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Full Length Research Paper

Anti-inflammatory effects of ethanol extract from *Melilotus suaveolens Ledeb*: Involvement of pro- and anti-inflammatory cytokines and mediators

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***Melilotus suaveolens Ledeb* is an annual or biennial plant of *Melilotus* genus in the Leguminosae family, which was used for treatment of inflammation-related diseases. The paper aims to investigate the anti-inflammatory mechanisms of ethanol extracts from *M. suaveolens Ledeb.* RAW 264.7 cells were stimulated with 10 ng/ml lipopolysaccharide (LPS) and intervened with 1 to 10 μg/ml ethanol extracts of *M. suaveolens Ledeb*. Several pro-inflammatory cytokines and mediators including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin (IL)-1β and tumour necrosis factor-α (TNF-α) were studied by the method of enzyme-linked immunosorbent assay (ELISA) analysis, real time – polymerase chain reaction (RT-PCR) and western blot, respectively. The anti-inflammatory cytokines and mediators, such as IL-10 and heme oxygenase-1 (HO-1), were also investigated by the same method. The activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) was studied using immunocytochemistry assay. Meanwhile, high performance liquid chromatography (HPLC) fingerprint was used to elucidate the active compounds in the extracts. The results showed that the extracts of *M. suaveolens Ledeb* have an anti-inflammatory effect by down-regulation of pro-inflammatory cytokines and mediators, such as iNOS, COX-2, IL-1β and TNF-α gene expression through the suppression of NF-κB activation. At the same time, the extracts increased anti-inflammatory cytokines and mediators IL-10 and HO-1. HPLC fingerprint chromatography of the *M. suaveolens Ledeb* extracts showed that coumarin is one of the main anti-inflammation compounds present in the plant, which is likely to be the basis of its anti-inflammation effect. The findings demonstrated that ethanol extracts from *M. suaveolens Ledeb* have great anti-inflammatory activities, which may be due to its dual effects on antagonizing pro-inflammatory and augmenting anti-inflammatory mediators.**

Key words: *Melilotus suaveolens Ledeb*, ethanol extract, anti-inflammatory effects, pro-inflammatory cytokines and mediators, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), anti-inflammatory cytokines and mediators.

INTRODUCTION

Melilotus suaveolens Ledeb is an annual or biennial plant of *Melilotus* genus in the Leguminosae family, which has long been prescribed in traditional medicine in China due to its anti-inflammatory property. Traditionally, *M. suaveolens Ledeb* was applied to treat the inflammation-related conditions such as tonsillitis and diphtheria (Plesca-Manea et al., 2002). Up to date, it might be

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developed into a prospective drug, so it is very necessary to explore its anti-inflammatory mechanisms.

Inflammation is a beneficial host response to a foreign challenge or tissue injury that leads ultimately to the restoration of normal tissue structure and function. During this response, macrophages play an important role in inflammatory conditions (Hernández-Ledesma et al., 2009). Once stimulated, macrophages produce a number of pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) (Kim et al., 2003; Shao et al., 2010) and pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1β (Ikeda et al., 2008) and IL-6 (Moriyama et al., 2006), which are essential for the inflammatory response to pathogenic germs or toxicants (Liew et al., 2003). In order to prevent inflammatory reaction and disorders, suppression of these mediators may be an effective therapeutic strategy (Yen et al., 2008).

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-Kb) is a nuclear transcription factor that regulates the expression of various genes, including IL-1β, TNF-α, iNOS and COX-2, which play critical roles in various autoimmune diseases and inflammation (Kim et al., 2003; Cao et al., 2006; Park et al., 2007). Meanwhile, when the pro-inflammatory factors occurred, anti-inflammatory cytokine IL-10 was secreted (Henry et al., 2009), and heme oxygenase-1 (HO-1) was synthesized as an anti-inflammatory mediator (Saluk-Juszczak and Wachowicz, 2005), since activation of pro-inflammatory, anti-inflammatory cytokines and mediators is the key procedure of inflammatory reaction and leads consequent inflammatory impairment and restoration (Hofman, 2004). Therefore, pro- and anti-inflammatory cytokines and mediators were investigated in order to elucidate the anti-inflammatory effect of *M. suaveolens Ledeb*.

In the past study, the lipopolysaccharide (LPS)-stimulated RAW 264.7 cells has been used as the inflammatory cellular model to study the effect of anti-inflammatory drugs and herbs (Shin et al., 2008; Rhule et al., 2006). Moreover, our previous studies have shown that *M. suaveolens Ledeb* contains numerous ingredients and possesses various functions. Various extract method for *M. suaveolens Ledeb* with different organic solvent may lead to the difference in effective ingredient. Ethanol extract contains some complicated active compounds with different polar solution fractions. According to the theory of traditional Chinese medicine (TCM), the therapeutic actions of herbal medicines are based on integral interaction of many kinds of ingredients combined rationally. However, the anti-inflammatory mechanisms of ethanol extract from *M. suaveolens Ledeb* have not been studied in depth until now as much as we known.

In this study, LPS-stimulated RAW 264.7 cells were intervened with the ethanol extract from *M. suaveolens Ledeb*. Subsequently, several pro-inflammatory cytokines and mediators, TNF-α, IL-1β, IL-6, iNOS and COX-2 were determined by the method of sandwich enzyme-linked immunosorbent assay (ELISA), real-time polymerase chain reaction (RT-PCR) and western blot analysis. The anti-inflammatory cytokines and mediators, such as IL-10 and HO-1, were also investigated by the same method. The activation of NF-κB was studied using immunocytochemistry assay. Because coumarin, rutin and hyperoside have been reported in *Melilotus* genus and owned anti-inflammatory effect (Bubenchikova and Drozdova, 2004), they were taken as standard substances to detect the contents of them in the extracts through high performance liquid chromatography (HPLC) fingerprint analysis. LPS-stimulated RAW 264.7 cell was selected as a cellular model. The aim of this study was to explore the anti-inflammatory effects of ethanol extract from *M. suaveolens Ledeb* in molecular levels.

