

## Anti-inflammatory Effects of Ethanol Extract of Various Korean Compositae Herbs in LPS-induced RAW 264.7 Macrophages

Min-gyu Seo<sup>1#</sup>, Yun-Mi Kang<sup>1#</sup>, Kyung-Sook Chung<sup>2</sup>, Se-Yun Cheon<sup>1</sup>,  
Jong Hyuk Park<sup>3</sup>, Young-Cheol Lee<sup>4</sup> and Hyo-Jin An<sup>1\*</sup>

1 : Department of Pharmacology, College of Korean Medicine, Sangji University, 83 Sangjidae-gil, Wonju-si, Gangwon-do 26339, Republic of Korea

2 : Catholic Precision Medicine Research Center, College of Medicine, The Catholic University of Korea, 222, Banpo-daero, Seocho-gu, Seoul, 06591, Republic of Korea

3 : Institute of Natural Cosmetic Industry for Namwon, Jeollabuk-do, 55801, Republic of Korea

4 : Department of Herbol, College of Korean Medicine, Sangji University, Wonju-si, Republic of Korea

### ABSTRACT

**Objective** : This study was designed to evaluate candidate materials as anti-inflammation agent from extracts of various Korean Compositae herbs in Hwaak mountain. Among Korea medicinal herbs, *Ainsliaea acerifolia* (AA) belongs to the Compositae family, has been used for the treatment of rheumatic arthritis. However, AA has not been previously reported to have an anti-inflammatory effect. Therefore, we investigated the anti-inflammatory effects of AA and its underlying molecular mechanisms in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages.

**Methods** : Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in RAW 264.7 macrophages. Nitric oxide (NO) was measured with Griess reagent and pro-inflammatory cytokines were detected by enzyme immunoassay (EIA) kits in LPS-stimulated RAW 264.7 macrophages. Protein expressions of inducible nitric oxide synthase, and cyclooxygenase-2 (COX-2) and p65 subunit of nuclear factor- $\kappa$ B (NF- $\kappa$ B) were determined by Western blot analysis.

**Results** : Among 8 extracts of Korean Compositae herbs tested, AA showed the inhibition of NO production without cytotoxicity. Consistent with the observation, AA reduced the expression levels of iNOS and COX-2 proteins in LPS-simulated RAW 264.7 macrophages in dose-dependent manner. In addition, AA inhibited the productions of TNF- $\alpha$  and IL-6 in LPS-simulated RAW 264.7 macrophages. However, AA did not inhibit activation of p65 NF- $\kappa$ B in LPS-simulated RAW 264.7 macrophages.

**Conclusion** : These results suggest that down-regulation of iNOS, COX-2 protein expression and TNF- $\alpha$  and IL-6 production by AA are responsible for its anti-inflammatory effects.

**Key words** : *Ainsliaea acerifolia* (AA), anti-inflammation, lipopolysaccharide (LPS), nitric oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2)

## I . INTRODUCTION

Inflammation is a defensive mechanism of a host to

various stimuli including cellular injury or infection with biological, chemical, or physical stimuli<sup>1)</sup>. Macrophages are key mediators of the early stages during the

\*Corresponding author : Hyo-Jin An, Department of Pharmacology, College of Korean Medicine, Sangji University, 83 Sangjidae-gil, Wonju-si, Gangwon-do, 26339, Republic of Korea.

· Tel : +82-33-738-7503 · Fax: +82-33-730-0679 · E-mail : hjan@sj.ac.kr

#First author : Min-gyu Seo, Department of Pharmacology, College of Korean Medicine, Sangji University, 83 Sangjidae-gil, Wonju-si, Gangwon-do, 26339, Republic of Korea.

· Tel : +82-33-738-7503 · Fax: +82-33-730-0679 · E-mail : namusup00@naver.com#Co-first author : Min-gyu Seo, Yun-Mi Kang, Department of Pharmacology, College of Korean Medicine, Sangji University, 83 Sangjidae-gil, Wonju-si, Gangwon-do, 26339, Republic of Korea.

· Tel : +82-33-738-7503 · Fax: +82-33-730-0679 · E-mail : yunmi6115@naver.com

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inflammatory process and their activation by bacterial products such as lipopolysaccharide (LPS) promotes the synthesis and release of nitric oxide (NO), pro-inflammatory cytokines, eicosanoids and bioactive lipids, all of them intermediaries involved in the inflammatory onset<sup>2)</sup>.

