

# Anti-inflammatory effect of patchouli alcohol isolated from *Pogostemonis Herba* in LPS-stimulated RAW264.7 macrophages

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**Abstract.** *Pogostemonis Herba* has long been used in traditional Chinese medicine for the treatment of inflammation-related disorders. Patchouli alcohol (PA) isolated from *Pogostemonis Herba* is a tricyclic sesquiterpene that is known to exert a variety of pharmacological activities. The present study aimed to investigate the anti-inflammatory effect of PA on lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Pre-treatment with PA at concentrations of 10, 20 or 40  $\mu$ M dose-dependently decreased the production of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6, nitric oxide (NO) and prostaglandin E<sub>2</sub> in LPS-stimulated RAW264.7 cells. In addition, PA treatment also reversed the increased mRNA expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 caused by LPS in RAW264.7 cells. These results indicate that PA is an important anti-inflammatory constituent of *Pogostemonis Herba* and that its anti-inflammatory effect may be mediated, at least in part, by down-regulation of the mRNA expression of a panel of inflammatory mediators, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, iNOS and COX-2.

## Introduction

*Pogostemonis Herba* is the dried aerial part of *Pogostemon cablin* (Blanco) Benth. (*Labiatae*), commonly known as 'Guang-Huo-Xiang' in Chinese or Cablin Patchouli in English. It has been traditionally used in Chinese medicine to remove dampness, relieve summer heat and exterior syndrome, and as an antiemetic and appetite stimulant (1). *Pogostemonis Herba*

is a common Chinese herb frequently used as a component in popular traditional formulae, such as Baoji Pill and Houdan Pill, for the treatment of inflammatory diseases (2,3). In addition, patchouli oil (essential oil of Patchouli) has been widely used in the cosmetic and oral hygiene industries to scent perfumes and flavor toothpaste. Recent research has demonstrated that patchouli oil has a variety of pharmacological activities, including antiemetic (4), anti-inflammatory (3,5), anti-allergic (6), immunomodulatory (7) and antimicrobial actions (8). Chemically, it has been reported that *Pogostemon cablin* contains sesquiterpenes (9), cytotoxic chalcones (10) and antimutagenic flavones (11). Patchouli alcohol (PA; chemical structure in Fig. 1), a tricyclic sesquiterpene, is a major active ingredient of *Pogostemonis Herba* and is the most odor-intensive component of patchouli oil (12). *Pogostemonis Herba* has been reported to contain 0.023-0.039% PA (12). In recent years, PA has attracted significant research attention due to its potential as a cognitive enhancing, learning impairment attenuating and neuroprotective agent (13,14). However, it remains to be determined whether PA is the active constituent responsible for the anti-inflammatory effect of *Pogostemonis Herba*.

Macrophages are the first line of host defense against bacterial infection and cancer growth, and are believed to play essential roles in the initiation, maintenance and resolution of inflammation. When stimulated by bacterial endotoxin, such as lipopolysaccharide (LPS), macrophages produce a number of cytokines, such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  and IL-6; chemokines, such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and nitric oxide (NO); inflammation-related enzymes, such as cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) for the primary protection of the host (15,16). These cytokines and chemokines are essential for the inflammatory response to pathogenic germs or toxicants (17). However, overproduction of these inflammatory mediators is associated with numerous diseases, such as rheumatoid arthritis and atherosclerosis (18-21). Thus, inhibition of the overproduction of these inflammatory mediators may have a beneficial effect on these inflammatory diseases.

RAW264.7, a mouse macrophage cell line, has been widely used as an *in vitro* inflammatory model (15,22,23). In the present study, we aimed to investigate whether PA has an anti-inflammatory effect on LPS-stimulated RAW264.7 cells. We also examined the molecular mechanisms underlying the anti-inflammatory effect of PA by evaluating the mRNA

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**Key words:** *Pogostemonis Herba*, patchouli alcohol, anti-inflammatory effect, lipopolysaccharide, RAW264.7 macrophages

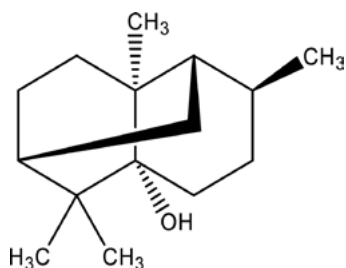


Figure 1. Chemical structure of patchouli alcohol.

expression of a number of inflammatory mediators, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, iNOS and COX-2.

