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Houttuynia cordata Thunb. Volatile Oil Exhibited Anti-inflammatory Effects *In Vivo* and Inhibited Nitric Oxide and Tumor Necrosis Factor-α Production in LPS-stimulated Mouse Peritoneal Macrophages *In Vitro*

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Houttuynia cordata **Thunb. (HC) is a medicinal herb that generally used in traditional Chinese medicine for treating allergic inflammation. The present study investigated the inhibitory effect of the volatile oil from HC Thunb. on animal models of inflammation and the production of inflammatory mediators *in vivo* and *in vitro*. *In vivo*, xylene-induced mouse ear edema, formaldehyde-induced paw edema and carrageenan-induced mice paw edema were significantly decreased by HC volatile oil. HC volatile oil showed pronounced inhibition of prostaglandin (PG) E_2 and malondialdehyde production in the edematous exudates. *In vitro* exposure of mouse resident peritoneal macrophages to 1, 10, 100 and 1000 μg/mL of HC volatile oil significantly suppressed lipopolysaccharide (LPS)-stimulated production of NO and tumor necrosis factor-α (TNF-α) in a dose-dependent manner. Exposure to HC volatile oil had no effect on cell viability and systemic toxicity. Furthermore, HC volatile oil inhibited the production of NO and TNF-α by down-regulating LPS-stimulated iNOS and TNF-α mRNA expression. Western blot analysis showed that HC volatile oil attenuated LPS-stimulated synthesis of iNOS and TNF-α protein in the macrophages, in parallel. These findings add a novel aspect to the biological profile of HC and clarify its anti-inflammatory mechanism. Copyright © 2012 John Wiley & Sons, Ltd.**

Keywords: Houttuynia cordata Thunb.; inflammation; prostaglandins; nitric oxide; tumor necrosis factor-α.

INTRODUCTION

Inflammation is a complex process, and various mediators, such as prostaglandins (PGs), leukotrienes and platelet activating factor, have been reported to be involved in the development of inflammatory diseases (Gryglewski, 1981). Tumor necrosis factor-α (TNF-α) plays a pivotal role in inflammation and host defense (Beutler, 1995). Persistent or inappropriately high TNF-α expression contributes to the inflammatory conditions, including septic shock, rheumatoid arthritis, multiple sclerosis and AIDS (Vassalli, 1992). Nitric oxide (NO), derived from Larginine, is produced by two types of NO synthase (NOS) (Forstermann *et al*., 1991). Various *in vivo* and *in vitro* experimental models have been set up to assess inhibitory effects of various natural products on these inflammatory mediators. The pro-inflammatory cytokine TNF-α and the reactive free radical NO synthesized by inducible NOS (iNOS) are the major macrophage-derived inflammatory mediators and also reported to be involved in the development of inflammatory diseases (Freeman and Natanson, 2000). Thus, the inhibition of the excessive productions of TNF-α and NO can be employed as criteria to evaluate anti-inflammatory effects of natural products. Many experimental results indicated that overproduction of TNF-α and NO resulted in excess inflammatory reactions deleterious to the human body in the inflammation process (Ialenti *et al*., 1992; Iuvone *et al*., 1994; Klosterhalfen and Bhardwaj, 1998; Li *et al*., 2007).

Houttuynia cordata Thunb. (HC) is a medicinal herb that is generally used in traditional Chinese medicine therapy. It possesses functions of clearing heat, eliminating toxins, reducing swelling, discharging pus and relieving stagnation. It is a perennial native herb, with a thin stalk and heart-like leaf known to be an effective drug for treating allergic inflammation. Previous study showed that the steam distillate prepared from fresh plants of HC possessed direct inhibitory activity against herpes simplex virus type 1, influenza virus and human immunodeficiency virus type 1 without showing cytotoxicity (Hayashi *et al*., 1995). Recent reports have demonstrated that HC is effective for treating anaphylaxis, cough, cancer and viral infection (Chiang *et al*., 2003; Zhou, 2003; Kwon *et al*., 2003; Li *et al*., 2005; Lu *et al*., 2006). Recently, several scientific studies have provided data to support and explain its anti-inflammatory activities (Lu *et al*., 2006; Ji *et al*., 2009; Park *et al*., 2005). It was chosen as one of the eight types of traditional Chinese medicine that play a unique role in severe acute respiratory syndrome, owing to its effect of diminishing inflammation (Lau *et al*., 2008; Zhang and Chen, 2008). Our previous studies clearly suggested that HC essential oil is able to inhibit the release of LPS-induced PG E_2 from mouse peritoneal macrophages,

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and the inhibitory activity of HC essential oil was due to a dose-dependent inhibition of COX-2 enzyme activity (Li *et al*., 2011a). Our previous study was to screen for the anti-inflammatory components from HC Thunb. using an analytical method combining cell membrane chromatography (CMC) with gas chromatography–mass spectrometry (GC–MS). The major component retained by CMC was identified as methyl nonyl ketone (MNK) by GC–MS (Li *et al*., 2011b). Some studies have attempted to characterize the essential oil composition of HC, extracting the essential oils by steam distillation and performing GC–MS (Xu *et al*., 2005; Lu *et al*., 2006).

In the current report, we explored the potential of HC essential oil as an anti-inflammatory drug. Although it has been shown that the modulation of COX-2 is a possible pathway by which HC essential oil may prevent various inflammatory responses, the precise molecular mechanism underlying the anti-inflammatory effect of HC worth us carrying on further studies. Medicinal properties claimed for the drug have been attributed to its volatile oil component (Hayashi *et al*., 1995; Lu *et al*., 2006). In this report, the anti-inflammatory activity of HC essential oil has been established in both acute and chronic inflammation models, *in vivo* and *in vitro*. Xylene-induced ear edema may involve inflammatory mediators such a shistamine, serotonin, bradykinin and PGs. These mediators induce ear edema by promoting vasodilation and increasing vascular permeability (Carlson *et al*., 1986). The formaldehyde-induced paw edema model is commonly used for screening anti-arthritic and anti-inflammatory agents because its pathology closely resembles human arthritis (Greenwald, 1991; Banerjee *et al*., 2000). Carrageenan-induced paw edema is a commonly used experimental model of acute inflammation, and this model has frequently been used to assess the anti-edematous effects associated with natural products (Panthong *et al*., 2003). To gain insight into the anti-inflammatory effect of the volatile oil from HC and its mechanisms of action, we investigated HC essential oil whether and how it was linked to the regulation of anti-inflammation in inflammatory models and LPS-induced murine peritoneal macrophages.

