Original Article

Anti-inflammatory effects of the ethyl acetate extract of *Aquilaria crassna* inhibits LPS-induced tumour necrosis factor-alpha production by attenuating P38 MAPK activation

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To study the inhibitory effect of the ethyl acetate extract of *Aquilaria crassna* on lipopolysaccharide (LPS)-induced tumour necrosis factor-alpha (TNF-α) secretion from isolated human peripheral blood mononuclear cells and its mechanisms of anti-inflammation so as to provide some evidence for its traditional use. Human peripheral blood mononuclear cells (hPBMCs) were isolated from healthy volunteers. Cells, at a concentration of 1×10^6 cell/ml, were induced to secrete TNF-α by exposure to 10 ng/ml LPS in the presence and absence of the ethyl acetate extract of *Aquilaria crassna*. The TNF-α secretion in the collection medium was measured by enzyme-linked immunosorbent assay and the TNF-α gene expression was measured by reverse transcriptase-polymerase chain reaction. Determination of ERK1/2 MAPK and p38 MAPK activation were performed by Western blot analysis using a specific phosphorylated form of ERK1/2, p38 MAPK antibody. LPS at a concentration of 10 ng/ml significantly increased in TNF-α secretion and was significantly inhibited when treated with 1.5 mg/ml ethyl acetate extract of *Aquilaria crassna* ($P<0.05$). Moreover, on treatment with 1.5 mg/ml, the extracts showed TNF-α gene expression inhibition. Co-treatment of the extract with LPS could not block p38 MAPK activation, but pre-treatment of the extracts significantly reduced the p38 MAPK phosphorylation without affecting the ERK1/2 MAPK activation ($P<0.05$). The ethyl acetate extract of *Aquilaria crassna* inhibits TNF-α gene expression and secretion in LPS-induced hPBMCs. This inhibitory effect apparently resulted from selectively attenuating the p38 MAPK activation.

Key words: Anti-inflammatory, *Aquilaria crassna*, p38 MAPK, PBMCs, TNF-α

INTRODUCTION

Agarwood (Aquilaria species) is heartwood of tropical tree belonging to the family Thymelecease and class Magnoliosida.[1] It can be found in many countries in the oriental regions, including Thailand, Indonesia, India, Laos, Malaysia, Philippines, Tibet, Korea and China.[1-3] There are many species of Aquilaria spp., including *Aquilaria sinensis, Aquilaria crassna, Aquilaria agallocha,* etc. It has been used for therapeutic purposes, as folk medical treatment for arthritis, gout and headache.[3] It was also used in the traditional treatment of vomiting, cough, asthma, cardiac disorders, leprosy and anorexia.[3] In addition, the plant extract was also reported as having anticancer activities,[4] and the benzene extracts of this plant contains an antidepressive activity.[5] In Thailand, the Aquilaria extract has been used as one of the compositions in Ya-hom, a traditional Thai herbal formulation for the treatment of fainting, and its dominant effect was known to increase in blood pressure, reflecting the cardiovascular effect of this folk formula.[6] Moreover, many evidences show that the extracts from the Agarwood plant exhibited a potentially anti-inflammatory effect.[2,7-10] However, information from those published works was demonstrated in an *in vivo* model, but still lacks some information describing the *in vitro* and cellular mechanisms of the Aquilaria heartwood extract to reduce inflammation.

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Phytomedicine of bioactive extracts, especially plant extracts, can provide an alternative means for inflammatory diseases treatment. Nowadays, in Thailand, numerous plant species have been screened for their pharmacological properties and toxicities. However, many of those still need to be investigated. Without such an understanding

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of the drug pharmacological properties, mechanisms and toxicities, there is, surely, a danger of introducing phytomedicine to patients. In the present study, we aimed to investigate the *in vitro* anti-inflammatory mechanism of Aquilaria extracts on the lipopolysaccharide (LPS)-induced tumour necrosis factor-alpha (TNF-α) production by human polymorphonuclear cells (hPBMC) and cell signaling response that was attenuated by Aquilaria extract treatment.

