The anti-inflammatory activities of *Ainsliaea fragrans* Champ. extract and its components in lipopolysaccharide-stimulated RAW264.7 macrophages through inhibition of NF-κB pathway

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1. Introduction

*Ainsliaea fragrans* Champ. (*A. fragrans*), belongs to the Asteraceae family (*Ainsliaea DC.*), is a folk herbal medicine named ‘Xingxiang Tuerfeng’ in China and has long history of medicinal practice in south China. The pharmacology studies have focused on its anti-inflammatory effects in vitro. More than 20 compounds (*Xing et al., 2006; Liu et al., 2007; Wang and Liu, 2007*) have been found over the last 30 years from *A. fragrans* including triterpenoids, flavonoids, sesquiterpenoids, (Wang et al., 2009), and phenolic compounds (*Zhang et al., 2006*), including 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid. The major bioactive constituents of *A. fragrans* are phenolic compounds (*Zhang et al., 2006*), including 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid.

Nowadays, the whole plant was applied as one of the key ingredients in preparations of ‘Xingxiang Tuerfeng’ tablet and ‘compound Xingxiang Tuerfeng granule’, which have been used for curing gynecological diseases like cervicitis, endometritis, and pelvic inflammatory activities of *A. fragrans* include components like 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid. It exhibits anti-inflammatory activities which has been used for the treatment of gynecological diseases for many years in China. The aims of the present study were to investigate the anti-inflammatory activities of *A. fragrans* and elucidate the underlying mechanisms with regard to its molecular basis of action for the best component.

**Materials and methods:** The anti-inflammatory effects of *A. fragrans* were studied by using lipopolysaccharide (LPS)-stimulated activation of nitric oxide (NO) in mouse RAW264.7 macrophages. Expression of inducible NO synthase (iNOS) and pro-inflammatory cytokines, inhibitory kB (IkBa) degradation and nuclear translocation of NF-κB p65 were further investigated.

**Results:** The present study demonstrated that *A. fragrans* could suppress the production of NO in LPS-stimulated RAW264.7 macrophages. Further investigations showed *A. fragrans* could suppress iNOS expression. *A. fragrans* also inhibited the expression of tumor necrosis factor-alpha and interleukin-6. *A. fragrans* significantly decreased the degradation of IkBa, reduced the level of nuclear translocation of p65. All these results suggested the inhibitory effects of *A. fragrans* on the production of inflammatory mediators through the inhibition of the NF-κB activation pathway.

**Conclusion:** Our results indicated that *A. fragrans* inhibited inflammatory events and iNOS expression in LPS-stimulated RAW264.7 cells through the inactivation of NF-κB pathway. This study gives scientific evidence that validate the use of *A. fragrans* in treatment of patients with gynecological diseases in clinical practice in traditional Chinese medicine.

**Abbreviations:** LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; IkBa, inhibitory kBα; NF-κB, nuclear factor-κB

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**Abstract**

*Ethnopharmacological relevance:* *Ainsliaea fragrans* Champ. (*A. fragrans*) is a traditional Chinese herbal medicine that contains components like 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid. It exhibits anti-inflammatory activities which has been used for the treatment of gynecological diseases for many years in China. The aims of the present study were to investigate the anti-inflammatory activities of *A. fragrans* and elucidate the underlying mechanisms with regard to its molecular basis of action for the best component.

*Materials and methods:* The anti-inflammatory effects of *A. fragrans* were studied by using lipopolysaccharide (LPS)-stimulated activation of nitric oxide (NO) in mouse RAW264.7 macrophages. Expression of inducible NO synthase (iNOS) and pro-inflammatory cytokines, inhibitory kBα (IkBa) degradation and nuclear translocation of NF-κB p65 were further investigated.

