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The production of nitric oxide and prostaglandin E_2 in peritoneal macrophages is inhibited by *Andrographis paniculata*, *Angelica sinensis* and *Morus alba* ethyl acetate fractions

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ABSTRACT

Aim of the study: Traditional Chinese medicine herbs (TCMHs) are used in medicines as well as in daily dietary supplements in Asia. In this study, we employed pNF-κB-Luc or pIFN-γ-Luc and BALB/c mice peritoneal macrophages or splenocytes to investigate both the immune and inflammatory effects of six selected plant species.
Materials and Methods: Specifically, we used ethyl acetate fractions of *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao (*Fabaceae*) (AM), *Andrographis paniculata* (Burm. f.) Nees (Acanthaceae) (AP), *Angelica sinensis* (Oliv.) Diels (*Apiaceae*) (AS), *Eucommia ulmodes* Oliv. (Eucommiaceae) leaves (EU leaves), *Isatis indigotica* Fort. (*Brassicaceae*) (II) and *Morus alba* L. (Moraceae) (MA).
Results: We found that ethyl acetate fractions of AP, AS and MA significantly decreased NF-κB luciferase activity and also the secretion of NO and PGE_2 in LPS/IFN-γ stimulated mouse peritoneal macrophages ($p < 0.05$). In contrast, they did not affect IFN-γ luciferase activity or IFN-γ production in concanavalin A (Con A)-activated mouse splenocytes. Our results indicated that the anti-inflammatory properties of these plant extracts might be resulted from the inhibition of pro-inflammatory mediators (e.g., NO and PGE_2), at least in part via suppression of a signaling pathway such as NF-κB.
Conclusions: Collectively, we have found that three potent bioactive TCMH species exerted significant NF-κB inhibitory activity and acted in a cell type dependent fashion.

1. Introduction

The purpose of this study was to investigate six selected plant species that may exert regulatory effects on the immune and inflammatory responses. In our preliminary study, we screened 22 selected traditional Chinese medicine herbs (TCMHs) using hexane, ethyl acetate (EtOAc) and H_2O fractions. We found that EtOAc fractions of *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao (*Fabaceae*), *Andrographis paniculata* (Burm. f.) Nees (Acanthaceae), *Angelica sinensis* (Oliv.) Diels (*Apiaceae*), *Eucommia ulmodes* Oliv. (Eucommiaceae) leaves, *Isatis indigotica* Fort. (*Brassicaceae*) and *Morus alba* L. (Moraceae) had potential anti-inflammatory effects (Chao et al., 2007).

TCMHs have been well known for their use as medical treatments and food supplements for many centuries. For example, *Astragalus membranaceus* (Fisch.) Bge. is amongst the most popular health promoting herbs in China. *Andrographis paniculata* is reputed to be effective in the treatment of infection, inflammation, cold, fever, diarrhea and venomous snake bites (Table 1). *Angelica sinensis* (Oliv.) Diels has been used to invigorate blood circulation and to treat menstrual disorders.

The major biologically active components of endotoxins are lipopolysaccharide (LPS), which are derived from the cell walls of Gram negative bacteria. LPS activates multiple signaling pathways in macrophages that enhance the production of inflammatory mediators (Calixto et al., 2003, 2004). One of the LPS-induced pathways involves nuclear factor kappa B (NF-κB). NF-κB exists within the cytoplasm in an inactive form associated with a regulatory protein called I-κB. When stimulated by the LPS signal cascade, this leads to phosphorylation of I-κB, thereby releasing the NF-κB dimers, which translocate to the nucleus (Yamamoto and Gaynor, 2004). Moreover, over-expression of the NF-κB pathway leads to the formation of NO and PGE_2 (Ghosh et al., 1998; Gilmore, 1999).

Interferon-γ (IFN-γ) is a key cytokine produced by activated T cells. IFN-γ expression has been reported to be regulated by many

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Table 1
Pharmacological activity assessed of the traditional Chinese medicine herbs studied here.

Botanical names	Family	Common names	Parts used	Pharmacological activity assessed	Reference
Astragalus membranaceus (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao	Fabaceae	Huang-qi	Root	Tonic, anti-pyretic	Cho and Leung (2007)
Andrographis paniculata (Burm. F.) Nees	Acanthaceae	Chuan-Chin-Lian	Aerial part	Anti-inflammatory, anti-infection, clearing heat and detoxicating	Roxas and Jurenka (2007)
Angelica sinensis (Oliv.) Diels	Apiaceae	Dang-Gui	Root	Anti-inflammatory	Wang et al. (2006)
Eucommia ulmodes Oliv. leaves	Eucommiaceae	Du-Zhong leaves	Leaves	Anti-diabetic activity	Park et al. (2006)
Isatis indigotica Fort.	Brassicaceae	Pei-Ban-Lan-Gen	Root	Anti-infection, anti-microbial action	Ko et al. (2006)
Morus alba L.	Moraceae		Root, bark	Anti-inflammatory anti-obesity	Hong et al. (2002); Lee et al. (2008)

nuclear factors such as NFAT, NF-κB, AP-1 and T-bet (Penix et al., 1996; Sica et al., 1997; Sweetser et al., 1998; Szabo et al., 2000). IFN-γ modulates a variety of immune responses including pathogen clearance, tumor eradication, T cell activation and inflammatory responses (Abbas et al., 1996). Transcriptional factors such as NF-κB and AP-1 have been reported to bind to the IFN-γ promoter and regulate IFN-γ transcription in T cells (Penix et al., 1996).

