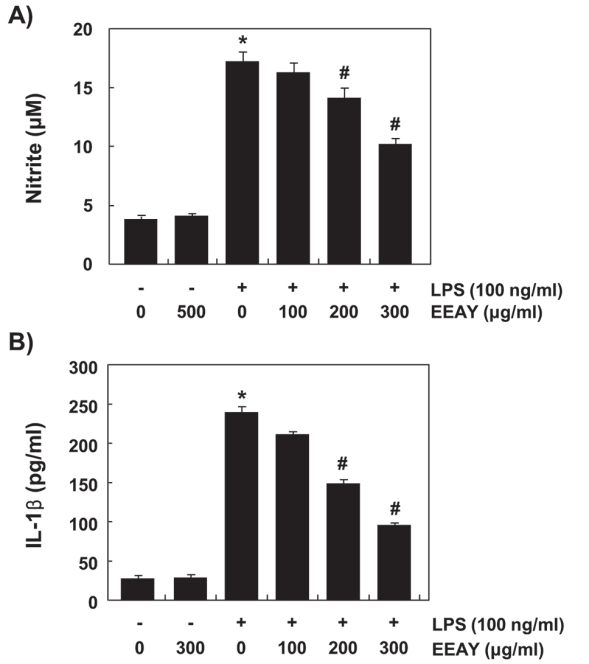

Figure 2. Inhibition of NO and IL-1β production by an ethanol extract of *Aster yomena* leaves (EEAY) in LPS-stimulated RAW 264.7 macrophages. Cells were pretreated with the indicated concentrations of EEAY for 1 h prior to incubation with 100 ng/mL LPS for 24 h. Levels of NO **(A)** and IL-1β **(B)** in culture media were measured by Griess assay and a commercial ELISA kit, respectively. Each value indicates the mean ± SD and is representative of the results obtained from three independent experiments ($^{*}p < 0.05$ compared to the control; $^{\#}p < 0.05$ compared to cells cultured with 100 ng/mL LPS).

with LPS markedly induced the production of NO and IL-1β when compared to cells not stimulated with LPS; however, EEAY significantly inhibited NO and IL-1β secretion in a concentration-dependent manner.

3.3. *EEAY suppresses LPS-induced iNOS and IL-1β expressions at the protein and mRNA levels in RAW 264.7 macrophages*

We subsequently used RT-PCR and Western blot analysis to investigate the possible relationship between the inhibitory effects of EEAY on NO and IL-1β production and regulation of expression of their synthesis enzymes. As indicated in Figure 3A and B, EEAY effectively inhibited protein and mRNA expression of iNOS and IL-1β in LPS-stimulated RAW 264.7 cells. Therefore, EEAY appeared to suppress NO and IL-1β production in LPS-stimulated RAW 264.7 cells by reducing expression of their encoding genes at the transcriptional level.

3.4. *EEAY blocks LPS-induced NF-κB activation in RAW 264.7 macrophages*

Active NF-κB translocates to the nucleus, where it activates its target genes including iNOS and IL-1β by binding to their promoter regions (*31,32*). We used immunoblotting of cytoplasmic and nuclear extracts to investigate the possibility that EEAY attenuates LPS-induced activation of the NF-κB signaling pathway. EEAY pretreatment inhibited nuclear accumulation of the NF-κB p65 subunit, as well as IκBα degradation, in LPS-stimulated RAW 264.7 cells (Figure 4A). Immunocytochemistry analysis also indicated that NF-κB p65 was normally sequestered in cytoplasm, whereas robust nuclear accumulation of NF-κB p65 was observed in RAW 264.7 microglial cells following stimulation with LPS. However, LPS-mediated nuclear translocation of NF-κB was effectively abolished by pretreatment with EEAY (Figure 4B). In agreement with the immunoblotting data, DNA-binding activity of NF-κB was markedly increased in response to LPS treatment, whereas pretreatment of the cells with EEAY significantly reduced DNA-binding activity of NF-κB (Figure 4C). These data indicate that EEAY inhibits LPS-induced NF-κB activation by attenuating the IκBα degradation.