## Materials and methods

**Plant materials and reagents.** The aerial parts of *Pogostemon cablin* were collected in June 2009 in Maoming, Guangdong, China. The plant was authenticated by one of the authors (X.-P.L., experienced in pharmacognosy) at the College of Chinese Medicines, Guangzhou University of Chinese Medicine, where a voucher specimen (no. 090612) was deposited.

LPS, RPMI-1640 culture medium, sulfanilamide and N-1-naphthylethylenediamine dihydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco (Grand Island, NY, USA). All other reagents and chemicals used in the study were of analytical grade.

**Extraction and isolation of PA.** The dried aerial parts of *Pogostemon cablin* (5 kg) were refluxed with 95% aqueous EtOH (40 liters  $\times$  2, 60 min each). The extract was evaporated under reduced pressure to obtain a residue (100 g). The residue was dissolved in acetone and subjected to silica gel column chromatography eluted with a petroleum ether-ethyl acetate-0.1% formic acid (20:1:0.1, 9:1:0.1, 8:3:0.1 and 7:4:0.1) gradient elution system. The fraction eluted with petroleum ether-ethyl acetate-0.1% formic acid (9:1:0.1) was combined and further evaporated to dryness, and a yellowish oily liquid was obtained. After crystallization from cyclohexane, white crystals of PA (540 mg, yield 0.011%) were finally obtained. The chemical structure of PA was identified by comparing its spectral data (MS,  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR) to those published previously (24). The purity of PA was found to be >98% based on gas chromatography (GC) analysis. PA was dissolved in dimethyl sulfoxide (DMSO) and the solvent concentration was <0.1% DMSO in all experiments.

**Cell culture and drug treatment.** The RAW264.7 cell line, derived from murine macrophages, was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were maintained in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) and 10% heat-inactivated FBS at 37°C in a humidified atmosphere of 95% air and 5% CO $_2$ . The cells were seeded onto 96-well culture plates at  $5 \times 10^4$  cells/well, unless otherwise specified. After seeding for 24 h, the cells were cultured in serum-free medium and incubated with different concentrations of PA (final concentrations:

0, 10, 20 and 40  $\mu\text{M}$ ) for 2 h. LPS at a final concentration of 100 ng/ml was then added for an additional 24 h.

**Cell viability assay.** Cell viability was measured by a CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation assay (Promega, Madison, WI, USA). In brief, the cells were washed with D-Hank's solution after drug treatment. Then, 100  $\mu\text{l}$  of serum-free medium and 20  $\mu\text{l}$  of CellTiter 96 AQueous One Solution were added to each well. The cells were further incubated at 37°C for 1 h. The quantity of formazan product, which is directly proportional to the number of living cells, was measured with a FLUOstar Optima microplate reader (BMG Labtech, Offenbury, Germany) at 490 nm. Cell viability was expressed as a percentage of the non-treated control.

**Nitrite oxide assay.** The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction method described elsewhere (15). Briefly, the supernatants were collected at the end of the drug treatment. A total of 100  $\mu\text{l}$  of each supernatant was mixed with the same volume of Griess reagent (50  $\mu\text{l}$  1% sulfanilamide in 5% phosphoric acid and 50  $\mu\text{l}$  0.1% N-1-naphthylethylenediamine dihydrochloride in water). After incubation for 10 min at room temperature, the absorbance was measured at 540 nm using a microplate reader. The content of nitrite was expressed as a percentage of the non-treated control.

**PGE $_2$  assay.** After PA treatment, the supernatants were collected and used for the PGE $_2$  assay. The PGE $_2$  concentration in the supernatant was determined using a commercially available PGE $_2$  EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, 50  $\mu\text{l}$  of diluted standard/samples was added to a 96-well plate pre-coated with goat polyclonal anti-mouse IgG antibody. Aliquots of a PGE $_2$  monoclonal antibody and PGE $_2$  acetylcholine esterase (AChE) conjugate were added to each well and allowed to incubate at room temperature for 60 min on an orbital shaker. After washing five times with wash buffer containing 0.05% Tween-20, 200  $\mu\text{l}$  of Ellman's reagent comprising acetylthiocholine and 5,5'-dithio-bis-(2-nitrobenzoic acid) was added to the wells. The plates were then incubated for 60 min at room temperature in the dark, then the absorbance was read at 405 nm using a microplate reader. The content of PGE $_2$  was expressed as a percentage of the non-treated control.

