Molecular Immunology 119 (2020) 123–131

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Anti-inflammatory effect of *Acalypha australis* L. via suppression of NF-κB signaling in LPS-stimulated RAW 264.7 macrophages and LPS-induced septic mice

Hyo-Jung Kim[a,1], Hae-In Joe[b,1], Zhiyun Zhang[c], Sang Woo Lee[d], Kyou-Young Lee[e], Yoon-Bum Kook[b,*], Hyo-Jin An[a,*]

[a] Department of Pharmacology, College of Korean Medicine, Sangji University, 83 Sangjidae-gil, Wonju-si, Gangwon-do 26339, Republic of Korea
[b] Department of Prescription, College of Korean Medicine, Sangji University, 83 Sangjidae-gil, Wonju-si, Gangwon-do 26339, Republic of Korea
[c] State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, the Chinese Academy of Sciences, Beijing 100093, PR China
[d] International Biological Material Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, 34141, Republic of Korea
[e] Department of Korean Ophthalmology and Otolaryngology and Dermatology, College of Korean Medicine, Sangji University, 83 Sangjidae-gil, Wonju-si, Gangwon-do 26339, Republic of Korea

ARTICLE INFO

Keywords:
Acalypha australis L.
Anti-inflammation
Lipopolysaccharide
NF-κB
RAW 264.7 macrophages
Septic shock

ABSTRACT

We evaluated the anti-inflammatory activity of methanol extracts of Chinese medicinal plants from Beijing and determined which extract was the most effective. We found the methanol extract of *Acalypha australis* L. (AAL) to be the most effective. AAL has been used for clearing heat, toxic material, and hemostasia in Chinese medicine. Although these uses are closely related to inflammation, the anti-inflammatory effect of AAL has not yet been described and its underlying mechanism remains unclear. Therefore, we aimed to identify anti-inflammatory effect of AAL and its underlying mechanism *in vitro* and *in vivo*. In RAW 264.7 macrophages, cytotoxicity was evaluated by MTT assay and nitric oxide (NO) was measured with Griess reagent. To confirm the production of pro-inflammatory cytokines and its mRNA expression, enzyme immunoassay (EIA) and quantitative real-time PCR (qRT-PCR) were performed. Further, protein expression was analyzed by western blotting. Septic shock was induced by intraperitoneal injection of LPS (25 mg/kg) in mice. One hour before LPS injection, AAL (25 and 50 mg/kg) was administered orally. In LPS-stimulated macrophages, AAL inhibited NO production at concentrations without cytotoxicity. Additionally, AAL reduced not only inducible nitric oxide synthase (iNOS) expression but the production of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) by attenuating nuclear factor-kappa B (NF-κB)-related proteins (NF-κB p65, phosphorylation of inhibitor κB-α; p-IκB-α, phosphorylation of inhibitor κB kinase-α/β; p-Ikk-α/β). Moreover, AAL enhanced the survival rate of mice through the inhibition of iNOS expression and IL-6 and interleukin-1β (IL-1β) production in LPS-induced septic mice. Furthermore, AAL also reduced the expression of NF-κB-related proteins. These finding suggest that AAL is related to the modulation of inflammatory reactions by blocking NF-κB activation in LPS-stimulated RAW 264.7 macrophages and LPS-induced septic mice.

1. Introduction

Inflammation plays a significant role in the complex immunological responses of the body, such as the removal of harmful stimulants like pathogens and damaged cells (Gatis-Carrazzoni et al., 2019). Moreover, excessive inflammation can cause multiple disorders, such as asthma, rheumatoid arthritis, and atherosclerosis (Guzik et al., 2003). Inflammation is triggered by various inflammatory cytokines and mediators, which are released from pro-inflammatory cells such as macrophages (Kim et al., 2017). Macrophages, which act as defense mechanisms against pathogens, play a crucial role in inducing host immune reactions to infection-related inflammation (Le et al., 2018). As

Abbreviations: LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β; NF-κB, nuclear factor-kappa B; p-IκB-α, phosphorylation of inhibitor κB-α; p-Ikk-α/β, phosphorylation of inhibitor κB kinase-α/β

* Corresponding Authors.
E-mail addresses: kook@sj.ac.kr (Y.-B. Kook), hjan@sj.ac.kr (H.-J. An).

[1] Hyo-Jung Kim and Hae-In Joe are equal contribution in this work.

https://doi.org/10.1016/j.molimm.2020.01.010
Received 30 September 2019; Received in revised form 23 December 2019; Accepted 16 January 2020

inflammation progresses, macrophages accumulate around the site of inflammation by invading agents (He et al., 2018b). Lipopolysaccharide (LPS), present in the external walls of gram-negative bacteria, can cause various inflammatory reactions, such as septic shock and death (Kanno et al., 2006). LPS-stimulated macrophages release cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), and mediators through the activation of the nuclear factor-kappa B (NF-κB) pathway (B. O. Cho et al., 2014). NF-κB pathway modulates inflammatory cytokine production and mediator gene expression. Under unstimulated conditions, heterodimers of NF-κB bind to inhibitor κB-α (IκB-α) and exist in an inactive form in the cytoplasm (Park et al., 2014). When the NF-κB pathway is activated by a stimulant that induces inflammation, the activation of the IκB kinase (IKK) complex induces IκB-α phosphorylation and degradation. Subsequently, the liberated subunits (p65/p50) of NF-κB enter the nucleus and induce gene expression. NF-κB pathway encodes various transcriptional genes and produces pro-inflammatory cytokines (S. H. Kim et al., 2015). Thus, targeting the NF-κB pathway is regarded as a possible therapeutic strategy against inflammatory disorders (He et al., 2018a). The overproduction of inflammatory mediators may cause life-threatening disorders, such as septic shock, which can lead to multi-organ dysfunction and death by systemic infection (Li et al., 2014). The treatment for septic shock remains a challenge despite the development of many pharmaceutical candidates. Thus, the inhibition of inflammation and septic shock is expected to possess a therapeutic potential. Chinese medicinal plants have not only been used as therapeutic materials for several disorders, but are also being actively studied to date (Le et al., 2018). In the current study, we sought to identify the anti-inflammatory activity of Chinese medicinal plant especially from Beijing and to confirm its potential in therapy. *Acalypha australis* L. (AAL), which has most inhibitory effect on the nitric oxide (NO) production in macrophage inflammatory model, belongs to the Euphorbiaceae family in Chinese medicine. The whole AAL plant is called “chul-hyun-chae”; it has been used for treating heat, dysentery, abdominal distension, diarrhea, uterus hemorrhage, eczema, and dermatitis (Seebaluck et al., 2015). Although the AAL extract has been already reported to show anti-cancer and anti-bacterial effects (Shin et al., 2013; Xiao et al., 2013), there was no evidence for its anti-inflammatory effects in LPS-stimulated RAW 264.7 macrophages. Therefore, we examined the anti-inflammatory effect of AAL in RAW 264.7 macrophages and LPS-induced septic mice.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco’s modified Eagle’s medium (DMEM), penicillin, streptomycin, and fetal bovine serum (FBS) were acquired from Life Technologies (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) was acquired from Junsei-Chemica (Tokyo, Japan). Lipopolysaccharide (Escherichia coli 055:B5), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Griess reagent, and NIL (N6-(1-Iminoethyl)lysine) were acquired from Sigma Chemical (St. Louis, MO, USA). The primary antibodies iNOS (M-19; cat. no. sc-650), IκB-α (C-15; cat. no. sc-203), IKK-α/β (H-470; cat. no. sc-7607), α-tubulin (TU-02; cat. no. sc-8035), and β-actin (ACTBD11B7; cat. no. sc-81178) were acquired from Santa Cruz (Santa Cruz, CA, USA). The primary antibodies NF-κB p65 (C22B4; cat. no. #4764), phospho-IKK-α/β (Ser176/180) (16A6; cat. no. #2697), and PARP (cat. no. #9542) were acquired from another company (Cell Signaling Technology, Danvers, MA, USA). Horseradish peroxidase-conjugated (HRP) secondary antibody was obtained from Jackson ImmunoResearch (West Grove, PA, USA). The enzyme immunoassay (EIA) kits for mouse IL-6 and TNF-α were acquired from R&D Systems (Minneapolis, MN, USA). Primers for IL-1β, IL-6, TNF-α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Bioneer Corporation (Daejeon, Republic of Korea) and PowerUp™ SYBR® green master mix was obtained from Applied Biosystem (Foster City, CA, USA). Other reagents were acquired from Sigma-Aldrich.

