Original Article

N-butanol Extract from Melilotus Suaveolens Ledeb Affects Pro- and Anti-Inflammatory Cytokines and Mediators

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Melilotus suaveolens Ledeb is a traditional medicinal plant for treating inflammation-related disease. This explores the inner anti-inflammatory mechanism of *n*-butanol extract from *M. suaveolens Ledeb*. Inflammatory cellular model was established by lipopolysaccharide intervention on RAW264.7 cell line. Levels of secreted cytokines TNF-α, IL-1β, IL-6, NO and IL-10 in supernatant, mRNA expression of TNF-α, COX-2, iNOS and HO-1, protein expression of COX-2 and HO-1, activation of NF-κB and ingredients in the extract were assayed by ELISA, real time quantitative PCR, western blot, immunocytochemical test and HPLC fingerprint test, respectively. As a result, the extract could not only markedly reduce the production of pro-inflammatory mediators to different extents by blocking NF-κB activation but also promote the release of anti-inflammatory mediator HO-1 significantly. Each 1 g extract contained 0.023531 mg coumarin and another two high polar ingredients, probably saponins. It can be concluded that the extract has similar effects on antagonizing pro-inflammatory mediators and cytokines like Dexamethasone, and has effects on promoting the production of anti-inflammatory mediators.

Keywords: *Melilotus suaveolens Ledeb* – *n*-butanol extract – RAW 264.7 cell line – NF-κB – HO-1

Introduction

Melilotus is a genus of plants including 20–25 species that are widely distributed all over the world. The main species used for medical purpose contain *Melilotus albus Desr, Melilotus officinalis Lam, Melilotus suaveolens Ledeb, Melilotus dentatus (W.&K.) Pers, Melilotus indica (L.) All, Melilotus italicus (L.) Lam, Melilotus volgicus Poirr, Melilotus hirsutus Lipcky (L.) Lam, Melilotus elegans Salzm, Melilotus salcatus Dest, Melilotus neopolitanus Ten*, etc. *Melilotus suaveolens Ledeb* is distributed in the Far East region and used as herbal medicine to treat inflammation and infection in throat and alimentary system in China (1).

In literatures, there were a few reports on *Melilotus*. It was documented that *Melilotus* is used to reduce spasm (2); its coumarinic extract have effects on lymphedema (3) and its polysaccharides have immuno-correcting, anti-anemia and adaptogenic effects (4). Although anti-inflammatory effect of *M. officinalis* was reported (5), exploration on *M. suaveolens Ledeb* on how to play an anti-inflammatory role at molecular level remained limited.

As discovered at present, NF-κB is a family of seven structurally related transcription factors that play a central role in inflammation by controlling gene network expression (6). Cyclooxygenease-2 (COX-2) is the key enzyme regulating the production of prostaglandins,

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the central mediators of inflammation. The expression of COX-2 is induced by several extra cellular signals including pro-inflammatory stimuli. COX-2 can be affected directly at its enzymatic activity by nitric oxide (NO) and inducible nitric oxide synthase (iNOS) (7). NO is recognized as a mediator and regulator of inflammatory responses. It possesses cytotoxic properties that are aimed against pathogenic microbes, but it can also have damaging effects on host tissues. NO plays a significant role in inflammation, where NO is produced in high amounts by iNOS, and then reactive oxygen species are synthesized by activated inflammatory cells (8). Several pro-inflammatory gene products have been identified that mediate a critical role in inflammation. Among these gene products are tumor necrosis factor (TNFα) and members of its superfamily, interleukin-1 beta (IL-1β), interleukin-6 (IL-6), etc. The expression of all these genes is mainly regulated by the transcription factor, nuclear factor-kappa B (NF-κB) (9). Therefore, whether suppressed or not, those pro-inflammatory cytokines and mediators are the key evaluation for novel anti-inflammatory agents.

Interleukin-10 (IL-10) has attached much attention because of its anti-inflammatory properties. Uniquely, among hematopoietic cytokines, IL-10 is a pleiotropic molecule that displays both immunostimulatory and immunoregulatory activities (10). Heme oxygenase-1 (HO-1), involved in the heme degradation process, is an important anti-inflammatory enzyme featured by its anti-oxidant activity (11). Experimental models of various diseases, including acute inflammation, have demonstrated that the induction of HO-1 can prevent or mitigate the symptoms associated with those ailments (12).