MATERIALS AND METHODS

Plant material

M. suaveolens Ledeb was collected from Longxian, Shanxi province, China and identified by Hubei College of Traditional Chinese Medicine.

Cell lines, chemicals and biochemicals

Murine macrophage cell line RAW 264.7 was obtained from the China Center for Typical Culture Collection (Wuhan, China). LPS (*Escherichia coli* O111:B4) and methyl thiazolyl tetrazolium (MTT) were obtained from Sigma (St Louis, USA). Goat anti-mouse HO-1 antibody was obtained from R&D Systems (Minneapolis, MN). Goat anti-mouse COX-2 antibody, rabbit anti-mouse NF-κB was obtained from Santa Cruz Biotechnology (CA, USA). β-actin was obtained from Ferments Company. Mouse TNF-α, L-1β and IL-6 ELISA kits were purchased from Quantikine, R&D Systems (Minneapolis, USA). Griess reagent nitric oxide (NO) assay kit was purchased from Jingmei Biotech Co., Ltd (Shenzhen, China). Mouse IL-10 ELISA kit was obtained from Bender Medsystem (Vienna, Austria).

Preparation of ethanol extracts from *Melilotus suaveolens Ledeb*

50 g air-dried whole aerial part of *M. suaveolens Ledeb* were powdered and extracted with 70% ethanol for three times at 85°C (3×500 ml, 1.5 h for each). Subsequently, the extracting liquid was filtered, combined and concentrated in vacuo. Then the concentrated liquid was diluted with deionized water to a concentration of 1 g/ml water. Finally, the liquid was diluted by 1640 medium to a concentration of 10, 5 and 1 μg/ml, which were used as intervene liquid for treatment on LPS-stimulated RAW 264.7 cells.

Establishments and treatments of lipopolysaccharide (LPS)-stimulated RAW 264.7 cellular model

Prior to stimulation by LPS, the cells were inoculated into 6, 24 and 96 micro-well plates. 24 h later when the cells were seen to be adhering to the bottom of well, the cell supernatants were disposed

Table 1. The note of control and intervene groups.

Sample	Note	Group
10+	10 μg/ml extract + 10 ng/ml LPS	High dose
5+	5 μg/ml extract + 10 ng/ml LPS	Middle dose
1+	1 μg/ml extract + 10 ng/ml LPS	Low dose
DM+	0.5 μg/ml Dexamethasone + 10 ng/ml LPS	Positive control
APS+	100 μg/ml APS + 10 ng/ml LPS	Negative control
0+	+ 10 ng/ml LPS	Blank control
0-	No stimulation and intervention	Normal control

and then 10 ng/ml LPS with prepared extract solution was added into the well. The supernatants of the cell culture and cells were harvested for sandwich ELISA, RT-PCR and western-blot analysis following stimulation and treatment lasted for various time.

Control establishment

There were four kinds of control groups, including positive control group, negative control group, blank control group and normal control group. Among them, cell treated with dexamethasone (DM, 0.5 μg/ml) was chosen as positive control group. Cell treated with astragalus polysaccharides (APS, 100 μg/ml) was selected as negative control group.

Cell only stimulated by LPS (10 ng/ml) was regarded as blank control group. Cell incubated by 1640 medium was regarded as normal control group (Table 1).

Methyl thiazolyl tetrazolium (MTT) assay for the measuring of cell proliferation

Cytotoxic effect of the extract was evaluated by conventional MTT assay. 20 μl of the MTT solution (5 mg/ml in a phosphate-buffered saline (PBS), pH 7.4) was added to the well before culture termination of 4 h when ethanol extract of *M. suaveolens Ledeb* intervened RAW 264.7 cell constantly during the process. 150 μl dimethyl sulfoxide (DMSO) was then added to each well for solubilization of the deposition of the cells, and the optical density of the cells at 490 nm was measured by a Spectramax 250 microplate reader.

Detection of cytokine tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, interleukin (IL)-6 and interleukin (IL)-10 production

Ethanol extract from *M. suaveolens Ledeb* intervened in RAW 264.7 cells stimulated by LPS for 24 h, and the supernatants of the cell culture were collected and assayed for TNF-α, IL-1β, IL-6 and IL-10 by ELISA kits according to the instructions provided by manufacturer.

Analysis of nitric oxide (NO) production

Levels of NO were determined by the Griess reaction. The samples were assayed by a nitrite detection kit according to the provided instructions, and a standard curve using $NaNO_2$ was generated in each experiment. Briefly, 100 μl of medium or standard $NaNO_2$ was mixed with 100 μl of Griess reagent in a 96-well plate. After 15 min, the optical density (OD) was measured at 540 nm in a microplate reader.

Quantitative real time polymerase chain reaction (RT-PCR) for detecting messenger ribonucleic acid (mRNA) of tumour necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1)

The total RNA from the LPS treated RAW 264.7 cells was extracted by adding TRIzol Reagent (Gibco BRL) according to manufacturer's protocol and stored at -80°C before use. The total RNA for detection of pro-inflammatory mediators TNF-α, iNOS and COX-2 were extracted at 4 h after the cells were stimulated with LPS and intervened, and the RNA of HO-1 was obtained at 18 h after the cells stimulation and intervention.

