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Acacia farnesiana extracts possess anti-inflammatory activity on LPS-induced macrophages

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ABSTRACT

Acacia farnesiana L. Willd (huizache, Mimosoideae, AF) is a leguminous plant possessing diuretic, anti-inflammatory and antimalaria properties. The aim of this study is to investigate the molecular pharmacological activity of aqueous and ethanol extract of AF in inflammatory response. Results showed that aqueous or ethanol extracts of AF reduced the productions of pro-inflammatory factors, including tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), nitrite oxide (NO) and prostaglandin E2 (PGE_2) with dose-dependent manner in murine marcrophage induced by 0.5 μg/ml lipopolysaccharide (LPS). Both aqueous and ethanol extracts did not exhibited cell cytotoxicity at the concentration up to 100 μg/ml. Reverse transcription polymerase chain reaction (RT-PCR) assay indicated that aqueous extract at 10 μg/ml completely inhibited the LPS-induced iNOS gene expression, and at 50 μg/ml suppressed cyclooxygenase 2 (COX-2), IL-6 and IL-1β gene expression. Ethanol extracts significantly blocked iNOS, COX-2, IL1-β and TNF-α gene expression as well, at the concentration of 5 μg/ml, 50 μg/ml and 100 μg/ml, respectively. The ethanol extracts showed more effectiveness in anti-inflammation activity compared with the same dose of aqueous extracts based on the inhibiting activity in iNOS expression. However, both types of extracts had no effect on the expression of COX-1. In conclusion, our present findings showed the anti-inflammatory activity of AF against RAW264.7 cells in vitro, and that both aqueous and ethanol AF extracts possess good anti-inflammatory activity. The molecular mechanism of the bioactivity of AF may be through down-regulating the gene expressions of inflammatory related factors.

Keywords: *Acacia farnesiana*, pro-inflammatory, nitrite oxide, marcrophage

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INTRODUCTION

Cytokines are intercellular signaling proteins released by both immune and non-immune cells [1]. Macrophages are the major type that initiates inflammatory responses against bacterial infection, pathologic stimuli, or injury. Macrophage-mediated responses production of the secretion of several pro-inflammatory cytokines, include NO, and upregulation of iNOS, as well as PGE_2 and COX-2 activity. These mediators along with TNF-α, IL-1β, IL-6 and other inflammatory mediators [2, 3]. Activated macrophages produce NO as a self-defense mechanism to protect the host against microbial pathogens. However, dysregulated production of these immune mediators by macrophages during prolonged inflammation is associated with many pathological conditions, such as cardiovascular diseases, autoimmune diseases, and cancer associated chronic diseases are involved in immune responses [4].

Acacia farnesiana (L.) Willd. (huizache, Mimosoideae, AF), known as "needle tree" or "sweet acacia", belongs to Leguminosae family and Minosoideae subfamily. It has been used anecdotally in South American and Chinese traditional medicine as therapy against malaria, as well as for reducing pain, draining pus, improving wound healing, alleviating common colds, and curing rheumarthritis arthritis [5]. Previous studies using human cancer cell lines have shown that AF produces that human cancer cell lines displayed moderate cytotoxic effects [5] and has antimalarial activity against the FcB2 strain [6]. Extracts of AF have been shown to possess chemotherapeutic activity against human hepatoma cells and to exhibit anti-inflammatory activity via the inhibition of superoxide anion generation and elastase release by human neutrophils. Macrophages are the major cell type that initiates inflammatory response during bacterial infection through the secretion of several pro-inflammatory cytokines. In the present study, we used a murine monocyte/macrophage cell line as an experimental model to evaluate the anti-inflammatory activity of aqueous and ethanol extracts of AF.

MATERIAL AND METHODS

Chemicals and biochemical

High glucose dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (p/s), and trypsin were purchased from GIBCO BRL (Grand Island, NY, USA). LPS, sodium bicarbonate, sulfanilamide, N-(1-naphthyl)ethylenediamine dihydrochloride, sodium nitrite were from Sigma Chemical (St. Louis, MO, USA). TRIzol reagent, SuperScript III kit were from Invitrogen-life Technologies (Carlsbad, CA, *USA*). Taq polymerase was from Takara (Bio Inc., Japan).