MATERIALS AND METHODS

Reagents. RPMI1640 medium was obtained from Gibco (Grand Island, NY, USA). Lipopolysaccharide (LPS, Escherichia coliserotype 055:B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoleum (MTT), dimethyl sulfoxide (DMSO) and thioglycolate broth were obtained from Sigma-Aldrich Biotechnology (St Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Lanzhoumin sea Biological Engineering Co., Ltd. (Lanzhou, China). Trypsin was purchased from Amresco Inc. (Solon, Ohio, USA). Sodium thioglycollate was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Anti-phospho-iNOS antibody, anti-phospho-TNF-α antibody and anti-β-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidene fluoride (PVDF) membranes were purchased from Pall Gelman Laboratory (Ann Arbor, MI, USA). NO kit and TNF-α kit were purchased from Nanjing Jiancheng Bio-engineering company (Nanjing, China).

Plant materials. Samples of wild HC were obtained commercially from Shaanxi Province, China. All the samples were authenticated by one of the authors, Prof. Niu Xiaofeng (School of Medicine, Xi'an Jiaotong University, Xi'an, China). The voucher specimens (No. HC2012005) for Shanxi were all deposited in the herbarium of School of Medicine, Xi'an Jiaotong University, Xi'an, China. Prior to use, samples were air dried, ground in a high-speed rotary cutting mill and then sieved to give fractions 150 μm in size. The particle size of the plant powder is important for steam distillation.

Steam distillation extraction. The volatile oil was prepared as follows: 100 g sample of 150 μm particle size was weighed into a 2000 mL distillation flask, 1000 mL deionized water was added and the mixture was distilled for 4 h. Oil was collected from the condenser, and 0.2 mL of oil was diluted with 2 mL of n-hexane. Then the extracts were dried with anhydrous sodium sulfate.

GC–MS analysis. The HPLC system consisted of a SPECTRA P200 chromatographic pump, a SPECTRA 100 detector (Thermo Separation Products, Fremont, CA, USA), a 7125 hand-sampling valve (Rheodyne Company, Berkeley, USA) and an ANASTAR chromatographic workstation (AOTAI Technology Ltd., Tianjin, China). SFE was carried out by using a HA220-50-06 extraction system (Hua'an SFE Ltd., Nantong, Jiangsu, China).

A capillary GC–MS instrument (GCMS-QP2010 Shimadzu, Kyoto, Japan) with a DB-5MS capillary column (30 m × 0.32 mm I.D., 0.25 μm film thicknesses, Agilent Technologies, Palo Alto, CA, USA) was used. The inlet temperature was maintained at 280 °C. The oven temperature was initially held at 140 °C for 2 min and was then programmed to 280 °C at 10 °C/min where it was held constant for 4 min. Helium was used as carrier gas at a constant flow rate of 2.0 mL/min. The source and electrodes of the quadrupole mass filter were both set to 200 °C. Ionization was carried out in electron impact ionization mode at 70 eV.

The identification of the components was performed by matching their recorded mass spectra with the standard mass spectra from the National Institute of Standards and Technology (NIST05.LIB) libraries data provided by the software of the GC–MS system, literature data and standards of the main components. The results were also confirmed based on their retention indices (determined with reference to a homologous series of normal alkanes) on DB-5MS capillary column. Quantitative analyses of each essential oil component (expressed as area percentage) were carried out by a peak area normalization measurement, calculated as mean values of three injections from each sample.

Animals. All experimental procedures utilizing mice were in accordance with the National Institute of Health guidelines.

Female (22–25 g) and male (20–25 g) Kunming mice were supplied by the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). Animals were housed in standard laboratory conditions under a temperature of 22 ± 3 °C, relative humidity 50–55% and 12 h light/dark cycle. All animals were provided with food and water *ad libitum*.

The xylene-induced ear edema of mice. According to the paper (Hosseinzadeh *et al*., 2003), the xylene was typically applied on the right ear to reproduce the ear edema model. Male mice ($n = 9$/group) were treated with different doses of HC volatile oil (20 and 40 mg/kg, i.p.) dissolved in 1% carboxymethyl cellulose (CMC), and dexamethasone (DEX, 5 mg/kg, i.p.) was used as the positive control, while the control group of animals received a suspension of 1% CMC in distilled water for seven consecutive days. Xylene (0.02 mL) was applied to the anterior and posterior surfaces of the right ear. The left ear was considered as control. One hour after xylene application, mice were killed by cervical dislocation, and both ears were removed. Ear biopsies of 7.0 mm in diameter were punched out and weighed. The extent of ear edema was evaluated by the weight difference between the right and the left ear biopsies of the same animal.

The formaldehyde-induced paw edema in mice. Male mice (9/group) were treated, i.p., for seven consecutive days with 20 or 40 mg/kg HC volatile oil dissolved in 1% CMC, 1 or 3 mg/kg MNK dissolved in 1% CMC and 5 mg/kg DEX (positive control), or a suspension of 1% CMC in distilled water. One hour after the last injection, these mice were injected directly with 30 μL 2.5% formaldehyde into the right hind paw. Paw thicknesses were measured by a digital Vernier caliper at several time points after the injection of formaldehyde. Edema is expressed in millimeters as the difference between the control and treated paws. Edema inhibitory activity was calculated according to the following formula:

$$\text{percentage inhibition} = (C_{\text{control}}\text{-}C_{\text{treated}})/C_{\text{control}} \times 100\%$$

The animals were sacrificed 4 h after the induction of inflammation, and the injected paws and the paws from healthy mice were collected for the PGE_2, NO and TNF-α measurement. Levels of NO were determined by a nitrite detection kit (Nanjing Jiancheng Bioengineering Institute) according to instructions provided by the manufacturer. The injected paws were weighed and homogenized three times with 0.9% saline solution, and the exudates were centrifuged at 3000 × g for 10 min. The nitrite concentration was measured with a spectrophotometer by absorbance at 570 nm. The levels of TNF-α were measured using commercially available ELISA kits.

The carrageenan-induced paw edema of mice. Mice were intraperitoneally injected with different doses of HC volatile oil (20 and 40 mg/kg), 5 mg/kg DEX or a similar volume of vehicle for seven consecutive days. One hour after the last injection, these mice were injected directly with 30 μL of 1% carrageenan dissolved in a saline solution into the foot pad of the right anterior paw. The thickness (mm) of the paw was measured at 1, 2, 3 and 4 h intervals after the administration of the carrageenan.

Edema ($\bigtriangledown$T) was calculated as follows:

$$^{\Delta}T = T_t\text{-}T_0$$

where T_t is the right hind paw thickness (mm) at time 't', T_0 is hind paw thickness (mm) before sub-plantar injection. After 4 h, mice were sacrificed, and the injected paws and the paws from healthy mice collected for the PGE_2 and malondialdehyde (MDA) measurement.