*Results:* The present study demonstrated that *A. fragrans* could suppress the production of NO in LPS-stimulated RAW264.7 macrophages. Further investigations showed *A. fragrans* could suppress iNOS expression. *A. fragrans* also inhibited the expression of tumor necrosis factor-alpha and interleukin-6. *A. fragrans* significantly decreased the degradation of IkBa, reduced the level of nuclear translocation of p65. All these results suggested the inhibitory effects of *A. fragrans* on the production of inflammatory mediators through the inhibition of the NF-κB activation pathway.

*Conclusion:* Our results indicated that *A. fragrans* inhibited inflammatory events and iNOS expression in LPS-stimulated RAW264.7 cells through the inactivation of NF-κB pathway. This study gives scientific evidence that validate the use of *A. fragrans* in treatment of patients with gynecological diseases in clinical practice in traditional Chinese medicine.

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inflammation with significant clinical effects (Chen and Wang, 2010; Cui et al., 2014). As a common over-the-counter (OTC) medicine in China, these preparations have been the first-line treatment of gynecological diseases (Su et al., 2014).

Inflammation is a local, protective response of the immune system. Excessive inflammatory responses can be harmful, as in diseases such as rheumatoid arthritis, Alzheimer's disease and septic shock syndrome (Tracey, 2002). Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, stimulates macrophages to produce pro-inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and inducible nitric oxide synthase (iNOS), which trigger a cascade responsible for the inflammatory response.

Nitric oxide (NO) participates in various physiological processes such as regulation of inflammation, neurotransmission, mitochondrial functions and apoptosis (Tennyson and Lippard, 2011). NO is synthesized from L-arginine by three types of NO synthases (NOSs): iNOS, neuronal NOS (nNOS), and endothelial NOS (eNOS) (Bredt, 1999). A great number of studies have showed that iNOS is the main enzyme to catalyze NO production in inflammatory condition (Nagy et al., 2007). Numerous studies have shown that in inflammatory conditions, iNOS gene transcription is chiefly modulated by the transcription factor nuclear factor (NF)-κB (Connelly et al., 2003).

The functions of NF-κB are performed in homo- or hetero-dimeric forms of Rel family proteins such as RelA (p65), RelB, cRel, p50 and p52. NF-κB is present in the cytoplasm in an inactive state, complexed with the inhibitory κB (κB) including κBα, κBβ, κBε, p105 and p100 (Beg et al., 1992; Ghosh and Hayden, 2008). Extracellular stimuli and/or pro-inflammatory cytokines can trigger NF-κB-activated pathways. These inducers can bind to cell surface receptors and activate the κB kinase (IKK) complex (Pennington et al., 2001). Activated IKK catalyzes κB phosphorylation, which is then ubiquitinated and proteasome-mediated degradation (Lee et al., 1998). After degraded, κB no longer binds with NF-κB, and the free NF-κB translocates into the nucleus where it induces the expression of multiple inflammatory genes, such as TNF-α and iNOS (Tian et al., 2005).

Although many studies have been previously reported about the medical effects of A. fragrans, the mechanism of the plant modulating molecular and cellular inflammatory responses has not been elucidated. In this study, we investigated the anti-inflammatory effects of A. fragrans Champ. Extract (AF-Ext) and its components 3,5-dicaffeoylquinic acid (AF-P1) and 4,5-dicaffeoylquinic acid (AF-P2) and the molecular targets in LPS-activated macrophages.

2. Materials and methods

2.1. Plant material

The whole herb of A. fragrans (‘Xingshang Tuerfeng’) was collected from Wuyuan city, Jiangxi Province, PR China, in June 2005, and authenticated by Prof. Minjian Qing (Dept. of Pharmacognosy, China Pharmaceutical University, Nanjing, China). A voucher specimen (no. 20050701) was deposited in the herbarium of China Pharmaceutical University, Nanjing, China.