Preparation of AF-H and AF-E extracts

AF was obtained from National Chiayi University, were used in this study. To prepare the aqueous extract, 100 g of AF powders was respectively extracted with 1 liter of boiling water for 3 hour. The extract was filtered with filter paper while the residue was re-extracted under the same conditions twice. The filtrates were combined, concentrated and lyophilized. The dried powdered extracts named AF-H was collected and stored at 4℃ until use.

For the ethanol extract, 100 g of AF powders was respectively soaked with 1 liter of ethanol (95%) at room temperature for 1 weeks. After filtering the extract with filter paper (Advantec No. 1, Japan), the filtrate collected was concentrated and lyophilized. The dried powdered-extracts named AF-E was collected and stored at 4℃ until use.

Determination of flavonoid and and polyphenolic contents

The total flavonoid and phenolic contents of AF extracts were determined by the colorimetric and Folin-Ciocalteu methods, respectively [7].

Cell culture

Mouse RAW 264.7 macrophage were purchased from Bioresource Collection and Research Center (BCRC, Taiwan) was routinely maintained in the DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37℃in an humidified atmosphere with 5% CO_2.

MTT assay for cell viability

Cell viability was examined using MTT and trypan blue assays. In brief, RAW264.7 cells were cultured at 5×105cells per well in 96-well plates containing 100 μl of DMEM medium. After an overnight incubation, cells were treated with LPS (0.5 μg/ml) alone or LPS (0.5 μg/ml) plus various concentrations of AF-H and AF-E (5, 10, 25, 50 and 100 μg/ml), followed by incubating the plates for 24 h. MTT assay before adding 10 μl containing 4 mg/ml MTT dissolved in PBS. After incubating at 37 ℃ for 4 h, the medium was discarded and the formazan blue that formed in the cells was dissolved in DMSO. The OD was measured at 570 nm.

NO generation analysis

After incubating the cells with either LPS (0.5 μg/ml) or LPS plus various concentrations of AF-H and AF-E (5, 10, 25, 50 and 100 μg/ml) for 24 hour, the supernatant was removed from the cultures. The nitrite accumulated in culture medium was measured as an indicator of NO production based on the Griess reaction [8]. Briefly, 100 μl of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethy lenediamine dihydrochloride in water, and then incubated at room temperature for 10 min, the

absorbance was measured at 540 nm using an ELISA reader. The amount of NO production in samples was determined basing on the sodium nitrite serial dilution standard curve.

RT-PCR analysis

The cells were placed on a 10 cm plate, divided to 5 × 10^5 cells/well and cultivated overnight. They were treated with LPS (0.5 µg/ml) and then with AF-H and AF-E (5, 10, 25, 50 and 100 µg/ml) for 24 hour. The cells were isolated using TRIzol reagent, chloroform, isopropyl, followed by 75% ethanol precipitation. Total RNA (1 µg) was reverse transcribed into DNA in a total volume 20 µl of 1 µg RNA, oligo (DT) primer, 2.5 mM of each dNTP was mixed for 65℃ at 30 min, adding 5× buffer, 0.1 M DTT, Reverse transcriptase. Extension 50 min at 50℃, and inactivated RT 15 min at 70℃. PCR amplification was conducted in a total volume of 20 µl of 10× PCR buffer containing 1.0 µl of the firststrand cDNA, 0.25 mM of each dNTP. Two micromolar of each primer and 0.4 µl of Taq DNA polymerase. The following oligonucleotides were used: β-actin, COX-2, iNOS, TNF-α, IL-6, IL-1β (Table 1). Resulting PCR products were separated in 1.2% agarose gel and visualized by ethidium bromide staining.

Table 1 Oligonucleotide sequences for primer sets used in RT-PCR analysis.

Gene	Forward primer (5‘-3‘)	Reverse primer (5‘-3‘)	Product size
18S	GGTCATAAGCTTGCGTTGAT	GAGGGCCTCACTAAACCATC	81
TNF-α	ATGAGCACAGAAAGCATGATC	TACAGGCTTGTCACTCGAATT	276
IL-6	ATGAAGTTCCTCTCTGCAAGAGACT	CACTAGGTTTGCCGAGTAGATCTC	683
iNOS	CTGGCAGCAGCGGCTCCATG	GAAAAGACCGCACCGAAAGAT	456
IL-1β	CAGGATGAGGACATGAGCACC	CTCTGCAGACTCAAACTCCAC	447
COX-2	TGTATCCCCCCACAGTCAAAGACAC	GTGCTCCCGAAGCCAGATGG	146

Cytokine measurements

Cytokine concentrations in the supernatants were determined by ELISA kits that were specific against murine cytokines. Culture medium was used as diluent and as blank to avoid medium phenol red interference. Levels of TNF-α, IL-1β and IL-6 were measured using ELISA kits from Pierce Endogen (R&D systems, Inc, USA). Assays were performed according to the manufacturer’s instructions.