**Cytokine determination.** After PA treatment, the supernatants were collected and used for TNF- $\alpha$ , IL-1 $\beta$  and IL-6 assays. The levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in the supernatant were measured using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen Co., Carlsbad, CA, USA) as per the manufacturer's instructions. Briefly, samples and biotinylated anti-TNF- $\alpha$ , anti-IL-1 $\beta$  and anti-IL-6 antibodies (Biotin Conjugate) were respectively added to the 96-well plates pre-coated with monoclonal anti-mouse TNF- $\alpha$ , anti-mouse IL-1 $\beta$  and anti-mouse IL-6 antibodies, and incubated for 90 min at room temperature, 90 min at 37°C and 2 h at room temperature, respectively.

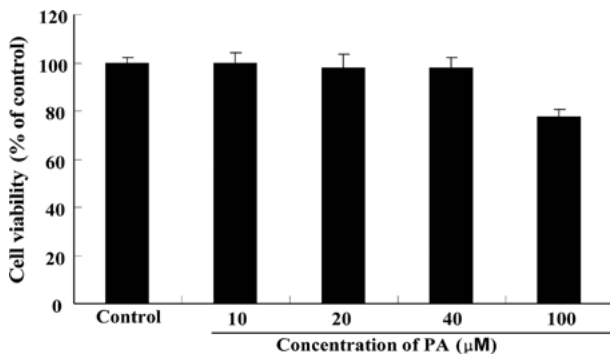


Figure 2. Effects of PA on the cell viability of RAW264.7 cells. The values shown represent the mean  $\pm$  SEM (n=6).

After washing four times, a streptavidin-HRP working solution was added and incubated for 30 min at room temperature. Then, tetramethylbenzidine (TMB) was added and incubated for 30 min at room temperature in the dark after washing. The reaction was stopped with stop solution and the absorbance was immediately recorded at 450 nm. The levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were expressed as a percentage of the non-treated control.

**Real-time PCR analysis.** The RAW264.7 cells were seeded at  $2 \times 10^6$  cells/well in 6-well plates. The cells were washed twice with D-Hank's solution after PA treatment. Total RNA was isolated from the cells with TRIzol reagent (Gibco). The concentration of extracted RNA was measured spectrophotometrically at 260 nm. The quality of RNA was assessed by the ratio of absorbance at 260 and 280 nm. The values of A260/A280 from 1.9 to 2.1 were considered acceptable. Total RNA (1.5  $\mu$ g) was used to synthesize cDNA using the High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Real-time PCR was performed with a Taq Man fast universal PCR master mix kit (2X) (Applied Biosystems) and mouse Taq Man gene expression assays were conducted (Applied Biosystems; assay ID: TNF- $\alpha$ , Mm00443258\_m1; IL-6, Mm99999064\_m1; IL-1 $\beta$ , Mm00434228\_m1; COX-2, Mm01307330\_g1; iNOS, Mm00440488\_m1 and  $\beta$ -actin, Mm02619580\_g1). The reactions were run at 50°C for 2 min and 95°C for 10 min, followed by 60 cycles at 95°C for 15 sec and 60°C for 1 min on the Applied Biosystems Step-One Fast Real-Time PCR system. Sequence Detection Software 2.0 (Applied Biosystems) was used for data analysis. The relative expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, COX-2 and iNOS mRNA was normalized to the amount of  $\beta$ -actin in the same cDNA using the relative quantification  $2^{-\Delta\Delta C_t}$  method (25). The fold change in target gene cDNA relative to the  $\beta$ -actin internal control was determined using the following formula: Fold change =  $2^{-\Delta\Delta C_t}$ , where  $\Delta\Delta C_t = (C_{t_{\text{target gene}}} - C_{t_{\beta\text{-actin}}}) - (C_{t_{\text{control}}} - C_{t_{\beta\text{-actin}}})$ .

**Statistical analysis.** Data were expressed as the mean  $\pm$  SEM. Multiple group comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test to detect differences between different treatment groups and the control. Differences were considered statistically significant at  $p < 0.05$ .