High performance liquid chromatography (HPLC) is a method recommended by World Health Organization (WHO) to assay herbal ingredients (13). Extraction by *n*-butanol is one of the most common methods to obtain organic substances that can not dissolve in water (14).

Based on those findings and methods, we at first extracted effective ingredients with *n*-butanol from *M. suaveolens Ledeb* and identified the active components by HPLC fingerprint. Then, we founded a classic inflammation cellular model (15) with RAW 264.7 mouse macrophage cell line stimulated by lipopolysaccharide (LPS). Subsequently, we chose the found inflammation cellular model to explore the anti-inflammatory effect of *n*-butanol extract from *M. suaveolens Ledeb* and clarified the inner anti-inflammatory mechanisms of that medicinal plant.

Methods

Plant Materials

Melilotus suaveolens Ledeb was collected in August 2006 from Long County, Shanxi Province, China and identified by Prof. Ke-Li Chen (School of Pharmacy, Hubei College of Traditional Chinese Medicine) according to *Drug Standard of Ministry of Health of the People's Republic of China* (*Tibetan Medicine*) (1). The plant materials were stored in the plant specimen department, school of pharmacy, Hubei College of Traditional Chinese Medicine.

Preparation of *n*-Butanol extract from *Melilotus suaveolens Ledeb*

Fifty grams air-dried aerial parts of *M. suaveolens Ledeb* were powdered and extracted by 70% ethanol for three times at $85°C$ (3×500 ml, 1.5 h each time). The extract solution was filtered and then combined and concentrated *in vacuo*. Subsequently, the concentrated solution was diluted by deionized water to the concentration of 1 g extracted substances per 1 ml water. Then the 5 ml concentrated liquid was accurately taken into a 100 ml separatory funnel by a 5 ml Mohr measuring pipette and was successively extracted with *n*-butanol to total dry weight of *n*-butanol subfraction 2.425 g. The dry material of *n*-butanol subfraction was re-suspended with deionized water to a concentration of 75 mg/ml for HPLC analysis. Then the solution was diluted by 1640 medium into concentration of $10 \mu g\ ml^{-1}$, $5 \mu g\ ml^{-1}$ and $1 \mu g\ ml^{-1}$ for interfering in inflammation cellular model.

HPLC Fingerprint for Analyzing the Herb Extract

HPLC fingerprint was used to analyze the ingredients of the herb extract (16). Balance (AB204-N, MAX 210 g, d = 0.1 mg, produced by MERRLER TOLEDO Group.) and UV-Detector (8450/HP, Agilent Science of Life and Chemistry Company) were used. The HPLC system consisted of a pump (model DIONEX P680 HPLC Pump, ASI-100 to form a high pressure gradient) with Automated Sample Injector facility, Chromeleon management system and UV–VIS (UVD 170U) model detector. The column was Kromasil C-18 (250×4.6 mm, 10 nm–5 μm, Hanbon Science &.Technology Co., Ltd). Chromatography conditions included: the gradient elution was acetonitrile and 0.05% H3PO4 of 1.0 ml min^{-1} flowing rate; 5 μl capacity per injection was used with UV detector at four kinds of wavelength of 220 nm (for coumarin), 254 ± 2 nm, 275 nm (for coumarin and rutin) and 363 nm (for hyperoside); the concentration of reference of coumarin, rutin and hyperoside was 0.007648 mg ml^{-1}, 0.2548 mg ml^{-1} and 0.2528 mg ml^{-1}, respectively; the column was placed in a column oven set at $25°C$. Then the petroleum ether subfraction was filtered through a 0.45 μm filter membrane (Hanbon Science & Technology Co., Ltd) and stored in the refrigerator before use. The multi-step gradient elution was carried out with acetonitrile and 0.05% H_3PO_4 solution. The procedure was as follows: 0–8 min

(5% acetonitrile, 95% H_3PO_4 solution), 8–25 min (5–30% acetonitrile, 95–70% H_3PO_4 solution), 25–35 min (30% acetonitrile, 70% H_3PO_4 solution), 35–60 min (30–70% acetonitrile, 70–30% H_3PO_4 solution), 60–70 min(30%–5% acetonitrile, 70–95% H_3PO_4 solution), 70–80 min(5% acetonitrile, 95% H_3PO_4 solution).