Quantitative RT-PCR was performed in a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) with the FastStart DNA Master SYBR Green I kit. Each 50 μl PCR contained 1/50th of the original complementary deoxyribonucleic acid (cDNA) synthesis reaction, 7 μl (25 mM) $MgCl_2$, 0.8 μl (20 pmol/μL) of each primer, 1 μl (10 mM) deoxynucleotide triphosphates (dNTP), 1 μl SYBR Green Ⅰ, 0.5 μl (5 U/μl) Taq and 5 μl 10×Buffer. 50 cycles of amplification were performed: after 94°C, 3 min, the annealing temperature was reduced from 94°C, 30 s, to 57°C, 30 s, then to 72°C, 30 s. The fluorescence signal was detected at the end of each cycle. Melting curve analysis was used to confirm the specificity of the products. The results were analyzed according to the previous study (Livak and Schmittgen, 2001).

Western blot analysis of cyclooxygenase-2 (COX-2) and heme oxygenase-1 (HO-1)

RAW 264.7 cells were incubated with or without LPS in the presence or absence of the extract of *M. suaveolens Ledeb* for 24 h. The cells were harvested, washed twice with ice-cold PBS, and re-suspended in PBS containing 0.1 mM phenylmethylsulfonyl fluoride. The cells were laid by three cycles of freezing and thawing in liquid nitrogen. The cytokine fraction was obtained from the supernatant by centrifugation (12,000×g) at 4°C for 20 min. Samples (30 μg protein) were separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Proteins were transferred

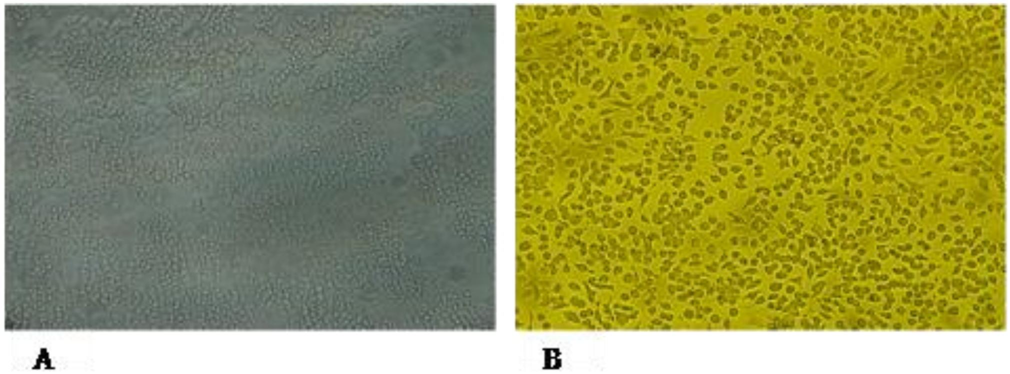


Figure 1. Cytopathic effects of pretreatment of ethanol extract from *M. suaveolens Ledeb*. A, Un-stimulated RAW264.7 cell before treatment; B, un-stimulated RAW 264.7 cell treatment with ethanol extract at the dose of 10 μg/ml for 24 h (1×40 times in microscope).

onto polyvinylidene difluoride membranes (Millipore, MA). The membranes were blocked with 5% nonfat milk in TBST (0.1%) for 1 h and then incubated with polyclonal antibody for goat HO-1(1:6000 dilutions) or polyclonal antibody for COX-2 (1:6000 dilutions) in TBST containing 1% nonfat milk for 1 h. After washing three times with TBST, the membrane was hybridized with secondary antibody conjugated with horseradish peroxidase for 1 h and washed five times with TBST (anti-rabbit and anti-mouse IgG-HRP, 1:2000 dilutions). The membrane was incubated with enhanced chemilumescent (ECL) detection kits (Pierce Biotechnology CO., Lit, US) for 2 min and exposed to X-ray film.

Immunocytochemistry assay for nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

Coverslips were soaked in polylysine for whole night. After cell crawling to the coverslips and subsequent LPS stimulation, and extract intervention, the cell was washed normally, fixed and blocked by turns. Then rabbit anti-mouse NF-κB Ig G (diluted to 1:100) was added. After having been placed for 12 h, the cell was incubated with biotin-conjugated goat anti-rabbit IgG (diluted to 1:75) at room temperature for 30 min. Then diaminobenzidine coloration, hematoxylin counterstain, ethanol dehydration (orderly by 75, 95 and 100%), dimethyl benzene clearance and mounting observation were performed one by one. Five visual fields were chosen randomly in microscope and positive cell was counted for each sample. The NF-κB was detected at 2 h after treatment with the extract.

High performance liquid chromatography (HPLC) fingerprint of ethanol extract from *Melilotus suaveolens Ledeb*

The 0.10% H_3PO_4 solution was prepared daily by diluting 0.5 ml H_3PO_4 with double distilled water to 500 ml, then filtrated through a 0.45 μm Nylon filter (Hanbon Science and Technology Co., Ltd). The reference substances of coumarin, rutin and hyperoside were accurately weighted, and they were prepared to a concentration of 7.6480 μg/ml, 0.2548 mg/ml and 0.2528 mg/ml, respectively.

Since coumarin, rutin and hyperoside have been reported in *Melilotus* genus and owned anti-inflammatory effect (Bubenchikova and Drozdova, 2004), they were taken as standard substances to detect the contents of them in the ethanol extracts from *M. suaveolens Ledeb*. During chromatography analysis, the gradient elution was acetonitrile and 0.10% H_3PO_4 at a flow rate of 1.0 ml/min. 5 μL capacity per injection was used with UV detector (UVD 170U) at four kinds of wavelengths of 220 nm (for coumarin), 254 nm (for general use), 275 nm (for coumarin and rutin) and 363 nm (for hyperoside), and the column (Kromasil C-18, 250 mm×4.6 mm, 10 nm-5 μm, Hanbon Science and Technology Co., Ltd) was placed in a column oven at 25°C.