Table 1
Extract information for Chinese medicinal plants.

Scientific name	Whole plant (g)	Yield (g)	Accession number
Digitaria sanguinalis (L.) Scop	46 g	4.3 g	0050065
Acalypha australis L.	46 g	3.57 g	0050064
Euphorbia maculata L.	35 g	7.07 g	0050074
Cenchrus echinatus L.	65 g	5.59 g	0050068
Chenopodium album L.	70 g	5.89 g	0050079
Cerastium caespitosum Gilib.	60 g	5.46 g	0050258

Table 2
Sequences of the oligonucleotide primer.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
IL-1β	GATCCACACTCTCCAGCTGCA	CAACCAACAAGTGATATTCTCCATG
IL-6	TTCCATCCAGTTGCCTTCTTG	GGGAGTGGTATCCTCTGTGAAGTC
TNF-α	ATGAGCACAGAAAGCATGAT	TACAGGCTTGTCACTCGAAT
GAPDH	GACGGCCGCATCTTCTTGT	CACACCGACCTTCACCATTTT

Table 3
Effect of methanol extracts of Chinese medicinal plants from Beijing on the cell viability in RAW 264.7 macrophages.

NO.	Scientific name	IC_{50} (μg/ml)
1	*Digitaria sanguinalis* (L.) Scop	267.8
2	*Acalypha australis* L.	307.2
3	*Euphorbia maculata* L.	> 500
4	*Cenchrus echinatus* L.	> 500
5	*Chenopodium album* L.	68.28
6	*Cerastium caespitosum* Gilib.	135.2

Each value represents the mean ± S.D. (n = 3).

Table 4
Effect of methanol extracts of Chinese medicinal plants from Beijing on NO production in LPS-stimulated RAW 264.7 macrophages.

NO.	Scientific name	IC_{50} (μg/ml)
1	*Digitaria sanguinalis* (L.) Scop	69.7477
2	*Acalypha australis* L.	59.9759
3	*Euphorbia maculata* L.	248.3546
4	*Cenchrus echinatus* L.	451.7357
5	*Chenopodium album* L.	106.9219
6	*Cerastium caespitosum* Gilib.	143.3705

Each value represents the mean ± S.D. (n = 3).

2.2. Preparation of methanol extracts of Chinese medicinal plants

Methanol extracts of Chinese medicinal plants were acquired from International Biological Material Research Center (Daejeon, Republic of Korea), which belongs to the Korea Research Institute of Bioscience and Biotechnology (KRIBB). *Digitaria sanguinalis* (L.) Scop (DSS), *Acalypha australis* L. (AAL), *Euphorbia maculata* L. (EML), *Cenchrus echinatus* L. (CEL), *Chenopodium album* L. (CAL), and *Cerastium caespitosum* Gilib. (CCG) were collected in Zheshang Park, Wuhu city, Anhui province in China and identified by Dr. Zhiyun Zhang of the Herbarium of the Institute of Botany of the Chinese Academy of Sciences in 2012. A voucher specimen (accession number) of the retained material is preserved at the KRIBB herbarium. Whole dried and refined Chinese medicinal plants were extracted with 1 l of 99.9 % (v/v) methanol with repeated sonication (15 min) and rest (2 h) for 3 days at 45 °C. The resultant product was filtered with non-fluorescence cottons and

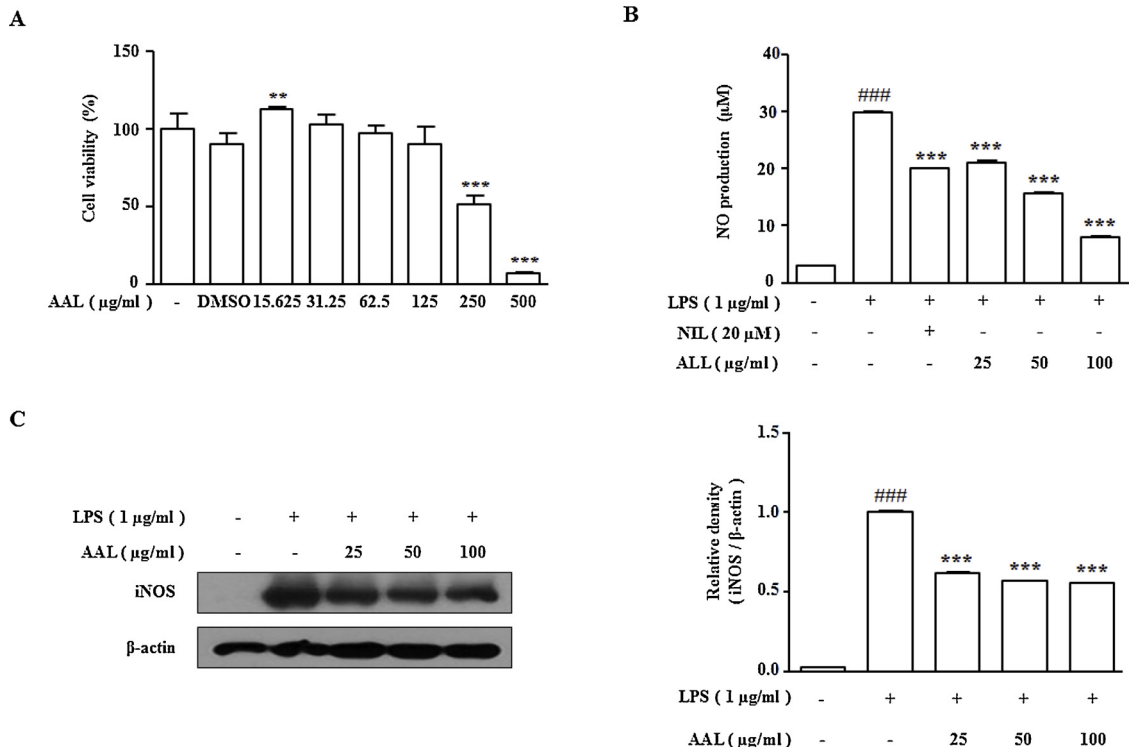

Fig. 1. Effect of AAL on NO production and iNOS expression in LPS-stimulated RAW 264.7 macrophages. (A) Cells were treated with different concentrations of AAL for 24 h, and cytotoxicity was determined using MTT assay. (B) NO production in the culture media was measured by Griess reagent. NIL (20 μM) was used as a positive control inhibitor. (C) iNOS expression was determined by western blot analysis using specific antibodies. β–actin was used as internal control. The values are represented as mean ± S.D. of three independent experiments. (A); ** $p < 0.01$, *** $p < 0.001$ vs. the DMSO-treated group, (B–C); ### $p < 0.001$ vs. the control group; *** $p < 0.001$ vs the LPS-treated group.

concentrated by a rotary evaporator (N-1000SWD, EYELA) under reduced pressure at 45 °C (Table 1). Finally, the total yields of methanol extracts were obtained by freeze-drying, and each sample was dissolved with a final concentration of 50 mg/ml in DMSO for subsequent bioassays.