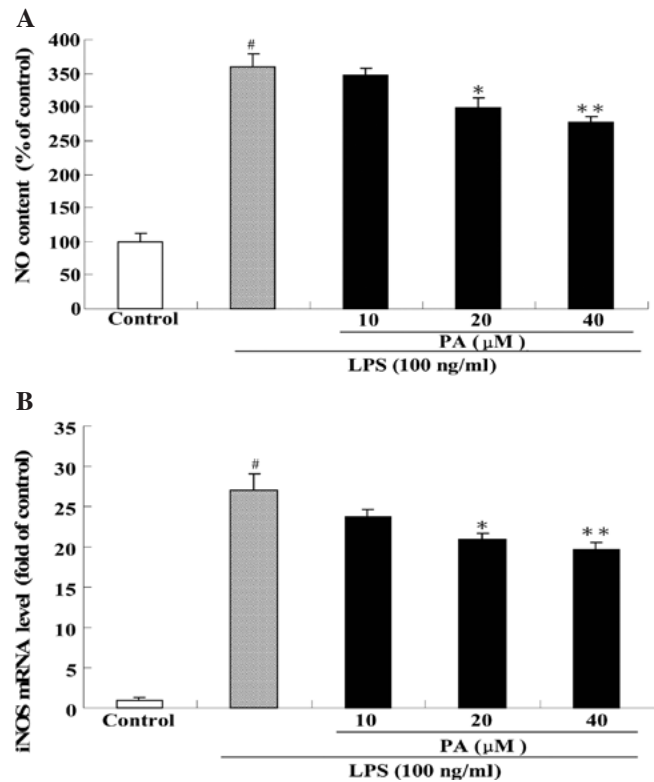


Figure 3. Effects of PA on the production of NO (A) and mRNA expression of iNOS (B) in LPS-stimulated RAW264.7 cells. The values shown represent the mean  $\pm$  SEM (n=3-6). <sup>#</sup> $p < 0.01$  compared to the control group; <sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$  and <sup>\*\*\*</sup> $p < 0.01$  compared to the LPS group.

## Results

**Cytotoxicity of PA on RAW264.7 cells.** As shown in Fig. 2, PA at concentrations up to 40  $\mu$ M did not show significant cytotoxicity to RAW264.7 cells when incubated for 24 h.

**Effect of PA on NO production and iNOS mRNA expression in LPS-stimulated RAW264.7 cells.** Fig. 3 shows the effect of PA on NO production (A) and iNOS mRNA expression (B) in LPS-stimulated RAW264.7 cells. After exposure of RAW264.7 cells to 100 ng/ml LPS for 24 h, the production of NO and the mRNA expression of iNOS were significantly increased by 3.6- and 27-fold, respectively, as compared to the control group. Pre-treatment with 20 and 40  $\mu$ M PA significantly decreased the production of NO by 17 and 23%, respectively, as compared to the LPS group. Pre-treatment with 20 and 40  $\mu$ M PA also significantly suppressed the mRNA expression of iNOS by 23 and 28%, respectively, as compared to the LPS group.

**Effect of PA on PGE<sub>2</sub> production and COX-2 mRNA expression in LPS-stimulated RAW264.7 cells.** Fig. 4 shows the effect of PA treatment on PGE<sub>2</sub> production (A) and COX-2 mRNA expression (B) in LPS-stimulated RAW264.7 cells. After exposure of RAW264.7 cells to 100 ng/ml LPS for 24 h, the production of PGE<sub>2</sub> and the mRNA expression of COX-2 were significantly increased by 39- and 180-fold, respectively, as compared to the control group. Pre-treatment with 20 and 40  $\mu$ M PA significantly decreased the production of PGE<sub>2</sub> by 19 and 21%, respectively, as compared to the LPS group. In