LPS is the main outer membrane component of gram-negative bacteria and plays a key role during severe gram-negative infection, sepsis, and shock. The most prominent LPS-sensitive cell population are cells of the monocyte/macrophages lineage<sup>3)</sup>. RAW 264.7 was found to be uniquely efficient for measuring infection-related pro-inflammatory mediators. It is a good model to study inflammatory responses, which can be activated by LPS and trigger the production of inflammatory mediators, such as leukotrienes, tumor necrosis factor alpha (TNF- $\alpha$ ), interleukins (ILs), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2)<sup>4)</sup>. These inflammatory mediators can be regulated by activation of the transcription factors<sup>5)</sup>.

Medicinal herbs have been used as therapeutics for centuries throughout the world. Phytochemicals derived from these medicinal plants are used to treat various neurological and immunological disorders<sup>6)</sup>. Korean medicinal herbs for alternative or complementary medicine are an important part of the culture and traditions of Korean populations. Most of the population in Korea have been reliant on herbal medicines for their health care needs. Many studies reported that Korean medicinal herbs possess various pharmacological effects, including immune-enhancing<sup>7)</sup>, anti-diabetic<sup>8)</sup> and anti-oxidant activities<sup>9)</sup>. Among Korea medicinal herbs, *Ainsliaea acerifolia* (AA) Sch.Bip. belongs to the Compositae family, has been used for the treatment of rheumatic arthritis and enteritis in the oriental folk medicine<sup>10)</sup>. It has been reported that sesquiterpenoids which are characteristic constituents of the *Ainsliaea* genus possess diverse biological activities, including cytotoxic, antiviral, antibacterial, and anti-inflammatory activities<sup>11)</sup>. Nonetheless, AA has not been previously reported to have an anti-inflammatory effect. In the present study, as a part of our screening project to evaluate the anti-inflammatory potentials of various Korean Compositae herbs from Hwaak mountain, we investigated the anti-inflammatory effect of AA in RAW 264.7 macrophage cell line, which can be stimulated with LPS to mimic the condition of inflammation.

## II. MATERIALS AND METHODS

### 1. Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal

bovine serum (FBS), penicillin, and streptomycin were purchased from Life Technologies Inc. (Grand Island, NY, USA). LPS (*Escherichia coli*, serotype 055:B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N6-(1-Iminoethyl)lysine (NIL), NS-398, and Griess reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). iNOS, COX-2 and  $\beta$ -actin monoclonal antibodies were purchased Santa Cruz Biotechnology (Santa Cruz, CA, USA). NF- $\kappa$ B and PARP antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The enzyme immunoassay kits for TNF- $\alpha$  and IL-6 were obtained from R&D Systems (Minneapolis, MN, USA).

### 2. Preparation of Ethanol Extracts of Various Korean Compositae Herbs

Extracts of various Korean Compositae herbs in Hwaak mountain were obtained from Institute of Natural Cosmetic Industry for Namwon (Namwon, Jeollabuk-do, Republic of Korea). The dried and powdered 8 plant materials were extracted with 95% EtOH three times by maceration. The extracts were evaporated *in vacuo* at 40°C and were filtered, freeze-dried *in vacuo*. The freeze-dried samples were dissolved in DMSO with the final concentration of 50 mg/ml for bioassays.

### 3. Cell Culture

The RAW 264.7 macrophage cell line was obtained from Korea Cell Line Bank (KCLB, Seoul, Republic of Korea). The cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml) in a 37 °C and 5% CO<sub>2</sub> incubator.

### 4. MTT Assay for Cell Viability

Cell viability was assessed using the MTT assay. Briefly, AA-treated cells were incubated for 24 h, and then cells were incubated with MTT solution (5 mg/ml) for 4 h at 37 °C. After washing out the supernatant, the insoluble formazan product was dissolved in DMSO. Cell viability was measured at 570 nm using an automatic microplate reader (Titertek Multiskan, Huntsville, AL, USA).