2.3. Cell culture and sample treatment

RAW 264.7 macrophages were acquired from Korea Cell Line Bank (Seoul, Republic of Korea). The cell line was cultivated in adequate incubator conditions (5 % CO_2, 37 °C) with DMEM supplemented with 10 % FBS (Gibco, Big Cabin, OK, USA) and 1 % penicillin/streptomycin. *In vitro*, cells were treated with or without AAL at non-cytotoxic concentrations (25, 50, and 100 μg/ml) for 1 h and stimulated with 1 μg/ml of LPS for the appropriate time at 37 °C incubator.

2.4. MTT assay

The cytotoxicity of AAL was analyzed by MTT assay. Under stable cultivation conditions, cells were seeded at a concentration of 1×10^5 cells per well in a 96-well plate. After cultivating in medium for 24 h, the cells were treated with medium containing DMSO and AAL (15.625, 31.25, 62.5, 125, 250, and 500 μg/ml) for 24 h. Subsequently, cells were treated with a 5 mg/ml MTT solution and cultivated for 4 h at 37 °C. After removing the culture medium, DMSO dissolved insoluble formazan. We analyzed the absorbance of formazan with an Epoch™ microplate spectrophotometer from BioTek (Winooski, VT, USA) at 570 nm.

2.5. NO assay

Cells were seeded at a concentration of 2×10^4 cells per well in 24-well plate, and treated with 20 μM of NIL and 25, 50, and 100 μg/ml of AAL for 1 h and stimulated with 1 μg/ml of LPS in for 48 h at 37 °C. The culture media were obtained from each well and NO concentrations were estimated using Griess reagent. Culture medium (50 μl) was mixed and incubated with Griess reagent (50 μl) for 15 min at 18 − 25 °C and assessed with an Epoch™ microplate spectrophotometer from BioTek at 540 nm.

2.6. EIA assay

IL-6 and TNF-α production were confirmed using EIA kits (R&D Systems, Minneapolis, MN, USA). RAW 264.7 macrophages were seeded in a 6-well plate at a concentration of 4×10^5 cells per well. Cells were treated with 25, 50, and 100 μg/ml of AAL for 1 h prior to the addition of LPS (1 μg/ml), and cells were further incubated for 24 h. Supernatants of culture media were obtained and measured for IL-6 and TNF-α production with an Epoch™ microplate spectrophotometer from BioTek.

2.7. Preparation of nuclear protein extraction

Nuclear extracts of the cells were prepared as follows. Cells (2×10^6 per well) were treated with AAL for 1 h before adding 1 μg/ml LPS, and cultivated for 30 min and washed with cold PBS. The nucleus extracts from the liver were obtained using NE-PER® Reagents (Thermo, Rockford, IL, USA).

2.8. Western blotting

Cells or liver tissues were homogenized and resuspended with PRO-PREP™ (iNtRON Biotechnology Inc., Gyeonggi-do, Republic of Korea) and maintained for 15 min at 4 °C. Cell debris was eliminated by micro-

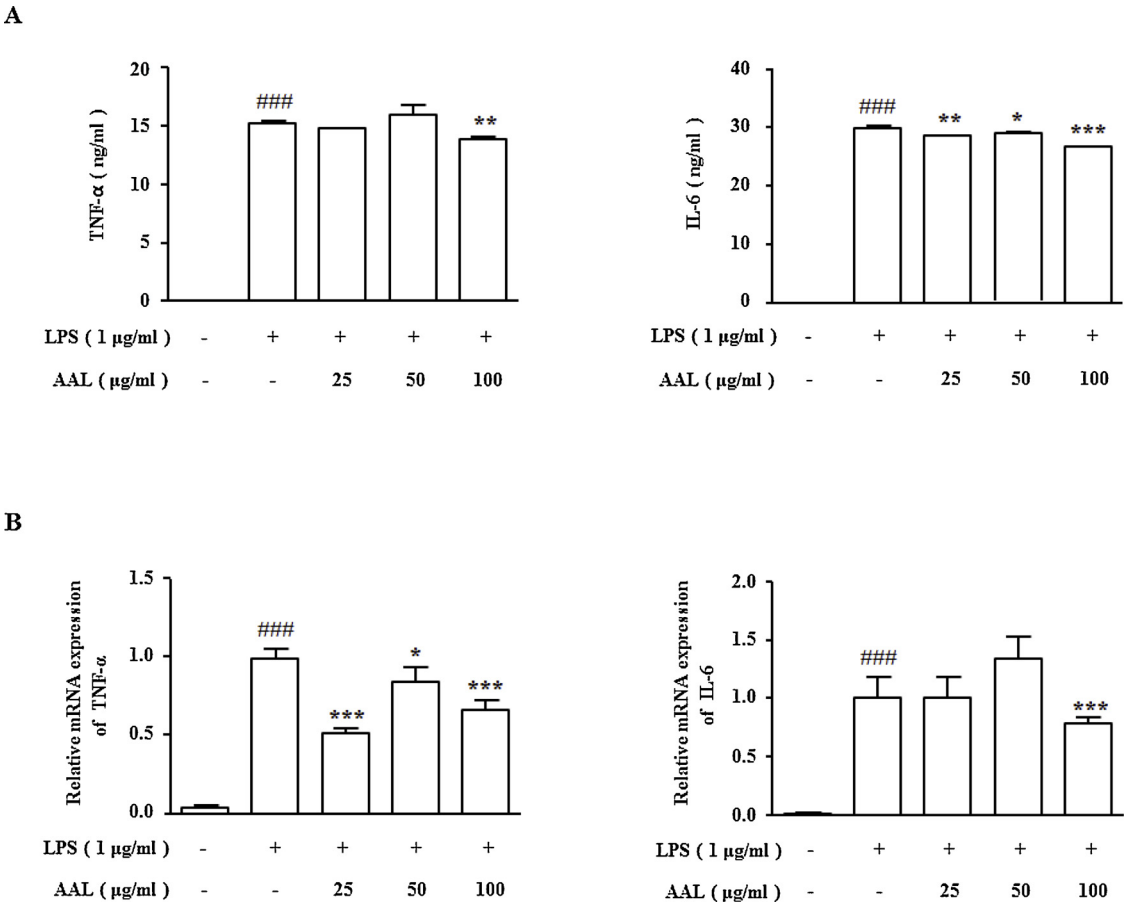

Fig. 2. Effect of AAL on production and mRNA expression of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages. The production of TNF-α and IL-6 (A) were detected in the culture media by using EIA kits. Detailed manner was described in material and method. The mRNA expression of TNF-α and IL-6 (B) were analyzed by qRT-PCR. Cells were treated with 25, 50, and 100 μg/ml AAL for 1 h prior to the addition of LPS (1 μg/ml), and the cells were further incubated for 6 h. The data shows quantification results normalized to GAPDH. The data are represented as mean ± S.D. of three independent experiments. $^{###}$ $p < 0.001$ vs. the control group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the LPS-treated group.

centrifugation (15,920 ×*g*, 30 min, 4 °C). Following the manufacturer's instructions, Bio-Rad dye reagent (Bio-Rad, Hercules, CA, USA) was used to examine protein concentrations. Each protein sample was separated on an 8–10 % poly-acrylamide gel and transmitted to a polyvinylidene fluoride (PVDF) membrane. Membranes were covered with 2.5 % or 5 % skim milk (30 min, 18 − 25 °C), followed by an overnight incubation at 4 °C with 1/1000 diluted primary antibody. Tween 20/tris-buffered saline (T/TBS) was used to wash the membranes three times followed by incubation for 2 h with a 1/2500 dilution of the secondary antibody (18 − 25 °C). Membranes were washed again three times and then detected using a WB detection kit, Ab Signal (AbClon, Seoul, Republic of Korea), in a dark room.

