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## *In vitro* anti-inflammatory activity of the *Artemisia montana* leaf ethanol extract in macrophage RAW 264.7 cells

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### ABSTRACT

*Artemisia* is one of the largest genera of the family Asteraceae or Compositae, consisting of 500 species. Some *Artemisia* species, such as *Artemisia afra*, *A. sacrorum*, and *A. annua*, have been widely used as traditional medicine to treat inflammatory and malarial diseases. However, the biological activity of *A. montana* has not been broadly studied. Therefore, in this study, we investigated the anti-inflammatory activity of *A. montana* leaf extract (ALE) and its molecular mechanisms in lipopolysaccharide-activated RAW 264.7 cells. Non-cytotoxic concentrations of ALE significantly reduced the expression of inducible nitric oxide (NO) synthase and cyclooxygenase-2, resulting in the decrease in NO and prostaglandin E<sub>2</sub>. Moreover, ALE inhibited the production of tumour necrosis factor- $\alpha$  and interleukin-6. We also observed that ALE treatment repressed mitogen-activated kinase pathways by inhibiting the phosphorylation of extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38, suggesting that ALE is a therapeutic candidate to treat inflammatory diseases.

### ARTICLE HISTORY

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### KEYWORDS

Inflammation; *Artemisia montana*; cytokines; nitric oxide synthase; cyclooxygenase

## Introduction

*Artemisia* is one of the largest genera of the family Asteraceae or Compositae, consisting of 500 species; it mainly grows in the temperate regions of Asia, Europe, and North America (Abad, Bedoya, Apaza, & Bermejo, 2012; Pandey & Singh, 2017). As a folk medicine, *Artemisia* species have shown a wide range of pharmacological activities, such as antitussive, analgesic, antihypertensive, antitoxic, antiviral, antimalarial, and anti-inflammatory activities (Bora & Sharma, 2011; Tan, Zheng, & Tang, 1998).

*Artemisia afra*, one of the most popular species in South Africa, contains rich terpenes and has valuable biological activities (Afolayan & Sunmonu, 2011; Ntutela et al., 2009). The methanol extract of *A. afra* aerial parts possesses suppressive activity against microorganisms and inflammation (Bora & Sharma, 2011; van Wyk, 2008). *A. asiatica* Nakai, a common species in East Asia, has been used as a food ingredient and traditional medicine. Studies have shown that ethanol extracts of *A. asiatica* have antioxidative, anti-inflammatory, and antiviral activities (Bora & Sharma, 2011; Lim, Chung, Lee, Lee, & Suk, 2008). *A. annua*,

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one of the most famous medicinal plants in China, has been used for treating malaria (Daddy et al., 2017). The Chinese pharmaceutical chemist, Tu Youyou, a Nobel laureate, extracted artemisinin from *A. annua* leaves, and found that it possessed antimalarial activity (Miller & Su, 2011; Tu, 2011). Artemisinin has a lactone structure with an unusual peroxide bridge, and artemisinin and its endoperoxide derivatives have been widely used for the treatment of malaria (Klonis et al., 2011; Miller & Su, 2011; Tu, 2011). In addition, ethanol extracts of *A. annua* exhibited antiulcerogenic activity in ethanol- and indomethacin-induced rat ulcer models (Bora & Sharma, 2011; Foglio et al., 2002; He et al., 2009).

*A. montana* is a perennial plant, growing in woods and grassy fields in Japan, China, and Korea. *A. montana* has been used in cuisine and as a moxibustion, a traditional heat therapy in China and Korea, which involves the burning of mugwort on the body to facilitate healing. In addition, aerial parts of *A. montana* have been used in folk medicine as antihypertensive, anti-hepatotoxic, and antihaemorrhoid agents (Kim, 1996). Previous studies have shown that *A. montana* has antioxidant and anti-obesity activities (Jung et al., 2011; Kim, Kim, Chung, & Choi, 2000). However, the anti-inflammatory activity of *A. montana* has only been tested by measuring nitric oxide (NO) production following *A. montana* extract treatment (Choi, Park, Lee, & Kim, 2013). Therefore, we have more comprehensively investigated the anti-inflammatory effects of *A. montana* leaf extract (ALE) and its underlying molecular mechanisms in murine macrophage cells.