### 5. NO Assay

NO content was determined indirectly by assaying the culture supernatants for nitrite using the Griess reagent (1% sulfanilamide in 5% phosphoric acid, 1%  $\alpha$ -

naphthylamide in H<sub>2</sub>O). NO production from RAW 264.7 macrophages was a form of NO<sub>2</sub> that exists in culture media. A 50  $\mu$ l amount of cell culture media was mixed with 50  $\mu$ l of Griess reagent in a 96-well plate, incubated at room temperature for 15 min, and then measured at 540 nm using an automatic microplate reader (Titertek Multiskan).

## 6. TNF- $\alpha$ and IL-6 Assays

Culture media were collected about 24 h after treatment with AA and then stored at -70°C. The levels of TNF- $\alpha$  and IL-6 were measured by enzyme immunoassay (EIA) kits according to the manufacturer's instructions.

## 7. Preparation of Nuclear Protein Extraction

The cells were treated with AA for 1 h prior to the addition of LPS (1  $\mu$ g/ml) for 1 h and washed with cold PBS. Nuclear extracts of the cells were prepared as described previously<sup>12)</sup>.

## 8. Western Blot Analysis

The cells were resuspended in a commercial lysis buffer (PRO-PREP, Intron Biotechnology, Seoul, Republic of Korea) and incubated for 20 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Aliquots of each protein sample (30  $\mu$ g) were separated on a sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane. Membranes were incubated for 1 h with 5% skim milk at room temperature, followed by incubation overnight with a 1:1000 dilution of primary antibody at 4°C. Blots were washed three times with Tween 20/Tris-buffered saline (T/TBS) and incubated with a 1:2500 dilution of horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Blots were again washed three times with T/TBS and then developed by enhanced chemiluminescence (GE Healthcare, Waukesha, WI, USA).

## 9. Statistical Analysis

Data are expressed as the mean  $\pm$  SD of triplicate experiments. Statistically significant values were compared using ANOVA and Dunnett's post hoc test, and *p*-values less than 0.05 were considered statistically significant.

# III. RESULTS

## 1. Anti-inflammatory Effects of Ethanol Extracts from Various Korean Compositae Herbs from Hwaak Mountain

To select candidate of anti-inflammation agents, we investigated the effect of various Korean Compositae herbs from Hwaak mountain on cell viability and LPS-induced NO production in RAW 264.7 macrophages. As shown in Table 1, most of the ethanol extract of herbs had no effect on the cell viability as determined by MTT assay at 500  $\mu$ g/ml except *A. stolonifera* Kom. These results suggested that ethanol extract of other 7 plants indicated nonspecific cytotoxicity in RAW 264.7 macrophages. In addition, as shown in Table 2, our data revealed that 5 plants (*S. deltooides* Nakai;SD, *L. obtusifolium* Siebold & Zucc.;LO, *A. keiskeana* Miq.;AK, *C. japonicum var. maackii* Matsum.;CJ, *Ainsliaea acerifolia* Sch.Bip.;AA) represent the IC<sub>50</sub> value in less than 200  $\mu$ g/ml and AA showed the best inhibitory effect on NO production. Considering all of these, AA was selected in Korean Compositae herbs from Hwaak mountain, and we investigated the anti-inflammatory effect of AA underlying molecular mechanisms in RAW 264.7 macrophages.

Table 1. Effect of Ethanol Extracts of Various Korea Compositae Herbs on the Cell Viability in RAW 264.7 Macrophages.

No.	Scientific name	IC <sub>50</sub> ( $\mu$ g/ml)
1	<i>Parasenecio auriculatus var. matsumurana</i> Nakai	>500
2	<i>Artemisia stolonifera</i> Kom.	458.94
3	<i>Synurus deltooides</i> Nakai	>500
4	<i>Ligustrum obtusifolium</i> Siebold & Zucc.	>500
5	<i>Artemisia dubia</i> Wall.	>500
6	<i>Artemisia keiskeana</i> Miq.	>500
7	<i>Cirsium japonicum var. maackii</i> Matsum.	>500
8	<i>Ainsliaea acerifolia</i> Sch.Bip.	>500

Each value represents the mean  $\pm$  SD (*n* = 3).

Table 2. Effect of Ethanol Extracts of Various Korea Compositae Herbs on the LPS-induced NO Production Level in RAW 264.7 Macrophages.