MATERIALS AND METHODS

Plant Material and Extraction

Aquilaria crassna Pierre ex Lecomte used in this experiment was obtained from Mr. Choosak Rerngrattanabhume. The plant was originally cultivated at the area in Pong Nam Ron district, Chantaburi province, Thailand, and, subsequently, identified by Dr. Pranee Nangngam, Department of Biology, Faculty of Science, Naresuan University. The specimen voucher number 002540 was kept at the Department of Biology herbarium, Faculty of Science, Naresuan University. The heartwood was sliced into small pieces. The dried plant (1 kg) was consecutively extracted with ethyl acetate (EtOAc) (800 ml reflux) for 2 days each. The resulting EtOAc solution was concentrated under reduced pressure to yield the EtOAc extract (950 mg).

Chemicals and Reagents

The chemicals and reagents used in the present study were Ficoll Hypaque (Pharmacia Biotech, Piscataway, NJ, USA), RPMI-1640 medium and foetal bovine serum (Gibco BRL; Life Technologies Inc., New York, NY, USA), 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Ameresco, Solon, Ohio, USA), LPS (from *Escherichia coli*) (Sigma, St. Louis, MO, USA), enzyme-linked immunosorbent assays (ELISAs) for human TNF-α (Invitrogen, Carlsbad, California, USA), Easy-RED™ total RNA extraction kit (iNtRON Biotechnology, Gyeonggi-do, Korea), cDNA synthesis kit (RevoScript RT Premix Kit with oligo (dT) primers #IP25084,iNtRON Biotechnology, Gyeonggi-do, Korea), Taq DNA polymerase from Maxime™ PCR Premix Kit (#25265, iNtRON Biotechnology, Gyeonggi-do, Korea), human TNF-α and GAPDH primers (Pacific Science, Bangkok, Thailand), total-p38 (T-p38) and diphospho-p38 (P-p38), total ERK1/2/42 MAPK (ERK1/2) and phospho-ERK1/2/42 MAPK (p-ERK1/2) antibody (Cell Signaling Technology, Danvers, Massachusetts, USA).

Isolation of Human PBMC and Culture

PBMC from healthy donors were isolated from EDTA blood by Ficoll-Hypaque gradient centrifugation. In short, peripheral blood from the donors was diluted with sterile phosphate buffer saline and overlaid on the Ficoll-Hypaque solution, and centrifugation was performed at 350 x g for 10 min at room temperature. The recovered PBMC were rested in RPMI-1640 and incubated at 37°C 95%O_2 + 5% CO_2 for 30 min before performing the experiments. Cell viability was determined by a Trypan blue dye exclusion assay. The percentages of cell viability were calculated by the ratio of Trypan blue excluding cells to total cell number.

LPS Stimulation and Incubation of the Aquilaria Extracts with PBMC

After the PBMC isolation and pre-incubation period, 200 l of 1×10^5 cells/ml PBMC were cultured in a 96-well polypropylene plate in serum-free RPMI-1640 medium with LPS at a final concentration of 10 ng/ml and various concentrations of the ethyl acetate extract of *Aquilaria crassna* (0.0–3.0 mg/ml, final concentration) in DMSO. In the control wells, cells were incubated with LPS and 0.01% DMSO vehicle. Cells in all conditions were incubated at 37°C, 95%O_2 + 5% CO_2 for 6 h. Cell viability was determined by Trypan blue dye exclusion and culture medium was collected and stored at -20°C until analysis for TNF-α production.

Determination of TNF-α Production

Supernatants were collected after an optimum incubation period and stored at -20°C until TNF-α ELISA was performed according to the manufacturer's instructions. Briefly, 100 µl of the collection medium and 50 µl of the detection antibody, conjugated with horseradish peroxidase, were added to 96-well plates pre-coated with capture antibody for 2 h at room temperature. TMB substrate was added to the reaction for 30 min at room temperature. The reactions were terminated by the addition of the stop solution before measuring the absorbance at 450 nm. The concentration of TNF-α can be calculated from the standard curve produced by the serial-diluted standard TNF-α.