2.2. Chemicals and reagents

All analytical grade solvents used for column chromatography and HPLC grade methanol for HPLC analysis were purchased from Jiangsu Hanbang Science and Technology Co., Ltd. (Nanjing, China). Purified water was afforded by a Milli-Q system (Millipore, USA). HPO-100 resins were purchased from Cangzhou Bon Adsorber Technology Co., Ltd. (Hebei, China). Sephadex LH-20 (40–70 μm) were purchased from Pharmacia Biotech AB (Uppsala, Sweden). Dimethyl sulfoxide (DMSO) and LPS from Escherichia coli 055: B5 were purchased from Sigma Chemical Co. (USA). NO detection kit was purchased from Beyotime Institute of Biotechnology (Haimen, China). Nuclear extract kit was purchased from Vazyme (Nanjing, China). Antibodies iNOS, κBα and NF-κB p65 were purchased from Cell Signaling Technology Inc. (USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat

![Fig. 1. Structures of compounds 1 and 2 isolated from A. fragrans.](image)

![Fig. 2. HPLC Chromatogram of A. fragrans Extract.](image)

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anti-mouse antibodies were purchased from Signalway Antibody LLC. (USA). Polysine film for fluorescence microscopy was purchased from Bio-Rad Laboratories, Inc. (USA). Immobilon western HRP substrate was purchased from Merck Millipore (Germany).

2.3. Preparation of plant extract and isolation of compounds 1 and 2

2.3.1. Preparation of plant extract

The dried aerial parts of A. fragrans (500 g) were powdered and extracted twice under reflux with 70% ethanol for 2 h. The combined extracts were concentrated under reduced pressure to afford a residue (50 g). The residue was further suspended in water and filtered, which was chromatographed using a HP-100 resin column eluted with a gradient system of EtOH-H2O (0: 100, 20: 80, 60: 40). The 60% aqueous ethanol elution were collected and evaporated under 60 °C in vacuum to give the dried extracts of A. fragrans (7.0 g).

2.3.2. Isolation of compounds 1 and 2

The extracts of A. fragrans (2.0 g) were further separated by Sephadex LH-20 column (MeOH-H2O, 60: 40) and preparative HPLC to afford 1 (85 mg) and 2 (60 mg). The structures of the compounds was determined to be over 95% by normalization of the fluorescence in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Wisen, Inc., Canada) in a humidified 5% CO2 atmosphere at 37 °C.

2.4. Cell culture

RAW264.7, a murine macrophages cell line, were purchased from American Type Culture Collection (ATCC; USA), and subcultured to confluence in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Wisen, Inc., Canada). Cells were incubated for 16 h, then alamarBlue® (Life Technologies Co., USA) was added to make the concentration of 10% (v/v). Cells were incubated for another 8 h. Subsequently, fluorescence was readed using a fluorescence excitation wavelength of 560 nm and emission of 600 nm by a fluorescence microplate reader Safire2™ (Tecan Group Ltd., Switzerland). The effects of drugs on cell viability were evaluated by comparing the fluorescence with that of control.

2.5. Cell viability test

RAW264.7 cells (1 x 10^{6} cells/mL) were cultured overnight. Then different concentrations of drugs were added and incubated for 16 h, then alamarBlue® (Life Technologies Co., USA) was added to make the concentration of 10% (v/v). Cells were incubated for another 8 h. Subsequently, fluorescence was readed using a fluorescence excitation wavelength of 560 nm and emission of 600 nm by a fluorescence microplate reader Safire2™ (Tecan Group Ltd., Switzerland). The effects of drugs on cell viability were evaluated by comparing the fluorescence with that of control.

2.6. Nitric oxide determination

RAW264.7 cells (1 x 10^{6} cells/mL) were pretreated with different concentrations of drugs for 2 h, and then incubated with 1 μg/mL LPS for 18 h. All control groups were not treated with anything. The nitrite accumulation in the supernatant was determined to be over 95% by normalization of the fluorescence.