Measurement of PGE_2 production. The injected paws were perfused with 0.9% saline solution and the exudates were centrifuged at 3000 × g for 10 min. Then, 0.25 mL of the supernatant was added to 1 mol/L KOH-CH_3OH, the samples were placed in a water bath at 50 °C for 20 min. At the end of this period, samples were transferred to a glass tube with 3 mL CH_3OH. PGE_2 activity was assessed in the exudates by measuring the change in the optical density (OD) at 278 nm; results were expressed as the change in absorbance (OD) per mg of tissue.

Measurement of MDA production. The injected paws were perfused with 0.45 mL 20% trichloracetic acid for 3 h, and the exudates were centrifuged at 3000 × g for 10 min. Then, the supernatants were added to the solution including 0.55 mL thiobarbituric acid and 1 mL 0.1 mol/L HCl. MDA activity was assessed by measuring the change in the OD at 532 nm; results were expressed as the change in absorbance (OD) per mg of tissue.

Peritoneal macrophages isolation and cell culture. Peritoneal macrophages were obtained from mice euthanized by cervical dislocation. The peritonea of the animals were surgically exposed using a midline incision. The experimental mice were injected (i.p.) 2 mL 3% (m/v) sulfur hydroxy acetate. Three days later, mice were sacrificed using cervical dislocation, and peritoneal macrophage was harvested by injecting 4 mL of ice-cold phosphate buffered saline. Cell suspensions were washed twice by centrifugation. Cells were incubated in RPMI1640 complete medium containing 10% FBS for 4 h at 37 °C under 5% CO_2 in a humidified chamber.

Cell viability assay. The cytotoxicity of HC volatile oil was evaluated using a MTT assay. Succinate dehydrogenase of living cells mitochondria convert MTT to insoluble blue-purple crystals formazan which can be solubilized and quantified by spectrophotometric means. In a certain range of cells, MTT crystals formed are proportional to volume and cell number. Mouse peritoneal macrophages (5×10^5 cells/well) were plated into 96-well plates, cells/well to a final volume of 200 μL, incubated at 37 °C for 24 h, and given a fresh change of medium. The cells were treated with HC volatile oil (1, 10, 100, 1000 μg/mL) dissolved in DMSO, and with LPS (final concentration10 μg/mL) alone or in combination with HC volatile oil dissolved in DMSO. This DMSO percentage allows the optimal solubilization of HC volatile oil in aqueous solution. The control and LPS wells received the same amount of DMSO. After 24 h of incubation at 37 °C, 20 μL of MTT (5 mg/mL) was added to each well, and 4 h later, the cells were lysed with 150 μL DMSO. The plate was shaken for 10 min, and, 30 min later, the optical densities (OD_{490}) for the volatile oil were compared with the OD of the control or LPS-stimulated wells to assess the cytotoxicity (Mosmann, 1983).

NO assay. Nitrite, the stable product of NO, is often indicative of NO production. Nitrite concentration was determined using the Griess reagent (Green *et al*., 1982). The mouse peritoneal macrophages were cultured

in 24-well culture plate at a density of 2×10^6 cells/well. The cells were further cultured for 24 h on treatment with LPS (final concentration 10 μg/mL) alone or in combination with different concentrations of HC volatile oil and MNK (10 μg/mL) dissolved in DMSO. The control and LPS wells received the same amount of DMSO. Briefly, 100 μL supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-napthylethylenediamine dihydrochloride in water) at room temperature for 10 min. The absorbance was measured at 540 nm by a microplate reader (Tecan, Germany). Nitrite concentration in the supernatants was determined from a standard curve that was generated using known concentrations of sodium nitrite.

TNF-α assay. For the cytokine immunoassay, the mouse peritoneal macrophages (2×10^6 cells/well) that are in a garithmic phase were inoculated in 24-well culture plate, to a final volume of 500 μL, incubated at 37 °C in 5% CO_2 for 3 h. The cells were further cultured for 24 h on treatment with LPS (final concentration 10 μg/mL) alone or in combination with different concentrations of HC volatile oil and MNK (10 μg/mL) dissolved in DMSO. The control and LPS wells received the same amount of DMSO. The supernatants were then collected and assayed for TNF-α using mouse TNF-α ELISA kit as described previously (Kim *et al.*, 1998).

iNOS assay. Cells were incubated with different concentrations of HC, and, after the supernatants were collected, the cells were rinsed with PBS and lysed by ultra-sonication with 500 μL lysis buffer (50 mM Tris-HCI pH 8.0, 5 mM EDTA Na_2, 0.2 M NaCl, 1% Triton X-100). The cell lysate was centrifuged at 15 000 × g for 25 min at 4 °C. The supernatants were then collected and assayed for the iNOS (U/mL) using iNOS detection kit (enzymic method).

Real-time-PCR analysis. Macrophages (5×10^6 cells/well) were incubated for 24 h with or without various concentrations of HC volatile oil and LPS (10 μg/mL). After washing with PBS twice, total RNA was isolated from the cell pellet using the RNAfast200 isolation kit (Fastagen Biotech, Shanghai, China) according to the manufacturer's directions. Quantitative real-time PCR was performed (Livak and Schmittgen, 2000) in a Light Cycler instrument (Roche Diagnostics, Mannheim, Germany) with the FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany), and results were analyzed with the LDCA software supplied with the machine. Each 100 μL PCR reaction contained 1/50 th of the original cDNA synthesis reaction, 7 μL (25 mM) $MgCl_2$, 0.8 μL (20 pmol/L) of each primer, 1 μL (10 mM) dNTPs, 1 μL SYBR Green I Mix, 0.5 μL (5U/L) *Taq* and 5 μL Buffer. A total of 10 × 50 cycles of amplification were performed. The primers for iNOS were 5′-CAT GGC TTG CCC CTG GAA GTT TCT CTT CAA AG-3′(sense), and 5′-GCA GCA TCC CCT CTG ATG GTG CCA TCG-3′, (antisense); TNF-α were 5′-TTC TGT CCC TTT CAC TCA CTG G-3′ (sense), 5′-TTG GTG GTT TGC TAC GAC GTG G-3′(antisense). β-actin was also amplified as a control for total RNA content for each sample in a similar way using the following primers: 5′-AGG GAA ATC GTG CGT GAC ATC AAA-3′ (sense), 5′-ACT CAT CGT ACT CCT GCT TGC TGA-3′ (antisense). The PCR amplification was carried out by the following temperature profile: 40 cycles of denaturation at 95 °C for 15 s, annealing at 59 °C (for iNOS) or 57 °C (for TNF-α) for 30 s; and primer extension at 72 °C for 30 s, using the Px2 thermal cycler (Thermo Electron Corporation, Waltham, MA, USA). The final extension step was performed at 72 °C for 10 min. The fluorescence signal was detected at the end of each cycle. Melting curve analysis was used to confirm the specificity of the products.