2.9. Quantitative real-time PCR analysis

After homogenizing the cells or liver tissue, an easy-BLUE™ kit (iNtRON Biotechnology, Inc.) was used to isolate total RNA. An Epoch® micro-volume Spectrophotometer system was procured from BioTek and used to quantify total RNA. Additionally, cDNA was obtained by synthesis from extracted RNA. The expression of relative genes with PowerUp™ SYBR® green master mix was measured by using a Real Time PCR System 7500 (Applied Biosystems, Foster City, CA, USA). The sequences of oligonucleotide primers (IL-1β, IL-6, TNF-α, GAPDH; mouse) are described in Table 2. A house-keeping gene, GAPDH, was used to express the results as the ratio of optimal density.

2.10. Experimental animals

Six-week-old male C57BL/6 mice (20 g) were acquired from Daehan Biolink CO. (Eumsung, Republic of Korea). Six mice were allocated into four groups and adapted under steady conditions (light cycle, 12 h; temperature, 20 − 25 °C; humidity, 40–60 %). Before starting the study, all experimental procedures were cleared by the Institutional Animal Care and Use Committee (IACUC) of Sangji University (approbation number #2018-9). The mice were administered AAL (25 and 50 mg/kg) orally for 1 h and injected intraperitoneally with PBS or 25 mg/kg LPS dissolved with PBS. After administration, the survival rate of mice was observed for 72 h. Liver samples were obtained 4 h after injection and immediately frozen (− 80 °C).

2.11. Statistical analysis

Each result was tested in triplicate and visualized as the mean ± S.D. Significance was compared by Dunnett's post hoc test for ANOVA using GraphPad Prism software (version 5.01) and p-values < 0.05 were considered to be statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

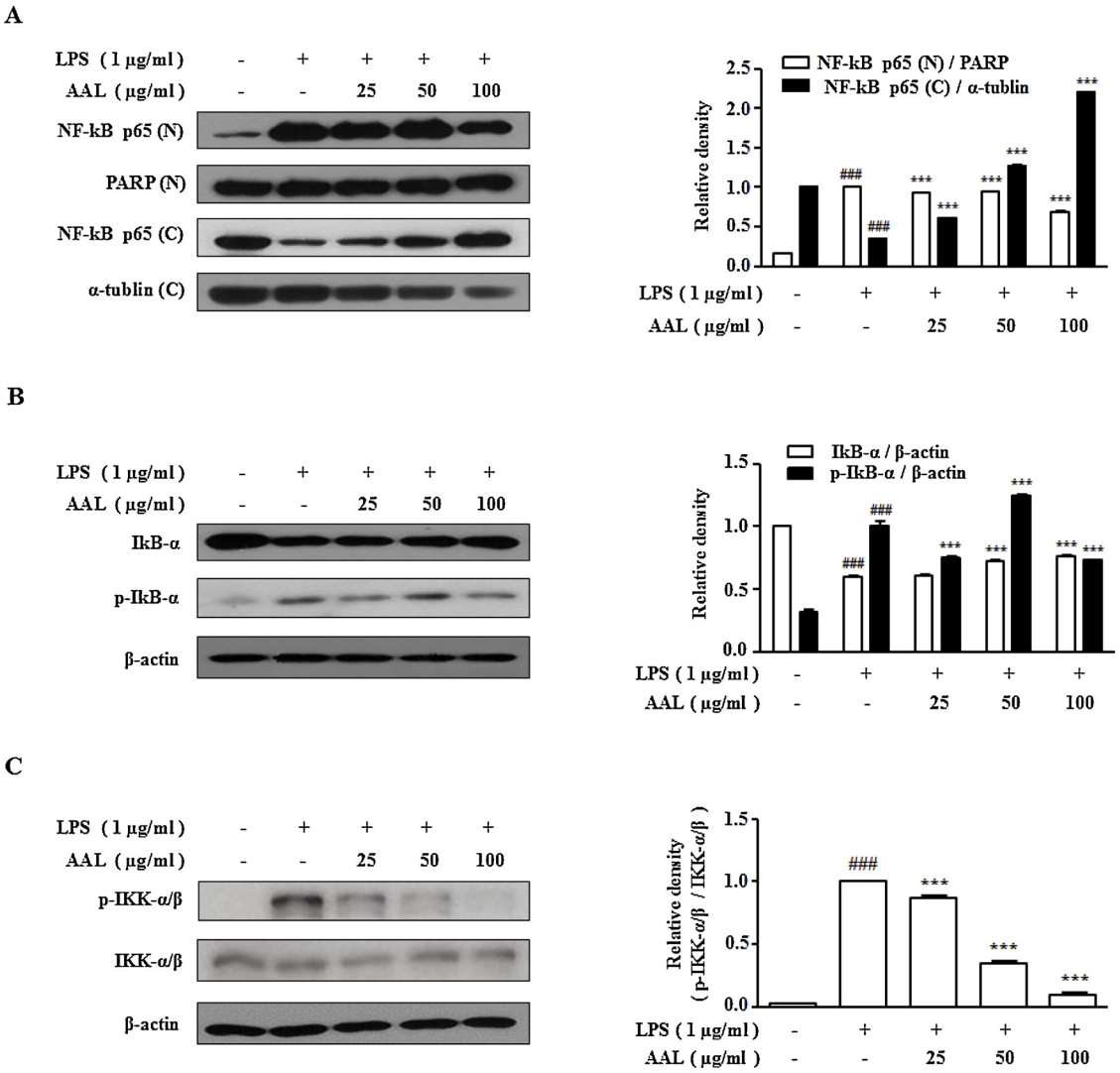

Fig. 3. Effect of AAL on the NF-κB activation in LPS-stimulated RAW 264.7 macrophages. In common, cells were pre-treated with AAL for 1 h prior to the addition of LPS (1 μg/ml): (A) Cells were further incubated for 30 min. Nuclear and cytosolic extracts were isolated and the p65 expression in each fraction was determined by western blot analysis. PARP and α-tubulin were used as internal controls for nuclear and cytosolic fractions, respectively. Cells were further incubated for (B) 15 min and (C) 7 min, respectively. β-actin was used as internal control. Densitometric analysis was performed using Bio-Rad Quantity One software. The data are represented as mean ± S.D. of three independent experiments. $^{###}p < 0.001$ vs. the control group; $^{***}p < 0.001$ vs. the LPS-treated group.