## Materials and methods

### Cell culture

RAW 264.7 cells, derived from murine macrophages, were cultured in Dulbecco's Modified Eagle's Medium (Welgene, Gyeongsan-si, Gyeongsangbuk-do, Korea) supplemented with 10% foetal bovine serum (Welgene), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, Grand Island, NY, USA) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### *A. montana* (Nakai) Pamp leaf ethanol extract (ALE)

An 80% ethanol extract of *A. montana* (Nakai) Pamp leaves was obtained from Jeju Biodiversity Research Institute (JBRI) (Code No: JBRI-10666). The extract was dissolved in dimethyl sulfoxide (DMSO; Duchefa Biochemie, Haarlem, The Netherlands) to prepare an 80 mg/mL stock solution. The stock solution was diluted to produce final concentrations ≤200 µg/mL. The final concentration of DMSO in the culture medium did not exceed 0.5%.

### Cell viability assay

RAW 264.7 cells were seeded overnight at a density of  $5 \times 10^4$  cells/well in a 96-well plate in 100 µL cell culture medium. The cells were treated with 0–200 µg/mL of ALE for 4 h, washed with cold phosphate-buffered saline (PBS), and stimulated with 1 µg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) for 20 h. Ten microliters of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) was added to each well, followed by 100 µL of 0.04 N HCl in isopropanol, 5 h later.

The optical density was measured at 540 nm to detect viable cells using an EMax<sup>®</sup> microplate reader (Molecular Devices, Sunnyvale, CA, USA).

### Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

RAW 264.7 cells were treated with ALE and LPS, and lysed using RNAiso (TAKARA BIO INC., Otsu, Shiga, Japan). Total RNA was then extracted and reverse transcribed into complementary DNA (cDNA) using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). TOPreal<sup>™</sup> qPCR 2X Premix (Enzynomics, Daejeon, Korea) and a CFX Connect<sup>™</sup> Real-Time PCR Detection System (Bio-Rad Laboratories, San Francisco, CA, USA) were used to perform qPCR as directed by the manufacturer's instructions. The sequences of primers used for qPCR are listed in Table 1.

### Measurement of NO production

RAW 264.7 cells were treated with ALE for 4 h, washed with cold PBS, and stimulated with LPS for 20 h. Fifty microliters of culture supernatant was then collected and mixed with 50  $\mu$ L of Griess reagent (Sigma-Aldrich). The optical density was measured at 540 nm, and NO production was quantified using sodium nitrite (NaNO<sub>2</sub>; Junsei Chemical CO., Chuo-ko, Tokyo, Japan) to generate a nitrite standard reference curve.

### Enzyme-linked immunosorbent assay (ELISA)

A 96-well immunoplate was coated with purified rat anti-mouse interleukin-6 (IL-6) antibody (Ab; eBioscience, San Diego, CA, USA) or purified Armenian hamster anti-mouse/rat tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) Ab (eBioscience) at 4°C overnight. The wells were rinsed with PBST (PBS with 0.05% Tween<sup>®</sup> 20), blocked with PBS containing 3% bovine serum albumin (BSA; MP Biomedicals, Solon OH, USA) at 25°C for 2 h, and washed with PBST. After washing, cytokine standards and samples were loaded into each well, incubated at 4°C overnight, and washed with PBST. Biotinylated anti-mouse IL-6 Ab (BD Biosciences) or biotinylated anti-mouse TNF- $\alpha$  Ab (eBioscience) were added to each well, incubated for 45 min, and washed with PBST. Streptavidin-conjugated AKP (alkaline phosphatase; BD Biosciences, San Diego, USA) was added for 30 min, followed by washing and incubation with phosphatase substrate, p-nitrophenyl phosphate (Sigma-Aldrich). The optical density was measured at 405 nm. The level of each cytokine was quantified using a standard curve generated with recombinant murine TNF- $\alpha$  (Peprotech, Rocky Hill, NJ, USA) or IL-6 (Peprotech).