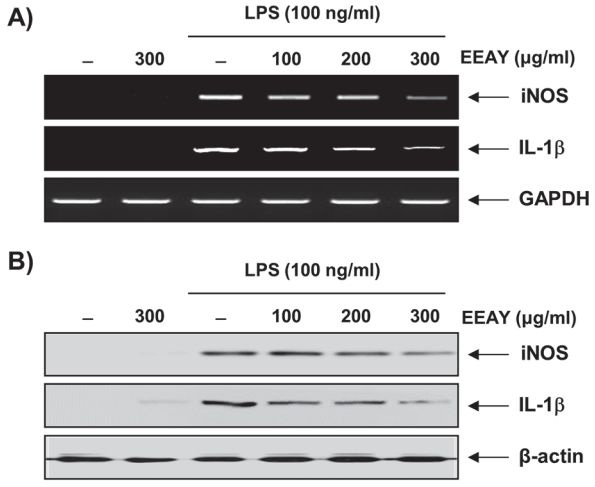

Figure 3. Suppression of iNOS and IL-1β expression by an ethanol extract of *Aster yomena* leaves (EEAY) in LPS-stimulated RAW 264.7 macrophages. Cells were pretreated with different concentrations of EEAY for 1 h, followed by stimulation with 100 ng/mL LPS for 24 h. **(A)** Total RNAs were prepared for RT-PCR analysis of the iNOS and IL-1β mRNA expression using the indicated primers. **(B)** Cell lysates were prepared for Western blot analysis with antibodies specific for murine iNOS and COX-2, and an enhanced chemiluminescence (ECL) detection system. Experiments were repeated three times and similar results were obtained. GAPDH and β-actin were used as internal controls for the RT-PCR and Western blot analysis, respectively.

3.5. *EEAY attenuates LPS-induced activation of PI3K/Akt and MAPK signaling pathways in RAW 264.7 macrophages*

LPS-induced NF-κB activation has a known association with activation of the PI3K/Akt and MAPK signaling pathways (*7-9*). Therefore, we examined the effect of EEAY on LPS-induced activation of Akt, a downstream kinase of PI3K, and three kinases of the MAPK pathway: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. As shown in Figure 5, the level of unphosphorylated Akt was unaffected by either LPS or EEAY treatment, whereas phosphorylation of Akt showed a marked increase within 30 min following stimulation with LPS. Stimulation of the cells with LPS induced rapid activation of ERK, JNK, and p38 MAPK, with peak levels of each phosphorylated MAPK occurring 30 min after addition of LPS; the unphosphorylated forms were unaltered. However, pretreatment with EEAY resulted in significant blockage of LPS-induced phosphorylation of Akt, as well as of the MAPKs. These results revealed that EEAY is capable of disrupting the PI3K/Akt and MAPK signal transduction pathways that are activated by LPS in RAW 264.7 cells and that EEAY might be involved in inhibition of inflammatory mediator release.

3.6. *EEAY inhibits LPS-induced TLR4 expression and the interaction between LPS and TLR4 in RAW 264.7 macrophages*

We next assessed the effects of EEAY on the LPS-activated TLR4 signaling pathway. As indicated in Figure 6A, the expression of TLR4 protein was markedly up-regulated in LPS-treated RAW 264.7 cells compared with untreated cells. However, pretreatment of the cells with EEAY induced a concentration-dependent inhibition of this increased expression of TLR4. We also used AF-conjugated LPS to examine the influence of EEAY on LPS binding to TLR4 on the RAW 264.7 macrophage