In order to study the anti-inflammatory properties and immunomodulatory functions of the six TCMHs, we used luciferase-expressing plasmids containing NF-κB response elements or IFN-γ promoter reporter gene bioassays and BALB/c mice peritoneal macrophages and splenocytes.

2. Materials and methods

2.1. Preparation of extracts

Six kinds of TCMH made up of *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao (*Fabaceae*) (AM), *Andrographis paniculata* (Burm. f.) Nees (Acanthaceae) (AP), *Angelica sinensis* (Oliv.) Diels (*Apiaceae*) (AS), *Eucommia ulmodes* Oliv. (Eucommiaceae) leaves (EU leaves), *Isatis indigotica* Fort. (*Brassicaceae*) (II) and *Morus alba* L. (Moraceae) (MA) were purchased from a licensed Chinese herbal drug store in Taipei City. In total 10 g of each TCMH was extracted with 300 ml of 95% ethanol at 50 °C for 3 h twice. The total crude extract was partitioned with hexane and ethyl acetate to obtain hexane, EtOAc and water fractions. The botanical identification of AM and MA was done at the Industrial Technology Research Institute of Taiwan, using internal transcribed spacer (ITS) of the nuclear ribosomal RNA gene sequenced from these samples (Chen et al., 2005; Cheng et al., 2000). The identification of II was authenticated by Dr. Wei-Chu Li (Sheng Chang Pharmaceutical Co., LTD) by pharmacognostical anatomical analysis. The identification of AS, AP and EU leaves were carried out using HPLC analysis described as following.

2.2. Quantitative analysis of marker substance in the Angelica sinensis, Andrographis paniculata and Eucommia ulmodes Oliv. leaves EtOAc fractions

Ferulic acid (3-(4-hydroxy-3-methoxyphenyl)-2-propenic acid) and Z-ligustilide (3-butylidene-4,5-dihydro-1(3H)-isobenzofuranone, solution), two marker substances of AS were purchased from Fluka (Sigma, St. Louis, MO, USA) and Chroma-Dex (Santa Ana, CA, USA), respectively. Both ferulic acid accurately weighed and dissolved in methanol, and standard solution of Z-ligustilide were diluted to give various concentrations and injections (10 μl aliquots) were performed for each level. HPLC was performed in Merck LiChroCART 250-4 HPLC cartridge (LiChrospher 100 RP-18e) (Merck KGaA, Darmstadt, Germany), using a Jasco UV-2075 plus Intelligent UV/VIS detector and Jasco PU-2089 plus Quaternary Gradient pump (Jasco, Tokyo, Japan). The solvents were (A) 1% acetic acid in water and (B) 100% acetonitrile. The gradient of A:B was 0–10 min, 95:5 gradient up to 65:35; 10–30 min, gradient up to 30:70; 30–40 min, 95:5. The flow rate of mobile phase was kept constant at 0.8 ml/min and the system operated at 30 °C (Colbox column oven). The peaks were detected at UV 284 nm (Lu et al., 2005; Zhao et al., 2003).

Andrographolide, a marker substance of AP, was purchased from Aldrich (Sigma). Standard of andrographolide was accurately prepared as described above. The solvents were methanol–water–acetonitrile (50:50:10, v/v). The flow rate of mobile phase was kept constant at 0.7 ml/min and detected at 230 nm (Du et al., 2003).

Standard of chlorogenic acid (Sigma), a marker substance of EU leaves, was accurately prepared as described above. The solvents were methanol–water–acetic acid (20.3:78.7:1, v/v). The flow rate of mobile phase was kept constant at 1 ml/min and detected at 280 nm (Li et al., 2005a,b). The software SISC 32 3.1 was used for the hierarchical clustering analysis of the samples.

2.3. NF-κB-luciferase plasmid (p3xκB/GL3) and reporter gene assay

The murine macrophage cell line RAW 264.7 were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY, USA) seeded on 24-well plates (Nunc, Roskilde, Denmark) at a concentration of 5×10^4 cells/well, then incubated for overnight. The cells were cotransfected with 0.3 μg of the NF-κB-promoted luciferase reporter gene plasmid pNF-κB-Luc (Kashiwada et al., 1998) was kindly provided to us by Professor Bor-Luen Chiang (Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University) and 0.1 μg of Renilla luciferase reporter plasmid pRL-tk (Promega Corp., Madison, WI, USA) for 48 h. These cells were divided into two groups, vehicle or pre-treated for 1 h with an appropriate concentration (2, 5, 10 μg/ml) of AM, AP, AS, EU leaves, II, MA EtOAc fractions. These two groups of cells were then either treated with LPS (100 ng/ml, Sigma)/IFN-γ (1000 units/ml, Sigma) for 8 h or left untreated as a control. Luciferase expression was then analyzed using the Dual-Glo™ luciferase reporter assay system (Promega) as previously reported (Chao et al., 2007).