The production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) was measured using a PGE<sub>2</sub> ELISA kit (Cayman Chemical, Ann Arbor, MI, USA), following the manufacturer's instructions.

**Table 1.** The list of primers used for quantitative PCR.

| Gene name (Accession No.)                      | Direction | Sequence (5'–3')         |
|------------------------------------------------|-----------|--------------------------|
| <b><math>\beta</math>-Actin</b><br>(NM_007393) | Forward   | ACCCACACTGTGCCCATCTAC    |
|                                                | Reverse   | GCCATCTCCTGCTCGAAGTC     |
| <b>iNOS</b><br>(NM_010927)                     | Forward   | AGACGGATAGGCAGAGATTGG    |
|                                                | Reverse   | ACTGACACTTCGGACAAAGC     |
| <b>COX-2</b><br>(NM_011198)                    | Forward   | AAGAAGAAAGTTCATTCTGATCCC |
|                                                | Reverse   | TGACTGTGGGAGGATACATCTCTC |

## Western blotting

RAW 264.7 cells were seeded at a density of  $1 \times 10^7$  cells/mL per well in a 6-well plate, pretreated with ALE, and stimulated with LPS for the indicated length of time. The cells were then lysed and protein was extracted using a RIPA lysis and extraction buffer (Thermo Fisher Scientific, Rockford, IL, USA). Protein concentration was determined by using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), and the western blotting protocol was continued as previously described (Kim, Jeong, Lee, & Min, 2017). Each membrane was incubated with appropriate primary antibodies. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Bio-Rad) was used as a secondary antibody, and West-Q Femto Clean ECL Solution (Gen Depot, Barker, TX, USA) was used as a substrate. To detect and quantify protein bands on the membranes, a ChemiDoc™ XRS densitometer and Quantity One software (Bio-Rad) were used. The primary antibodies that were used detected: inducible NO synthase (iNOS; Santa Cruz Biotechnology, Dallas, TX, USA), cyclooxygenase-2 (COX-2; Bioss Antibodies, Woburn, MA, USA),  $\beta$ -tubulin (Cell Signalling, Danvers, MA, USA), extracellular signal-regulated kinase (ERK, Cell Signalling), phosphorylated ERK (p-ERK, Cell Signalling), c-Jun N-terminal kinase (JNK, Cell Signalling), phosphorylated JNK (p-JNK, Cell Signalling), p38 (Cell Signalling), phosphorylated p38 (p-p38, Cell Signalling), inhibitor of  $\kappa$ Ba (I $\kappa$ Ba, Cell Signalling), phosphorylated I $\kappa$ Ba (p-I $\kappa$ Ba; Thermo Fisher Scientific), signal transducer and activator of transcription 3 (STAT-3, Cell Signalling), and phosphorylated STAT-3 (p-STAT-3, Cell Signalling).

## Statistical analysis

All data are shown as means  $\pm$  standard deviations (S.D). Experiments were repeated four to six times, and representative images were chosen. Difference between treatments was tested using a one-way analysis of variance (ANOVA) followed by Tukey's *post-hock* test.  $p < .05$  was considered statistically significant.