Reverse Transcription Polymerase Chain Reaction

Cells (1.5×10^6/ml) were stimulated with 10 ng of LPS per milliliter in the presence or absence of the extracts, at inhibitory concentration, or vehicle control for 3 h at 37°C in a humidified environment with 5% CO_2. Total RNA was isolated from 10^7 monocytes/sample with the Easy-RED™ total RNA Extraction Kit. In brief, cell pellets were resuspended in 250 µl of phosphate-buffered saline and then lysed with 750 µl of the Easy-RED™ solution for 5 min at room temperature. The suspensions were treated with 200 µl of chloroform for 5 min at room temperature and the samples were centrifuged at 13,000 rpm for 15 min at 4°C. To precipitate RNA, an equal volume of isopropanol was added and incubated for 10 min at room temperature. The RNA was pelleted by centrifugation at 13,000 rpm at 4°C for 10 min. The pellet was washed with 70% ethanol and dissolved in 20–50 µl RNase-free water.

First strand cDNA was prepared using a RevoScript RT

Premix Kit with oligo (dT) primers. In brief, 1 µg of total RNA, which was extracted as described previously, was mixed with the RevoScript RT Premix solution to a final volume of 20 µl. The reactions were performed at 50°C for 1 h followed by RTase inactivation at 95°C for 5 min. After the reaction, Polymerase Chain Reaction (PCR) was performed using a Maxime™ PCR Premix Kit with primer pairs for human TNF-α or GAPDH [Table 1]. For each PCR reaction, 2 µg of cDNA was added to the mixture to a final volume of 20 µl. PCR was carried out on a thermal cycler (Perkin-Elmer, Norwalk, CN, USA) for 30 cycles for both TNF-α and GAPDH, with a single cycle consisting of 1 min at 94°C (denaturation), 1 min at 61°C (annealing) and 1 min at 72°C (extension). The final cycle was completed with an additional extension of 7 min at 72°C. The PCR products were subjected to 1% agarose gel electrophoresis and visualized under ultraviolet gel documentation. The intensity of the PCR product bands was measured and expressed in relative fold of expression compared with the level of GAPDH expression.

Measurement of p38 MAPK Activation

Isolated hPBMC (2 x 10^6 cells/sample) in serum-free RPMI-1640 medium were pre-incubated with the ethyl acetate extract of *Aquilaria crassna*, at inhibitory concentration, or vehicle control for 60 min prior to treatment with a final concentration of 10 ng/ml LPS, at 37°C, in a humidified environment with 5% CO_2 for 15 min. After incubation, the cells were pelleted and resuspended in sodium dodecyl sulphate (SDS) sample buffer (containing 2% SDS, 2% β-mercaptoethanol and 10% glycerol in 300 mM Tris-Cl, pH 6.8) and boiled for 5 min. The protein lysates were separated on 12% SDS–polyacrylamide gels at 110 V and transferred to Polyvinylidene fluoride (PVDF) membranes (Hybond-P, GE Health Care, Pittsburgh, Philadelphia, USA) for 1 h at 20 mV. The transferred protein on PVDF were incubated for 1 h with 5% non-fat milk + 1% bovine serum albumin in TRIS-buffered saline (pH 7.4) containing 0.1% Triton (TBST), for avoiding non-specific binding, and probed overnight at 4°C with the appropriate primary antibody recognizing p38 protein (T-p38), phosphorylated form of p38 (P-p38) and phosphorylated ERK1/2 MAPK (P-ERK1/2) or ERK1/2. After washing and exposure for 1 h at room temperature to horseradish peroxidase-conjugated secondary antibody, the membranes were developed using an enhanced chemiluminescence technique (GE Health Care, Pittsburgh, Philadelphia, USA). Bands corresponding to the detected protein of interest appeared as lighter regions when measured on a FluorChem™ SP imager (Alpha Innotech, Cell Biosciences, Santa Clara California, USA), which were scanned, and all band densities were measured. Band densities in different lanes can be compared, providing information on the relative abundance of the protein of interest.