2.4. Construction of the IFN-γ-luciferase plasmid (pIFN-γ/GL3) and reporter gene assay

The murine IFN-γ-pUC18 promoter (Fox et al., 1991) has been submitted to Genbank (accession no. M28381); this plasmid has a 3.45 kb promoter fragment and was kindly provided by Profes-

sor Shie-Liang Hsieh (Department and Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan). The promoter sequence was directly cloned using Xma I and Hind III (Promega) restriction sites into the pGL_3-Basic vector (Promega) to produce the reporter construct pIFN-γ-Luc. For the transient transfection assay, EL-4 T cells were grown in DMEM supplemented with 10% FBS. EL-4 T cells were seeded on 24-well plates at a concentration of 4×10^5 cells/well. The EL-4 T cells were transiently transfected using Opti-MEM® I medium containing Lipofectamin 2000 transfection reagent (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) together with 0.9 μg of pIFN-γ-luciferase plasmid and 0.1 μg of internal control pRL-tk plasmid for 5 h, then given a fresh change of medium. EL-4 transfectants were left untreated or were treated with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma)/Ionomycin (1000 ng/ml, Sigma) for 24 h, either following a 1 h pre-treatment with the EtOAc fractions (2, 5, 10 μg/ml) or treatment with vehicle. Cell lysis was performed 24 h after treatment and luciferase activity measured as described above and previously reported (Chao et al., 2007).

2.5. Cell viability test (MTT assay)

Six kinds of TCMH extract were dissolved in DMSO (Sigma) to give a final concentrations of 2, 5, 10 μg/ml in the medium. These media were then used with RAW cells, EL-4 T cells, peritoneal macrophages and splenocytes and the cells incubated for 24 h. 3-(4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide, MTT, Sigma) in DMEM medium was added into each well and incubated at 37 °C for 3 h. The MTT formazan crystals formed from dye reduction by viable cells were dissolved using 0.04N HCl–isopropanol, which was mixed in at room temperature. After 30 min, an index of cell viability was calculated by measuring the optical density (OD) of the color at 540 nm (Carmichael et al., 1986).

2.6. Mouse peritoneal macrophages cell culture

The female BALB/c mice (20 ± 2 g) were purchased from Laboratory Animal Center at National Taiwan University, College of Medicine (Taipei, Taiwan). The mice used in this experiment were 10–12 weeks of age. To harvest the mouse peritoneal macrophages, the mice were killed by excessive exposure to anaesthetic ether. After thorough washing of the peritoneal cavity of mice with 9 ml of cold Hank's balanced salt solution (HBSS; Life Technologies, Paisley, UK) by i.p. injection, peritoneal fluid was collected. The peritoneal macrophages were centrifuged and washed twice before use. The peritoneal macrophages were grown in RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 2% TCM (mouse serum replacement, Celox Corp., Hopkins, MN, USA) and antibiotic-antimycotic (Life Technologies). Cells were inoculated into 96-well plates (Nunc) at a concentration of 2×10^5 cells/well. After adhering to plates for 3 h, non-adherent cells were washed off, and the cells were pre-incubated with the six kinds of TCMH extract or vehicle for 1 h, and then stimulated with LPS (10 μg/ml)/IFN-γ (50 units/ml). The cells were then incubated for 48 h before the culture supernatant was collected and frozen at −70 °C. This supernatant was used for the cytokines assay.

2.7. Mouse splenocytes cell culture

The female BALB/c mice splenocytes were removed and crushed into a single cell suspension and the red blood cells lysed with RBC lysis buffer before washing with HBSS. Splenocytes were inoculated onto 48-well plates at a concentration of 2.5×10^6 cells/well. The cells were preincubated with six kinds of TCMH extract or vehicle for 1 h, and then stimulated with Con A (5 μg/ml, Sigma). Incubation was continued for 24 h and then the culture supernatant was collected and frozen at −70 °C. This supernatant was used for the cytokines assay.

2.8. NO determination (Griess assay)

Griess reagent was freshly prepared from reagent A and B at a ratio of 1:1 (reagent A: 1% sulfanilamide dissolved in 2.5% phosphoric acid; reagent B: 0.1% N-1-naphthylethylene diamide dihydrochloride dissolved in 2.5% phosphoric acid. After incubation for 10 min, the plate was read on a ELISA reader at 540 nm. Using a standard curve, the NO concentration for each unknown samples were determined (Calixto et al., 2004).

2.9. PGE_2 determination

The PGE_2 concentration in the supernatants was determined with a Correlate-EIA™ prostaglandin E_2 kit (Assay Designs, Inc., Ann Arbor, MI, USA) according to the manufacturer's protocol.

2.10. Cytokine analysis

Mouse IL-6, IFN-γ ELISA kits (PharMingen, San Diego, CA, USA) and mouse TNF-α ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) were used and the cytokine concentration was assayed according to the manufacturer's cytokine ELISA protocol.