Western blot analysis of iNOS and TNF-α. Peritoneal macrophages (5×10^6 cells/well) that had been grown to confluence in six-well plates were incubated with or without LPS in the absence or presence of the test agents. Cells were washed with ice-cold PBS and stored at −70 °C until further analysis. Frozen cells were thawed and lysed in a solution of 1% Triton X-100, 0.15 M NaCl and 10 mM Tris–HCl, pH 7.4, for 30 min. Proteins in the cell lysates were then separated by 7% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were blocked with 5% fat-free dry milk in TBS-T, pH 8.0 (Tris-buffered saline [50 mM Tris, pH 8.0, and 150 mM NaCl] with 0.1% Tween 20) and then incubated with a mouse immunoglobulin G_1 against either iNOS or TNF-α, or monoclonal anti-β-actin antibody (1: 2500 dilutions), and incubated overnight at 4 °C. After washing three times with TBS-T, iNOS and TNF-α were visualized by using an anti-mouse IgG: horseradish peroxidase conjugate and the enhanced chemiluminescence system (ECL™; Amersham Pharmacia Biotech, Piscataway, NJ, USA). Signal intensities were evaluated by densitometric analysis (Kodak Digital Science™ Image Station 2000R; Life Science Products, Rochester NY, USA).

Statistical analysis. Data shown represent the mean and standard deviation (SD). Comparison between the two groups was made using the independent-samples *t*-test. All analyses were performed using SPSS software (Chicago, IL, USA). A p value <0.05 was considered to be statistically significant.

RESULTS

Identification of volatile compounds in HC

In this work, the volatile oils extracted from HC were analyzed by GC–MS (Fig. 1). The peak area was chosen as the analytical signal for the relative content, and these identified components are listed in Table 1. As outlined in Table 1, there were 15 components that were observed in the samples. The anti-inflammatory active component in extracts of HC was MNK (6.17%), which was screened from HC using an analytical method combining CMC with GC–MS (Li *et al.*, 2011b).

Effect of HC volatile oil on xylene-induced ear edema in mice

Figure 2 shows the effect of HC volatile oil on xylene-induced ear edema in mice. Treatment with HC volatile oil (20 and 40 mg/kg) significantly inhibited ear edema

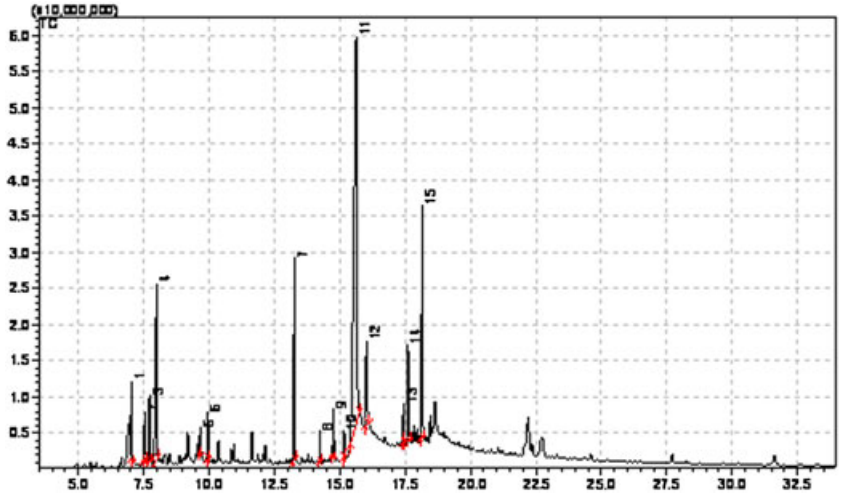

Figure 1. Chromatograms of *Houttuynia cordata* Thunb. volatile oil using the GC–MS method.

Table 1. Chemical compositions of the volatile oil from *Houttuynia cordata* Thunb.

The common peaks	Compound	Molecular weight	Similarity degree (%)	Peak area percentage (%)
1	2-Undecanone	170	97	6.17
2	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)]-	220	87	2.36
3	Caryophyllene oxide	220	94	5.71
4	n-Decanoic acid	172	96	9.20
5	Androstan-17-one, 3-ethyl-3-hydroxy-, (5.alpha.)-	318	81	3.42
6	Longifolenaldehyde	220	82	3.87
7	2-Pentadecanone, 6,10,14-trimethyl-	268	94	8.44
8	2-Nonadecanone	282	82	2.25
9	Hexadecanoic acid, methyl ester	270	96	1.91
10	Isophytol	296	96	1.81
11	l-(+)-Ascorbic acid 2,6-dihexadecanoate	652	93	24.17
12	n-Hexadecanoic acid	256	91	7.01
13	Decanoic acid, decyl ester	312	85	5.27
14	1-Octadecanol	270	96	8.16
15	Phytol	296	97	10.24

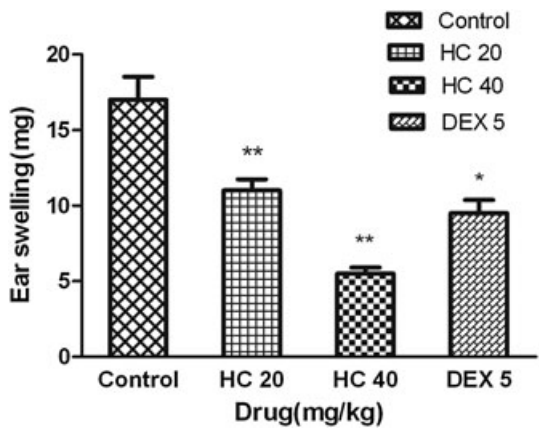

Figure 2. The effect of *Houttuynia cordata* Thunb. volatile oil on xylene-induced ear edema. Data are presented as mean ± SD ($n = 9$). * $p < 0.05$, ** $p < 0.01$ as compared with the control group. HC stands for HC volatile oil, DEX stands for dexamethasone and MNK stands for methyl nonyl ketone.

formation in a dose-dependent manner, and the inhibition rates were 35.3% and 67.6% (data not shown), respectively, as compared to the vehicle control. The positive control DEX (5 mg/kg) significantly inhibited the xylene-induced ear edema by 44.1% (data not shown) when compared to vehicle control.