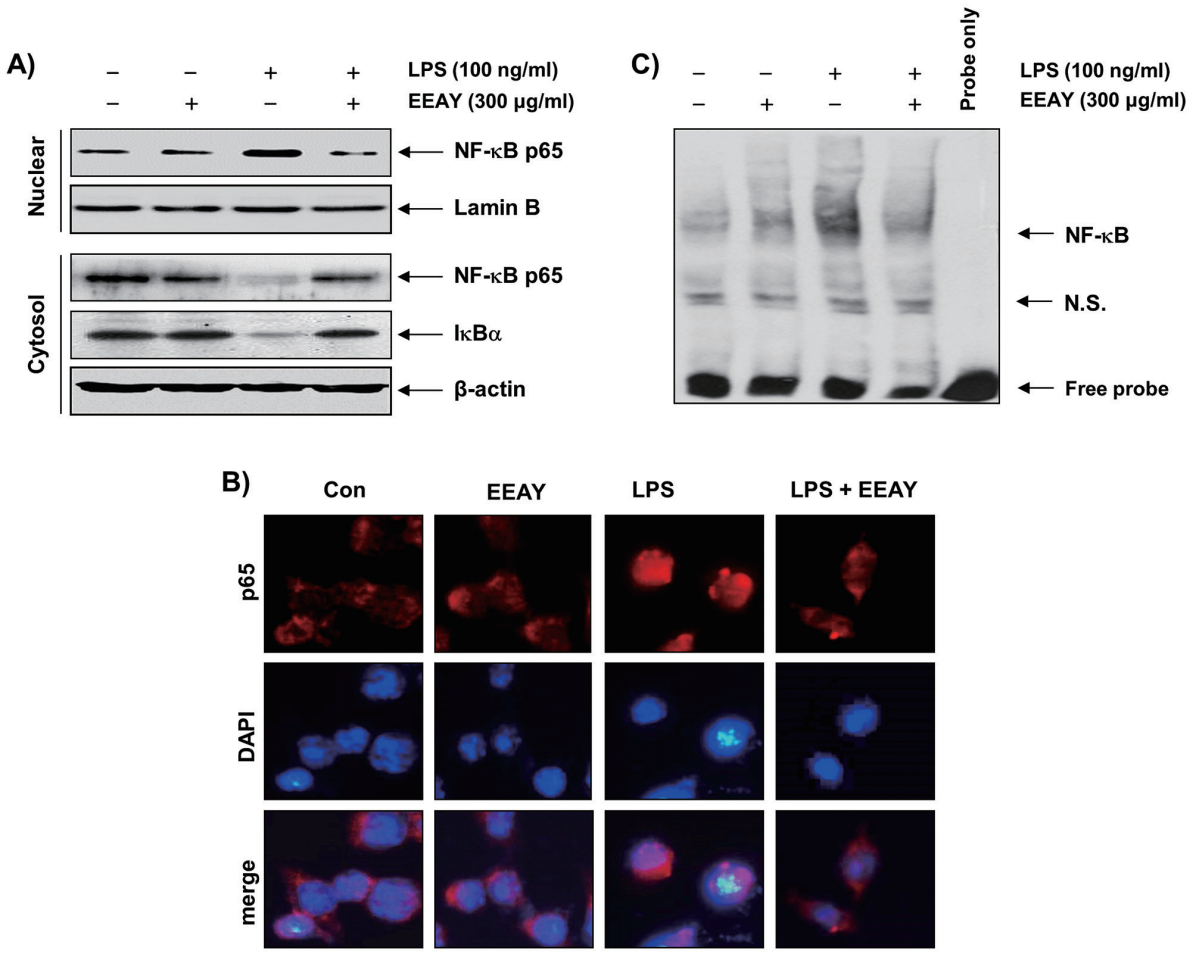

Figure 4. Inhibition of NF-κB activation by an ethanol extract of *Aster yomena* leaves (EEAY) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. (A) Cells were preincubated with 300 μg/mL EEAY for 1 h before 100 ng/mL LPS treatment for 30 min. Nuclear and cytosolic proteins were prepared for Western blot analysis using anti-NF-κB p65 and anti-IκB-α antibodies, and an enhanced chemiluminescence (ECL) detection system. Lamin B and β-actin were used as internal controls for the nuclear and cytosolic fractions, respectively. **(B)** Cells were pretreated with 300 μg/mL EEAY for 1 h before stimulation with LPS for 30 min. Nuclear extracts were subsequently assayed for NF-κB activity by an electrophoretic mobility assay (EMSA). **(C)** Cells were pretreated with 100 μg/mL EEAY for 1 h before 100 ng/mL LPS treatment. After 30 min of incubation, the localization of NF-κB p65 was visualized with fluorescence microscopy after immunofluorescence staining with anti-NF-κB p65 antibody and a fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG antibody (green). Cells were also stained with DAPI to visualize the nuclei (blue). Results are representative of those obtained from three independent experiments.

surface. As indicated in Figure 6B, treatment of cells with AF-LPS alone resulted in increased fluorescence of AF-LPS and TLR4 on the macrophage surface when compared with the control group. However, this binding of AF-LPS to the membrane of RAW 264.7 cells was blocked by pretreatment with EEAY, as indicated by attenuation of the fluorescence intensity. Therefore, EEAY might block LPS-induced activation of the TLR4 signaling pathway by suppression of LPS binding to the cell surface.