No.	Scientific name	Inhibition ratio(%)	IC <sub>50</sub> ( $\mu$ g/ml)
1	<i>P. auriculatus var. matsumurana</i> Nakai	17.59 $\pm$ 6.76**	>500
2	<i>A. stolonifera</i> Kom.	18.62 $\pm$ 3.23**	>500
3	<i>S. deltooides</i> Nakai	26.52 $\pm$ 4.18***	196.62
4	<i>L. obtusifolium</i> Siebold & Zucc.	19.43 $\pm$ 11.06**	172.02
5	<i>A. dubia</i> Wall.	16.81 $\pm$ 3.35**	>500
6	<i>A. keiskeana</i> Miq.	26.32 $\pm$ 3.80***	137.31
7	<i>C. japonicum var. maackii</i> Matsum.	32.68 $\pm$ 0.99***	195.42
8	<i>A. acerifolia</i> Sch.Bip.	38.93 $\pm$ 0.95***	59.42

Inhibition ratio evaluated at dose of 31.25 $\mu$ g/ml. Each value represents the mean  $\pm$  SD (*n*=3). \*\* *p* < 0.01, \*\*\* *p* < 0.001

## 2. Effect of AA on Cell Viability in RAW 264.7 Macrophages

The cell viability of AA was examined by MTT assay in RAW 264.7 macrophages. As shown in Figure 1, treatment with AA (15, 30 and 60  $\mu\text{g}/\text{mL}$ ) for 24h had nonspecific cytotoxicity in RAW 264.7 macrophages (cell viability at the highest concentration was 84.96%). Accordingly, we investigated the anti-inflammatory effects of AA with concentration 15, 30 and 60  $\mu\text{g}/\text{mL}$  in LPS-stimulated RAW 264.7 macrophages.

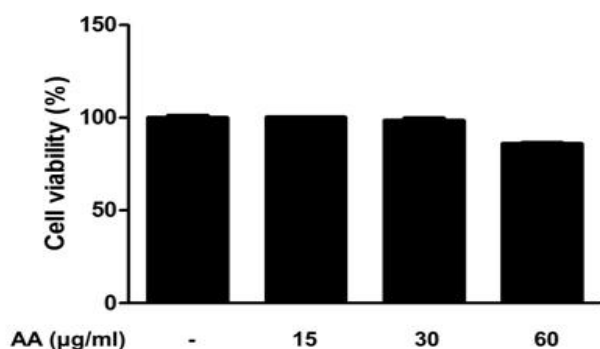


Figure 1. Effect of AA on the cell viability in RAW 264.7 macrophages. RAW 264.7 macrophages were treated with different concentrations of AA for 24 h, and their viability was determined using MTT assay. Values represent mean  $\pm$  S.D. of three independent experiments.

## 3. Effect of AA on LPS-induced NO Production in RAW 264.7 Macrophages

To determine the inhibitory effects of AA on LPS-induced NO production in RAW 264.7 macrophages, cells were treated with or without AA (15, 30 and 60  $\mu\text{g}/\text{mL}$ ) for 1h and then treated with LPS (1  $\mu\text{g}/\text{mL}$ ) for 24h. As shown in Figure 2, AA inhibited significantly the LPS-induced NO production in a concentration-dependent manner and its effect at the highest concentration is similar to NIL used as a positive control inhibitor.

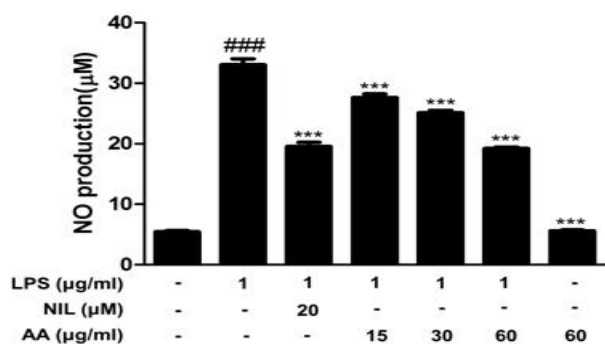


Figure 2. Effect of AA on LPS-induced NO production in RAW 264.7 macrophages. Cells were treated with different concentrations of AA for 1 h prior to the addition of LPS (1  $\mu\text{g}/\text{mL}$ ), and the cells were further incubated for 24 h. NO levels were determined with Griess reagent. NIL (20  $\mu\text{M}$ ) was used as a positive control inhibitor. The data shown represent the mean  $\pm$  SD of three independent experiments. ###  $p < 0.001$  vs the control group; \*\*\*  $p < 0.001$  vs the LPS-treated group.