Determination of prostaglandin E_2 (PGE_2)

Culture medium was used as diluent and as blank to avoid medium phenol red interference. The level of PGE2 in the supernatant of the culture medium was measured using an ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). Assays were performed according to the manufacturer’s instruction.

Statistical analysis

mRNA expression analysis using RT-PCR was conducted at least three times to ensure that the same result was derived, and this study used the representative diagram. data were presented as means±standard deviations (SD). Results were evaluated by oneway ANOVA, followed by Duncan's multiple range tests using the SPSS for Windows version 10.0 (Statistical Program for Social Sciences, SPSS Corp., Chicago, IL). Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

The present study demonstrates that both AF-H and AF-E significantly inhibit NO production, PGE_2 production, cytokine (TNF-α, IL-1β and IL-6) secretion and expression, and iNOS and COX-2 expression in LPS-stimulated macrophage. AF-H was a more potent inhibitor than AF-E. This is the first time that AF was shown to possess potent anti-inflammatory activity and to selectively inhibit COX-2 expression. These activities were not attributable to the cytotoxicity of AF as assessed by the MTT assay.

Table 2 shows the flavonoids and polyphenolic contents of AF-H and AF-E. The results showed that AF-Hpossessed a higher polyphenolic content (216.28±7.82 mg/g) than AF-E (161.39±3.34 mg/g). However, AF-H showed a significantly lower total flavonoid contents (1.49±0.04 mg/g) than AF-E (13.49±2.22 mg/g). Reactive oxygen species (ROS) could induce biological damage and pathological events contribute to products of chronic inflammation, carcinogenesis and aging. Natural components, such as the polyphenols of plant extracts can exhibit antioxidants and regular cellular redox states. Chinese herbal extracts have been demonstrated to possess strong antioxidant and anti-inflammatory properties. Extracts of Chinese materia medica [9] possessed significant DPPH radical scavenging and anti-oxidant activities. Chinese medicals formulae from shengmai san [10], sho-saiko-to [11], *Scutellaria baicalensis* [12], and *Osmanthus fragrans* flowers [13] were reported to exhibit potent antioxidant and free radical scavenging activities, as well as anti-inflammatory properties *in vitro* and *in vivo.* Therefore, it is possible that the antioxidant and anti-inflammatory activities of AF correlates with its total phenolic contents. Phenolic acids and flavonoid compounds have also been reported to powerfully affect other biological systems [7, 10-17]. We showed that AF-E and AF-H possessed excellent superoxide radical scavenging activity, suggesting that these extracts could effectively treat oxidative stress-related diseases (data not show). In this study, AF-H contained a significantly higher level of phenolics than the AF-E extracts. This suggests that the high antioxidant and free radical scavenging activities of AF-H may be due to the presence of phenolic compounds.

AF-H: aqueous *Acacia farnesiana* extract; AF-E: ethanolic *Acacia farnesiana* extract. Data are presented as mean±SD (n = 3). [a] Values are expressed as milligrams quercetin equivalent per gram dried AF extract. [b] Values are expressed as milligrams gallic acid equivalent per gram dried AF extract. * Significant difference ($P < 0.05$) between AF-H and AF-E as analyzed by Student's paired t-test.

Table 2 Total flavonoids and total polyphenolic contents of different AF extracts.

	AF-H	AF-E
Total flavonoids (mg/g)[a]	1.49±0.04	13.49±2.22*
Total phenols (mg/g)[b]	216.28±7.82*	161.39±3.34

In order to determine whether AF-H and AF-E is toxic to on RAW264.7 cells, these cells were treated with various concentrations of AF-H and AF-E at 5, 10, 25, 50, and 100 µg/ml in the presence or absence of 0.5 µg/ml LPS, and cell viability was examined using the MTT assay. The results showed that AF-H and AF-E (5–l00 µg/ml) did not cause significant cytotoxicity to RAW264.7 cells either in the presence or absence of 0.5 µg/ml LPS (Figures 1A and 1B).