Chemicals and Reagents

RPMI1640 was from Gibico (Grand Island, NY, USA). LPS (*Escherichia coli* O111:B4) and methyl thiazolyl tetrazolium (MTT) were obtained from Sigma (St Louis, MO, USA). Affinity-purified goat anti-mouse HO-1 antibody was obtained from R&D Systems (Minneapolis, MN, USA). Affinity-purified goat anti-mouse COX-2 antibody and rabbit anti-mouse NF-κB p65 IgG antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse TNF-α, IL-1β and IL-6 ELISA kits were purchased from Quantikine, R&D Systems (Minneapolis, MN, USA). Griess reagent nitric oxide assay kit was from Beyotime Biotech (Jiangsu, P. R. China). Mouse IL-10 ELISA kit was obtained from Bender Medsystem (Vienna, Austria). Trizol was purchased from Gibico (Grand Island, NY, USA). M-MLV Reverse Transcriptase was afforded from Promega (Madison, WI, USA). SYBR GreenI was taken from Biotium (Hayward, CA, USA). The Oligo(dT18) and primers were synthesized by Shanghai Invitrogen (Shanghai, China). The dNTP was obtained from Promega (Madison, WI, USA).

Cell Culture

Murine macrophage cell line RAW264.7 was obtained from China Center for Typical Culture Collection (CCTCC) (Wuhan, China). The cell was maintained in RPMI1640 medium supplemented with 100 U ml^{-1} of penicillin, 100 μg ml^{-1} of streptomycin and 10% fetal bovine serum and was cultured at 37°C and 5% CO_2 in humidified air.

Cellular Model Establishment and Intervention

Twenty four hours prior to LPS treatment, the cells were inoculated into 6, 24 or 96 micro-well plates. Twenty four hours later, when the cells were observed for adherence at the bottom of well, cell supernatants were disposed and 10 ng ml^{-1} LPS with prepared exact solution were added into the well. The stimulation and intervention lasted for different hours and the supernatants and cells were harvested for ELISA, real-time PCR, western-blot and immunocytochemical test.

Control Establishment

Dexamethasone, which is known as a classic gluco-corticosteroid drug widely used in clinical practice, was chosen as positive control with concentration of 0.5 μg ml^{-1}. Astragalus polysaccharides (APS) is an immunomodulator to reinforce immune response on TNF-α, IL-1β and IL-6 (17). According to the literature (17), APS with concentration of 100 μg ml^{-1} was selected as negative control for monitoring the procedure. Cells stimulated by LPS without any intervention were observed as blank control. Cells incubated by 1640 medium were as normal control.

MTT Assay for Measuring Cell Proliferation

Based on the instruction from the American Type Culture Collection (ATCC), cytotoxic effect of the extract was evaluated by MTT assay. At 4 h prior to culture termination, 20 μl MTT solution (5 mg ml^{-1} in a phosphate-buffered saline, pH 7.4) was added. When cell culture was terminated, 150 μl dimethyl sulfoxide was added into each well for solubilization. The optical density at 490 nm was measured by a Spectramax 250 microplate reader.

Cytopathic Effect (CPE) Test for Cell Morphous Observation

After cell model interfered with the extract for 24 h, CPE test was performed for observing cell morphous.

Detection of TNF-α, IL-1β and IL-6 in Supernatant

Inhibitory effects of 10 μg ml^{-1}, 5 μg ml^{-1} and 1 μg ml^{-1} extract on the cytokine TNF-α, IL-1β and IL-6 production from LPS-treated RAW264.7 cells were determined by sandwich ELISA. After stimulation and intervention on RAW264.7 cells for 24 h, supernatant was harvested and assayed for TNF-α, IL-1β and IL-6 by respective ELISA kits. The procedure obeyed to instructions from related kits. Results of three independent experiments were used for statistical analysis.

Analysis of Nitric Oxide

Levels of the NO derivative nitrite were determined with the Griess reaction. The nitrite detection kit was used according to instructions provided by the manufacturer. The samples were assayed in triplicate, and a standard curve using $NaNO_2$ was generated for each experiment for quantification. 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, optical density was read in a microplate reader at 540 nm.