## 4. Effects of AA on the LPS-induced iNOS and COX-2 Protein Expression in RAW 264.7 Macrophages

In macrophages exposed to LPS, the expression of iNOS and COX-2 is modulated, which ultimately results in overproduction of NO<sup>13</sup>. Therefore, we investigated the effect of AA on the levels of iNOS and COX-2 protein expressions by Western blot analysis. In unstimulated RAW 264.7 macrophages, the levels of iNOS and COX-2 protein expressions were a little. However, the expression levels of iNOS and COX-2 proteins were significantly up-regulated in response to LPS (1  $\mu\text{g}/\text{mL}$ ), while pretreatment with AA inhibited the expression levels of iNOS and COX-2 proteins in a dose-dependent manner.

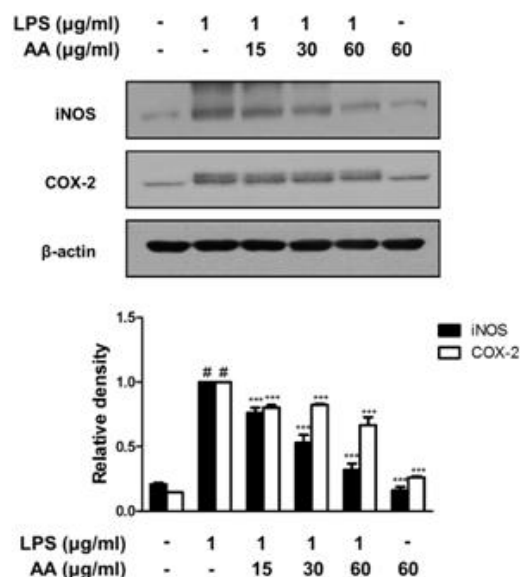


Figure 3. Effects of AA on the LPS-induced iNOS and COX-2 protein expression in RAW 264.7 macrophages. Cells were treated with 15, 30, and 60  $\mu\text{g}/\text{mL}$  AA for 1 h prior to the addition of LPS (1  $\mu\text{g}/\text{mL}$ ), and the cells were further incubated for 1 h. The protein levels of iNOS and COX-2 were determined by Western blot analysis using specific antibodies. Densitometric analysis was performed using Bio-Rad Quantity One software. The data shown represent the mean  $\pm$  SD of three independent experiments. #  $p < 0.05$  vs the control group; \*\*\*  $p < 0.001$  vs the LPS-treated group.

## 5. Effects of AA on the LPS-induced Production of Pro-inflammatory Cytokines in RAW 264.7 Macrophages

Cytokines are local protein mediators, known to be involved in biological processes, including cell growth and activation, inflammation, immunity, and differentiation. Thus, they have a role in an autoimmune disease, in which chronic inflammation<sup>14</sup>. To examine the inhibitory effects of AA on pro-inflammatory cytokine production induced by LPS, we investigated its effects on LPS-induced

TNF- $\alpha$  and IL-6 productions by using EIA kits. As shown in Figure 4, LPS significantly increased TNF- $\alpha$  and IL-6 productions ( $P < 0.001$ ), while pretreatment with AA reduced the LPS-induced these cytokines productions ( $P < 0.001$ ).