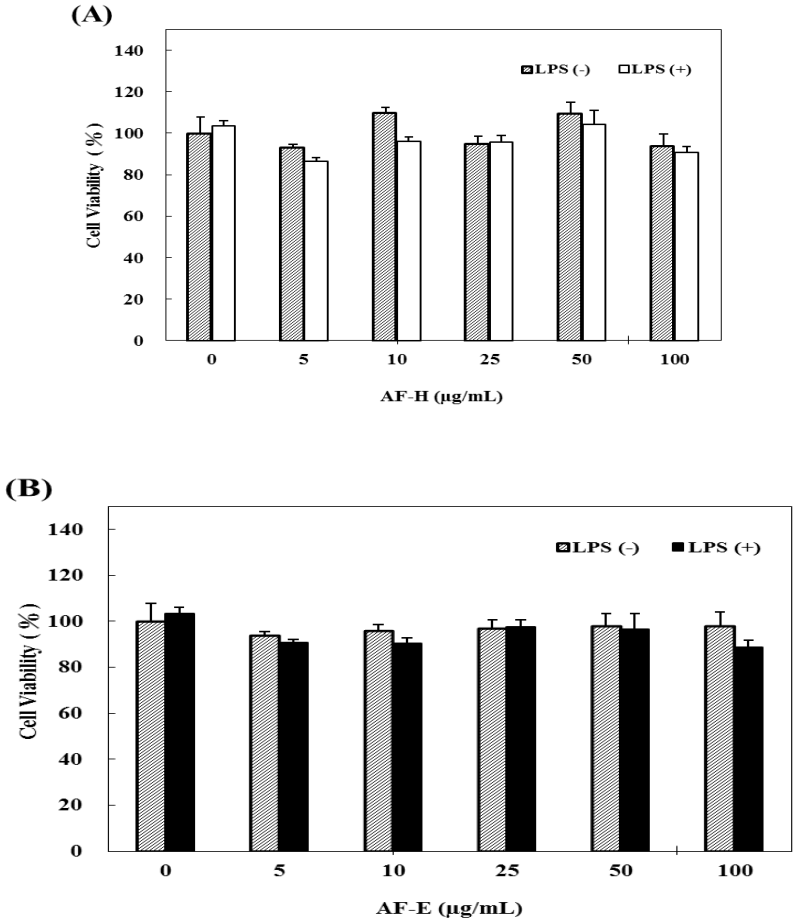

Figure 1. Effect of different AF-H and AF-E concentrations on RAW 264.7 cells macrophages viability.

RAW264.7 cells were cultured with indicated concentrations of (A) AF-H and (B) AF-E alone or in combination with LPS (0.5 μg/ml) at 37℃ in a 96-well plate for 24 h. Cell viability was determined by MTT assay. Each data represents the mean ± SD of three independent experiments. Bars not denoted by the same letter differ significantly at $P < 0.05$ as analyzed by Duncan's multiple range test.

Nitric oxide (NO) production increases in several inflammatory disorders and NO appears to act as a secondary mediator of pro inflammatory cytokines signaling. To determine whether the observed decrease in cytokine secretion resulted from the cytotoxic effects of NO, the effects of AF-H and AF-E on NO and PGE_2 production were determined in unstimulated RAW264.7 cells and LPS-induced RAW264.7 cells. Table. 3 shows that RAW264.7 cells in the resting state released 0.89±0.80 μM NO and 39.54±21.12 pg/ml PGE_2, a stable metabolite of NO, during incubation for 24 h. In contrast, cells stimulated with LPS markedly increased NO and PGE_2 production up to 27.32±1.14 μM and 192.28±03.76 pg/ml, respectively. AF-H and AF-E slightly inhibited LPS-induced NO and PGE_2 production at low concentrations, but these extracts strongly inhibited NO and PGE_2 production in a dose-dependent manner at concentrations of 10-100 μg/ml. The AF-H and AF-E extracts at 50 μg/ml inhibited NO production more than 42.12% and 78.94%, respectively, and 100 μg/ml of AF-H or AF-E produced 95.79 % and 99.57% NO inhibition, respectively, compared with the untreated control. However, the AF-H and AF-E extracts at 50 μg/ml inhibited PGE_2 production more than 17.74% and 44.16%, respectively, and 100 μg/ml of AF-H or AF-E1 86.55% and 61%, respectively, compared with the untreated control. AF-H and AF-E at concentrations below 5-100 μg/ml did not show significant cytotoxic effects toward the RAW 264.7 cells, indicating that the inhibitory effect of the compounds on LPS-induced NO production was not attributable to non-specific cell toxicity. These results show that AF-E more strongly inhibits LPS-induced NO production than AF-H does. However, cells treated with the AF-H extract produced significantly less PGE_2 than those treated with AF-E did.