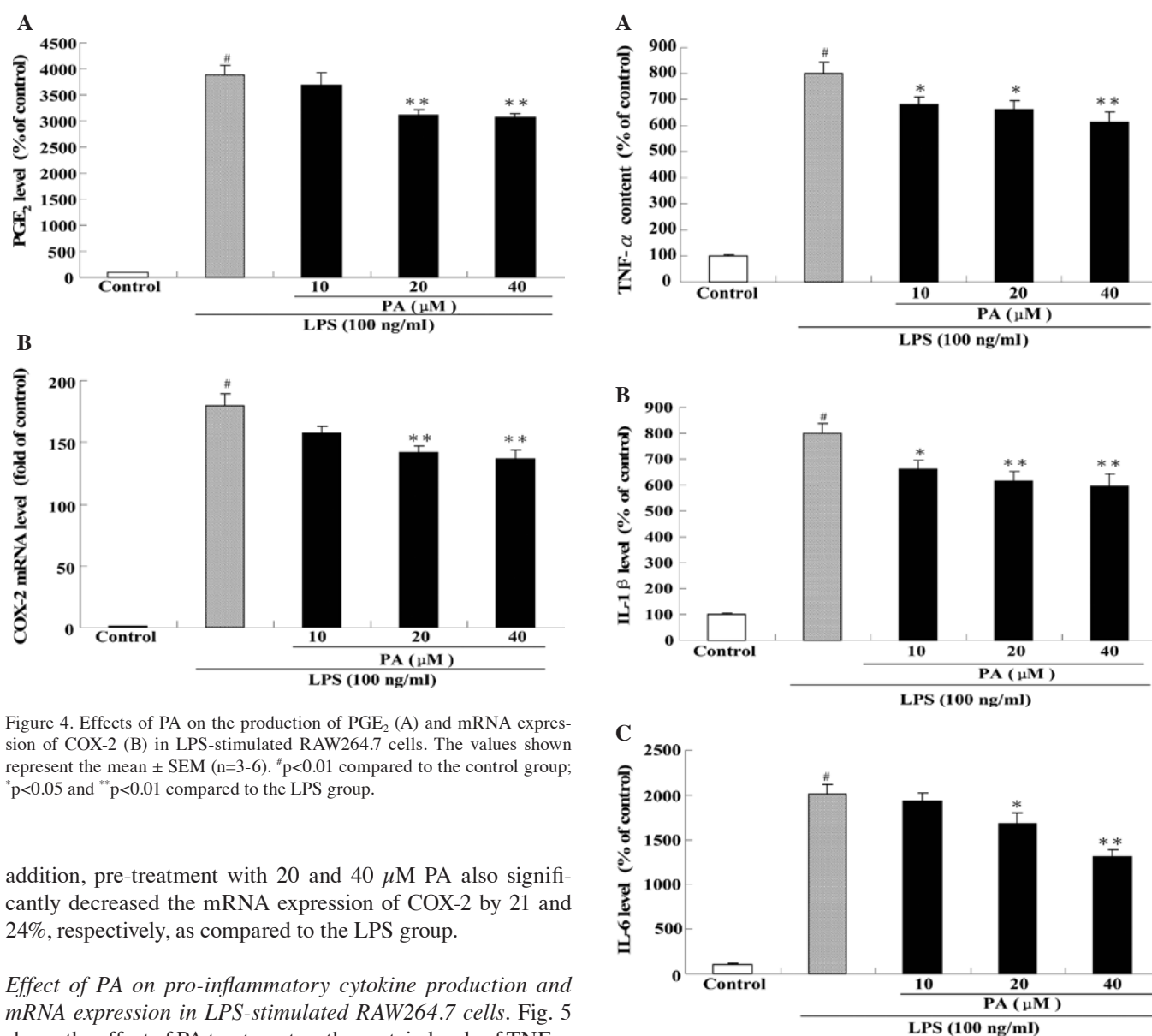


Figure 4. Effects of PA on the production of PGE<sub>2</sub> (A) and mRNA expression of COX-2 (B) in LPS-stimulated RAW264.7 cells. The values shown represent the mean  $\pm$  SEM (n=3-6). <sup>#</sup>p<0.01 compared to the control group; \*p<0.05 and \*\*p<0.01 compared to the LPS group.

addition, pre-treatment with 20 and 40  $\mu$ M PA also significantly decreased the mRNA expression of COX-2 by 21 and 24%, respectively, as compared to the LPS group.

*Effect of PA on pro-inflammatory cytokine production and mRNA expression in LPS-stimulated RAW264.7 cells.* Fig. 5 shows the effect of PA treatment on the protein levels of TNF- $\alpha$  (A), IL-1 $\beta$  (B) and IL-6 (C) in LPS-stimulated RAW264.7 cells. After exposure of RAW264.7 cells to 100 ng/ml LPS for 24 h, the protein levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were significantly increased by 8-, 8- and 20-fold, respectively, as compared to the control group. Pre-treatment with 10, 20 and 40  $\mu$ M PA dose-dependently suppressed the protein level of TNF- $\alpha$  by 15, 17 and 23%, respectively, as compared to the LPS group. In a similar fashion, pre-treatment with 10, 20 and 40  $\mu$ M PA dose-dependently decreased the protein level of IL-1 $\beta$  by 17, 23 and 25%, respectively, as compared to the LPS group. In addition, pre-treatment with 20 and 40  $\mu$ M PA also significantly reduced the protein level of IL-6 by 17 and 35%, respectively, as compared to the LPS group. Consistent with the results of the protein levels, real-time PCR revealed that pre-treatment with PA also significantly inhibited the mRNA expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in LPS-stimulated RAW264.7 cells (Fig. 6).