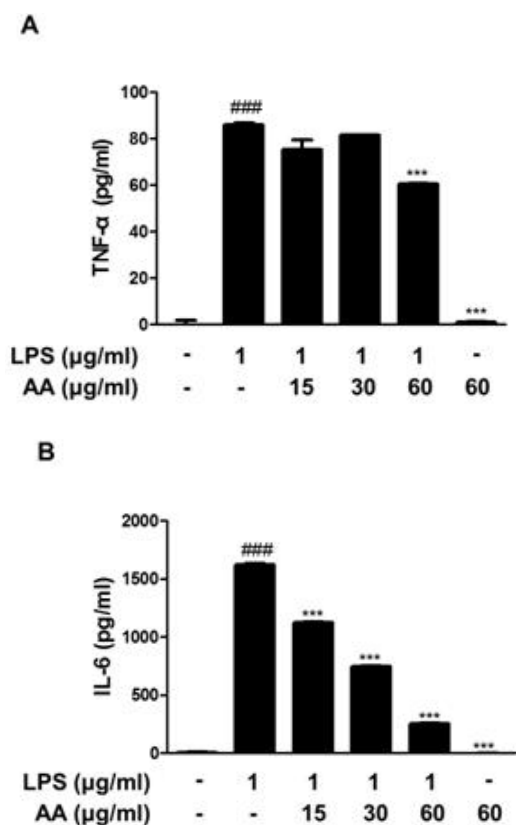


Figure 4. Effects of AA on the LPS-induced production of pro-inflammatory cytokines in RAW 264,7 macrophages. Cells were treated with 15, 30, or 60  $\mu\text{g/ml}$  AA for 1 h prior to the addition of LPS (1  $\mu\text{g/ml}$ ), and the cells were further incubated for 24 h. TNF- $\alpha$  and IL-6 levels were determined by using EIA kits. The data shown represent mean  $\pm$  SD of three independent experiments. ###  $p < 0.001$  vs the control group; \*\*\*  $p < 0.001$  vs the LPS-treated group.

## 6. Effects of AA on the LPS-induced NF- $\kappa$ B Activation and the Nuclear Translocation

Independent of the initial pro-inflammatory stimulus, cellular responses are related to gene modulation mediated by changes in the activation of transcription factors<sup>15</sup>. Because NF- $\kappa$ B is an important transcriptional factor in inflammatory response<sup>16</sup>, we examined the effect of AA on the nuclear translocation of the p65 subunit of NF- $\kappa$ B in RAW 264,7 macrophages. As shown in Figure 5, LPS induced the translocation of p65 NF- $\kappa$ B to the nucleus. However, pretreatment with AA could not suppress this process in LPS-simulated RAW 264,7 macrophages. These data suggested that the concerted action of multiple distinct transcription factors might be strongly associated with the expression

of inflammatory-related genes in AA-treated RAW 264,7 macrophages.

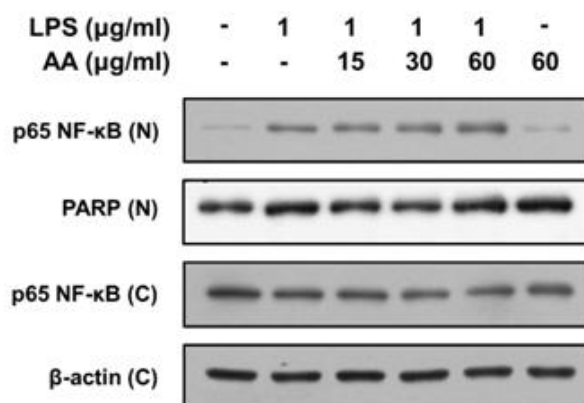


Figure 5. Effect of AA on LPS-induced NF- $\kappa$ B activation in RAW 264,7 macrophages. Cells were pretreated with AA for 1 h prior to the addition of LPS (1  $\mu\text{g/ml}$ ) for 1 h. Nuclear (N) and cytosolic (C) extracts were isolated, and the levels of p65 in each fraction were determined by Western blot analysis. PARP and  $\alpha$ -tubulin were used as internal controls.

## IV. DISCUSSION

Activated macrophages rapidly activate the expression of genes responsible for the high-output synthesis of reactive oxygen and nitrogen species and bioactive lipids derived from arachidonic acid. All of these agents contribute to the regulation of the inflammatory response<sup>17</sup>. The recognition of invading pathogens by host cells starts with the binding of specific cellular receptors to pathogen molecules with distinct patterns<sup>18</sup>.

LPS is an endotoxin, an integral outer membrane component used to assess Gram-negative bacteria, and triggers the most potent microbial initiators of inflammatory response, including septic shock, fever, and microbial invasion<sup>19</sup>. In response to LPS, macrophages synthesize and release inflammatory mediators. One of the major inflammatory mediators NO is markedly up-regulated by iNOS. Macrophages also produce pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-6<sup>20</sup>.