2.7. Real-time polymerase chain reaction

RAW264.7 cells (1 x 10^{6} cells/mL) were pretreated with different concentrations of drugs for 2 h, followed by treatment with LPS (1 μg/mL) and incubation for an additional 6 h. Total RNA from RAW264.7 cells was extracted using Total RNA Extraction Reagent (Vazyme, China) according to the manufacturer’s instructions. The concentration and purity of RNA were measured by spectrophotometric analysis with a GeneQuant Pro spectrophotometer (Amerham Biosciences, USA). Total RNA (2 μg) was reverse-transcribed to cDNA by using the HiScript™ Q RT SuperMix (Vazyme, China) in a total volume of 20 μL. qRT-PCR was performed on a iCycler IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA) with SYBR® Green Master Mix (Vazyme, China). The sequences of primers used for qRT-PCR analyses are shown below. Amplification conditions were as follows: 95 °C initial denaturation for 5 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Relative expression levels of the target genes were calculated based on 2^{-ΔΔCT} according to the manufacturer's specifications by using the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as a reference housekeeping gene.

2.8. Preparation of cytoplasmic and nuclear protein extracts

RAW264.7 cells (1 x 10^{6} cells/mL) were pretreated with different concentrations of drugs for 2 h, and then incubated with 1 μg/mL LPS for 18 h (for iNOS and NF-κB p65). Cytoplasmic and nuclear protein extracts were prepared following the manufacturer's instructions. Briefly, 1 x 10^{7} cells were centrifuged for 3 min at 500g at 4 °C before 200 μL of lysis buffer A was added. After vortexed, the cells were centrifuged for 5 min at 16,000g at 4 °C. The supernatant (cytoplasmic protein extracts) was collected and stored at −80 °C. The pellet was used for nuclear fraction collection. Nuclear pellet was resuspended in 200 μL lysis buffer B, vortexed and incubated for 40 min on ice and then centrifuged for 10 min at 16,000g at 4 °C. The supernatant (nuclear protein extracts) was collected and stored at −80 °C.

2.9. Western blot

RAW264.7 cells (1 x 10^{6} cells/mL) were pretreated with different concentrations of drugs for 2 h, and then incubated with 1 μg/mL LPS for 18 h (for iNOS). The cellular protein was extracted by RIPA lysis buffer (Vazyme, China). The quantity of proteins was measured using the BCA (Beyotime, China) assay. Equal amounts of protein samples were separated by sodium dodecyl sulfate polyacrylamide gel (6% for iNOS and 10% for IkBα and p65) electrophoresis, and then transferred onto PVDF membranes. After blocking with 5% non-fat milk for 1 h at room temperature, the PVDF membranes were incubated with the primary antibodies at 4 °C overnight and subsequently with HRP-conjugated secondary antibodies at room temperature for 1 h. The protein bands were detected using ECL reagents. Chemiluminescent signals were detected and analyzed using the ChemiDoc XRS imaging system (Bio-Rad, USA).

2.10. Statistical analyses

Statistical analyses were performed with GraphPad Prism 5.0 software (GraphPad, USA). The results are expressed as the mean ± SD of 3 repeats.
individual values from three independent experiments. Data were compared by one-way ANOVA followed by a Dunnett’s Multiple Comparison Test. The differences were considered statistically significant when \( p < 0.05 \).

3. Results

3.1. Effect of A. fragrans on cell proliferation

To precisely determine any cytotoxic effect of A. fragrans, RAW264.7 cells were incubated with various concentrations of AF-Ext, AF-P1 and AF-P2 for 24 h and cell proliferation was examined. Cell viability of RAW264.7 cells treated with AF-Ext, AF-P1 and AF-P2 were without cytotoxicity below the concentration of 130.1 \( \mu g/mL \), 101.3 \( \mu g/mL \) and 134.2 \( \mu g/mL \), respectively (Fig. 3). Then all subsequent experiments were conducted at nontoxic concentrations (25–100 \( \mu g/mL \)).