Real-time PCR for Detecting mRNA of TNF-α, COX-2, iNOS and HO-1

The total RNA from stimulated and interfered cell model was prepared by adding TRIzol Reagent according

to manufacturer's protocol. The RNA on TNF-α, inducible iNOS and COX-2 was extracted at 4 h after stimulation and intervention and the RNA on HO-1 was obtained at 18 h after stimulation and intervention. Quantitative PCR was performed in ABI-7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). The reverse transcription was performed with M-MLV Reverse Transcriptase. The reverse transcription reaction system included: 5.5 μl H_2O, 1.0 μl Oligo(dT18) (50 μg ml^{-1}), total RNA 6.0 μl, 70°C 5 min to ice for unfolding secondary structure of mRNA; 0.5 μl RNasin (40 U μl^{-1}), 4.0 μl 5 × buffer, 2.0 μl dNTP (10 mM), 1.0 μl RTase (200 U μl^{-1}), 42°C 60 min to 95°C 5 min to 4°C at the end. Real time PCR reaction was performed with SYBR GreenI fluorochrome. The standard curve of each sample was obtained and cycle threshold (Ct) value was calculated. Each 50 μl PCR system contained 1/50 of the original cDNA synthesis reaction, 7 μl (25 mM) $MgCl_2$, 0.8 μl (20 pmol μl^{-1}) of each primer, 1 μl (10 mM) dNTP, 1 μl SYBR GreenI, 0.5 μl (5 U μl^{-1}) Taq and 5 μl 10 × Buffer. Fifty cycles of amplification were performed: after 94°C 3 min, reaction cycle with 94°C, 30 min, to 57°C, 30 s, then to 72°C, 30 s was carried out for 50 times. The fluorescence signal was detected at the end of each cycle. Melting curve analysis was used to confirm the specificity of the products. The $2^{-\Delta\Delta CT}$ method was performed to analyze the results (18). The primer was as below:

Mus-COX-2:
  Forward: 5′-GAAGTCTTTGGTCTGGTGCCTG-3′,
  Reverse: 5′-GTCTGCTGGTTTGGAATAGTTGC-3′;

Mus-iNos:
  Forward: 5′-GGAGCGAGTTGTGGATTGTC-3′,
  Reverse: 5′-GTGAGGGCTTGGCTGAGTGAG-3′;

Mus-TNF-α:
  Forward: 5′-GTGGAACTGGCAGAAGAGGC-3′,
  Reverse: 5′-AGACAGAAGAGCGTGGTGGC-3′;

Mus-HO-1:
  Forward: 5′-CACAGATGGCGTCACTTCGTC-3′,
  Reverse: 5′-GTGAGGACCCACTGGAGGAG-3′.

Mus-β-actin:
  Forward: 5′-GCTACAGCTTCACCACCACAG-3′,
  Reverse: 5′-GGTCTTTACGGATGTCAACGTC-3′.

Western Blot Analysis of COX-2 and HO-1

The treated cells were harvested and incubated on ice for 15 min in a lysis buffer of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 100 mg ml^{-1} phenyl methylsulfonyl fluoride, 1 mg ml^{-1} aprotinin and 1% Triton X-100. Cell debris was removed by centrifugation at 10 000 r.p.m. and 4°C for 10 min. The protein concentration of each cell lysate was determined with a Bio-Rad (Hercules, CA, USA) protein assay kit. To each tube, an equivalent volume of 2 × sodium dodecyl sulfate (SDS) loading buffer (100 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerine, 10% b-mercaptoethanol and 0.2% bromophenol blue) was added and mixed again. The mixtures were then denatured at 95°C for 10 min, and about 10 mg of the protein mixture was loaded and separated in each well on 10% SDS-polyacrylamide electrophoresis gels. After separation for about 80 min, the proteins were transblotted onto nitrocellulose membranes (Bio-Rad), and the membranes were saturated and blocked with 5% fat-free milk at 37°C for 1 h. Membranes were probed with goat polyclonal anti-COX-2, anti-HO-1 (1:6000) and then with horse-radish peroxidase-conjugated secondary immunoglobulin G (IgG, Kangcheng, Shanghai, P.R. China). The membranes were then treated with an enhanced chemiluminescence reagent (Amersham, Piscataway, NJ, USA), and the signals were detected by exposure of the membranes to X-ray films (Kodak, Rochester, NY, USA). The relative signal intensity was quantified by densitometry with Gel pro3.0 image software (Media Cybernetics, Silverspring, MD, USA) for an IBM-compatible personal computer. All experiments were performed three times independently.