TNF- $\alpha$  and IL-6 are major pro-inflammatory cytokines produced by various immune cells such as macrophages, monocytes, and T cells. TNF- $\alpha$  production is crucial for the synergistic induction of NO synthesis in IFN ( $\gamma$ ) and/or LPS-stimulated macrophages. TNF- $\alpha$  elicits a number of physiological effects, such as septic shock, inflammation, cachexia, and cytotoxicity<sup>21</sup>. IL-6 contributes to a multitude of physiological and pathophysiological processes. IL-6 represents a keystone cytokine in infection, cancer and inflammation, in which it drives disease progression or supports the maintenance of immunological reactions<sup>22</sup>. Raised levels

of IL-6 have been described in a number of acute conditions such as burns, major surgery, and sepsis<sup>23</sup>. In the present study, it was found that *Ainsliaea acerifolia* Sch.Bip. (AA) is a potent inhibitor on the LPS-induced pro-inflammatory molecules, including NO, TNF- $\alpha$  and IL-6 in RAW 264.7 macrophages (Figure 2 and 4).

Pro-inflammatory mediators, iNOS and COX-2, play key roles in the pathogenesis of various acute and chronic inflammatory diseases<sup>24</sup>. iNOS and COX-2 are important enzymes that regulate inflammatory processes. NO is overproduced endogenously by iNOS which is induced in response to pro-inflammatory cytokines and LPS. COX-2 is also induced by several stimuli and is responsible for the production of large amounts of pro-inflammatory prostaglandins at the inflammatory sites<sup>25</sup>. The pharmacological blockade of LPS-inducible inflammatory mediators is an attractive therapeutic strategy for these inflammatory disease. In this study, our data indicated that AA suppressed the expression levels of iNOS and COX-2 proteins in LPS-stimulated RAW 264.7 macrophages (Figure 3).

Moreover, LPS activates TLR4, which activates MyD88- and TRIF-dependent pathways, in addition to activating NF- $\kappa$ B, phosphorylation of mitogen activated protein kinase (MAPK) and activator protein 1(AP-1)<sup>26, 27</sup>. NF- $\kappa$ B and MAPK pathways, two important inflammatory signaling pathways in macrophages, induce the expression and release of a wide range of inflammatory mediators that orchestrate the host's inflammatory responses. A number of previous studies have shown that various naturally derived substances exert anti-inflammatory effects by inhibiting the NF- $\kappa$ B and MAPK signaling pathways<sup>28</sup>. Activated NF- $\kappa$ B is translocated from the cytoplasm to the nucleus, where it binds to specific sequences in the promoter regions of many genes including iNOS, COX-2, inflammatory cytokines, and chemokines involved in immune and inflammatory responses<sup>29</sup>. However, in the study, no significant decrease was observed in the levels of translocation of p65 from cytosol to the nucleus (Figure 5). In this regard, we conclude that the NF- $\kappa$ B signaling pathway does not significantly participate in AA-mediated anti-inflammatory effect.

Instead of NF- $\kappa$ B signaling pathway, we assumed that MAPK signaling pathway or other factors have an impact on AA-mediated anti-inflammatory effect. MAPK signaling are evolutionarily conserved regulators that mediate signal transduction and play essential roles in various physiological processes. Three main families of MAPKs exist in mammalian species, grouped by their structures and functions: ERK (extracellular

signal-regulated kinase), JNK (c-Jun NH2-terminal kinases) and p38 kinase<sup>30</sup>. MAPK signaling cascades are activated by LPS through binding to its TLR4 receptor on cell membranes, leading to the regulation of inflammatory mediators<sup>31</sup>. These signaling pathways in turn mediate multiple downstream events leading to the activation of AP-1 and NF- $\kappa$ B, which coordinate the induction of a range of inflammatory proteins. Therefore, treatment aimed at inhibiting MAPKs may have potential therapeutic advantage for inflammatory diseases<sup>32</sup>. It is worth further studying whether MAPKs indeed participate in regulating LPS-induced pro-inflammatory mediators, and whether AA exhibit anti-inflammatory activity by modulating MAPK activation.

## V. CONCLUSION

The present study demonstrates that AA inhibits the LPS-induced inflammatory response in RAW 264.7 macrophages. The results showed that AA exhibited NO inhibitory activity and AA also inhibited the levels iNOS and COX-2 protein expressions in LPS-stimulated RAW 264.7 macrophages. Thus, our findings suggest that AA represent a potential bioactive source that can be considered a potent candidate treatment for inflammatory diseases.

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