Table 3. Effects of different AF-H and AF-E concentrations on LPS (0.5 μg/ml)-induced NO release and PGE_2 production in RAW 264.7 cells macrophages.

Sample	**Nitric oxide (μM)**		**PGE_2 (ng/ml)**	
	H_2O	EtOH	H_2O	EtOH
Control	0.89±0.80^{d}	0.89±0.80^{c}	39.54±21.12^{d}	39.54±21.12^{c}
LPS	27.32±1.14^{a}	27.32±1.14^{a}	192.28±03.76^{a}	192.28±03.76^{a}
LPS＋5 μg/mL AF	25.22±1.94^{a}	25.52±3.53^{a}	189.78±00.68^{a}	188.64±10.05^{ab}
LPS＋10 μg/mL AF	25.22±1.94^{a}	25.52±3.53^{a}	189.78±00.68^{a}	188.64±10.05^{ab}
LPS＋25 μg/mL AF	24.57±0.95^{a}	11.53±2.96^{b*}	182.21±04.53a	186.81±00.79^{ab}

LPS＋50 μg/mL AF	15.81±2.85^{b}	1.15±2.20^{c}*	158.17±05.23^{b}*	179.68±00.40^{b}
LPS＋100 μg/mL AF	5.75±0.12^{c}	0.12±0.51^{c}*	107.38±14.50^{c}*	156.50±07.15^{b}

Data are presented as mean±SD (n = 3). Values in the same column with the same superscript letter indicate no significant difference ($P < 0.05$) as analyzed by Duncan's multiple range test.

* Significant difference ($P < 0.05$) between AF-H and AF-E as analyzed by Student's paired t-test.