Statistical Analysis

The results are expressed as mean±SD. Data sets were analysed by one-way analysis of variance followed by Tukey's multiple comparison test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Effect of the Ethyl Acetate Extract of *Aquilaria crassna* on LPS-Induced TNF-α Production

In order to activate TNF- production, isolated hPBMCs were exposed to 10 ng/ml LPS for 6, 12 and 24 h. The optimum condition for LPS-induced TNF-α production was 6 h [Figure 1]. Then, the effect of the ethyl acetate extract of *Aquilaria crassna* on anti-inflammation was tested by the addition of various concentrations of the Aquilaria extracts. Exposure of the Aquilaria extracts reduced the TNF-α level in a dose-dependent manner [Figure 2]. The final concentration of the extract at 1.5 mg/ml significantly reduced the TNF-α level. We then tested whether the reduction of TNF-α when treated with the extracts, observed in the previous results, was not due to the toxicity of the extract. Cell viability was performed by a Trypan blue dye exclusion assay. Exposure of hPBMCs to the Aquilaria extracts did not reduce the percentages of cell viability at all concentrations [Figure 3].

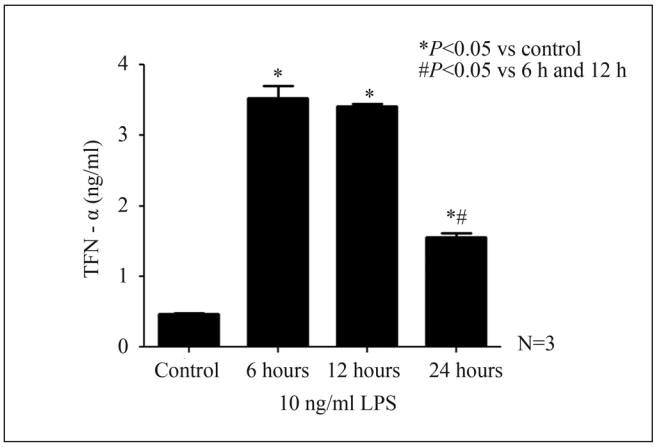

Figure 1: Lipopolysaccharide-induced tumour necrosis factor-alpha production

Table 1: Primer sequences for RT-PCR determine human TNF-α expression

Primer	Orientation	Sequences (5'>3')	Expected size (bp)
TNF-α	Sense	TCGGGCCAATGCCCTCCTGGCCAA	468
	Antisense	GTAGACCTGCCCAGACTCGGCAAA	
GAPDH	Sense	GGCATGGCCTTCCGTGTCCC	352
	Antisense	GCTCTTGCTGGGGCTGGTGG	

Effect of the Ethyl Acetate Extract of *Aquilaria crassna* on LPS-Induced TNF-α Expression

Evaluation of the effect of the ethyl acetate extract of the *Aquilaria crassna* extract on LPS-induced TNF-α mRNA expression was performed. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed on hPBMCs treated with 10 ng/ml LPS in the presence and absence of 1.5 mg/ml of the extract and the concentration that showed significantly inhibition of TNF-α secretion. The RT-PCR product fragment for TNF-α, and housekeeping gene GAPDH, were quantified and presented as expression intensity [Figure 4a] and as relative fold induction [Figure 4b]. The LPS-induced TNF-α mRNA expression was significantly higher than that of the vehicle control. Treatment with 1.5 mg/ml of the extract significantly reduced the LPS-induced TNF-α mRNA expression (P<0.05) without a background expression reduction (Aquilaria extract alone).