Immunocytochemistry Assay for NF-κB

SP immunocytochemical assay was employed to detect expression of the nuclear translocation of NF-κB. Coverslips were soaked in polylysine for whole night. After cell crawling to the coverslips and subsequent LPS stimulation and extract intervention, the cells were fixed by acetone. Then the slides with cells were soaked in 3% H_2O_2–methanol solution for 20 min, in order to block endogenous peroxydase. Next, 1% Triton X-100 was added at 37°C for 5 min, followed by PBS washing. After incubated with normal goat serum at room temperature for 20 min, rabbit anti-mouse NF-κB p65 IgG antibody was drop wise and the slides were stored at 4°C for whole night. Next day following PBS washing, biotin-conjugated goat anti-rabbit IgG was added drop wise and the incubation lasted 30 min at 37°C. Then with PBS washing again, streptavidin-HRP was added and incubated with cells for 30 min at 37°C. Subsequently, with PBS washing for 5 min, three times, DAB coloration was performed. Following normal dehydration, lucidification and mounting, the slides were pictured.

Statistical Analysis

Data were presented as means ± SD of three separate experiments. Comparisons between multiple groups were performed with one-way ANOVA test. Statistical significance was considered significant when $P < 0.05$.

A: at 220nm wavelength;

B: at 254nm wavelength;

C: at 275nm wavelength;

D: at 363nm wavelength.
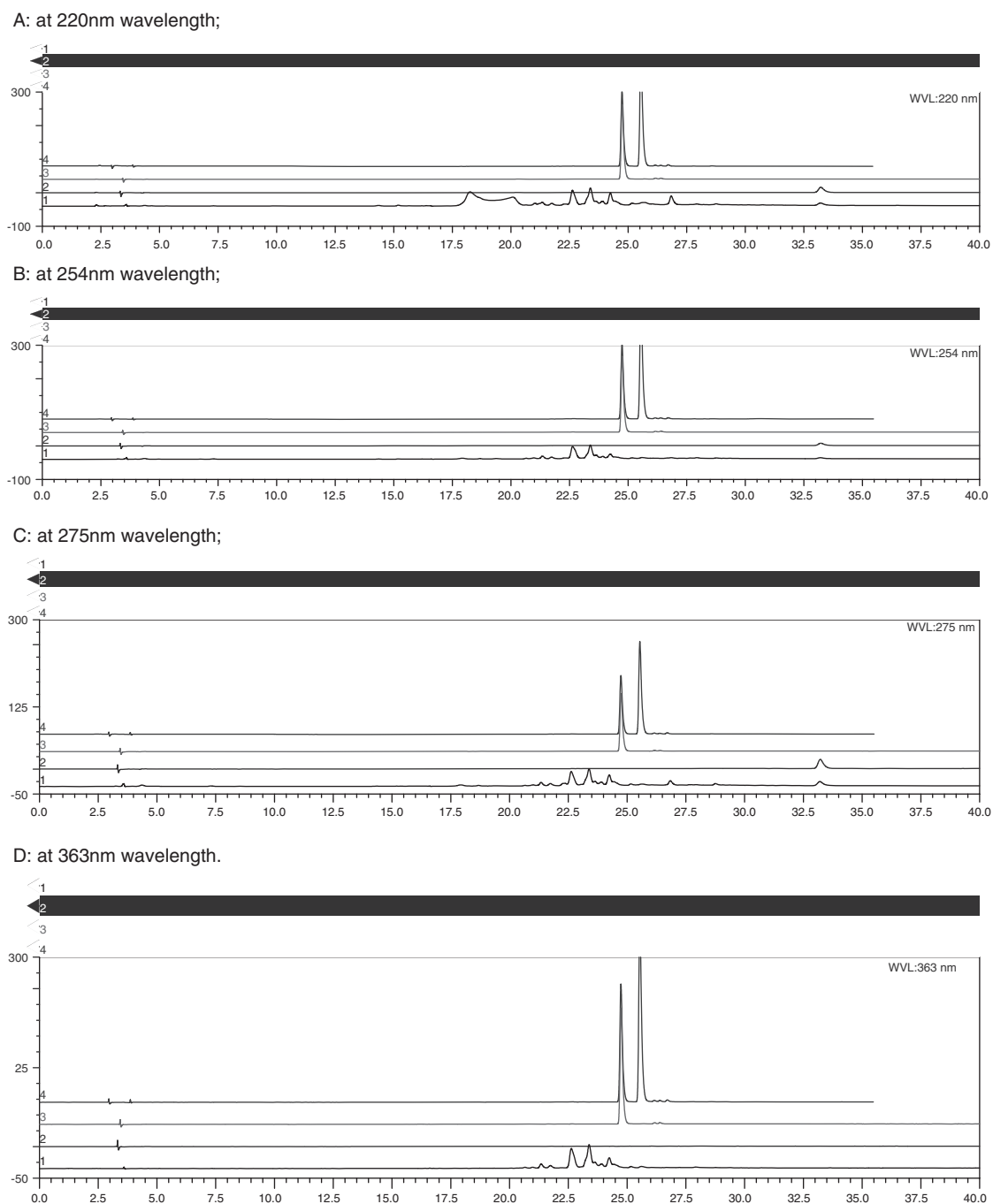
Figure 1. HPLC fingerprint of the *n*-butanol extract from *M. suaveolens Ledeb*: line 1 represents *n*-butanol solution; line 2 represents standard substances of coumarin; line 3 represents rutin and line 4 represents rutin and hyperoside.