Effect of HC volatile oil on formaldehyde-induced paw edema

In the formaldehyde-induced animal tests, treatment with HC volatile oil (20 and 40 mg/kg) and MNK (1 and 3 mg/kg) significantly and dose dependently suppressed the paw edema compared with the control groups, which was measured 3 h and 4 h after injection with maximum inhibition of 24.6% and 11.9%, 21.5% and 6.9 % respectively. Its activity was less than DEX (5 mg/kg) with an inhibition percentage of 35.4% 3 h after the injection with formaldehyde (see Table 2). The anti-inflammatory effect of HC volatile oil and

Table 2. Effects of *Houttuynia cordata* Thunb. volatile oil on inflammation caused by formaldehyde in mice[a]

Group	Dose (mg/kg)	Thickness of the injected foot (mm)				Inhibition/%	
		1 h[b]	2 h[b]	3 h[b]	4 h[b]	3 h	4 h
Control		4.69 ± 0.07	5.47 ± 0.05	6.22 ± 0.05	5.79 ± 0.07		
DEX	5	3.75 ± 0.05	3.84 ± 0.08	4.02 ± 0.09*	3.78 ± 0.06*	35.4	34.3
HC	40	4.24 ± 0.04*	4.48 ± 0.12	4.67 ± 0.04*	4.55 ± 0.06*	24.6	21.4
	20	4.43 ± 0.05	5.00 ± 0.05	5.53 ± 0.11*	5.19 ± 0.10*	11.9	10.4
MNK	3	4.47 ± 0.12	4.89 ± 0.17	4.88 ± 0.04*	4.74 ± 0.06*	21.5	18.1
	1	4.78 ± 0.05	5.21 ± 0.09	5.79 ± 0.11	5.48 ± 0.10*	6.9	5.3

[a]Each value is the mean ± SD of nine mice.

[b]Time after formaldehyde injection.

*$p < 0.05$, compared with control group; Student–Newman–Keuls test.

**$p < 0.01$, compared with control group; Student–Newman–Keuls test.

MNK was assessed by measuring the increase in the release of PGE_2, NO and TNF-α in exudates following stimulation by formaldehyde. Formaldehyde stimulated the right hind paw of mice significantly increased the excretion of the PGE_2, NO and TNF-α. HC volatile oil inhibited PGE_2, NO and TNF-a production in a dose-dependent manner, and MNK also inhibited PGE_2, NO and TNF-a production in a dose-dependent manner (see Fig. 3(A), (B) and (C). Similarly, when tested at a dose of 5 mg/kg, the DEX treatment also greatly reduced the release of PGE_2, NO and TNF-α according to assessment by ultraviolet spectrophotometer.

Effect of HC volatile oil on carrageenan-induced paw edema

The maximal increase in paw volume was observed at 3 h after carrageenan administration (maximal in paw volume:1.16 + 0.27 mm, data not shown). However, carrageenan-induced paw edema was significantly and dose dependently reduced by treatment with HC volatile oil (20 and 40 mg/kg) at all time points (Fig. 4A). Paw tissue was examined for PGE_2 and MDA production, in order to estimate inflammatory mediator and lipid peroxidation. As shown in Figure 4B and 4C, PGE_2 production and MDA levels were significantly increased in the paw at 4 h after carrageenan injection when compared to blank groups. PGE_2 production and MDA levels were significantly reduced, in a dose-dependent manner, by HC volatile oil (20 and 40 mg/paw) treatment (Figure 4B and 4C)

Cell viability

The cytotoxicity of compounds was assessed by MTT assay. As shown in Figure 5A, the volatile oil showed no cytotoxicity on cell proliferation at concentrations ranging from 1 μg/mL to 1000 μg/mL. In the MTT assay, however, HC volatile oil also inhibited the cell viability in a dose-dependent manner at concentrations higher than 1000 μg/mL. The concentrations of HC volatile oil applied in subsequent experiments were less than 1000 μg/mL. LPS alone or in combination with HC volatile oil (1–1000 μg/mL) showed no cytotoxicity on cell proliferation as well.

Effect of HC volatile oil on LPS-induced NO and TNF-α production by mouse peritoneal macrophages

To assess the effect of HC volatile oil and MNK on NO and TNF-α production by LPS-stimulated mouse peritoneal macrophages, peritoneal macrophages of KM mice were collected and treated with 10 μg/mL LPS and various concentrations of HC volatile oil (1, 10, 100 and 1000 μg/mL) and MNK (10 μg/mL) for 24 h. The nitrite and TNF-α content within the media was determined. As shown in Figure 5B, HC volatile oil inhibited NO production and about 68.62% ($p < 0.01$, data not shown) of the NO production at 1000 μg/mL, compared with the control (0 μg/mL HC Thunb. volatile oil). And MNK (10 μg/mL) inhibited NO production and about 37.45% ($p < 0.01$, data not shown) compared with the control. LPS-stimulated cells significantly increased the excretion of nitrite in the culture medium, and this increase was inhibited by HC volatile oil in a dose-dependent manner. No effect on the viability of peritoneal macrophages exposed to the HC volatile oil was observed. MNK showed no cytotoxicity at concentrations ranging from 0.1 to 100 (μg/mL) (Li *et al.*, 2011a, 2011b).With HC volatile oil included at 1000 μg/mL, TNF-α production was reduced ($p < 0.01$) by 77.13% (data not shown), compared with 0 μg/mL (Figure 5C). Inhibition was apparently dose dependent, with modest inhibitions by the 100 μg/mL ($p < 0.01$) HC volatile oil treatments. And MNK (10 μg/mL) inhibited TNF-α production and about 59.05% ($p < 0.01$, data not shown) compared with the control. As mentioned before, no effect of exposure to the volatile oil of HC and MNK (10 μg/mL) on the viability of peritoneal macrophages was observed (in all treatments, viabilities were > 95% of the control).