## Discussion

The present study clearly demonstrated that treatment of RAW264.7 cells with LPS caused a significant increase in

Figure 5. Effects of PA on the production of TNF- $\alpha$  (A), IL-6 (B) and IL-1 $\beta$  (C) in LPS-stimulated RAW264.7 cells. The values shown represent the mean  $\pm$  SEM (n=6). <sup>#</sup>p<0.01 compared to the control group; \*p<0.05 and \*\*p<0.01 compared to the LPS group.

the protein and mRNA levels of iNOS, COX-2, TNF- $\alpha$ , IL-6 and IL-1 $\beta$ . However, pre-treatment with PA at concentrations of 10, 20 and 40  $\mu$ M dose-dependently decreased the protein and mRNA levels of NO, PGE<sub>2</sub>, TNF- $\alpha$  and IL-1 $\beta$  in LPS-stimulated RAW264.7 cells.

NO, an important messenger molecule, is intimately involved in the inflammatory response (26,27). NO is generated by a family of enzymes called NO synthase (NOS) (28). It is known that NO plays a beneficial role in anti-tumor and anti-virus replication, and in anti-inflammatory processes (29,30). However, overproduction of NO by macrophages has been implicated in bacterial septic shock (31), and in certain inflammatory and autoimmune diseases (32,33). For this reason, down-regulation of the overproduction of NO has become a new therapeutic strategy for the treatment of chronic inflammatory diseases (34,35). In the present study, we found

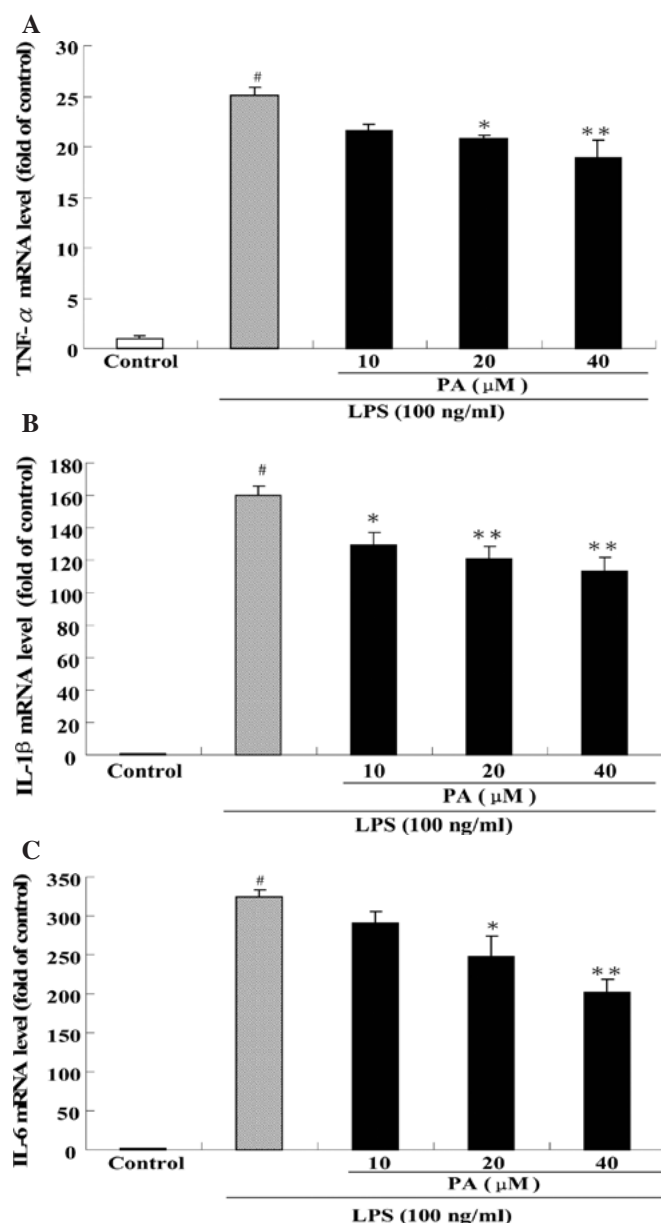


Figure 6. Effects of PA on the mRNA expression of TNF- $\alpha$  (A), IL-6 (B) and IL-1 $\beta$  (C) in LPS-stimulated RAW264.7 cells. The values shown represent the mean  $\pm$  SEM (n=3). <sup>#</sup>p<0.01 compared to the control group; <sup>\*</sup>p<0.05 and <sup>\*\*</sup>p<0.01 compared to the LPS group.