Procedure

1 ml concentrated liquid of 1 g/ml was accurately place in a 10 ml volumetric flask using a 1 ml Mohr measuring pipette and diluted to the scale mark, then 3 ml liquid was diluted in a 25 ml volumetric flask to a concentration of 12 mg/ml (mg herb weight per 1 ml solution), filtered through a 0.45 μm filter (Hanbon Science and. Technology Co., Ltd) and stored in the refrigerator before analyzing by HPLC.

The multi-step gradient elution involved as follows: C and D was acetonitrile and 0.10% H_3PO_4 respectively, 0 to 8 min (5%C, 95%D), 8 to 25 min (5%C to 30%C and 95%D to 70%D), 25 to 35 min (30%C, 70%D), 35 to 60 min (30%C to 70%C, 70%D to 30%D), 60 to 70 min (30%C to 5%C, 70%D to 95%D), and 70 to 80 min (5%C, 95%D).

Statistical analysis

The student's t-test and one-way analysis of variance (ANOVA) were used to determine the statistical significance of differences for the values between the various experimental and control groups. Data were expressed as means ± SD and P-values of 0.05 or less were considered to be statistically significance.

RESULTS

In vitro cytotoxicity of ethanol extract from *Melilotus suaveolens Ledeb*

Pretreatment of unstimulated RAW 264.7 cell lines with

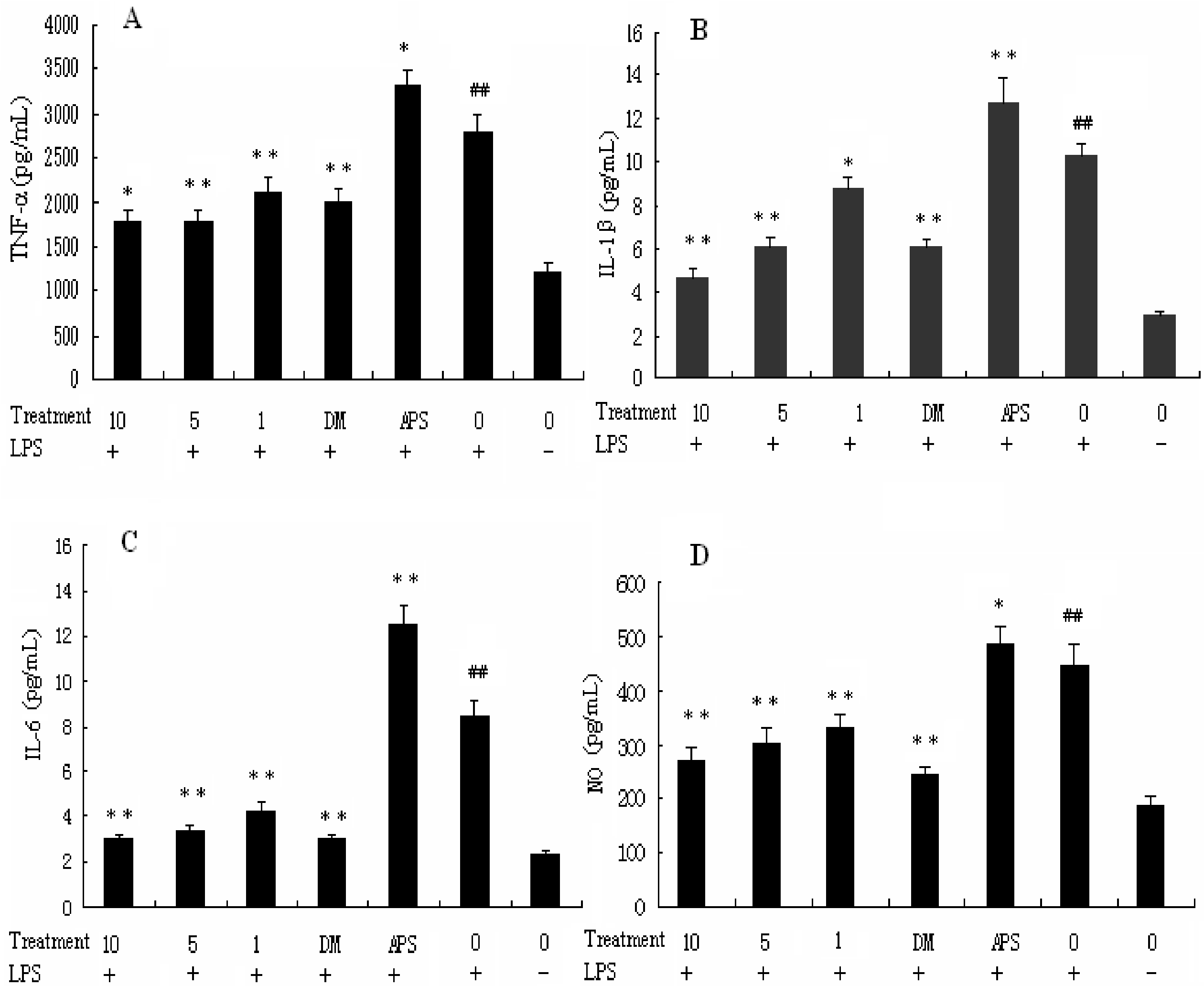


Figure 2. Effects of different concentrations of ethanol extract from *M. suaveolens Ledeb* on pro-inflammatory cytokines and mediators production. A, TNF-α; B, IL-1β; C, IL-6; D, NO. Data were shown as the mean ±SD (*n*, 3). $^{*}P<0.05$ or $^{**}P<0.01$ versus LPS alone, $^{\#}P<0.05$ or $^{\#\#}P<0.01$ compared to normal cell.

the prepared solution from ethanol extract of *M. suaveolens Ledeb* for 24 h did not significantly affect cell viability (data not shown, Figure 1).