Effect of HC volatile oil on LPS-induced iNOS activity by mouse peritoneal macrophages

To examine whether the inhibitory effect of HC volatile oil on LPS-stimulated NO production was attributable to iNOS activity, we measured it using iNOS detection kit. iNOS activity was very low in the resting peritoneal macrophages, but pronounced iNOS activity was induced upon exposure to LPS alone (Figure 5D). There was no significant difference in the iNOS activity between the resting peritoneal macrophages and the cells treated with HC volatile oil (1000 μg/mL) alone. HC volatile oil inhibited LPS-induced iNOS activity

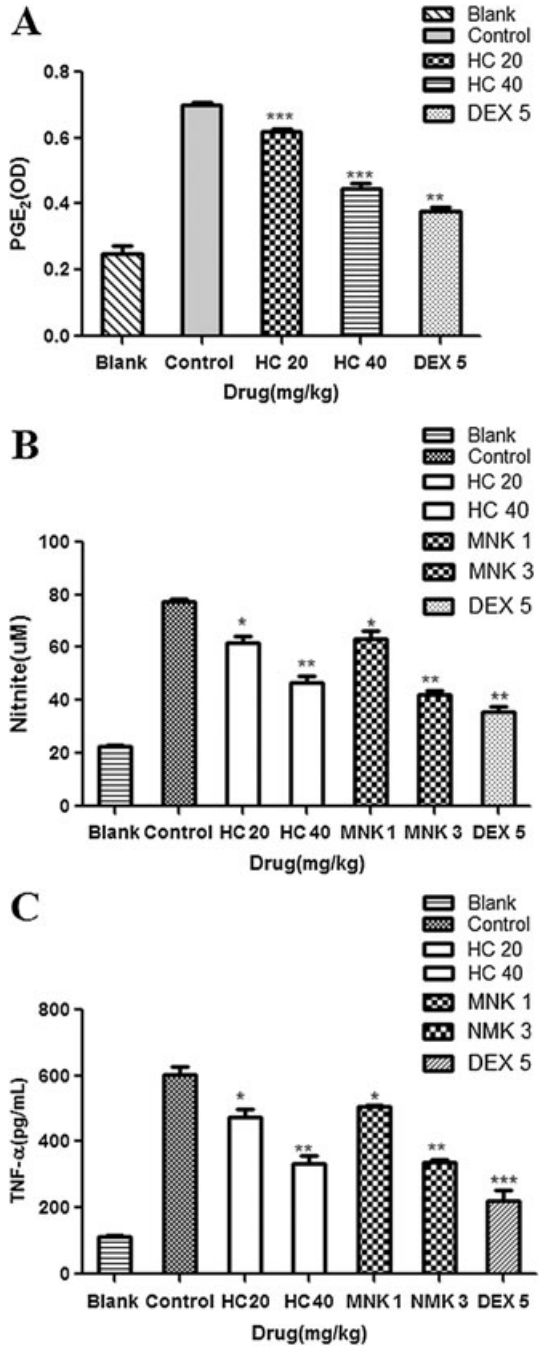

Figure 3. Effects of *Houttuynia cordata* Thunb. volatile oil on formaldehyde-induced PGE_2 (A), NO (B) and TNF-α (C) accumulation in mice (n = 9/group). Each value is represented as mean ± SD. ** $p < 0.01$, *** $p < 0.001$ as compared with the control group. Blank stands for healthy mice, HC stands for HC volatile oil, DEX stands for dexamethasone and MNK stands for methyl nonyl ketone.

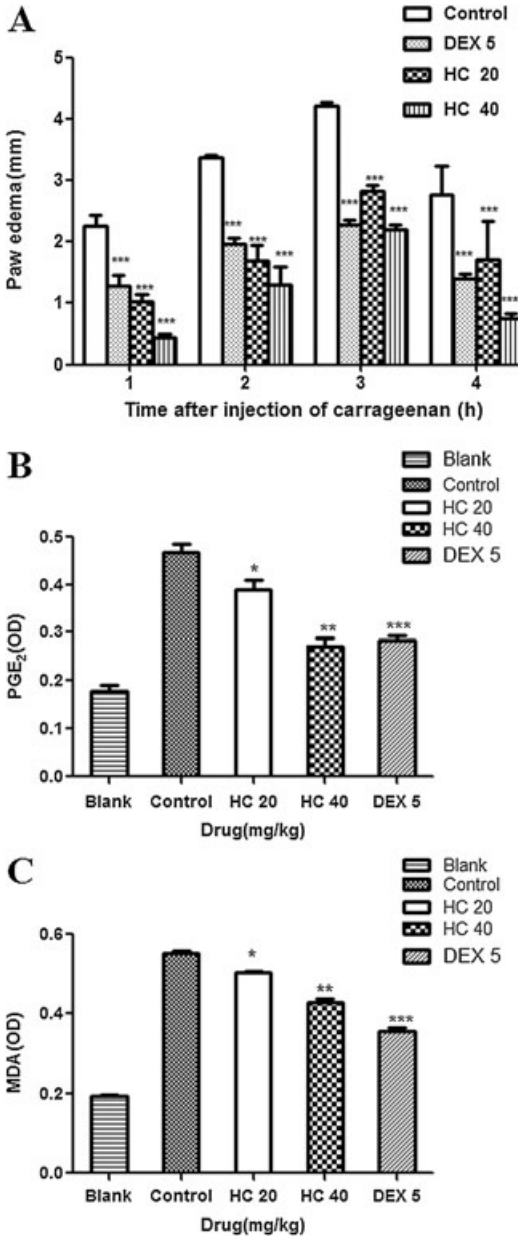

Figure 4. The effect of *Houttuynia cordata* Thunb. volatile oil treatment on carrageenan-induced hind paw edema (A), PGE_2 (B) and MDA (C) accumulation in mice (n = 9/group). Male mice were treated, i.p., with 20 or 40 mg/kg, or 5 mg/kg dexamethasone, 1 h prior to a sub-plantar injection of carrageenan. The thickness (mm) of the paw was measured at 1 h, 2 h, 3 h and 4 h after carrageenan treatment. Data are presented as mean ± SD (n = 9). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared with the control group. Blank stands for healthy mice, HC stands for HC volatile oil, DEX stands for dexamethasone and MNK stands for methyl nonyl ketone.

in a dose-dependent manner, showing IC_{50} values of 562.3 μg/mL (Figure 5D).

Transcriptional suppression of LPS-induced TNF-α and iNOS mRNA expression by HC volatile oil

Semi-quantitative RT-PCR was carried out to understand whether HC volatile oil could influence the decline in expression of TNF-α and iNOS mRNAs transcripts. The amount of TNF-α and iNOS transcript in the steady state was markedly increased by treatment of peritoneal macrophages with LPS alone (Fig. 6A). HC volatile oil inhibited LPS-induced synthesis of TNF-α and iNOS transcript in a dose-dependent manner, showing IC_{50} values of 481.4 μg/mL and 467.9 μg/mL, respectively (Fig. 6A). However, synthesis of housekeeping β-actin transcript was not affected by LPS, HC Thunb. volatile oil.