3. Results

3.1. Anti-inflammatory effects of methanol extracts of Chinese medicinal plants in LPS-stimulated RAW 264.7 macrophages

Prior to screening the anti-inflammatory activity of several extracts of Chinese medicinal plants from Beijing, six extracts were selected that were extracted from methanol using the whole part of each plant. We measured the cytotoxicity and NO production in LPS-stimulated RAW 264.7 macrophages to examine the anti-inflammatory activity of six methanol extracts (Table 3). Based on these data, NO production was observed at non-cytotoxic concentrations for each extract. NO production of six methanol extracts is represented by the half maximal inhibitory concentration (IC_{50}) and the value of AAL was the lowest among them (Table 4). Thus, AAL was investigated as a candidate anti-inflammatory agent given that AAL significantly inhibited NO production without cytotoxicity.

3.2. Effect of AAL on NO production and iNOS expression in LPS-stimulated RAW 264.7 macrophages

To deduce appropriate concentrations, we first examined the cytotoxicity of AAL in RAW 264.7 macrophages and found that AAL had no cytotoxicity up to 125 μg/ml (Fig. 1A). Based on this, concentrations of 25, 50, and 100 μg/ml were selected for further experiments. We sought to determine whether AAL inhibits the amount of NO released by LPS stimulation. NO production was notably reduced by AAL in a dose-dependent manner and its effect was superior to NIL, which was used as a positive control (Fig. 1B). Furthermore, we assessed whether the inhibitory effect of AAL on NO production was achieved through iNOS modulation. Pre-treatment with AAL considerably reduced iNOS expression compared to the LPS group (Fig. 1C).

3.3. Effect of AAL on production and mRNA expression of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages

To investigate the effect of AAL on pro-inflammatory cytokine release, we examined its production and mRNA expression. A significant difference was observed in the release of TNF-α and IL-6 between non-

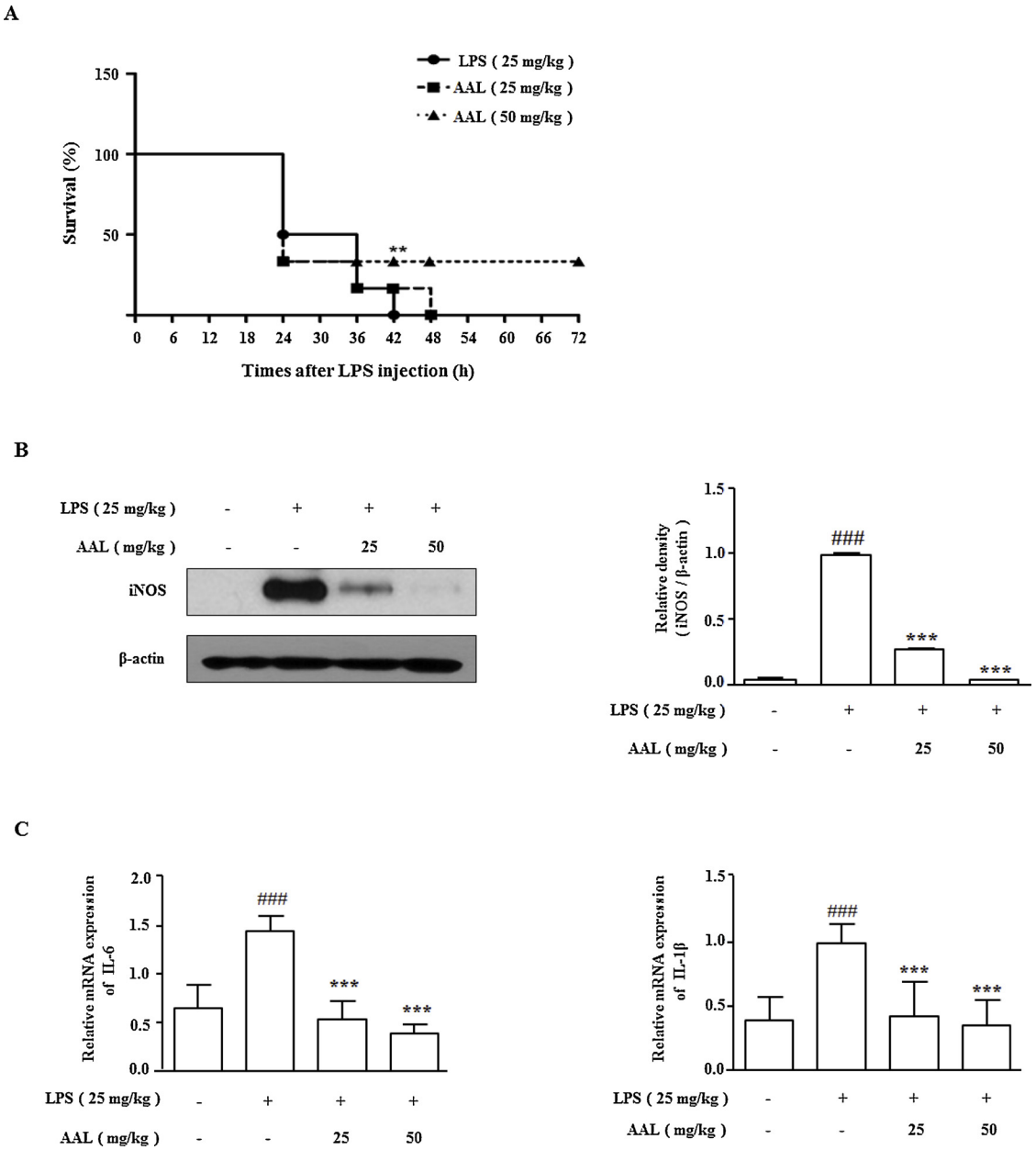

Fig. 4. Effect of AAL on LPS-induced septic mice. Mice (n = 6 per group) were injected with AAL (25 or 50 mg/kg, p.o.) or vehicle (PBS) for 1 h before LPS injection (25 mg/kg, i.p.). (A) Survival rate of mice was monitored for 72 h. (B) The protein expression of iNOS was determined by western blot analysis using specific antibodies. β–actin was used as internal control. (C) The mRNA expression of IL-6 and IL-1β was determined by qRT-PCR. Densitometric analysis was performed using Bio-Rad Quantity One software. The data are represented as mean ± S.D. of three independent experiments. $^{###}p < 0.001$ vs. the control group; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the LPS-treated group.

treated cells and LPS-treated cells. LPS stimulation significantly induced the release of TNF-α and IL-6 in RAW 264.7 macrophages. However, pre-treatment with AAL attenuated TNF-α and IL-6 production (Fig. 2A) and mRNA expression (Fig. 2B).

3.4. Effect of AAL on NF-κB activation in LPS-stimulated RAW 264.7 macrophages

To clarify the underlying AAL regulatory mechanism, we investigated whether AAL prevents the translocation of NF-κB dimers from the cytosol to the nucleus. LPS induced the translocation of NF-κB p65 to the nucleus, whereas pre-treatment with AAL effectively reduced this translocation in LPS-stimulated RAW 264.7 macrophages (Fig. 3A). Since the translocation of the NF-κB dimers into the nucleus is accomplished by the phosphorylation of IκB-α and degradation in the proteasome (G. Yang and An, 2014), we confirmed the effect of AAL on the expression of IκB-α. We found that AAL significantly reduced IκB-α phosphorylation and restored IκB-α degradation in LPS-stimulated RAW 264.7 macrophages (Fig. 3B). We further identified IKK-α/β, which is the upstream kinase of IκB-α (Shin et al., 2016). LPS-stimulated IKK-α/β phosphorylation was markedly reduced by AAL treatment (Fig. 3C). However, the total amount of IKK-α/β was not affected. These data indicate that inactivation of the NF-κB pathway was related to a reduction of inflammatory reactions in LPS-stimulated RAW 264.7 macrophages.