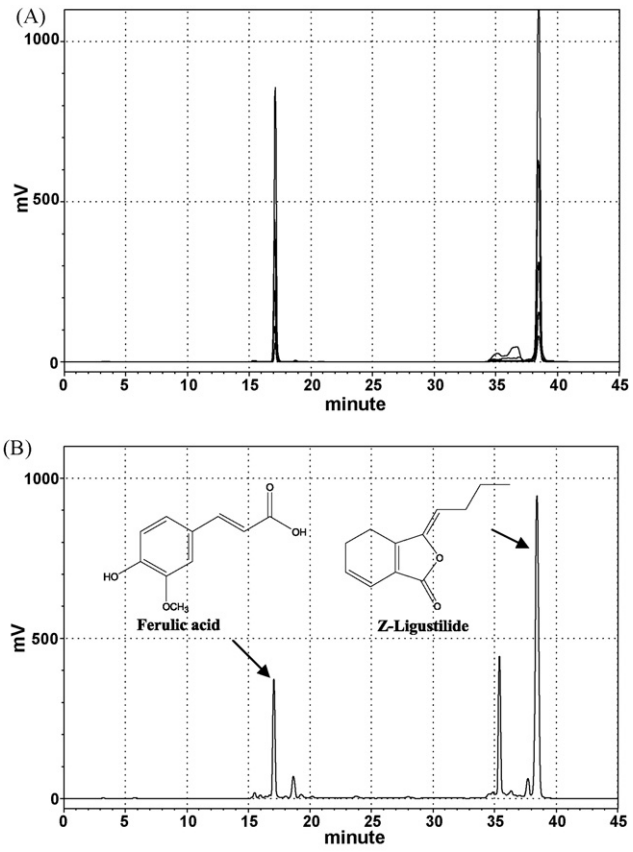

Fig. 1. HPLC analyses of ferulic acid and Z-ligustilide from AS EtOAc fraction. (A) Representative chromatogram (284 nm) of ferulic acid and Z-ligustilide. (B) Representative chromatogram (284 nm) of the AS EtOAc fraction.

2.11. Statistical analysis

Data from three independent experiments are presented as mean ± S.E.M. The Student's *t*-test was used for statistical analysis and $p < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Identification of TCMH by pharmacognostical anatomical or HPLC analysis

To verify the species of TCMH used in this study, quantitative analysis of marker substance was carried out for AS, AP and EU leaves which species are seldom mistaken in the market. Fig. 1 showed the components of AS EtOAC fraction were identified as ferulic acid and Z-ligustilide. The amount of ferulic acid and Z-ligustilide in AS EtOAc fraction were calculated as 3.75 and 15.95 mg/g dry weight, similar to those reported previously (Zhao et al., 2003). Z-ligustilide has been shown to inhibit LPS-induced TNF-α production in monocytes, and TNF-α mediated NF-κB activation in HeLa cell lines (Liu et al., 2005).

The components of AP EtOAC fraction were identified as andrographolide (Fig. 2), which is recognized as the most medicinally active phytochemical in AP (Chen et al., 2007). Based on standard curves in Fig. 2A, the amount of andrographolide in AP EtOAc fraction were calculated as 25 mg/g dry weight. Andrographolide was shown to suppress LPS/IFN-γ induced NF-κB transcriptional activity in a dose-dependent manner (Xia et al., 2004). The component of EU leaves EtOAc fraction was identified as chlorogenic acid (Fig. 3).

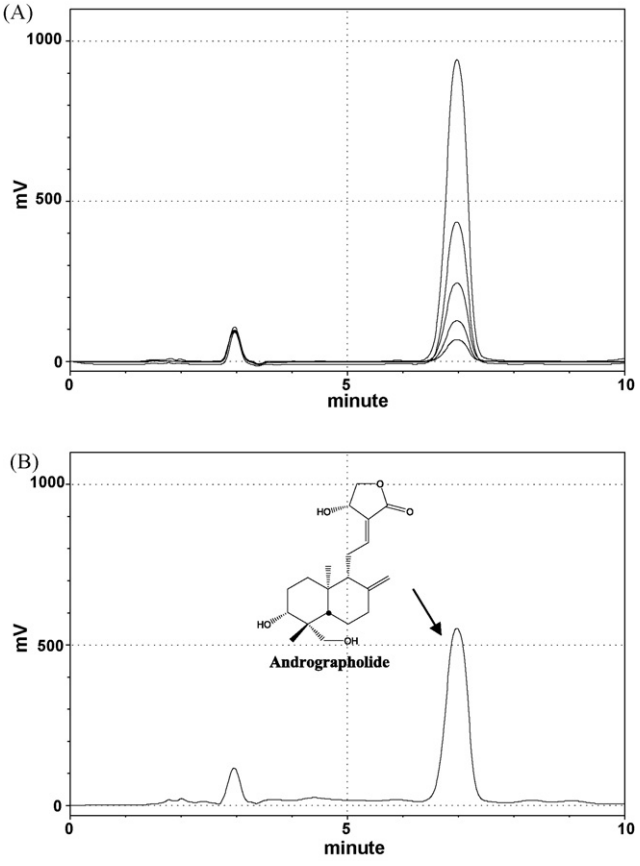

Fig. 2. HPLC analyses of AP EtOAc fraction. (A) Representative chromatogram (230 nm) of andrographolide. (B) Representative chromatogram (230 nm) of the AP EtOAc fraction. Andrographolide identified from AP EtOAc fraction had a retention time of 6.97 min.