3.7. *EEAY reduces LPS-induced accumulation of ROS in RAW 264.7 macrophages*

Oxidative stress is partially responsible for the initiation of inflammation (*11,14*). Therefore, we used DCF-DA staining to examine the effects of EEAY on LPS-induced generation of ROS in RAW 264.7 cells. The flow cytometry results indicated an accumulation of intracellular ROS at 1 h, and the ROS levels continued to increase up to 3 h following LPS treatment (Figure 7A). However, this increase in LPS-stimulated ROS production was markedly attenuated by pretreatment with EEAY (Figure 7B). The positive control, generated by treatment with the ROS scavenger N-acetyl-l-cysteine (NAC), also showed effective attenuation of LPS-induced ROS generation, and EEAY itself did not contribute to ROS generation. Taken together, the results suggest that the antioxidant properties of EEAY may be responsible for its anti-inflammatory properties in RAW 264.7 cells.

4. Discussion

When cells are infected by Gram-negative bacteria, LPS acts as a prototypical ligand of the membrane-bound

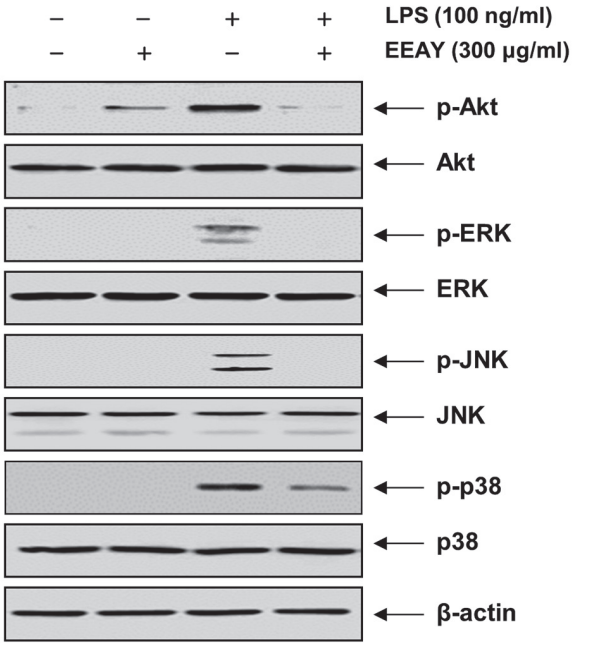

Figure 5. Effect of an ethanol extract of *Aster yomena* leaves (EEAY) on lipopolysaccharide (LPS)-induced phosphorylation of Akt and MAPKs in RAW 264.7 macrophages. Cells were pretreated with 300 μg/ml EEAY for 1 h prior to exposure to LPS for 30 min, and total proteins were isolated. The proteins were subjected to SDS-polyacrylamide gels, followed by Western blot analysis using the indicated antibodies, and an enhanced chemiluminescence (ECL) detection system. β-actin was used as the internal control. The experiments were repeated three times and similar results were obtained.