3.5. Effect of AAL on LPS-induced septic mice

To evaluate the anti-inflammatory effect of AAL *in vivo*, we used a well-known laboratory animal model for septic shock, which is the most

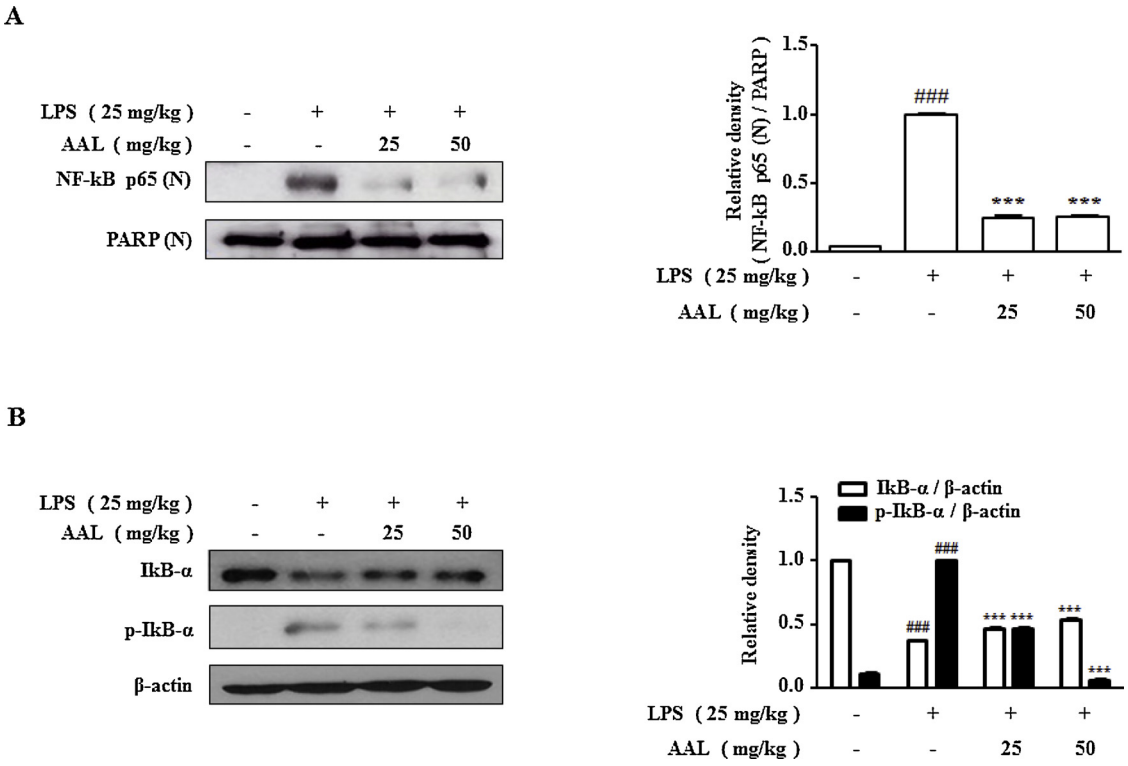

Fig. 5. Effect of AAL on NF-κB activation in liver of septic mice. (A) Nuclear extracts were isolated and p65 expression was determined by western blot analysis. PARP was used as internal controls for nuclear fraction. (B) The protein expression of p-IκB-α and IκB-α was determined by western blot analysis using specific antibodies. β–actin was used as internal control. Densitometric analysis was performed using Bio-Rad Quantity One software. The data are represented as mean ± S.D. of three independent experiments. $^{###}p < 0.001$ vs. the control group; $^{***}p < 0.001$ vs. the LPS-treated group.

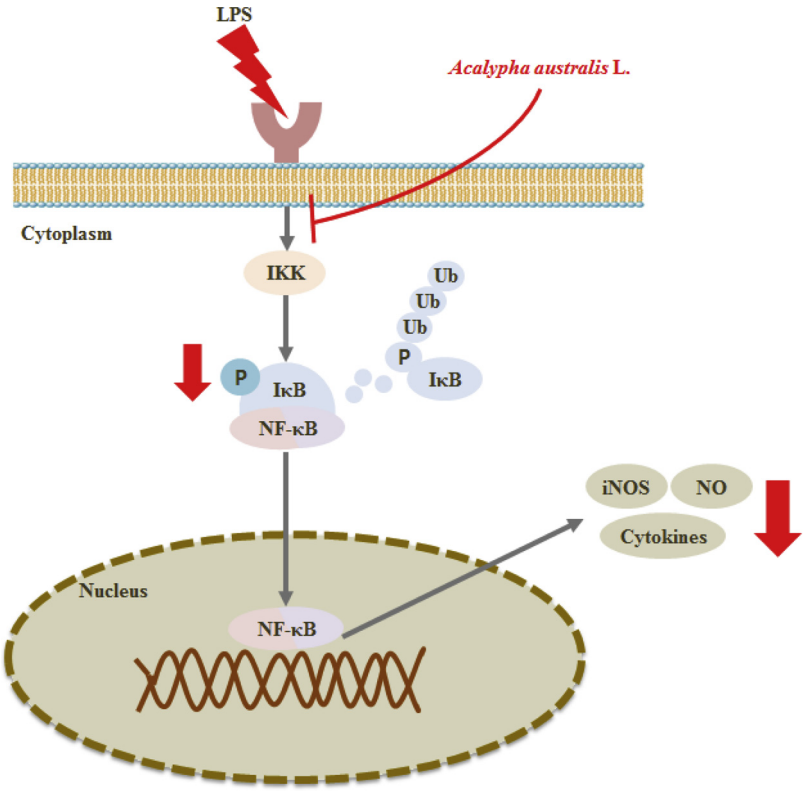

Fig. 6. Schematic diagram of the underlying mechanism of AAL's anti-inflammatory activity. AAL blocked the activation of NF-κB pathway and attenuated the release of pro-inflammatory cytokines.

extreme form of LPS-mediated inflammatory disorders (Yang et al., 2002). Stimulation by LPS indicated 100 % mortality within 42 h and pre-treatment with a low-dose of AAL (25 mg/kg) showed 100 % mortality within 48 h post-injection (Fig. 4A). However, pre-treatment with high-dose of AAL (50 mg/kg) reduced this mortality rate to 30 %. As septic shock progresses, pro-inflammatory cytokines are over-expressed and result in multiple tissue damage. Among organs, the liver is known to be the most affected by septic shock (Checker et al., 2014). Therefore, we evaluated the anti-inflammatory effect of AAL in the livers of septic mice. We confirmed the iNOS expression in liver samples and that AAL considerably attenuated the iNOS expression when compared to the LPS group (Fig. 4B). Furthermore, we investigated the mRNA expression of pro-inflammatory cytokines in liver tissue. IL-6 and IL-1β mRNA expression in the livers of mice was upregulated by LPS treatment and remarkably reduced by AAL treatment (Fig. 4C).