Inflammatory model establishment and procedure monitoring

As shown in Figure 2, the values of pro-inflammatory cytokines and mediators TNF-α, IL-1β, IL-6 and NO in LPS-stimulated cells were significantly higher than those in normal cells, which illuminated that the cellular inflammation model was established successfully (p<0.01). Meanwhile, the value of these cytokines and mediators in cell intervened by Dexamethasone was significantly lower than that from single LPS stimulation (p<0.01). Moreover, as APS is an immunemodulator for enhancing immune response, the results showed the level of pro-inflammatory mediator in cell interfered by LPS with APS was marked higher than that by single LPS, which elucidated the experimental procedure was proper (p < 0.01 or p < 0.05).

Effect of ethanol extract from *Melilotus suaveolens Ledeb* on the pro-inflammatory cytokines and mediators produced by lipopolysaccharide (LPS) stimulation

The secretion of TNF-α, IL-1β, IL-6 and NO was significantly decreased in LPS-stimulated RAW264.7

Figure 3. Effects of different concentrations of ethanol extract from *M. suaveolens Ledeb* on COX-2 protein production.

cells treated with ethanol extract compared with that in single LPS stimulation cells (p<0.01 or p <0.05) (Figure 2). Furthermore, the higher concentration of ethanol extracts, the greater influence on antagonizing pro-inflammatory cytokines, showed a concentration-dependent manner of extract on anti-inflammatory effect. However, the secretion of these cytokines from LPS-stimulated RAW 264.7 cells was not restored to the value of normal condition. At the same time, the ethanol extract caused strikingly decreased level of COX-2 protein, TNF-α and COX-2 mRNA compared to blank control (Figures 3 and 4), which suggested that ethanol extract might control TNF-α and COX-2 production not only at levels of proteins but also by transcriptional and translational levels. Moreover, effect of *M. suaveolens Ledeb* ethanol extract on mRNA of iNOS (Figure 4) was coincidence with that on NO production.

Effect of ethanol extract from *Melilotus suaveolens Ledeb* on nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

As seen from Figure 5, NF-κB activation was significantly blocked by ethanol extract from *M. suaveolens Ledeb*. It suggested that suppression of IL-1β, TNF-α, iNOS and COX-2 gene expression by the extract might be due to the attenuation of NF-κB activation.

Effect of ethanol extract from *Melilotus suaveolens Ledeb* on anti-inflammatory cytokines and mediators produced by lipopolysaccharide (LPS) stimulation

The IL-10 levels of cells increased by ethanol extract from *M. suaveolens Ledeb* were similar to that of DM treatment, which showed the inhibitory effect of ethanol extract on inflammatory cytokines like DM (Figure 6). The levels of HO-1 mRNA and protein treated by 10 and 5.0 μg/ml of ethanol extract, DM and APS were significantly higher than those in single LPS stimulation and normal cells (p<0.05) (Figure 7). Furthermore, the higher concentration of *M. suaveolens Ledeb* extract, the greater its effect on expression of HO-1 mRNA and protein, which suggested that ethanol extract from *M. suaveolens Ledeb* might promote regression of inflammation and control anti-inflammatory mediator production in protein level.

High performance liquid chromatography (HPLC) fingerprint of ethanol extract from *Melilotus suaveolens Ledeb*

The HPLC fingerprint (Figure 8) showed that the ethanol extract mainly contained lower polar compounds, including coumarin (retention time was 33.485 min), but neither hyperoside nor rutin (retention time was 24.535 min and 25.407 min, respectively). Coumarin that isolated from *Melilotus officinalis* has been shown to result a good anti-inflammatory effect (Li et al., 2005). So we could draw a conclusion that coumarin might be one of the main anti-inflammatory compounds in ethanol extract (12 mg/ml). As shown in Table 2, the content of which was 11.5397 μg/ml was calculated by one point external standard method. The contents of coumarin in 10, 5 and 1 μg/ml intervention sample prepared from the *M. suaveolens Ledeb* extract were 9.6164, 4.8082 and 0.9617 ng/ml, respectively.

DISCUSSION

Inflammation is an important part of immuno-pathogenesis. During inflammatory process, macrophages produce excess amounts of pro-inflammatory cytokines and mediators (Jung et al., 2007). TNF-α, IL-1β and IL-6 are well known to be pro-inflammatory cytokines that possess a multitude of biological activities linked to the acute or chronic inflammatory diseases (Bertolini et al., 2001), and these cytokines were usually studied to explain the anti-inflammatory mechanisms of many drugs by other researchers. It found that the ethanol extract of *M. suaveolens Ledeb* could inhibit the production of TNF-α, IL-1β and IL-6 in LPS-stimulated RAW264.7 cell, which suggested that the extract might have an anti-inflammatory effect through down regulation of these pro-inflammation cytokines.