3.2. Effect of A. fragrans on NO production

Subsequently, we investigated the effect of A. fragrans on LPS-induced NO production in RAW264.7 cells. The level of NO significantly increased after LPS-stimulation for 18 h in RAW264.7 cells (1.408 ± 0.2112 \( \mumol/L \) vs. 8.557 ± 0.6950 \( \mumol/L \), \( p < 0.001 \)). Dose-dependent response was observed in A. fragrans pretreated group (Fig. 4). AF-Ext and AF-P2 at 50 and 100 \( \mu g/mL \) significantly reduced the NO production to 2.608 ± 0.4032 \( \mumol/L \) and 1.733 ± 0.2412 \( \mumol/L \), 4.588 ± 0.8085 \( \mumol/L \) and 2.192 ± 0.4454 \( \mumol/L \), respectively, compared to the LPS alone-stimulated group (\( p < 0.001 \)). AF-Ext at 25 \( \mu g/mL \) and AF-P1 at 100 \( \mu g/mL \) also decreased NO production to 7.133 ± 0.9478 \( \mumol/L \) and 6.075 ± 0.5831 \( \mumol/L \), respectively, compared to the LPS alone-stimulated group (\( p < 0.05 \)).

3.3. Effect of A. fragrans on iNOS expression

Expression of iNOS mRNA and protein were minute in unstimulated RAW264.7 cells, but the expression of iNOS mRNA and protein were considerably upregulated over 272 folds and 367\% upon exposure to LPS for 6 h and 18 h, respectively. AF-Ext and AF-P1 at 25–100 \( \mu g/mL \) and AF-P2 at 50 and 100 \( \mu g/mL \) markedly decreased iNOS mRNA expression (by 47\%, 75\% and 95\% for AF-Ext; 36\%, 43\% and 86\% for AF-P1; 83\% and 87\%), after LPS-stimulation for 6 h in RAW264.7 cells (Fig. 5A–C, \( p < 0.001 \)). AF-Ext at 25–100 \( \mu g/mL \) and AF-P2 at 100 \( \mu g/mL \) markedly decreased iNOS protein expression, by 40\%, 57\% and 64\% for AF-Ext and 37\% for AF-P2, respectively, after LPS stimulation for 18 h in RAW264.7 cells (\( p < 0.001 \)). AF-P1 at 100 \( \mu g/mL \) and AF-P2 at 50 \( \mu g/mL \) decreased iNOS protein expression, by 32\% and 29\%, after LPS stimulation for 18 h in RAW264.7 cells (\( p < 0.01 \)). AF-P2 at 25 \( \mu g/mL \) also decreased iNOS protein expression, by 18\%, after LPS stimulation for 18 h in RAW264.7 cells (\( p < 0.05 \)).

3.4. Effect of A. fragrans on TNF-\( \alpha \) and IL-6 mRNA expression

To investigate the effects of A. fragrans on pro-inflammatory response at the transcription level, we examined TNF-\( \alpha \) and IL-6 mRNA expression in LPS-stimulated RAW264.7 cells. The levels of TNF-\( \alpha \) and IL-6 mRNA expression significantly increased after LPS-stimulation for 6 h in RAW264.7 cells (over 44 and 15786 folds, \( p < 0.001 \)). Dose-dependent responses were observed in AF-Ext pretreated groups (Fig. 6). AF-Ext and AF-P2 at 25–100 \( \mu g/mL \) significantly reduced the TNF-\( \alpha \) (by 25\%, 56\% and 81\% for AF-Ext; 19\%, 41\% and 72\% for AF-P1; 38\%, 53\% and 72\% for AF-P2) and IL-6 (by 60\%, 80\% and 97\% for AF-Ext; 24\%, 70\% and 95\% for AF-P1; 72\%, 86\% and 93\% for AF-P2) mRNA expression, respectively, compared to the LPS alone-stimulated group.