3.6. Effect of AAL on NF-κB activation in liver of septic mice

To investigate whether the *in vivo* mechanisms are similar to *in vitro* observations, we examined the protein expression of NF-κB-related genes in liver samples. LPS markedly translocated NF-κB p65 to the nucleus, whereas pre-treatment with AAL suppressed the translocation of NF-κB p65 (Fig. 5A). Additionally, the expression of IκB-α, which regulates the translocation of NF-κB dimers to nucleus (Tocmo and Parkin, 2018), was confirmed. AAL considerably inhibited IκB-α phosphorylation and restored IκB-α degradation in the livers of septic mice (Fig. 5B).

4. Discussion

The genus *Acalypha*, one of the largest genera in the Euphorbiaceae family, contains more than 450 species. Among them, *Acalypha australis* L. (AAL), an annual plant, is widespread across roadsides and farmlands in the tropical and subtropical regions of America, Africa, and Asia (Onocha et al., 2011). In current study, we identified the anti-inflammatory effect and the underlying mechanism of AAL in LPS-stimulated RAW 264.7 macrophages and LPS-induced septic mice. Inflammation is a normal immune response that repairs and returns damaged tissue to a healthy state in the presence of infection or tissue injury (Dunster, 2016). Lipopolysaccharides act as a potent initiator of inflammation by inducing the expression of many pro-inflammatory mediators in macrophages, which is one of the important cell types involved in septic shock (Guha and Mackman, 2002). Macrophages are pivotal cells in immune reactions and prevent invading pathogens by releasing several pro-inflammatory cytokines and mediators (Chen et al., 2016; Shen et al., 2018). As a defense mechanism by stimulation, macrophages express iNOS and induce the production of a massive amount of NO. Excessive NO has been reported to be associated with some inflammatory diseases (Liu et al., 2012). Since NO inhibition is a crucial part anti-inflammatory therapies, we examined the modulatory effect of the methanol extract of AAL on NO production in RAW 264.7 macrophages. We found that AAL significantly reduced NO production without cytotoxicity in LPS-stimulated RAW 264.7 macrophages. Additionally, the expression of iNOS, an enzyme that catalyzes NO formation, was consistent with the results of NO production (Jahani-Asl and Bonni, 2013). Thus, we identified that AAL inhibited NO that was induced by the suppression of iNOS expression in LPS-stimulated RAW 264.7 macrophages. Pro-inflammatory cytokines, such as TNF-α and IL-6, are predominantly generated by activating macrophages and are related to excessive inflammatory reactions (Cho et al., 2011). Therefore, the suppression of large quantities of pro-inflammatory cytokines is a potential therapeutic strategy for modulating inflammation-related disorders. Herein, we estimated the effect of AAL at a transcriptional level and found that AAL reduced TNF-α and IL-6 production as well as mRNA levels, which were increased by LPS stimulation in RAW 264.7 macrophages. Inflammatory systems operate through numerous signaling pathways, among which the most important pathway, NF-κB has been long studied as a major target for anti-inflammatory treatments (Lawrence, 2009). To elucidate the mechanisms underlying AAL inflammatory responses, we examined the effect of AAL on the NF-κB pathway. We found that AAL blocked the nuclear translocation of p65 by inhibiting IκB-α phosphorylation and degradation, which was caused by IKK-α/β activation in LPS-stimulated RAW 264.7 macrophages. The results demonstrate that the anti-inflammatory effect of AAL appears to block the activation of NF-κB by inhibiting the translocation of the p65 protein to the nucleus in LPS-stimulated RAW 264.7 macrophages. Septic shock is a life-threatening disorder caused by gram-negative bacterial infection that leads to multi-organ dysfunction and eventually death (Chang et al., 2013). To estimate the anti-inflammatory effect of AAL *in vivo*, we observed the survival rate of mice LPS-induced sepsis. AAL administration enhanced the survival rate compared to the LPS group. These results show that AAL protected against septic shock in LPS-induced mice. We further investigated whether the AAL anti-septic effect is present via the same mechanisms *in vitro*. iNOS overexpression can cause the overproduction of NO, which is known to cause a severe septic shock (Yen et al., 2018). From the livers of septic shock mice, we confirmed that AAL attenuated not only iNOS expression, but the mRNA expression of pro-inflammatory cytokines through the inhibition the NF-κB-related genes. Together, we demonstrated the anti-inflammatory effect of AAL through the regulation of inflammatory actions in LPS-stimulated RAW 264.7 macrophages and LPS-induced septic mice.

5. Conclusions

In conclusion, AAL displayed anti-inflammatory activity that attenuated the production of cytokines, mediators, and the expression of inflammation-related genes by blocking the NF-κB pathway in both *in vitro* and *in vivo* experiments, in which an inflammatory environment was established by LPS stimulation (Fig. 6). We propose that the methanol extract of AAL could be useful in potential treatments for inflammatory diseases.

Declaration of Competing Interest

None of the authors have any competing interests regarding this study.

CRediT authorship contribution statement

Hyo-Jung Kim: Writing - original draft, Visualization, Investigation, Software. **Hae-In Joe:** Writing - review & editing, Data curation. **Zhiyun Zhang:** Resources. **Sang Woo Lee:** Resources. **Kyou-Young Lee:** Funding acquisition. **Yoon-Bum Kook:** Conceptualization, Methodology, Supervision. **Hyo-Jin An:** Conceptualization, Methodology, Supervision, Project administration.

Acknowledgments

Funding: This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP; Ministry of Science, ICT & Future Planning) (No. NRF-2018R1C1B5041014).

References

Chang, Y.C., Tsai, M.H., Sheu, W.H., Hsieh, S.C., Chiang, A.N., 2013. The therapeutic potential and mechanisms of action of quercetin in relation to lipopolysaccharide-induced sepsis in vitro and in vivo. PLoS One 8, e80744.

Checker, R., Patwardhan, R.S., Sharma, D., Menon, J., Thoh, M., Sandur, S.K., Sainis, K.B., Poduval, T.B., 2014. Plumbagin, a vitamin K3 analogue, abrogates lipopolysaccharide-induced oxidative stress, inflammation and endotoxic shock via NF-kappaB suppression. Inflammation 37, 542–554.