that exposure of RAW264.7 cells to LPS caused a significant increase in NO production and iNOS mRNA expression. This observation is consistent with the findings previously reported by other researchers (15,36). Pre-treatment with PA markedly reduced NO production and iNOS mRNA expression in LPS-stimulated RAW264.7 cells, suggesting that PA is capable of exerting anti-inflammatory action by inhibiting iNOS mRNA expression.

PGE<sub>2</sub> is considered to be one of the most potent inflammatory mediators in the inflammatory response and plays a major role in the pathogenesis of various inflammatory diseases, edema, angiogenesis, tumor growth and invasion (15,37). PGE<sub>2</sub> is transformed from arachidonic acid via a COX-2 catalytic reaction (38). COX-2 has been demonstrated to be a critical pro-inflammatory enzyme which contributes to the development of many chronic inflammatory diseases,

such as vascular atherosclerosis and rheumatoid arthritis (39). Recent years have seen an increasing interest in the use of COX-2 inhibitors as novel anti-inflammatory agents. Indeed, non-steroidal anti-inflammatory drugs (NSAIDs) exert their antipyretic, anti-inflammatory and analgesic effects through the inhibition of COX activity and the reduction of the production of inflammatory mediators, such as PGE<sub>2</sub> (15). Our present study demonstrated that PGE<sub>2</sub> production and COX-2 mRNA expression were markedly increased in LPS-stimulated RAW264.7 cells. However, pre-treatment with PA significantly mitigated the augmented PGE<sub>2</sub> production and COX-2 mRNA expression in these cells. These results suggest that PA exhibits a selective inhibitory effect on COX-2 similar to traditional NSAIDs. Thus, PA is worthy of further development as a new anti-inflammatory agent.

Cytokines play important roles in the regulation of inflammation. TNF- $\alpha$ , IL-1 $\beta$  and IL-6 are multifunctional pro-inflammatory cytokines and exhibit various pro-inflammatory effects in chronic inflammatory diseases, such as rheumatoid arthritis and atherosclerosis (40-42). TNF- $\alpha$  has long been considered as a key mediator for the induction of apoptosis and the development of the humoral immune response. At a high concentration, however, it elicits detrimental effects, such as causing tissue injury and potentiating septic shock (43,44). It has been reported that TNF- $\alpha$  elicits downstream pro-inflammatory events, such as the release of IL-6, another inflammatory cytokine (45). IL-6 plays essential roles in host defense, acute phase reactions, immune responses and nerve cell functions (46-49). A high level of IL-6 has been observed in various pathological conditions, including bacterial and viral infections, trauma, autoimmune diseases and inflammations (47,48). IL-1 $\beta$  is mainly produced by macrophages, monocytes and T cells and is also involved in immune defense against infection. In the present study, we clearly demonstrated that the protein and mRNA levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were significantly increased in LPS-stimulated RAW264.7 cells, and pre-treatment with PA dose-dependently reduced the protein and mRNA levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in these cells. These findings indicate that the anti-inflammatory effect of PA is associated with the down-regulation of the mRNA expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6.

In conclusion, the present experimental results demonstrated that PA exerts an anti-inflammatory effect on LPS-stimulated RAW264.7 cells. The observed anti-inflammatory action of PA may be mediated, at least in part, by down-regulation of the mRNA expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, iNOS and COX-2 in LPS-stimulated RAW264.7 cells. These promising experimental findings not only justify the use of *Pogostemonis Herba* in Chinese medicine for inflammatory conditions, but also suggest that PA, an essential active constituent of *Pogostemonis Herba*, to be a potential chemical agent for the treatment of inflammatory diseases. Further investigation to elucidate the underlying mechanism of action associated with the anti-inflammatory effect of PA is currently in progress at our laboratory.

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