TNF-α and IL-1β can also induce the synthesis of COX-2 and iNOS enzymes. COX-2 is the central mediators of inflammation which involved in inflammatory processes and markedly stimulated by LPS and cytokines (Cho et al., 2004). Induction of COX-2 and iNOS can elevate the levels of NO, which is produced from the guanidino nitrogen of L-arginine oxidized by NO synthase (NOS) (Yoon et al., 2009; Martel-Pelletier et al., 2003). NO is essential for host innate immune responses to pathogens such as bacteria, viruses and fungi (Bogdan et al., 2000). However, excessive NO production can also do great

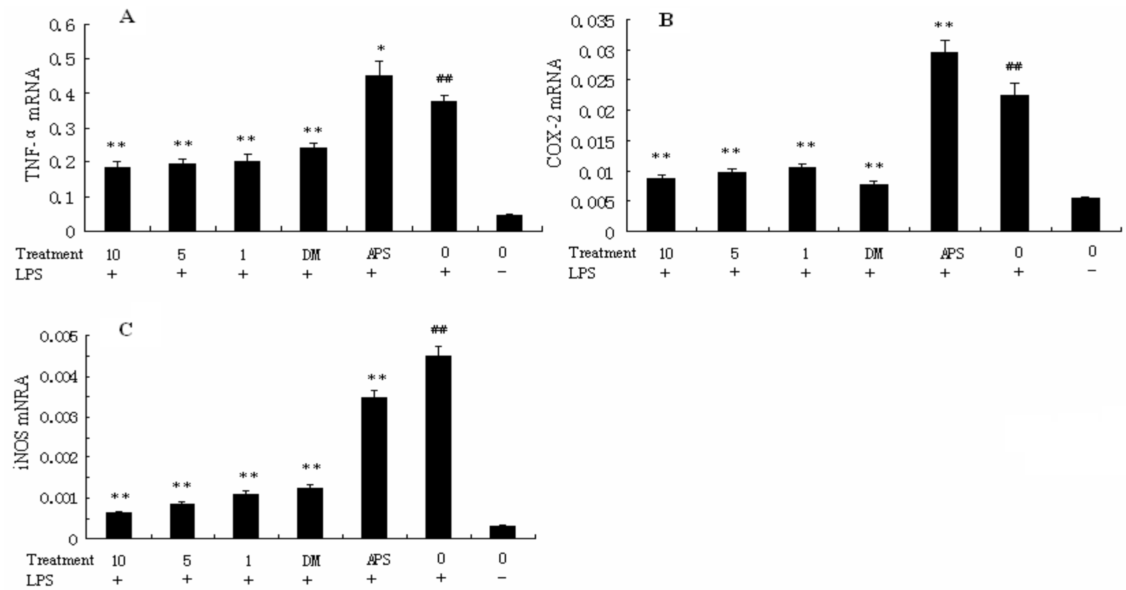


Figure 4. Effects of different concentrations of ethanol extract from *M. suaveolens Ledeb* on pro-inflammatory cytokines and mediators mRNA expression. A, TNF-α; B, COX-2; C, iNOS. Data were shown as the mean ± SD (*n*, 3). **P*<0.05 or ***P*<0.01 versus LPS alone, #*P*<0.05 or ##*P*<0.01 compared to normal cell.

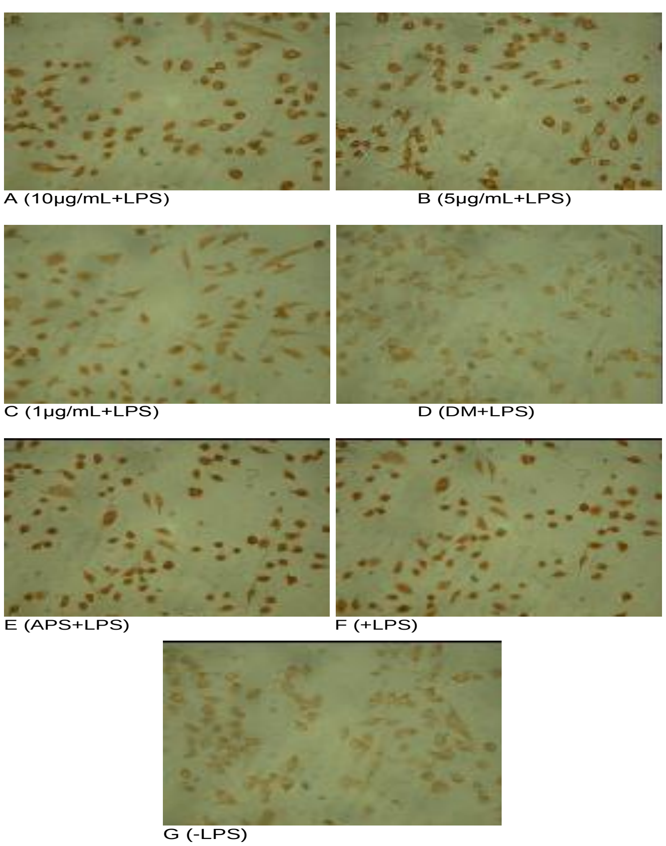


Figure 5. Effects of ethanol extract from *M. suaveolens Ledeb* on NF-κB levels in the culture media of LPS-stimulated cells. A, High dose group; B, middle dose group; C, low dose group; D, positive control; E, negative control; F, blank control; G, normal control.

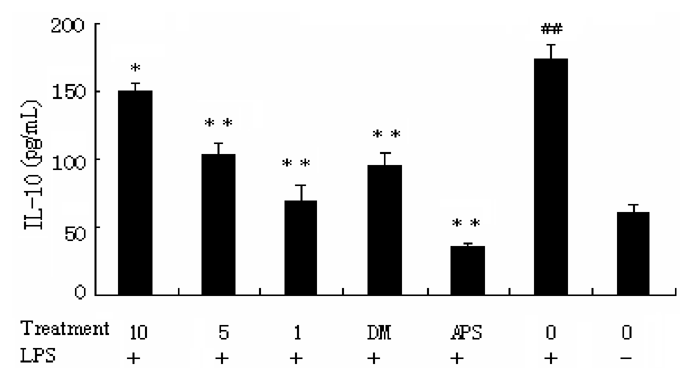


Figure 6. Effects of different concentrations of ethanol extract from *M. suaveolens Ledeb* on IL-10 production. Data were shown as the mean ± SD (*n*, 3). **P*<0.05 or ***P*<0.01 versus LPS alone, #*P*<0.05 or ##*P*<0.01 compared to normal cell.