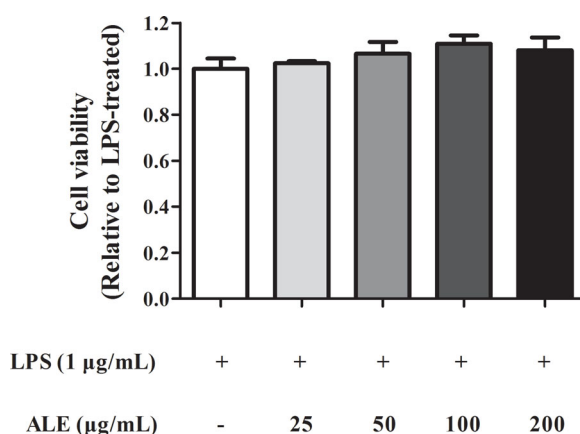
## Results and discussion

### Cytotoxicity assay of ALE

An MTT assay was performed to determine the cytotoxicity of ALE in RAW 264.7 cells. As shown in Figure 1, ALE did not show cytotoxic effects at concentrations up to 200  $\mu$ g/mL, compared to that of DMSO controls. Thus, the anti-inflammatory effects of ALE were subsequently evaluated at concentrations  $\leq 200$   $\mu$ g/mL.

### Inhibition of iNOS expression and NO production

Upon microbial infection or inflammation, activated macrophages produce iNOS leading to a massive generation of NO, a major inflammatory mediator (Bogdan, Röllinghoff, & Diefenbach, 2000; Suleria, Addepalli, Masci, Gobe, & Osborne, 2017). To test the anti-inflammatory effects of ALE, iNOS expression was measured using RT-qPCR and western blot. The results show that ALE significantly reduced the expression of iNOS both at the mRNA and protein levels in RAW 264.7 cells upon LPS stimulation (Figure 2A



**Figure 1.** Cytotoxicity of ALE. RAW 264.7 cells were treated with 25, 50, 100, or 200 µg/mL of ALE for 4 h and stimulated with LPS (1 µg/mL) for 20 h. Cell viability was measured by MTT assay and plotted as fold changes relative to LPS-treated cells (white bar), which was arbitrarily set to a value of 1.0. The data are expressed as means  $\pm$  S.D. ( $n = 4$ ).

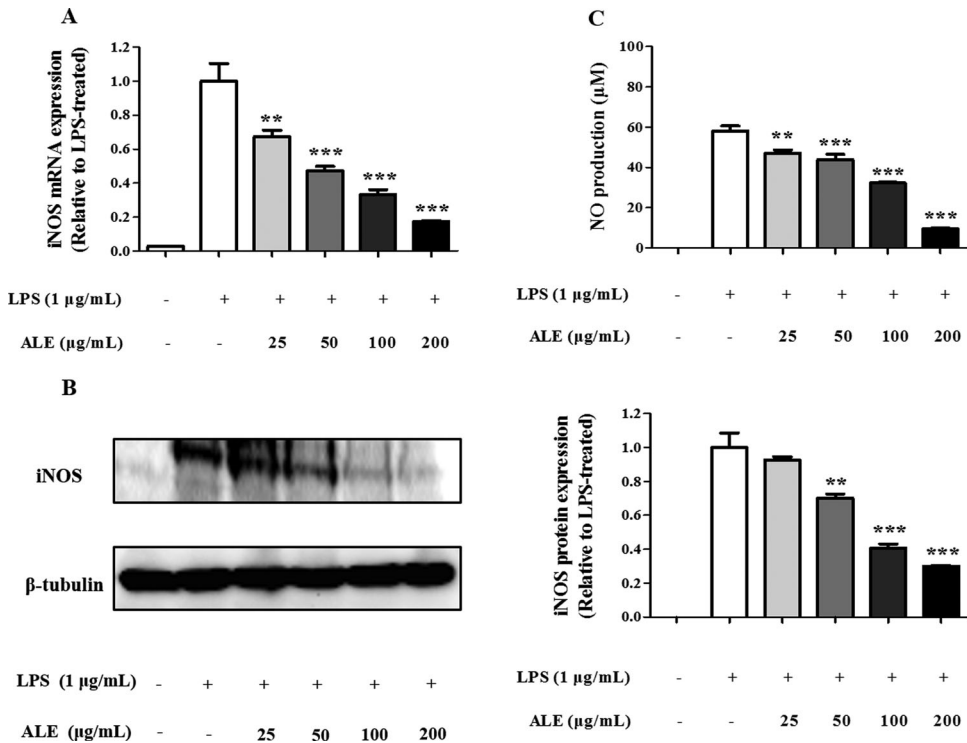
and B). In addition, ALE treatment decreased the production of NO, a major mediator and indicator of inflammation, in LPS-stimulated cells in a concentration-dependent manner (Figure 2C).