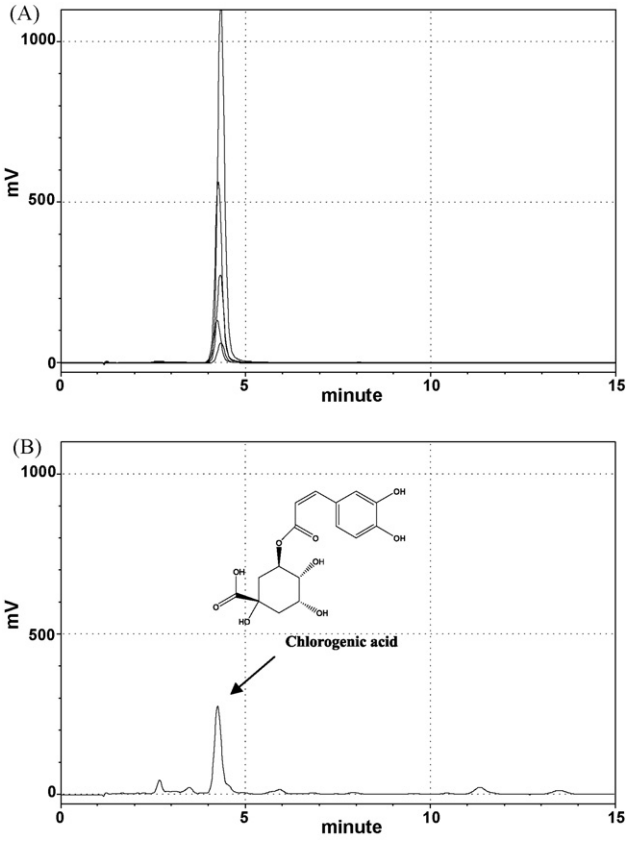

Fig. 3. HPLC analyses of EU leaves EtOAc fraction. (A) Representative chromatogram (280 nm) of chlorogenic acid. (B) Representative chromatogram (280 nm) of the EU leaves EtOAc fraction. Chlorogenic acid identified from EtOAc fraction had a retention time of 4.25 min.

The amount of chlorogenic acid in EU leaves EtOAc fraction was calculated as 2.14 mg/g dry weight. Chlorogenic acid is an important bioactive compounds and rich in EU leaves with anti-bacterial activity (Li et al., 2005a,b).

II used in this study was identified by pharmacognostical anatomical method compared with the microscopic characteristics of stem representation (data not shown). Another two TCMH used in this study, AM and MA, were identified as their ITS sequence were 100% identical to *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao and *Morus alba* L., respectively (data not shown).

3.2. Effect of six kinds of TCMH on NF-κB dependent luciferase gene expression

The cell viability assay indicated that there is no cytotoxicity at a high concentration (10 μg/ml; data not shown). In order to investigate the potential regulatory effects of the six kinds of TCMH EtOAc fractions on NF-κB driven reporter gene expression, we transiently transfected RAW 264.7 cells with LPS/IFN-γ for 8 h and this increased luciferase activity by 4-fold. In contrast, the NF-κB inhibitor helenalin significantly inhibited NF-κB transcriptional activity (Fig. 4). The results shown that AP, AS, EU leaves, II and MA caused a significant inhibition of LPS/IFN-γ-stimulated NF-κB dependent reporter gene expression ($p < 0.05$).

Inflammation, an important component of innate immunity, is dependent on the activation of NF-κB. The NF-κB/IκB system can exert transcriptional regulation on pro-inflammatory genes. Based on the finding that activation by LPS is able to cause NF-κB

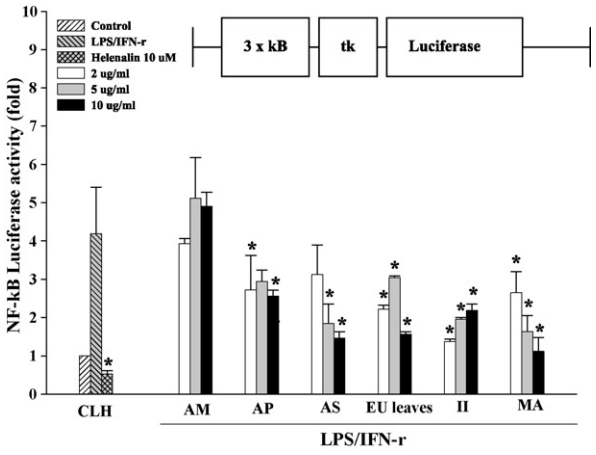

Fig. 4. The effects of the EtOAc fractions from ethanol extracts of six kinds of TCMH on NF-κB dependent luciferase gene expression in LPS/IFN-γ stimulated RAW 264.7 cells. C: control; L: LPS (100 ng/ml)/IFN-γ (1000 units/ml); H (Helenalin, NF-κB inhibitor): 10 μM. The data are reported as the means ± S.E.M. from three independent experiments. *: $p < 0.05$ compared with the L.

activation in culture (Medzhitov et al., 1997), transfection based reporter assays have been widely applied to analyze LPS signaling pathways (Byrd-Leifer et al., 2001). Our results showed that the luciferase reporter assay is relatively rapid, sensitive and is able to serve as a rapid pre-screen tool to identify bioactive herbs.