damage to host tissues such as inflammatory diseases (O'Shea et al., 2002). Thus, inhibition of NO production is a major target for anti-inflammatory agent development, and the potential inhibitors of iNOS and COX-2 have been considered to be anti-inflammatory drugs (Plutzky et al., 2001). In this study, the ethanol extract from *M. suaveolens Ledeb* significantly inhibits the gene expression of iNOS and COX-2, and the down regulation of iNOS and COX-2 protein; and mRNA expression may be responsible for the inhibition of NO production in RAW 264.7 cells stimulated with LPS. Some researchers suggested that the relevant compounds that inhibit the expression of these pro-inflammatory mediators is a

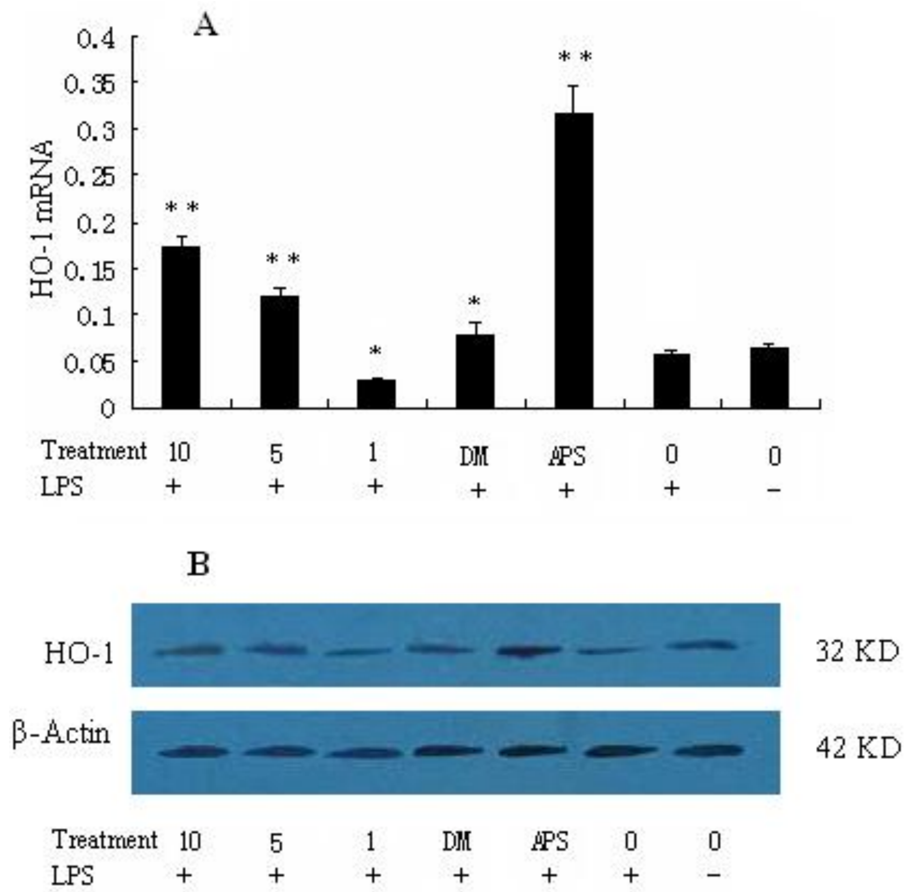


Figure 7. Effects of different concentrations of ethanol extract from *M. suaveolens Ledeb* on HO-1 mRNA (A) and protein production (B). Data were shown as the mean ± SD (*n*, 3). **P*<0.05 or ***P*<0.01 versus LPS alone, $^{\#}P$<0.05 or $^{\#\#}P$<0.01 compared to normal cell.

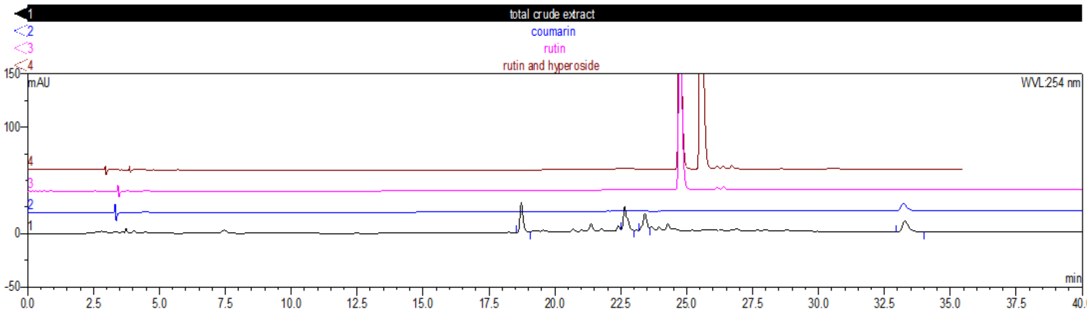
Table 2. The content of coumarin in analysis liquid and intervention samples.