Effects of HC volatile oil on LPS-induced TNF-α synthesis and iNOS expression

Because HC volatile oil suppressed the expressions of TNF-α and iNOS genes in LPS-stimulated

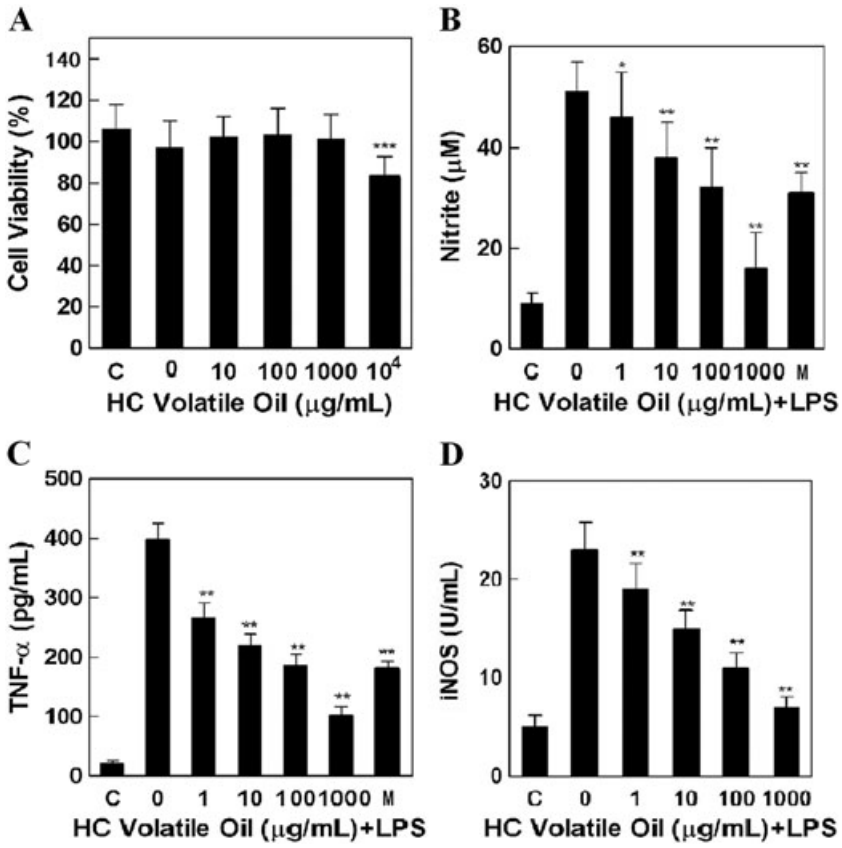

Figure 5. Effect of *Houttuynia cordata* Thunb. volatile oil on cell viability by MTT (A), LPS-induced NO (B), TNF-α (C) production and iNOS activity (D) on mouse peritoneal macrophages. Peritoneal macrophages were incubated with 10 μg/mL LPS, various concentrations of *Houttuynia cordata* Thunb. volatile oil (1, 10, 100 and 1000 μg/mL) and NMK (10 μg/mL) for 24 h. The nitrite and TNF-α content of culture media was analyzed. Activity of iNOS was measured with the supernatants of cell lysate. Values are mean ± SD ($n = 5$). * $p < 0.05$, ** $p < 0.01$ compared with the LPS treated (second column). C stands for control, 0 stands for LPS alone, 10, 100, 1000 stands for LPS (10 μg/mL) and 10, 100, 1000 μg/mL HC volatile oil, respectively, and M stands for methyl nonyl ketone (10 μg/mL).

peritoneal macrophages, we examined whether HC volatile oil could inhibit intracellular TNF-α protein synthesis and iNOS protein expression. As shown in Figure 6B, HC volatile oil inhibited intracellular TNF-α synthesis and iNOS expression in a dose-dependent manner.

DISCUSSION

HC is a traditional herb in China, Russia and many other Asian countries. For thousands of years, HC has been well known as a therapeutic drug and

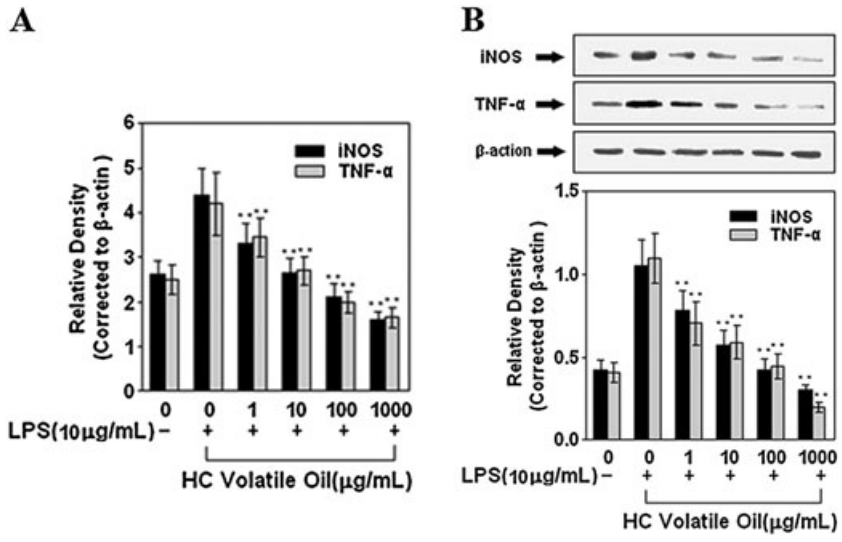

Figure 6. Effect of *Houttuynia cordata* Thunb. volatile oil on TNF-α and iNOS expression in peritoneal macrophages. (A) mRNA expression of TNF-α and iNOS in peritoneal macrophages stimulated with LPS and LPS plus HC essential oil, and (B) Western blots for TNF-α synthesis and iNOS expression. mRNA and protein levels of TNF-α and iNOS were normalized to β-actin loading control. Bars represent mean ± SD of at least three independent experiments; the picture is of a single representative experiment. ** $p < 0.01$.

used to treat various inflammatory diseases such as pneumonia, bronchitis, enteritis, postoperative infection and so on. However, little is known about the mechanisms of the anti-inflammatory effects of HC. This study investigated the effect of the volatile oil of HC on animal models of inflammation and NO and TNF-α production to clarify their anti-inflammatory mechanism.

In the present work, we have demonstrated that HC essential oil exerts anti-inflammatory activities and inhibits NO and TNF-α production and TNF-α and iNOS protein expression inflammatory tests. This can be supported by the following findings: (i) Xylene-induced mouse ear edema, formaldehyde-induced paw edema and carrageenan-induced mice paw edema were significantly decreased by HC essential oil; (ii) HC essential oil showed pronounced inhibition of PGE_2 and MDA production in the edematous exudates; (iii) We also observed inhibition of NO and TNF-α production with or without LPS and TNF-α and iNOS protein expression in the mouse peritoneal macrophages. All these findings clearly demonstrate that HC essential oil is an effective inhibitor.