3.3. Effect of six kinds of TCMH on NO production in mouse peritoneal macrophages

BALB/c mice peritoneal macrophages stimulated with LPS/IFN-γ produced 20.27 ± 2.88 μM of NO over a 48 h period (Fig. 5A). When treated with the EtOAc fractions of AP, AS or MA, there was a significant decrease in NO release and this occurred in a dose-dependent manner ($p < 0.05$). AM, EU leaves and MA decreased NO production only in high dose. The results also showed that NF-κB luciferase activity and NO production had a strong positive correlation ($r = 0.72$, $p = 0.0001$) as shown in Table 2. As has been previously shown, NF-κB is the main transcription factor regulating the promoters responsible for NO and PGE_2 (Ghosh et al., 1998; Gilmore, 1999). As shown in Figs. 4 and 5A, the decrease in luciferase expression levels occurred in parallel to the decrease in NO production in the mouse peritoneal macrophages. This is consistent with the dose-dependent inhibition of NO production through a suppression of NF-κB gene transcription.

NO is an important mediator in acute and chronic inflammation. The inflammatory responses are largely controlled through regulation of NF-κB (Musiek et al., 2005). Wang et al. reported that a low molecular weight (<10 kDa) fraction found in AS extract significantly attenuated endotoxin induced HMGB1 release and also exerted a protective effect against lethal endotoxemia (Wang et al., 2006). Andrographolide, a major compound of AP, has been reported to suppress the expression of inducible nitric oxide synthase (iNOS) (Chiou et al., 2000). Previous studies have shown that MA inhibited LPS-induced NO and PGE_2 productions in RAW264.7 cells (Chung et al., 2003; Hong et al., 2002). The inhibition was also shown in primary peritoneal macrophages in our study.

3.4. Effect of six kinds of TCMH on PGE_2 production in mouse peritoneal macrophages

When primary peritoneal cells were activated with LPS/IFN-γ for 48 h, a substantial increase in PGE_2 production was observed

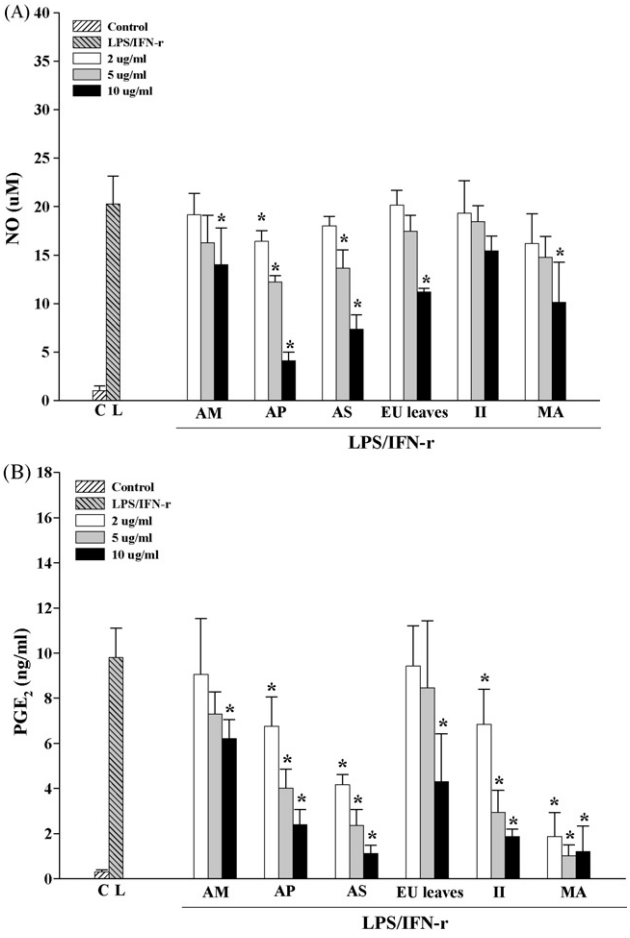

Fig. 5. The effects of the EtOAc fractions from ethanol extracts of six kinds of TCMH on LPS/IFN-γ induced NO (A), PGE_2 (B) production in BALB/c mice peritoneal macrophages. C: control; L: LPS (10 μg/ml)/IFN-γ (50 units/ml). Data representative of three independent experiments were expressed as mean ± S.E.M. *: $p < 0.05$ is compared with the L.

(~10 ng/ml, Fig. 5B). The EtOAc fractions of AP, AS, II and MA significantly decreased PGE_2 production in a dose-dependent manner ($p < 0.05$). AM and EU leaves also reached significant suppression of PGE_2 production at high dose. Our results shown that NF-κB luciferase activity and PGE_2 production have a strong positive correlation ($r = 0.43$, $p = 0.0001$, Table 2). In the transient transfection experiments, LPS/IFN-γ stimulated luciferase expression through (B-response elements and this could be correlated with high level of iNOS and COX-2 promoter activity in LPS/IFN-γ treated macrophages (Gilmore, 1999).

Ho and Tsai (2004) reported that 'hot' herbs, such as *Zingiber officinale* and *Cinnamomum cassia*, induced PGE_2 production and COX-2 expression in unactivated cells. In contrast, LPS-induced PGE_2 production and COX-2 expression was inhibited by 'cold' herbs, such as *Coptis chinensis* and *Scutellaria baicalensis*. In traditional Chinese medicine, AP is a 'cold' herb and is used to rid the body of heat, for example fevers and acute infections, and to dispel

Table 2
The correlation between NF-κB luciferase activity and pro-inflammatory mediator secretion.