Sample/content	Analysis of ethanol extract (mg/ml)	Treatment samples (ug/ml)		
	12	10	5	1
In analysis liquid (ug/ml)	11.5397			
In treatment samples (ng/ml)		9.6164	4.8082	0.9617

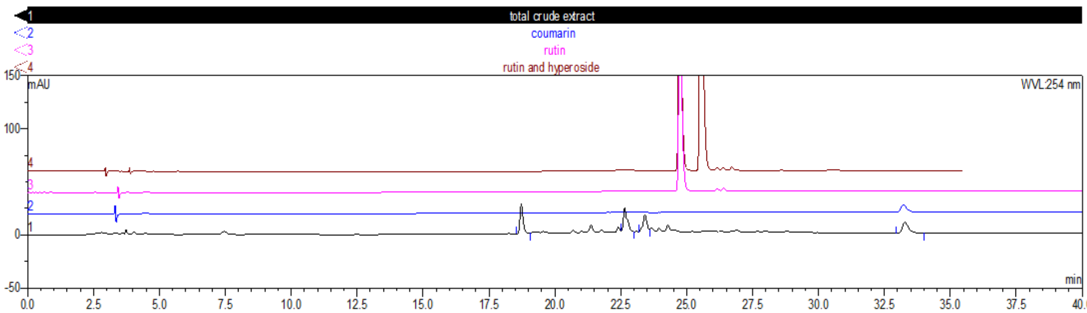
promising treatment strategy for managing the inflammation-related disease. The results in this study could be used to explain the anti-inflammatory effect due to degrade of the production of some key pro-inflammatory mediators, such as iNOS and COX-2.

NF-κB is one of the most ubiquitous transcription factors that regulates the expression of various genes involved in inflammatory responses. The activation of NF-κB has been reported to induce the transcriptions of multiple pro-inflammatory mediators (Rahman et al., 2004). Therefore, we focused on the NF-kB pathway, because of its involvement in the inflammatory response mediated by macrophages. The results confirmed that ethanol extract from *M. suaveolens Ledeb* could inhibit the production of NO, iNOS, COX-2, IL-1β and TNF-α in LPS-stimulated RAW 264.7 cells via suppression of NF-κB activation. Such results could be used to explain its successful use in treating inflammatory conditions.

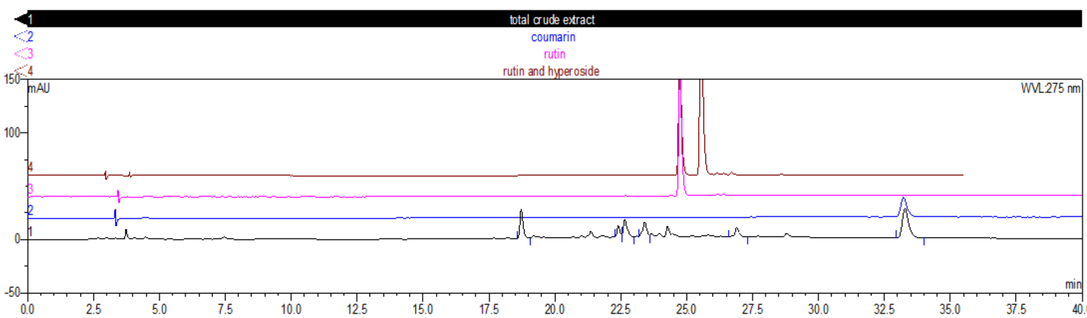
IL-10 is a cytokine of potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1β by activated macrophages. HO-1, one of the three isoforms of HO, is a member of the heat shock protein family and can be induced by various stimuli including pro-inflammatory cytokines. HO-1 has been shown to mediate the anti-inflammatory effect of IL-10 (Lee et al., 2002). Blockage



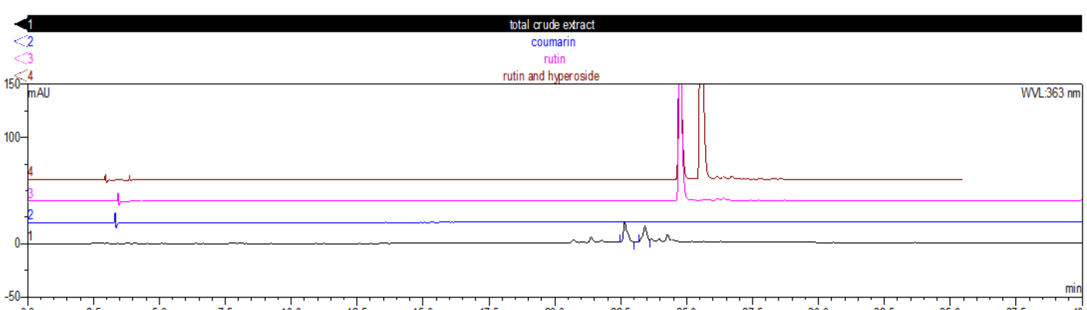
A, 220 nm wavelength



B, 254 nm wavelength



C, 275 nm wavelength



D, 363 nm wavelength

Figure 8. HPLC fingerprint chromatography of the ethanol extract. (Note: from below to up was total ethanol extraction, standard substances of coumarin, rutin, rutin and hyperoside, respectively).

HO-1 with inhibitors blocks the anti-inflammatory actions of IL-10 (Sanjabi et al., 2009). As shown in Figures 7 and 8, ethanol extract from *M. suaveolens Ledeb* can also increase anti-inflammatory cytokines and mediator production including IL-10 and HO-1.

These results collectively suggested that ethanol extract from *M. suaveolens Ledeb* exerted the dual effects on antagonizing pro-inflammatory and augmenting anti-inflammatory mediators. The HPLC fingerprint demonstrated that neither rutin nor haperoside, but only coumarin existed in the extraction, and the concentration change of coumarin in ethanol extract from *M. suaveolens Ledeb* might attribute to their different anti-inflammation activity. However, there also might be some other anti-inflammatory ingredients in *M. suaveolens Ledeb*. Characteristics of the exact active compounds that mediated the anti-inflammatory activity of *M. suaveolens Ledeb* are under investigation.

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