A previous study indicated that methanol extracts of various *Artemisia* species, such as *A. stolonifera*, *A. selengensis*, *A. capillaris*, and *A. keiskeana*, decreased NO production in LPS-stimulated RAW 264.7 cells (Choi et al., 2013). However, a methanol extract of *A. montana* did not affect NO production, while ALE, an ethanol extract of *A. montana* leaves, exerted a significant inhibitory effect on NO production, even at 25 µg/mL, suggesting that differences in extraction solvent and plant parts result in distinct biological activities.

### **Inhibition of COX-2 expression and PGE<sub>2</sub> production**

In addition to NO, PGE<sub>2</sub> is another inflammatory mediator that induces fever and vasodilation. PGE<sub>2</sub> is synthesized by sequential oxygenation of arachidonic acid by cyclooxygenases (COX-1 and COX-2) and prostaglandin E synthase during inflammation (Ahn et al., 2015; Echizen, Hirose, Maeda, & Oshima, 2016; Yu, Lao, & Zheng, 2016). As shown in Figure 3, ALE remarkably reduced the expression of COX-2 in LPS-stimulated macrophages at both mRNA and protein levels in a concentration-dependent manner. In accordance with the decreased COX-2 expression, the level of PGE<sub>2</sub> (PGE<sub>2</sub>) produced by COX-2 decreased from  $631.0 \pm 70.53$  pg/mL to  $248.33 \pm 47.65$  pg/mL after treatment with 200 µg/mL ALE.

The decrease in PGE<sub>2</sub> production was also observed with a methanol extract of a leaf mixture of *A. argyi* and *A. princeps*, an ethanol extract of *A. asiatica* Nakai, and a supercritical carbon dioxide extract of *A. annua*. These findings support the prevailing anti-inflammatory activity of *Artemisia* species, including *A. montana*, through the suppression of PGE<sub>2</sub> production (Hunt, Yoshida, Davis, Greenhill, & Davis, 2015; Jeong et al., 2014; Yun et al., 2016).



**Figure 2.** Decrease in iNOS expression and NO production. RAW 264.7 cells were treated with 25, 50, 100, or 200 µg/mL of ALE for 4 h and stimulated with LPS (1 µg/mL) for 20 h. Total RNA and protein were extracted from the cells and analysed for iNOS mRNA (A) and protein (B) expression, respectively. The cell culture media was also collected for measuring NO using the Griess reaction (C). Representative western blots are shown in part (B). The data are expressed as means  $\pm$  S.D. ( $n = 4$ ). \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$  compared to LPS-treated cells.

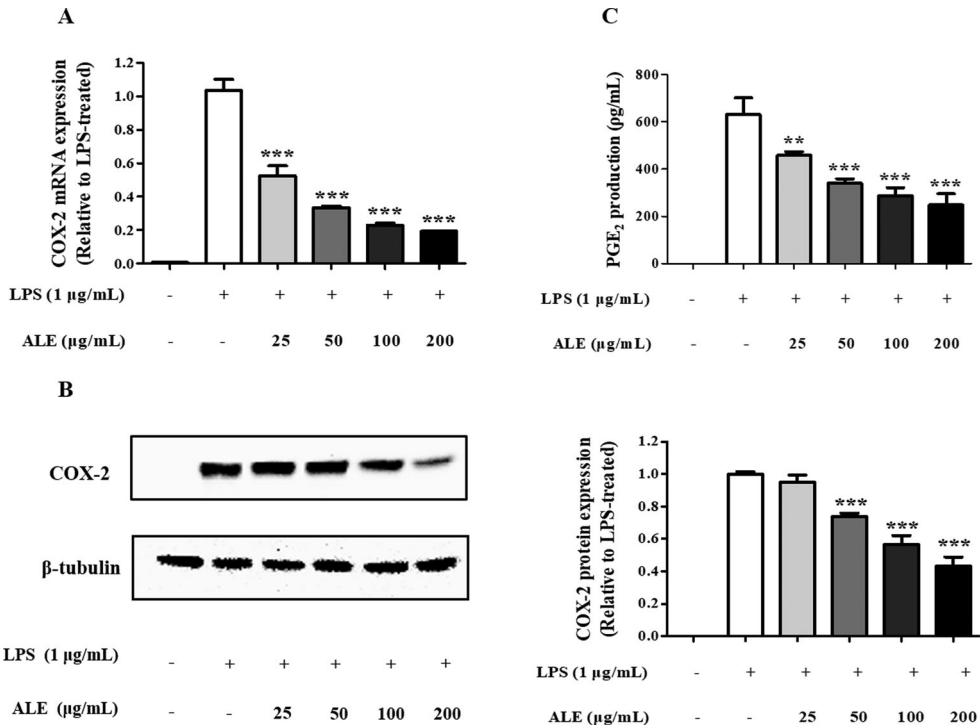
### Inhibition of proinflammatory cytokine production