Ethyl Acetate Extract of *Aquilaria crassna* Attenuates LPS-Induced p38 MAPK Activation

It has been shown in many experimental settings that LPS induces the ERK1/2 MAPK (ERK1/2) and p38 MAPK activation.[11] Therefore, we hypothesized that the inhibitory effect of the ethyl acetate extract of *Aquilaria crassna* possibly results from an attenuation of activation of these MAPKs. To facilitate this hypothesis, cells were exposed to LPS in the presence and absence of 1.5 mg/ml of the extract for 15 min or pre-treatment with 1.5 mg/ml of the extract for 1 h prior to exposure to LPS. The results showed that LPS enhanced the ERK1/2 MAPK and p38 MAPK phosphorylation. Treatment of 1.5 mg/ml of the ethyl acetate extract of *Aquilaria crassna* together with LPS did not reduce the ERK1/2 and p38 MAPK activation [Figure 5a]. However, pre-treatment of the ethyl acetate extract of *Aquilaria crassna* for 1 h selectively prevented the phosphorylation of p38 MAPK, but not ERK1/2 [Figure 5b].

DISCUSSION

Aquilaria spp. or agarwood has been used for therapeutic purposes, as a traditional medical treatment for many diseases,[3] including inflammation.[2,7-10] However, the underlying mechanisms of this plant extract still need to be elucidated in an attempt to increase an understanding of the actual effect and possible toxicity of using the plant extract.

In the present study, the anti-inflammatory effect of the ethyl acetate extract of *Aquilaria crassna* was demonstrated in an *in vitro* model, which focused on the inhibitory effect of the extract on pro-inflammatory cytokine secretion. Bacterial LPS has been proven to induce TNF-α secretion from many cell types such as murine macrophage cell line RAW 264.7, isolated human monocytes, THP-1 and NK cells.[12,13] This model is a good model for screening the anti-inflammatory effect of chemicals or natural extracts, which are candidates for new therapeutic agents. In this work, we

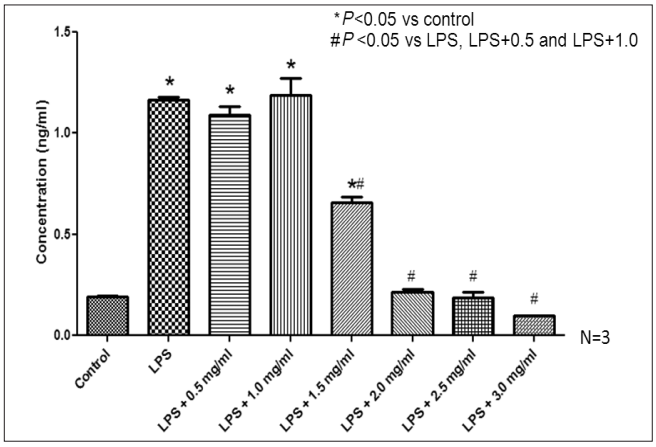

Figure 2: Effect of the Aquilaria extract on lipopolysaccharide-induced tumour necrosis factor-alpha production