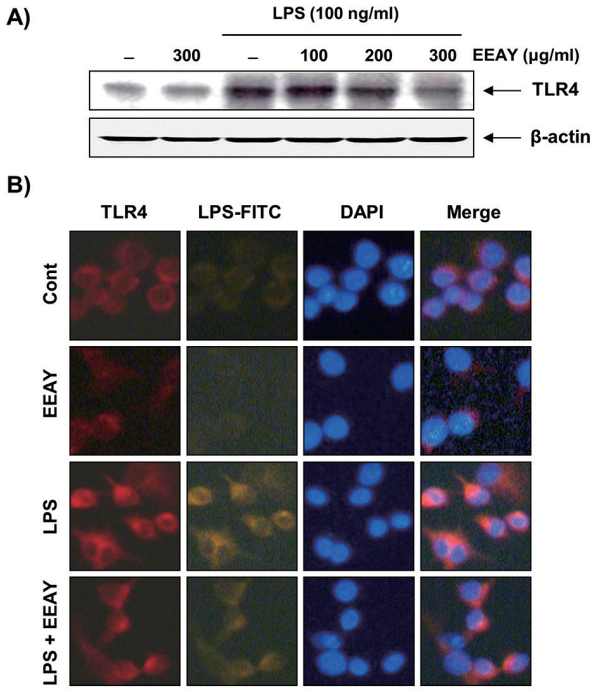

Figure 6. Attenuation of lipopolysaccharide (LPS)-induced TLR4 expression, and interaction between LPS and TLR4 by an ethanol extract of *Aster yomena* leaves (EEAY) in LPS-stimulated RAW 264.7 macrophages. (A) Cells were pretreated with the indicated concentrations of EEAY for 1 h prior to LPS treatment, and total protein was isolated at 6 h after LPS treatment. Levels of TLR4 protein were assessed by Western blot analysis using anti-TLR4 antibody, and an enhanced chemiluminescence (ECL) detection system. β-actin was used as the internal control. **(B)** Cells were incubated with Alexa Fluor 488-conjugated LPS (AF-LPS) for 1 h in the absence or presence of 300 μg/mL EEAY, and then distribution of AF-LPS and TLR4 was detected by a fluorescence microscopy.

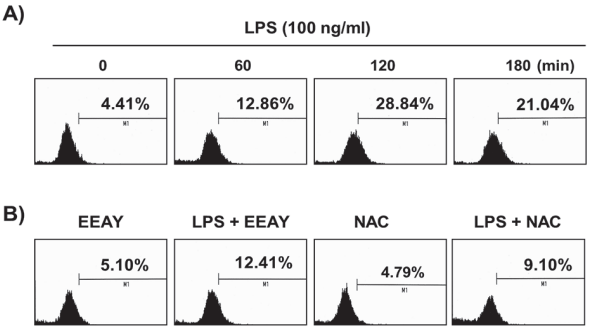

Figure 7. Effect of an ethanol extract of *Aster yomena* leaves (EEAY) on LPS-induced reactive oxygen species (ROS) production in RAW 264.7 macrophages. RAW 264.7 cells were treated with 100 ng/mL lipopolysaccharide (LPS) for the indicated time **(A)** or pre-incubated with or without 300 μg/mL EEAY or 10 mM NAC for 1 h and then stimulated with 100 ng/mL LPS for 2 h **(B)**. Cells were incubated with 10 μM 2',7'-dichlorofluorescein diacetate (DCF-DA) for 30 min at 37°C. Cells were collected and dichlorofluorescein (DCF) fluorescence was measured by flow cytometry. Values were represented as means ± SD of two independent experiments.

TLR4 pattern recognition receptor in macrophages (*5,6*). The activation of the TLR4 pathway triggers intracellular signaling pathways that culminate in the activation of several signaling molecules, such as NF-κB, PI3K/Akt, and MAPKs (*8,9*). This activation of macrophages then triggers inflammation through the production and release of pro-inflammatory mediators and cytokines, like NO and IL-1β, respectively, which play a central role in initiating and sustaining the inflammatory response (*4,6*). Stimulation of macrophages with LPS increases expression of the iNOS gene and promotes excessive NO release *via* decomposition of L-arginine. iNOS is also activated in response to inflammatory stimuli such as cytokines, IL, and bacterial endotoxin (*33,34*). In addition to its role in NO release, IL-1β also serves as one of the major pro-inflammatory cytokines released following LPS stimulation of macrophages, and its excessive production has been linked to development of chronic inflammatory diseases (*35,36*). In the present study, EEAY pretreatment prevented the LPS-stimulated production of NO and IL-1β in RAW 264.7 macrophages by potent inhibition of iNOS and IL-1β expression at both the protein and mRNA levels. This inhibition occurred without cytotoxicity, supporting EEAY as a promising target for inhibiting the early steps in inflammatory pathways.

Transcription factor NF-κB plays a significant role in the LPS-induced expression of many inflammation-related enzymes and cytokines (*31,32*). NF-κB is normally retained in the cytoplasm due to its association

with its endogenous inhibitor, IκB-α, which renders NF-κB inactive. Once activated by inflammatory stimuli, such as LPS, IκB-α is rapidly phosphorylated and degraded by an ubiquitin-proteasome pathway. Thereafter, NF-κB translocates to the nucleus, where it binds to target gene promoters and drives transcriptional activation of various pro-inflammatory genes (*6,32*). Therefore, agents that effectively modulate NF-κB activation are promising candidates for treatment of various inflammatory diseases. Our results revealed that LPS stimulation resulted in nuclear translocation of NF-κB p65 in RAW 264.7 macrophages; however, EEAY attenuated this nuclear translocation by inhibiting LPS-induced IκB-α degradation. In addition, EEAY suppressed binding of NF-κB to DNA. Consequently, inactivation of the NF-κB signaling pathway by EEAY might lead to down-regulation of pro-inflammatory factors and a consequent reduction in their release. This would prevent inflammation and might be the molecular mechanism underlying the anti-inflammatory effects of EEAY.