One of the hallmarks of an inflammatory response is the production of proinflammatory cytokines, such as TNF- $\alpha$  and IL-6, by macrophages (Im et al., 2016). Similarly, LPS treatment was shown previously to stimulate the production of TNF- $\alpha$  and IL-6 in RAW 264.7 cells (Wu & Schauss, 2012). In order to confirm the anti-inflammatory effects of ALE, the secretion of TNF- $\alpha$  and IL-6 was measured by ELISA. As shown in Figure 4, LPS treatment induced the secretion of TNF- $\alpha$  and IL-6. However, the induction of these pro-inflammatory cytokines was repressed by ALE treatment in a concentration-dependent manner, which has been detected in other *Artemisia* species, such as *A. argyi*, *A. princeps*, *A. absinthium*, and *A. asiatica* (Jeong et al., 2014; Krebs, Omer, & Omer, 2010; Yun et al., 2016).

### Effects of ALE on mitogen-activated kinase (MAPK) signalling pathways

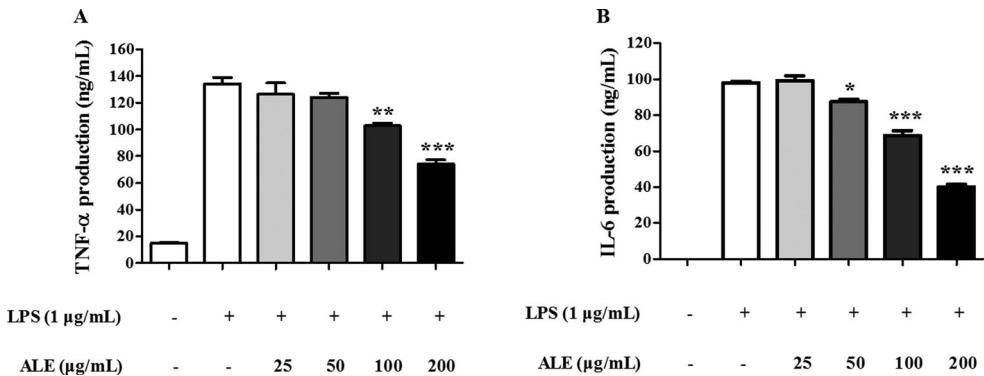
To investigate the effects of ALE on intracellular signalling pathways, the phosphorylation of MAPKs, including ERK, JNK, and p38 was assessed in ALE-treated RAW 264.7 cells. In addition, a downstream transcription factor, STAT-3, and I $\kappa$ B $\alpha$ , which mediates the