Based on published studies Lau *et al*. (2008), we examined acute intraperitoneal toxicity associated with HC essential oil. Briefly, normal healthy male each weighed between 20 and 25 g were used. Twelve hours prior to dosing, all food was removed to fast the animals before initiating the test. On the day of the test, animals were identified and body weights recorded. The dosage to be administered was calculated based on the animal's body weight. The dosage of HC essential oil was 40 mg/kg bodyweight. The control group was treated with same amount of distilled water. Animals were closely observed for gross toxicological effects at 1, 3 and 6 h immediately after a single dose administration of the sample and then daily for a 7-day observation period. All animals appeared normal throughout the 7-day observation period. There was no difference of body weights between HC group and control group. HC essential oil was tested as specified and considered to be essentially non-toxic to laboratory animals following intraperitoneal at 40 mg/kg. Treatment with 20 or 40 mg/kg HC essential oil dose-dependently inhibited the formation of xylene-induced ear edema. Sub-cutaneous injection of formaldehyde into the hind paw of a mouse produces localized inflammation, and this was successfully inhibited by treatment with either 20 or 40 mg/kg HC essential oil or 5 mg/kg DEX. Carrageenan-induced paw edema is a commonly used experimental model of inflammation, and this model has frequently been used to assess the anti-edematous effects associated with natural products (Panthong *et al*., 2003). The result of the present study indicates that HC essential oil plays an important role in protection against carrageenan-induced inflammation.

NO is an effector molecule with multiple effects on various organ systems. The most prominent physiological actions of NO as a biological mediator include cGMP-dependent vasodilation, neurotransmission and cytotoxicity against pathogens in the nonspecific immune defense (Moncada *et al*., 1991). And accumulating evidences indicate that excessive production of NO plays a pathogenic role in both acute and chronic inflammations (Clancy and Abramson, 1995). NO is produced in physiological and pathophysiological conditions by three distinct isoforms of NOS: endothelial NOS (eNOS or NOS III), iNOS (or NOS II) and neuronal NOS (nNOS or NOS I) (Marletta, 1993; Nathan and Xie, 1993). While eNOS and nNOS are constitutively expressed and regulated by Ca^{2+}-calmodulin, the activity of iNOS is regulated at the transcriptional level by mediators such as IL-2, IFN-γ and inflammatory stimuli including bacterial LPS. LPS (endotoxin), highly conserved outer membrane component of gram-negative bacteria, triggers many biological responses such as fever, septic shock and even death (Morrison and Ryan, 1987). Murine and human macrophages exhibit a particularly vigorous response to LPS, which induces a variety of inflammatory modulators such as NO, interleukin-1b (IL-1b), TNF-α, IL-6 and PGs (Adams and Hamilton, 1984). Among these, NO is a cytotoxic mediator and contributes to the antimicrobial, antitumor activity of these cells. It is also known that TNF-α is produced by LPS and plays a role in inflammation. Although TNF-α is not an inducer of iNOS, it is crucial for synergistic induction of NO synthesis in IFN-γ and/or LPS-stimulated murine peritoneal macrophages and regulates NO synthesis *in vivo* (Harbrecht *et al*., 1994).

In this paper, the volatile oil of HC was discovered to have a dose-dependent inhibitory effect on NO production in mouse peritoneal macrophages induced by LPS. In parallel with an inhibition of TNF-α, the volatile oil of HC (1, 10, 100 and 1000 μg/mL) significantly inhibited extracellular TNF-α production in mouse peritoneal macrophages induced by LPS in a concentration-dependent manner *in vitro*. Furthermore, the volatile oil of HC attenuated the LPS-induced synthesis of iNOS and TNF-α mRNA, in parallel. These results indicated that HC volatile oil could down-regulate LPS-induced iNOS and TNF-α expression at the transcription level. As a molecular mechanism for the anti-inflammatory action shown by HC volatile oil, suppression of NO and TNF-α production has been demonstrated. HC volatile oil inhibited LPS-induced NO and TNF-α production in a dose-dependent manner. Furthermore, inhibition of LPS-induced iNOS activity and synthesis of iNOS protein and TNF-α protein expression by HC volatile oil is similar to inhibition of nitrite and TNF-α production by them, so they possibly inhibited the production of NO and TNF-α by down-regulating LPS-induced iNOS expression and TNF-α protein synthesis.

In addition, we reported previously that HC was able to inhibit the release of LPS-induced PG E_2 from mouse peritoneal macrophages and the inhibitory activity of HC essential oil elicited a dose-dependent inhibition of cyclooxygenase-2 enzyme activity (Li *et al*., 2011a). PGs of the E series, in particular PGE_2, are known regulators of TNF-α production (Kunkel *et al*., 1988). In this regard, it has been shown that inhibition of NO production decreases PG production by cultured macrophages (Salvemini *et al*., 1993) or by inflammatory exudate cells (Salvemini *et al*., 1995) suggesting that NO activates the induced form of cyclooxygenase. In this situation, inhibition of NO would be indirectly expected to partially release TNF-α production by PGE_2-suppressed macrophages as we observed in our model.

Peritoneal macrophages constitute an important class of immune cells. Receptors expressed at the cell surface include the toll-like receptors (TLRs), and the TLRs are the most important membrane receptors in relation to inflammatory processes in peritoneal macrophages

(O'Neil, 2003). The TLR_4 pathway was involved in the expression of TNF-α and iNOS after LPS challenge in macrophages (Medzhitov *et al*., 1997). Consequently, from a therapeutic standpoint, inhibition of the TLR_4 pathway could provide an efficacious strategy for inhibiting LPS-induced inflammatory mediator production. It was reported previously to act on white blood cell membranes and TLR_4, and its anti-inflammatory activity was related to antagonizing TLR_4 (Li and He, 2006). In the present study, it was demonstrated the effect of volatile oil of HC on various animal models of inflammation and production of NO and TNF-α in LPS-stimulated murine macrophages. The suppressive effect was not due to cytotoxicity or systemic toxicity. The overproduction of NO and TNF-α has been implicated in autoimmunity and inflammatory diseases. These results suggest that the inhibitory NO and TNF-α production of the volatile oil of HC is partly responsible for the anti-inflammatory function. Taken together, the volatile oil of HC could be a candidate for the development of new drugs to treat inflammatory diseases accompanied by the overproduction of TNF-α and NO, in addition to as an antagonist of TLR_4. Further identification of the active components of volatile oil of HC and the mechanisms are necessary fully to explain the observations.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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