Chen, C.C., Lin, M.W., Liang, C.J., Wang, S.H., 2016. The anti-inflammatory effects and mechanisms of Eupafolin in lipopolysaccharide-induced inflammatory responses in RAW264.7 macrophages. PLoS One 11, e0158662.
Cho, E.J., An, H.J., Shin, J.S., Choi, H.E., Ko, J., Cho, Y.W., Kim, H.M., Choi, J.H., Lee, K.T., 2011. Roxatidine suppresses inflammatory responses via inhibition of NF-kappaB and p38 MAPK activation in LPS-induced RAW 264.7 macrophages. J. Cell. Biochem. 112, 3648–3659.
Cho, B.O., So, Y., Jin, C.H., Nam, B.M., Yee, S.T., Jeong, I.Y., 2014. 3-deoxysilybin exerts anti-inflammatory effects by suppressing NF-kappaB activation in lipopolysaccharide-stimulated RAW264.7 macrophages. Biosci. Biotechnol. Biochem. 78, 2051–2058.
Dunster, J.L., 2016. The macrophage and its role in inflammation and tissue repair: mathematical and systems biology approaches. Wiley interdisciplinary reviews. Systems biology and medicine 8, 87–99.
Gatis-Carrazzoni, A., Mota, F.V.B., Leite, T.C.C., de Oliveira, T.B., da Silva, S.C., Bastos, I.V.A., de Souza Maia, M.B., Pereira, P.S., Neto, P.P.M., de Oliveira Chagas, E.C., Silva, T.M.S., do Nascimento, M.S., da Silva, T.G., 2019. Anti-inflammatory and antinociceptive activities of the leaf methanol extract of Miconia minutiflora (Bonpl.) DC. And characterization of compounds by UPLC-DAD-QTOF-MS/MS. Naunyn Schmiedebergs Arch. Pharmacol. 392, 55–68.
Guha, M., Mackman, N., 2002. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. J. Biol. Chem. 277, 32124–32132.
Guzik, T.J., Korbut, R., Adamek-Guzik, T., 2003. Nitric oxide and superoxide in inflammation and immune regulation. J. Physiol. Pharmacol. 54, 469–487.
He, J., Li, J., Liu, H., Yang, Z., Zhou, F., Wei, T., Dong, Y., Xue, H., Tang, L., Liu, M., 2018a. Scandoside exerts anti-inflammatory effect via suppressing NF-kappaB and MAPK signaling pathways in LPS-Induced RAW 264.7 macrophages. Int. J. Mol. Sci. 19.
He, J., Lu, X., Wei, T., Dong, Y., Cai, Z., Tang, L., Liu, M., 2018b. Asperuloside and asperulosidic acid exert an anti-inflammatory effect via suppression of the NF-kappaB and MAPK signaling pathways in LPS-Induced RAW 264.7 macrophages. Int. J. Mol. Sci. 19.
Jahani-Asl, A., Bonni, A., 2013. iNOS: a potential therapeutic target for malignant glioma. Curr. Mol. Med. 13, 1241–1249.
Kanno, S., Shouji, A., Tomizawa, A., Hiura, T., Osanai, Y., Ujibe, M., Obara, Y., Nakahata, N., Ishikawa, M., 2006. Inhibitory effect of naringin on lipopolysaccharide (LPS)-induced endotoxin shock in mice and nitric oxide production in RAW 264.7 macrophages. Life Sci. 78, 673–681.
Kim, S.H., Lee, T.H., Lee, S.M., Park, J.H., Park, K.H., Jung, M., Jung, H., Mohamed, M.A., Baek, N.I., Chung, I.S., Kim, J., 2015. Cynandione A attenuates lipopolysaccharide-induced production of inflammatory mediators via MAPK inhibition and NF-kappaB inactivation in RAW264.7 macrophages and protects mice against endotoxin shock. Exp. Biol. Med. 240, 946–954.
Kim, Y.S., Shin, W.B., Dong, X., Kim, E.K., Nawarathna, W., Kim, H., Park, P.J., 2017. Anti-inflammatory effect of the extract from fermented Asterina pectinifera with Cordyceps militaris mycelia in LPS-induced RAW264.7 macrophages. Food Sci. Biotechnol. 26, 1633–1640.
Lawrence, T., 2009. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb. Perspect. Biol. 1, a001651.
Le, H.T.T., Cho, Y.C., Cho, S., 2018. Methanol extract of Guettarda speciosa Linn. Inhibits the production of inflammatory mediators through the inactivation of Syk and JNK in macrophages. Int. J. Mol. Med. 41, 1783–1791.
Li, Y.C., Xian, Y.F., Su, Z.R., Ip, S.P., Xie, J.H., Liao, J.B., Wu, D.W., Li, C.W., Chen, J.N., Lin, Z.X., Lai, X.P., 2014. Pogostone suppresses proinflammatory mediator production and protects against endotoxic shock in mice. J. Ethnopharmacol. 157, 212–221.
Liu, T., Xiao, N., Liu, Y., Wilson, B., Li, J., Wang, Y., Cao, X., Liu, Z., 2012. The anti-inflammatory effects of ethyl acetate on Lipopolysaccharide/D-galactosamine challenged mice and Lipopolysaccharide activated RAW264.7 cells. Biochem. Biophys. Res. Commun. 427, 518–524.
Onocha, P.A., Oloyede, G.K., Afolabi, Q.O., 2011. Phytochemical investigation, cytotoxicity and free radical scavenging activities of non-polar fractions of Acalypha hispida (leaves and twigs). EXCLI J. 10, 1–8.
Park, J.H., Jun, J.G., Kim, J.K., 2014. (E)-3-(3,4-dihydroxy-2-methoxyphenyl)-1-(2,4-dihydroxyphenyl)prop-2-en-1-one, a novel licochalcone B derivative compound, suppresses lipopolysaccharide-stimulated inflammatory reactions in RAW264.7 cells and endotoxin shock in mice. Chem. Biol. Interact. 224, 142–148.
Seebaluck, R., Gurib-Fakim, A., Mahomoodally, F., 2015. Medicinal plants from the genus Acalypha (Euphorbiaceae)–a review of their ethnopharmacology and phytochemistry. J. Ethnopharmacol. 159, 137–157.
Shen, C., Chen, J.H., Lee, Y., Hassan, M.M., Kim, S.J., Choi, E.Y., Hong, S.T., Park, B.H., Park, J.H., 2018. mTOR- and SGK-Mediated connexin 43 expression participates in lipopolysaccharide-stimulated macrophage migration through the iNOS/Src/FAK Axis. J. Immunol. 201, 2986–2997.
Shin, J.A., Kim, J.J., Choi, E.S., Shim, J.H., Ryu, M.H., Kwon, K.H., Park, H.M., Seo, J.Y., Lee, S.Y., Lim, D.W., Cho, N.P., Cho, S.D., 2013. In vitro apoptotic effects of methanol extracts of Dianthus chinensis and Acalypha australis L. Targeting specificity protein 1 in human oral cancer cells. Head Neck 35, 992–998.
Shin, J.S., Lee, K.G., Lee, H.H., Lee, H.J., An, H.J., Nam, J.H., Jang, D.S., Lee, K.T., 2016. Alpha-solanine isolated from Solanum tuberosum L. Cv Jayoung abrogates LPS-Induced inflammatory responses via NF-kappaB inactivation in RAW 264.7 macrophages and endotoxin-induced shock model in mice. J. Cell. Biochem. 117, 2327–2339.
Tocmo, R., Parkin, K., 2018. S-Alk(en)ylmercaptocysteine suppresses LPS-induced pro-inflammatory responses in murine macrophages through inhibition of NF-kappaB pathway and modulation of thiol redox status. Free Radic. Biol. Med. 129, 548–558.
Xiao, S., Zhang, L.F., Zhang, X., Li, S.M., Xue, F.Q., 2013. Tracing antibacterial compounds from Acalypha australis Linn. By spectrum-effect relationships and semi-preparative HPLC. J. Sep. Sci. 36, 1667–1676.
Yang, G., An, H.J., 2014. beta-sitosteryl-3-O-beta-glucopyranoside isolated from the bark of Sorbus commixta ameliorates pro-inflammatory mediators in RAW 264.7 macrophages. Immunopharmacol. Immunotoxicol. 36, 70–77.
Yang, F., Comtois, A.S., Fang, L., Hartman, N.G., Blaise, G., 2002. Nitric oxide-derived nitrate anion contributes to endotoxic shock and multiple organ injury/dysfunction. Crit. Care Med. 30, 650–657.
Yen, I.C., Shi, L.S., Chung, M.C., Ahmetaj-Shala, B., Chang, T.C., Lee, S.Y., 2018. Antrolone, a Novel Benzoid Derived from Antrodia cinnamomea, Inhibits the LPS-Induced Inflammatory Response in RAW264.7 Macrophage Cells by Balancing the NF-[Formula: see text]B and Nrf2 Pathways. Am. J Chinese med. 46, 1297–1313.