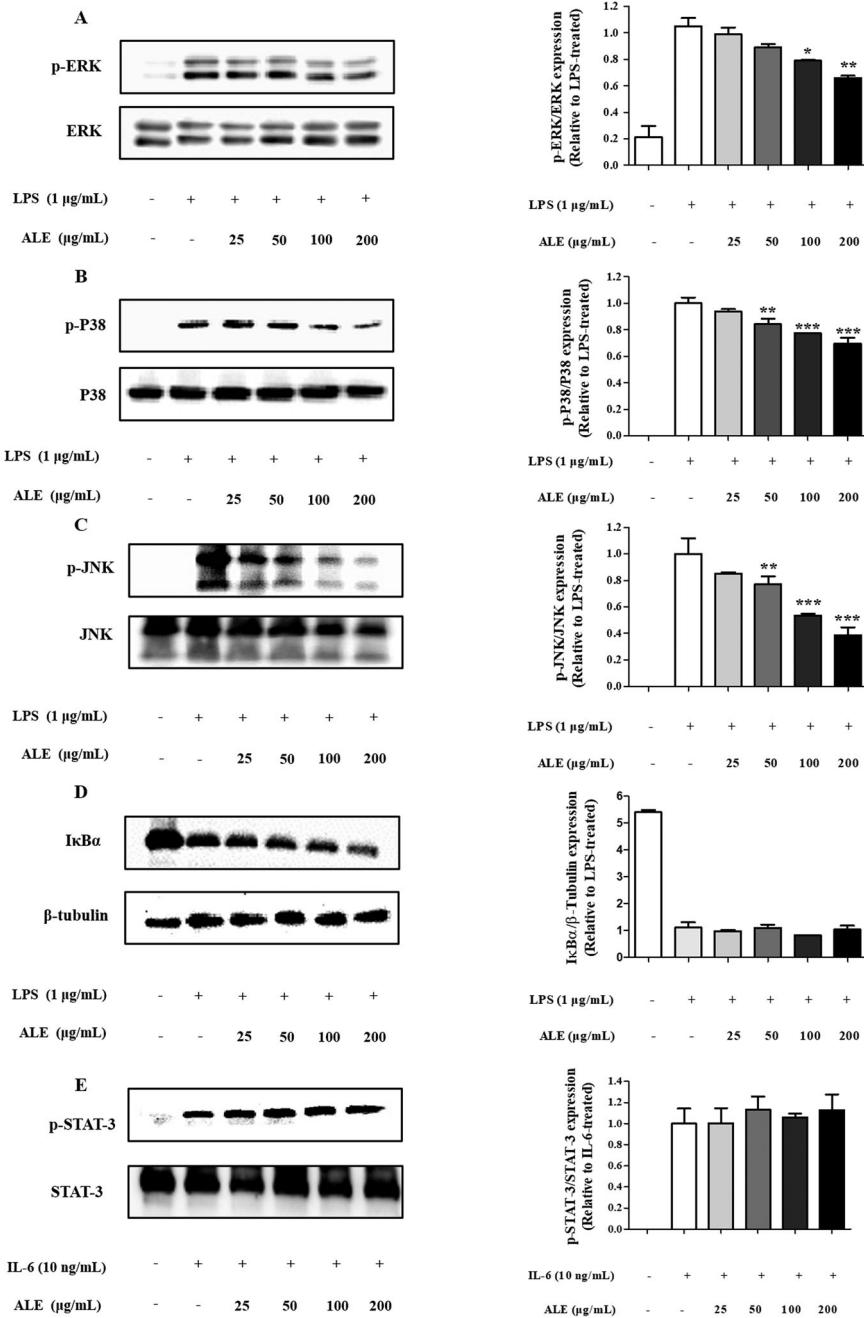
**Figure 3.** Decrease in COX-2 expression and PGE<sub>2</sub> production. ALE suppressed the expression of COX-2 at the mRNA (A) and protein (B) levels, and the production of PGE<sub>2</sub> (C). PGE<sub>2</sub> present in the cell culture media was measured by ELISA. Representative western blots are shown in part (B). The data are expressed as means ± S.D. (n = 4). \*p < .05, \*\*p < .01, \*\*\*p < .001 compared to LPS-treated cells.