Mediators	NF-κB luciferase activity (fold)	
	r	*p*
NO (μM)	0.72	0.0001
PGE_2 (ng/ml)	0.43	0.0001

toxins from the body. AP is also shown to affect the secretion of the pro-inflammatory chemokine RANTES in human bronchial epithelial cells infected with influenza A virus H1N1 (Ko et al., 2006). The root of II has been used ethnomedically to treat erysipelas, influenza, carbuncles, epidemic hepatitis and epidermic encephalitis B for hundreds of years in China (Wu et al., 1997). It is also an antipyretic and shown to attenuate LPS-induced pyrexia and to inhibit carrageenan-induced paw edema in rats (Ho and Chang, 2002).

3.5. Effect of six kinds of TCMH on IL-6 and TNF-α production in peritoneal macrophages

Stimulation of peritoneal macrophages from BALB/c mice with LPS/IFN-γ for 48 h caused a substantial increase in the release of the pro-inflammatory cytokine IL-6 (4.61 ± 0.28 ng/ml, Fig. 6A). The EtOAc fraction of MA significantly decreased IL-6 production when presented at 10 μg/ml ($p < 0.05$). The other TCMHs did not affect LPS/IFN-γ induced IL-6 production (Fig. 6A). IL-6 is the predominant mediator of the acute phase response, an innate immune mechanism that is triggered by infection and inflammation. Several consensus sequences, including those for NF-κB, AP-1, NF-IL-6 and CREB, are present in the 5′ promoter region of the IL-6 gene (Grassl et al., 1999). Our results revealed that the EtOAc fractions of MA significantly decreased IL-6 production in high dose ($p < 0.05$).

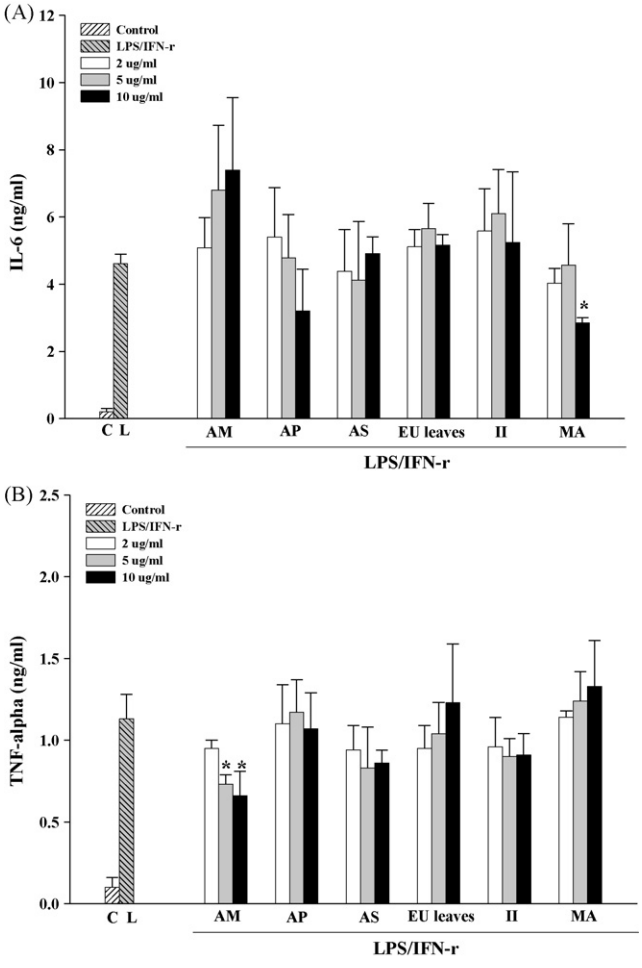

Fig. 6. The effects of the EtOAc fractions from ethanol extracts of six kinds of TCMH on LPS/IFN-γ induced (A) IL-6 and (B) TNF-α production in BALB/c mice peritoneal macrophages. C: control; L: LPS (10 μg/ml)/IFN-γ (50 units/ml). Data representative of three independent experiments were expressed as mean ± S.E.M. *: $p < 0.05$ is compared with the L.