One response to inflammatory stimuli is the phosphorylation of intracellular signaling molecules belonging to the PI3K/Akt and MAPK signaling pathways; this often accelerates production of pro-inflammatory mediators and cytokines (*8,9*). The rate of phosphorylation of these molecules, which act as upstream regulators of NF-κB, is also well recognized to increase in LPS-stimulated macrophages, so that phosphorylation plays a critical role in progression of inflammation (*31,37*). The effects of EEAY on LPS-induced phosphorylation of Akt and MAPKs were therefore examined to further explore mechanisms underlying its anti-inflammatory effects. Immunoblotting experiments confirmed that EEAY treatment attenuated the extent of Akt phosphorylation, indicating inactivation of the PI3K/Akt signaling pathway. EEAY treatment also down-regulated LPS-triggered activation of all three MAPKs (ERK, JNK, and p38 MAPK) studied in the RAW 264.7 macrophages. Therefore, EEAY suppressed PI3K/Akt and MAPK signaling pathways in LPS-treated RAW 264.7 macrophages, and then inactivated activation of the NF-κB signaling pathway to prevent activation of inflammation-related genes.

Many studies have indicated that the TLR family of pattern recognition receptors includes central mediators of the inflammatory response. Accumulating evidence now indicates that TLR4 specifically mediates signaling induced by LPS (*37,38*). A first step in the LPS/TLR4-mediated inflammatory signaling pathway is binding of LPS to TLR4, in conjunction with MYD88 adapters at the plasma membrane; this then initiates intracellular signaling cascades (*4,5*). Activation of TLR4 by LPS induces phosphorylation of PI3K/Akt and MAPK signaling molecules, as well as the IB kinase complex, eventually resulting in activation of NF-κB (*40,41*). Therefore, expression levels of TLR4 are significant as they enable optimal LPS responsiveness. In the present study, we found that TLR4 protein expression was highly stimulated by LPS and concentration-dependently inhibited by EEAY. Treatment with EEAY also markedly reduced formation of the complexes of LPS with TLR4, indicating that EEAY treatment could disrupt association of TLR4 with its adaptors, leading to inactivation of TLR4 in LPS-stimulated RAW 264.7 macrophages. Therefore, EEAY could inhibit an initial step of the intracellular signaling cascades by blocking the TLR4 signaling pathway and subsequently suppressing activation of NF-κB.

ROS accumulation and the compensatory response from the endogenous antioxidant network results in a redox imbalance that causes oxidative stress (*12,15*). Overwhelming generation of ROS is strongly associated with many other pathological conditions, including inflammation. Chronic inflammation causes an amplification in inflammatory signals in macrophages through activation of the NF-κB signaling pathway and over-expression of inflammation-associated genes (*41,42*). The inflammatory mediators and cytokines also promote an influx of macrophages that, in turn, accelerate intracellular accumulation of ROS (*11,14*). In addition, ROS, acting as a secondary messenger, participates in the TLR4-mediated inflammatory signaling pathway (*43*). Therefore, the profound ability of EEAY to suppress ROS in LPS-stimulated RAW 264.7 macrophages might be attributable to its ability to scavenge free radicals. The EEAY-mediated inhibition of ROS generation might also potentially inhibit the intracellular signaling cascade-dependent expression of pro-inflammatory mediators and cytokines, thereby explaining EEAY‘s strong anti-inflammatory properties.

In conclusion, the results presented here demonstrate that EEAY exerts potent anti-inflammatory effects in RAW 264.7 macrophages. Treatment of LPS-stimulated RAW 264.7 macrophages with EEAY significantly attenuated production of NO and IL-1β by reducing their corresponding gene expression. Anti-inflammatory effects of EEAY were mediated by suppression of NF-κB activation and subsequent attenuation of PI3K/Akt and MAPK signaling pathways. The ability of EEAY to inhibit the inflammatory response was associated, at least partially, with suppression of activation of TLR4 and a reduction of intracellular ROS production. The results of this study support use of EEAY as an alternative candidate for safe and effective treatment of inflammatory diseases.

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