nuclear factor-κB (NF-κB) signalling pathway, were evaluated in ALE-treated RAW 264.7 cells. As shown in Figure 5A–C, the phosphorylation of ERK, p38, and JNK was decreased by ALE treatment in response to LPS stimulation. However, ALE treatment had no effect



**Figure 4.** The inhibitory effect of ALE on cytokine production. RAW 264.7 cells were treated with 25, 50, 100, or 200 µg/mL of ALE for 4 h followed by LPS stimulation. ELISAs were used to measure the production of the proinflammatory cytokines, TNF-α (A) and IL-6 (B). The data are expressed as means ± S.D. (n = 4). \*\*p < .01, \*\*\*p < .001 compared to LPS-treated cells.





**Figure 5.** The inhibitory effects of ALE on the activation of MAPK. ALE inhibited the phosphorylation of MAPK including ERK, p38, and JNK. Cells were treated with ALE for 4 h, followed by LPS stimulation (1 μg/mL) for 15 min. For STAT3 analysis, cells were treated with 10 ng/mL of IL-6 for 30 min. Total cell lysates were used, and the expression levels of ERK, p-ERK (A), p38, p-p38 (B) JNK, p-JNK (C), IκB, p-IκB (D), and STAT-3, p-STAT-3 (E) were determined by western blot analysis. p-MAPK expression was normalized to the corresponding total MAPK level. IκBα, p-IκBα, STAT-3, and p-STAT-3 expression levels were normalized to the corresponding β-tubulin level. The images shown are representative of three independent experiments with similar results. The intensity of protein bands from the western blots was quantified and expressed as bar graphs. Data are expressed as the means ± S.D. (n = 3). \*p < .05, \*\*p < .01 versus LPS-treated cells.

on the activation of I $\kappa$ B $\alpha$  and STAT-3, indicating that ALE controls inflammatory responses by inhibiting the activation of MAPKs, such as ERK, p38, and JNK.

Although various *Artemisia* species have been shown to possess anti-inflammatory activities in common, the molecular mechanisms for these effects are varied. For example, a leaf extract of *A. argyi* and *A. princeps* inhibited phosphorylation of p38 but had no effect on the NF- $\kappa$ B pathway (Yun et al., 2016). However, *A. asiatica* ethanol extract suppressed the Src/Syk/NF- $\kappa$ B and TRAF6/JNK/AP-1 signalling pathways and JNK phosphorylation without affecting p38 or ERK. In addition, an ethyl acetate extract of *A. anomala* S. Moore inhibited the activation of MAPK signalling pathways, including JNK, ERK, and p38, along with the NF- $\kappa$ B signalling pathway (Tan, Wang, Yang, & Zhang, 2014). The disparity in underlying molecular mechanisms among diverse *Artemisia* species could be due to the differences in chemical constituents. For example, artemisinin, isolated from *A. annua*, exerts anti-inflammatory effects by repressing the NF- $\kappa$ B and MAPK pathways, accompanied by a decrease in phosphorylation of p38 and ERK without affecting JNK (Wang et al., 2017). Jaceosidin, which is present in *A. argyi* and leaves of *A. montana*, blocks the phosphorylation of ERK-1 and ERK-2 (Jeong, Lee, Yoon, & Lee, 2007; Kim, Lee, & Kim, 2013). Similarly, eupatilin, derived from *A. asiatica* and leaves of *A. montana*, was shown to inhibit the activation of Akt, as well as ERK-1 and ERK-2 (Kim et al., 2005; Kim et al., 2013). The combination of numerous compounds present in *Artemisia* extracts would result in anti-inflammatory activity through diverse molecular mechanisms.

In conclusion, this study shows that ALE has anti-inflammatory activity in LPS-induced RAW 264.7 cells. ALE significantly reduced the production of NO, PGE<sub>2</sub>, and proinflammatory cytokines by repressing MAPK pathways without affecting I $\kappa$ B $\alpha$  and STAT-3, suggesting a potential therapeutic use of ALE for treating inflammatory diseases.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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