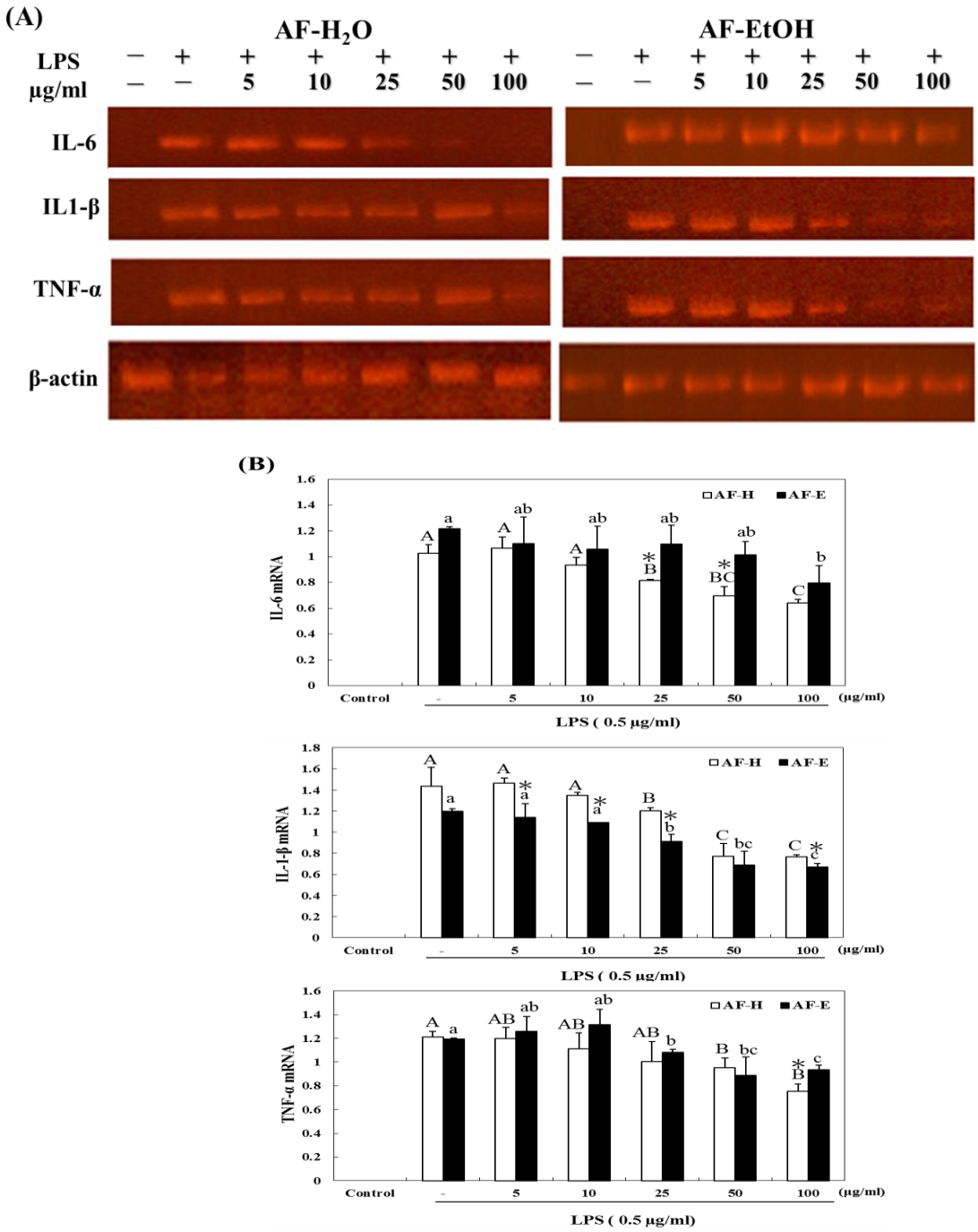

Figure 2. Effect of different AF concentrations inhibited LPS-induced inflammatory cytokine expression in RAW 264.7 cells macrophages.

To investigate the effects of AF on the production of pro inflammatory cytokines such as IL-1β, IL-6, and TNF-α, cells were treated with control vehicle (0.1% DMSO) and different concentrations of AF-H and AF-E (25, 50, and 100 µg/ml) for 24 h in the presence or absence of 0.5 µg/ml LPS. The levels and expression of IL-1β and TNF-α were measured using ELISA and RT-PCR. As shown in Table 4 and Fig. 2, 50 µg/ml AF-H and AF-E significantly inhibited both TNF-α cytokine production and mRNA expression, and the level of inhibition increased in a dose-dependent manner. AF-H and AF-E significantly decreased IL-6, TNF-α, and IL-1β mRNA expression in a dose-dependent manner in LPS-stimulated macrophages. Consistent with our TNF-α results, AF dose-dependently inhibited the production of IL-1β and IL-6 cytokines and mRNA expression as presented in Table 4 and Figure. 2. These results show that both AF-H and AF-E significantly inhibit cytokine secretion and expression.

IL-6, IL1-β and TNF-α mRNA expression in RAW 264.7 cells macrophages were stimulated by LPS (0.5 µg/ml) for 24 hr with or without AF-H and AF-E at different concentrations.(A) Representative gel image of gene expression related to inflammatory cytokine detected by RT-PCR. (B) Quantitation of RT-PCR products. Data are presented as mean±SD (n = 3). Bar values of the same treatment

sharing the same superscript letter (A, B, C, D for AF-H and a, b, c, d for AF-E) indicate no significant difference ($P < 0.05$) as analyzed by Duncan's multiple range test. * Significant difference ($P < 0.05$) between AF-H and AF-E as analyzed by Student's paired t-test.

Table 4. Effects of different AF-H and AF-E concentrations on LPS (0.5 µg/ml)-induced IL-1β production and TNF-α production in RAW 264.7 cells macrophages.

Sample	IL-1β (ng/mL)		TNF-α (ng/mL)	
	H_2O	EtOH	H_2O	EtOH
Control	0.06 ± 0.02^{d}	0.06 ± 0.02^{d}	0.29 ± 0.17^{d}	0.29 ± 0.17^{c}
LPS	1.04 ± 0.01^{a}	1.04±0.01a	16.28 ± 3.26^{a}	16.28 ± 3.26^{a}
LPS＋25 µg/mL AF	0.84 ± 0.16^{ab}	0.46 ± 0.09^{b}*	12.53 ± 3.11^{ab}	14.39 ± 0.45^{a}
LPS＋50 µg/mL AF	0.63 ± 0.11^{b}	0.25 ± 0.03^{c}*	10.60 ± 1.62^{b}	12.67 ± 0.55^{a}
LPS＋100 µg/mL AF	0.42 ± 0.01^{c}	$0.18\pm0.04^{c*}$	6.96 ± 0.79^{c}*	9.01 ± 0.04^{b}

Data are presented as mean±SD (n = 3). Values in the same column with the same superscript letter indicate no significant difference ($P < 0.05$) as analyzed by Duncan's multiple range test. * Significant difference ($P < 0.05$) between AF-H and AF-E as analyzed by Student's paired t-test.