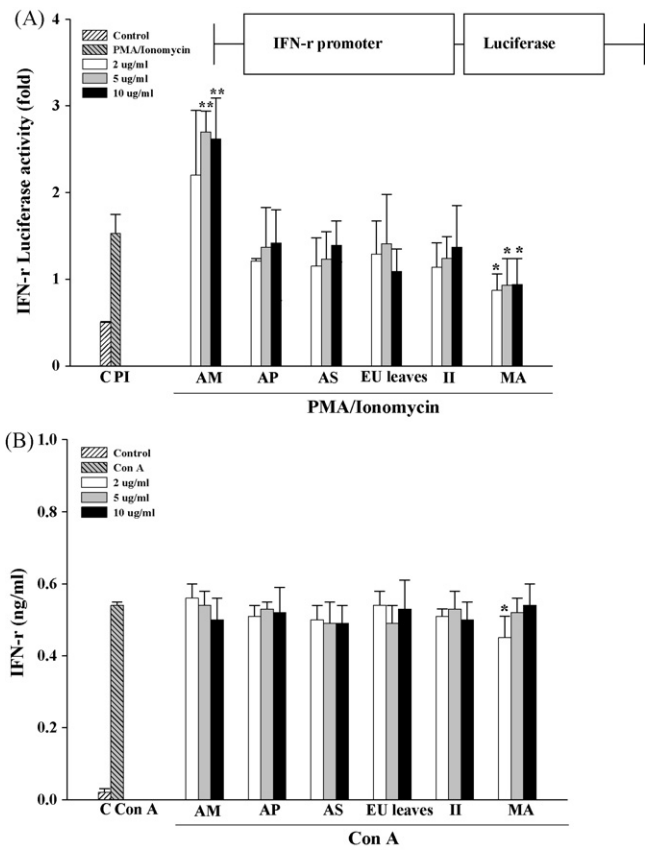

Fig. 7. (A) The effects of the EtOAc fractions from ethanol extracts of six kinds of TCMH on PMA/Ionomycin induced IFN-γ luciferase activity in transfected EL-4 T cells. C: control; PI: PMA (50 ng/ml)/Ionomycin (1000 ng/ml). (B) The effects of the EtOAc fractions from ethanol extracts of six kinds of TCMH on ConA induced IFN-γ production in BALB/c mouse splenocytes. C: control; Con A (5 μg/ml). Data representative of three independent experiments were expressed as mean ± S.E.M. *: $p < 0.05$ is compared with the Con A.

We also examined the effect of the EtOAc fractions from ethanol extracts of TCMHs on LPS/IFN-γ induced peritoneal macrophages TNF-α production (Fig. 6B). The result indicated that the EtOAc fraction of AM significantly decreased TNF-α production ($p < 0.05$). Several reports have shown that TNF-α is the most important cytokine mediator linked the pathogenesis of septic shock or lupus (Hong et al., 2009; Marks et al., 1990). It has also been shown that the production of TNF-α is crucial for the synergistic induction of NO synthesis in IFN-γ and/or LPS stimulated macrophages (Park et al., 2000). Our results show that the EtOAc fraction of AM significantly inhibited NO, PGE_2 and TNF-α generation by LPS/IFN-γ stimulated peritoneal macrophages (Figs. 5 and 6B). AM is a traditional Chinese medicinal herb and originated in Northern China. Several studies have focused on the plant's immunomodulatory properties such as increasing human lymphocyte proliferation, the cytotoxic T cell response and modulating macrophages to produce pro-inflammatory cytokines (Kang et al., 2004; Yesilada et al., 1997; Yoshida et al., 1997). AM stimulated TNF-α or NO productions by macrophages through enhancing NF-κB or iNOS mRNA expression (Bedir et al., 2000; Lee and Jeon, 2005), but exerted inhibitory effects when cells were stimulated with LPS (Clement-Kruzel et al., 2008; Ryu et al., 2008; Yesilada et al., 2005). Similar inhibition was also found in LPS-stimulated peritoneal macrophages pre-treated with AM EtOAc fraction in our study. Our results showed that AM can inhibit TNF-α but not IL-6, which might be due to dose concentration or fraction of AM different from other reports (Li et al., 2007; Ryu et al., 2008; Shon et al., 2002; Xu et al., 2007).

3.6. Effect of six kinds of TCMH on IFN-γ luciferase activity and on IFN-γ production in mouse splenocytes

We further employed an IFN-γ promoter driven luciferase reporter construct and murine splenocytes to investigate the immunomodulatory herbs on IFN-γ-driven luciferase reporter gene expression (Fig. 7). Incubation of EL-4 T cells with PMA/Ionomycin for 24 h increased IFN-γ transcription activity and AM further up-regulates this activity ($p < 0.05$) (Fig. 7A). However, the other herbs did not affect IFN-γ luciferase activity or IFN-γ production in concanavalin A (Con A)-activated splenocytes (Fig. 7B).

IFN-γ is an important immunoregulatory protein responsible for several immunological effects including regulation of cytokine gene expression, such as IL-6 and TNF-α, and the promotion of activation of immune effector cells, including B cells and monocytes (Collart et al., 1986). The 3.45 kb IFN-γ promoter (Fox et al., 1991) contains the *cis*-element responsible for IFN-γ expression in naïve and in memory $CD4^+$ and $CD8^+$ cells (Aune et al., 1997; Fox et al., 1991). Transcriptional factors such as NF-κB and AP-1 have been reported to bind to the IFN-γ promoter and regulate IFN-γ transcription in T cells (Chang et al., 2007; Penix et al., 1996). Therefore, we conclude that the EtOAc fraction of AM up-regulates IFN-γ transcription, but does not affect NF-κB transcriptional activity. On the other hand, the EtOAc fractions of AP, AS and MA significantly decreased NF-κB transcriptional activity, but did not affect IFN-γ luciferase activity. Our results support the hypothesis that these anti-inflammatory properties might result, at least in part, from inhibition of pro-inflammatory mediators such as NO and PGE_2 by suppression of the NF-κB signaling pathway.

4. Conclusion

Our results show that EtOAc fractions of AP, AS and MA have a significant inhibitory effect on NF-κB luciferase activity and the secretion of NO and PGE_2 in LPS/IFN-γ stimulated mouse peritoneal macrophages. In contrast, they did not affect IFN-γ luciferase activity or IFN-γ production in Con A activated mouse splenocytes. Collectively, we have demonstrated that these three species are potent bioactive TCMHs that show significant NF-κB inhibitory activity in a cell type dependent fashion.

Conflict of interest

The authors had no conflict of interest to this report.

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