Fig. 3. Effect of AF-Ext (A), AF-P1 (B) and AF-P2 (C) on cell viability of RAW264.7 cells. RAW264.7 cells were incubated with 1–2000 \( \mu g/mL \) of AF-Ext, AF-P1 and AF-P2, respectively, for 24 h. Cell viability was measured by alamarBlue\textsuperscript{\textregistered} assay. Three independent experiments were performed in duplicate.
3.5. Effect of A. fragrans on IkBα degradation

Since IkBα degradation was the key step in NF-κB activation, we investigated the effect of A. fragrans on IkBα degradation. Compared with the unstimulated RAW264.7 cells, content of IkBα protein quickly decreased by nearly 23% after 1 h of LPS-stimulation (p < 0.05), which suggested the fast degradation of IkBα protein by LPS-stimulation. Pre-treatment with AF-P1 and AF-P2 at 50 and 100 μg/mL significantly recovered the content of IkBα protein by 79% and 54% for AF-P1 and 93% and 125% for AF-P2 (Fig. 7, p < 0.001). AF-Ext at 25 and 100 μg/mL, AF-P1 and AF-P2 at 25 μg/mL recovered the content of IkBα protein by 35% and 50% for AF-Ext, 47% for AF-P1 and 56% for AF-P2 (p < 0.01), AF-Ext at 50 μg/mL also recovered the content of IkBα protein by 34% (p < 0.05). All these results suggested the inhibitory effect of AF-Ext on LPS-induced degradation of IkBα.

3.6. Effect of A. fragrans on p65 translocation

In the unstimulated RAW264.7 cells, slight amount of p65 translocated into nucleus. LPS induced an increment by nearly 35% of nuclear translocation of p65 compared with that under the unstimulated condition (p < 0.05, Fig. 8). AF-Ext at 100 μg/mL significantly reduced the nuclear translocation of p65 by over 30%. AF-P2 at 100 μg/mL also reduced the nuclear translocation of p65 by over 19%.

4. Discussion

There have been many studies on 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid. These two compounds both can activate murine macrophage and enhance the spreading and mobility of macrophages (Tatefuji et al., 1996). They can suppress NO/iNOS (Olmos et al., 2008; Park et al., 2009) and PGE2/COX-2 (Puangpraphant et al., 2011) pathways through inhibiting nucleus translocation of NF-κB and reduce the production of TNF-α (Zhong et al., 2015) in LPS-induced RAW264.7 macrophages. And 4,5-dicaffeoylquinic acid is more potent than 3,5-dicaffeoylquinic acid (Park et al., 2009).

In this context, we investigated the anti-inflammatory effects of A. fragrans Champ. Extract (AF-Ext) and its components 3,5-dicaffeoylquinic acid (AF-P1) and 4,5-dicaffeoylquinic acid (AF-P2) using LPS-stimulated murine macrophages RAW264.7 cells. We found that A. fragrans imposed significant inhibitory effects in NO production. Therefore, following studies of A. fragrans in inhibiting iNOS expression and NF-κB activation were performed in order to elucidate its underlying molecular mechanisms.

Over-production of NO is thought to be closely associated with inflammation. NO can mediate the functions of many types of cells at the site of inflammation, including T lymphocytes, monocytes/macrophages, endothelial cells, synovial fibroblasts and osteoclasts (Sakaguchi et al., 2004). Meanwhile, excessive NO may promote the production of cytokine and matrix metalloproteinase, mitochondrial dysfunctions and cell apoptosis which accelerate the development of inflammation (Jarvinen et al., 2008). Therefore, NO/iNOS has been considered as a promising therapeutic target for treatment of inflammatory diseases. In the present study, we first investigated the effect of A. fragrans on NO production in LPS-stimulated RAW264.7 cells. We found that AF-Ext and AF-P2 at the concentrations without significant cytotoxicity remarkably decreased the LPS-induced NO production in a dose-dependent manner. Therefore, suppressive effect of NO is not due to the cytotoxic effect. However, AF-P1 decreased the LPS-induced NO production might be due to its cytotoxicity.