Several reports have shown that LPS strongly activates macrophages to produce PGE_2 and the rate-limiting step in PGE_2 increased synthesis is catalyzed by COX-2[18]. Next, we investigated whether the inhibitory effect of AF on NO and PGE_2 production was related to the modulation of

iNOS and COX-2 expression. As shown in Fig. 3, both AF-H and AF-E strongly decreased the level of iNOS and COX-2 mRNA expression at concentrations of 10 or 25 μg/ml. In addition, LPS-induced COX-2 mRNA expression was significantly decreased by AF-H and AF-E in a dose-dependent manner. These results suggest that AF suppresses iNOS and COX-2 mRNA expression at the transcriptional level, resulting in the inhibition of NO and PGE_2 production.

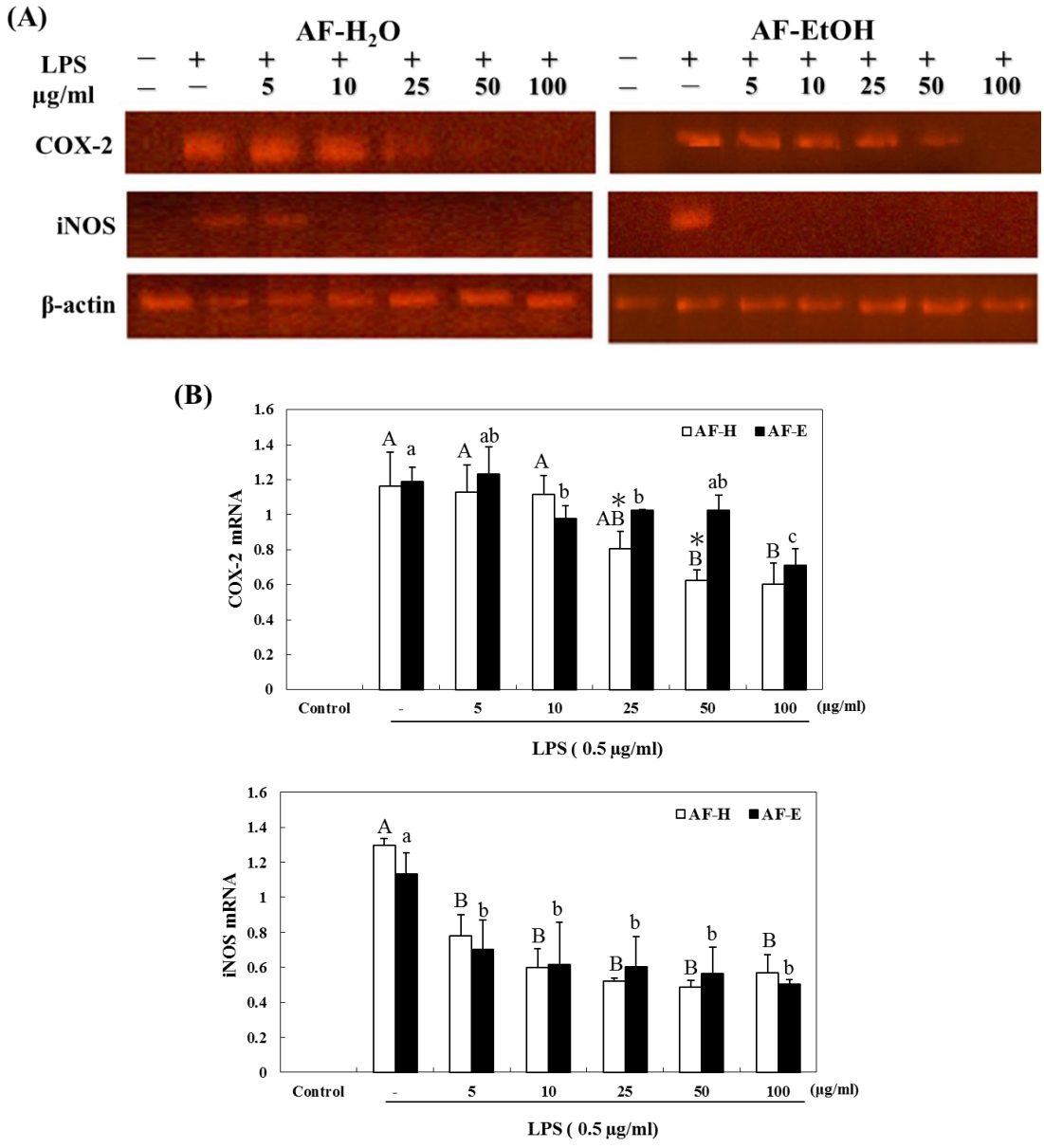

Figure 3. Effect of different AF concentrations inhibited LPS-induced iNOS and COX-2 expression in RAW 264.7 cells macrophages.

iNOS and COX-2 mRNA expression in RAW 264.7 cells macrophages were stimulated by LPS (0.5 μg/ml) for 24 hr with or without AF-H and AF-E at different concentrations.(A) Representative gel image of gene expression related to inflammatory cytokine detected by RT-

PCR. (B) Quantitation of RT-PCR products. Data are presented as mean±SD (n = 3). Bar values of the same treatment sharing the same superscript letter (A, B, C, D for AF-H and a, b, c, d for AF-E) indicate no significant difference ($P < 0.05$) as analyzed by Duncan's multiple range test. * Significant difference ($P < 0.05$) between AF-H and AF-E as analyzed by Student's paired t-test.

Accumulating evidence suggests that inflammation plays a critical role in the pathogenesis of various types of chronic inflammatory diseases, including cardiovascular diseases, autoimmune diseases, and cancers [4]. Studies have shown that natural antioxidants and anti-inflammatory compounds such as *Basella alba* fruits [17], curcumin [14], tocotrienol [7], Lycium Fruit [15], and theaflavin [16] exhibit inhibitory effects on LPS-induced iNOS and COX-2 expression and pro-inflammatory responses. As mentioned above, inhibitors of pro-inflammatory responses were considered to be potential effective therapeutics for preventing chronic inflammatory diseases. In this study, although both AF-H and AF-E significantly inhibited LPS-induced NO production and iNOS expression, particularly at low concentrations. This result indicates that a significant reduction in NO accumulation after AF treatment is consistent with the inhibition of iNOS expression.