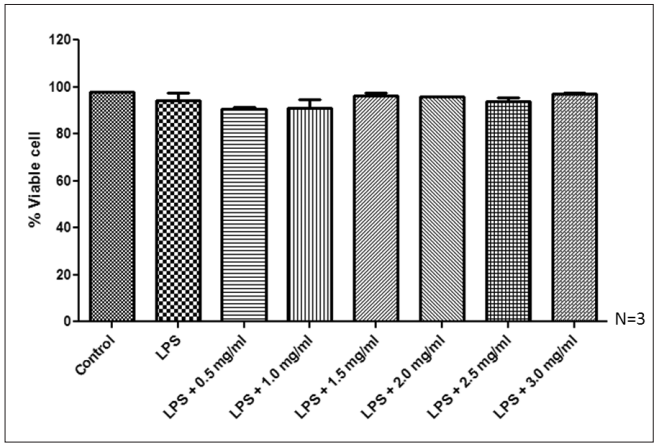

Figure 3: Effect of the Aquilaria extract on cell viability

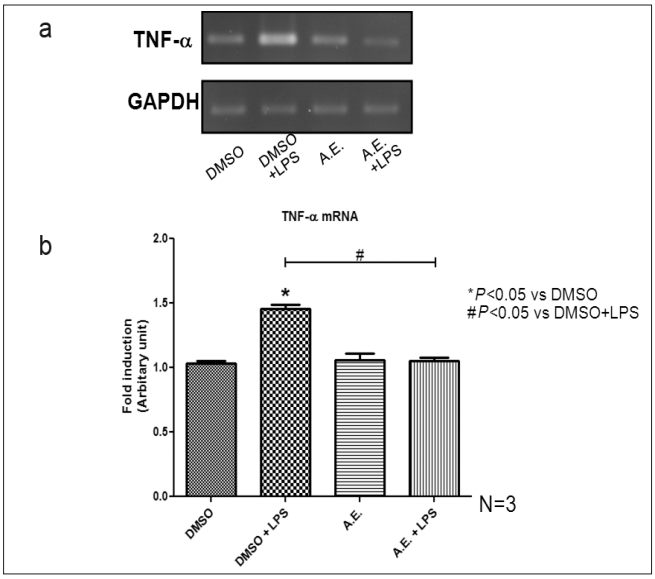

Figure 4: Effect of the Aquilaria extract on lipopolysaccharide-induced tumour necrosis factor-alpha expression

used LPS-induced TNF-α secretion in isolated hPBMCs, which is a model representing cellular responses in humans and is closely related to the physiological condition of inflammation in humans.

The results showed that 1.5 mg/ml of the ethyl acetate extract of *Aquilaria crassna* significantly reduced the TNF-α secretion from isolated hPBMCs, which was induced to secrete TNF-α by exposure to 10 ng/ml LPS. In addition, treatment with the Aquilaria extract was shown to inhibit the TNF-α gene expression when stimulated by LPS. However, the results also showed that treatment of this extract in non-stimulated cells did not show any difference when compared with the control. Interestingly, treatment with this dose, or an even higher dose, of the extract did not cause cellular cytotoxicity. These results suggest the anti-inflammatory effect of the Aquilaria extract, targeting on TNF-α production, and using this plant extract possibly did not result in adverse effects. However, the effect of this extract on other pro-inflammatory cytokines and the sensitivity to the extract of cells in different tissues needs to be elucidated.

The anti-inflammatory effect of the Aquilaria extract may not only target the TNF-α production, as Zhou *et al*.[10] demonstrated that the ethanol extract of Aquilaria leaves potentially inhibit the elevated nitric oxide (NO) level in LPS-stimulated NO release from macrophages.[11] Together with our findings, this work supports the anti-inflammatory effect and explains another mechanism of this plant extract.

p44/42 (ERK1/2) and p38 MAPK are activated in response to LPS,[14-16] and are also known to regulate TNF-α mRNA translation.[17-19] Treatment with a specific inhibitor of ERK1/2 and p38 MAPK inhibits the activation of these signaling proteins and reduces TNF-α secretion.[11] In our hands, this was the first evidence showing that LPS-induced p38 MAPK phosphorylation was inhibited by pre-treatment of the Aquilaria extract. However, co-treatment of the extract with LPS could not block p38 MAPK activation. Interestingly, the inhibitory effect of this extract on signal transduction seems to be selectively inhibiting p38 MAPK, not ERK1/2. This could be explained by the fact that the p38 MAPK pathway is the signaling pathway predominantly activated in response to stress and inflammation, than the ERK1/2 pathway, and the inhibitory effect of the Aquilaria extract on TNF-α levels predominates the p38 MAPK activation. Therefore, the inhibitory effect of the extract on p38 MAPK activation could be potentially fascinating for the treatment of other diseases such as ischaemic heart diseases.