Among the three types of NO synthases, iNOS is thought to produce much larger amounts of NO than nNOS and eNOS in inflammatory conditions (van’t Hof andRalston, 2001). Since we showed the inhibitory effect of A. fragrans on NO over-production in LPS-stimulated RAW264.7 cells, we investigated the effect of A. fragrans on the expression of iNOS in the level of mRNA and protein. We found that A. fragrans both significantly decreased the expression of iNOS in...
Independent experiments were performed in duplicate, was analyzed by Western blot, and was used as the reference. (A–C) INOS mRNA was analyzed by qRT-PCR, and GAPDH was used as the reference. (D) INOS protein was analyzed by Western blot, and β-actin was used as the reference. (E–G) The bar chart shows the quantitative evaluation of INOS bands by densitometry. Three independent experiments were performed in duplicate, **p < 0.01 and ***p < 0.001 vs. Control group.

Fig. 5. Effect of A. fragrans on iNOS mRNA (A–C) and protein (D–G) expression in LPS-stimulated RAW264.7 cells. RAW264.7 cells were incubated with 25–100 µg/mL AF-Ext (A and E), AF-P1 (B and F), and AF-P2 (C and G), and 1 µg/mL LPS for 6 h. (A–C) INOS mRNA was analyzed by qRT-PCR, and GAPDH was used as the reference. (D) INOS protein was analyzed by Western blot, and β-actin was used as the reference. (E–G) The bar chart shows the quantitative evaluation of INOS bands by densitometry. Three independent experiments were performed in duplicate, **p < 0.01 and ***p < 0.001 vs. Control group.

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Based on the above results, our current work was consistent with previous studies. AF-P1 can reduce the expression of inflammatory mediators, but little impact on NF-κB activation pathway. This suggested that AF-P1 regulates the expression of inflammatory mediators through other pathways, such as mitogen-activated protein kinase (MAPK). AF-P2 can inhibit the expression of inflammatory mediators by inhibiting NF-κB-activated pathway along with AF-Ext. Anti-inflammatory activity of AF-Ext was more potent than AF-P2 for reducing the production of NO, the mRNA expression of TNF-α and IL-6, and protein expression of iNOS, which suggested that AF-P2 may be one of the main ingredients of AF-Ext, but not the only one. Studing the remaining unknown components.

Fig. 6. Effects of *A. fragrans* on TNF-α (A–C) and IL-6 (D–F) mRNA expression in LPS-stimulated RAW264.7 cells. RAW264.7 cells were incubated with 25–100 μg/mL AF-Ext (A and D), AF-P1 (B and E) and AF-P2 (C and F), and 1 μg/mL LPS for 6 h. TNF-α and IL-6 mRNA were analyzed by qRT-PCR, and GAPDH was used as the reference. Three independent experiments were performed in duplicate, ***p < 0.001 vs. Control group, *p < 0.01 and **p < 0.001 vs. LPS group.

Fig. 7. Effect of *A. fragrans* on IκBα degradation in LPS-stimulated RAW264.7 cells. RAW264.7 cells were incubated with 25–100 μg/mL AF-Ext (B), AF-P1 (C) and AF-P2 (D), and 1 μg/mL LPS for 1 h. (A) IκBα degradation was analyzed by Western blot, and β-actin was used as the reference. (B–D) The bar chart shows the quantitative evaluation of IκBα bands by densitometry. Three independent experiments were performed in duplicate, #p < 0.05 vs. Control group, *p < 0.05, **p < 0.01 and ***p < 0.001 vs. LPS group.

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5. Conclusions

Taken together, our findings suggest that *A. fragrans* might reduce LPS-induced NO over-production in RAW264.7 macrophages through...
The inhibition of iNOS mRNA and protein expression. A. fragrans also inhibited the mRNA expression of pro-inflammatory cytokines such as TNF-α and IL-6. We demonstrated that A. fragrans suppressed LPS-induced NF-κB activation, particularly upon LPS degradation and nuclear translocation of p65. It would have been interesting to study the effects of A. fragrans on other related signaling pathways such as MAPK. And we also demonstrated that 4,5-dicaffeoylquinic acid as one of the major components of A. fragrans. Our current findings may provide scientific evidence validating the use of A. fragrans in treatment of patients with gynecological diseases in clinical practice in traditional Chinese medicine.

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