The inflammatory response is mediated by multiple factors including cytokine and non-cytokine proteins [19]. Nitric oxide and PGE_2 represent two major non-cytokine mediators, which are biosynthesized by NOS and COX, respectively [20]. PGE_2 and iNOS, the major metabolites of the COX-2 and NO pathways, respectively, have been considered important lipid mediators of inflammatory and immunoregulatory processes [21]. In fact, the overproduction of NO could induce cell damage as well as inflammation. In this study, AF-H and AF-E effectively down-regulated COX-2 and iNOS mRNA expression, suggesting that the inhibitory effect of AF on PGE_2 and NO production could be through the suppression of COX-2 and iNOS enzyme activity.

Pro-inflammatory cytokines such as TNF-α, IL-4, IL-6 and IL-8 mediate the tissue response during different phases of inflammation [3]. Pro-inflammatory cytokines have a multitude of biological activities linked to a number of inflammatory diseases such as septic shock, rheumatoid arthritis and autoimmune diseases. Thus, the phytochemical natural products that inhibit production of these cytokines may play an important role in controlling chronic inflammation [22]. The anti-inflammatory activity of AF was assessed *in vitro* by assessing AF inhibitory effects on the production of inflammatory mediators, including TNF-α, IL-1β and IL-6 in RAW 264.7 cells stimulated with LPS. In this study, AF effectively inhibited TNF-α, IL-1β and IL-6 secretion in LPS-stimulated macrophages, suggesting that the inhibition of NO production by either AF-H or AF-E could be indirectly due to the reduction in IL-1β and TNF-α secretion.

CONCLUSION

we have demonstrated that AF possesses potent anti-inflammatory activity as demonstrated by its inhibitory effects in LPS-stimulated macrophages on pro-inflammatory cytokine secretion, NO and PGE_2 production, iNOS and COX-2 expression, and TNF-α, IL-1β and IL-6 secretion and expression. These findings provide the first pharmacological evidence of the immunomodulating properties of AF, and also suggest the potential use of AF in the prevention of inflammatory diseases.

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