It has been known that myocardial ischaemia is a good stimulant of p38 MAPK, which resulted in many cellular responses predominantly causing cellular necrosis. There

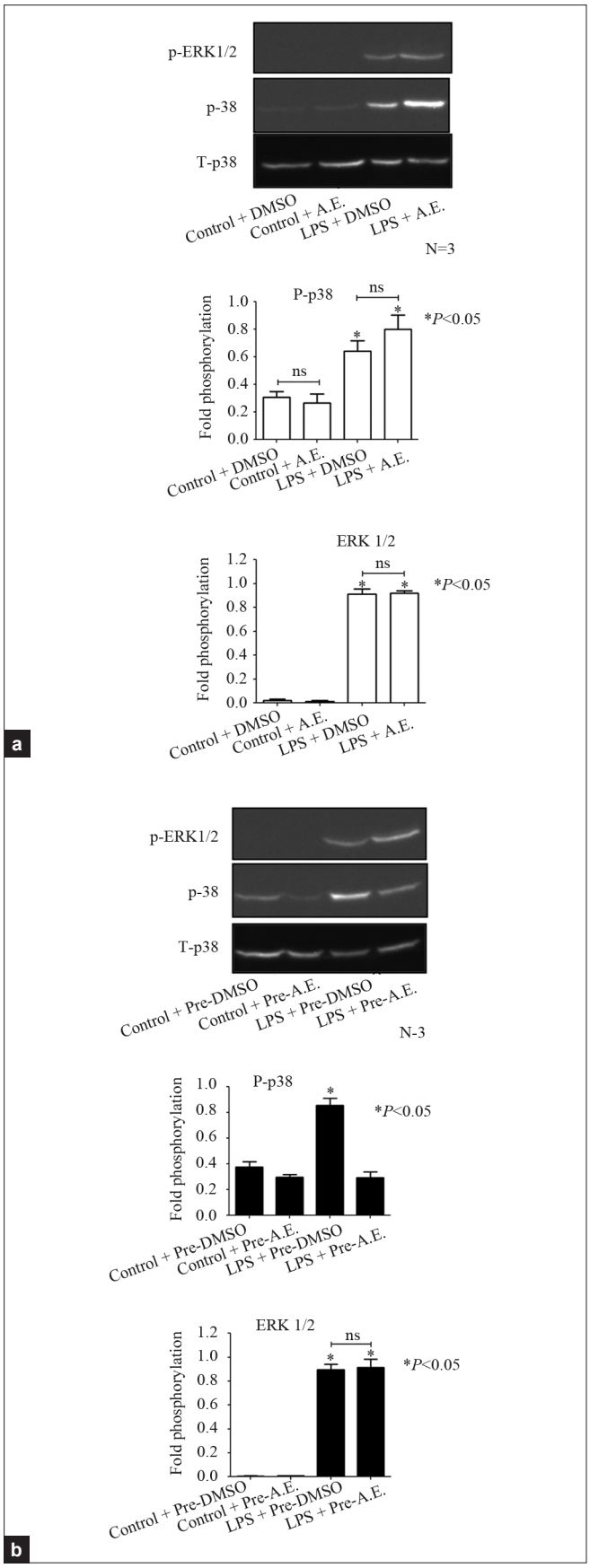

Figure 5: Effect of the Aquilaria extract on lipopolysaccharide-induced p38 MAPK activation

is increasing evidence from pre-clinical investigations that inhibition of p38 during prolonged ischaemia slows the rate of infarction/death and inhibits the production of inflammatory cytokines such as TNF-α, interleukin (IL)-1 and IL-8, which in turn aggravate ischaemic injury.[18,20] Inhibition of p38 MAPK activation and its activity, by the pharmacological inhibitor SB203580, is known to reduce the infarct size and improve cardiac function.[21] Interestingly, in Thailand, the Aquilaria extract has been used as one of the compositions in Ya-hom, a traditional Thai herbal formulation for the treatment of fainting, and its dominant effect was known to target the cardiovascular system.[22]

Our findings not only provided partial experimental evidence for an anti-inflammatory mechanism but were also beneficial to future studies about the effect of Aquilaria extract on other diseases. Identification of active compounds with therapeutic applications is a challenge and needs to be investigated further.

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