Results

HPLC Fingerprint of *n*-butanol Extract from *Melilotus suaveolens Ledeb*

As coumarin and hyperoside had been reported in *Melilotus* and coumarin was isolated from *M. suaveolens Ledeb* in our chemistry study, coumarin and hyperoside were taken as standard substance to perform fingerprint assay. Rutin is an anti-inflammatory flavone derivative commonly existing in many medical plants (19), so we also select rutin as standard substance in ingredient analysis test. As shown in Fig. 1, according to the peaks of HPLC fingerprint, it could be inferred that there was no rutin and hyperoside in the extract. There was 0.023531 mg coumarin in each 1 g extract. The coincidence of peaks of coumarin and ingredient in the extract at 275 nm showed that the coumarin was one of the main components. At 275 nm, there were
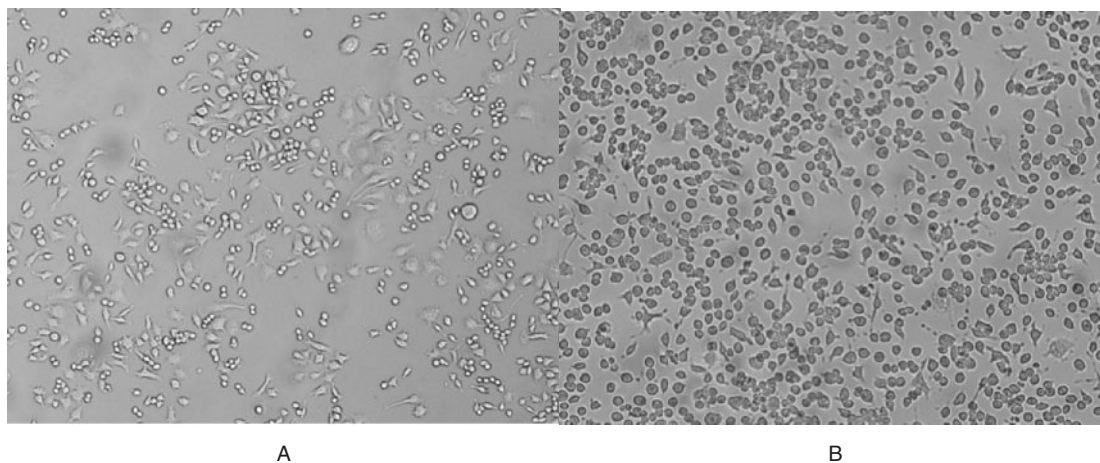
Figure 2. (A) The cells before intervention of *n*-butanol extract from *M. suaveolens Ledeb*; (B) the cells after intervention of $1\,\mu g\,ml^{-1}$ *n*-butanol extract from *M. suaveolens Ledeb* for 24 h.

another two peaks emerging before coumarin peak, which could infer that there were another two substances in the extract. The polarity of the two substances was higher than that of rutin, hyperoside and coumarin and according to the polar region in HPLC, they might be saponins.

In vitro Cytotoxicity of *n*-Butanol Extract from *Melilotus suaveolens Ledeb*

Based on MTT assay, it showed that pre-treatment on unstimulated RAW264.7 cells with prepared solution of *n*-butanol extract from *Melilotus suaveolens Ledeb* (concentration mentioned earlier) for 24 h did not significantly affect cell viability (data not shown). CPE test presented the same result (Fig. 2).

Inflammatory Model Establishment and Procedure Monitoring

As shown from figures on pro-inflammatory cytokines and mediators, the levels of those factors of cells stimulated by LPS were significantly higher than those of normal cells ($P < 0.01$), which implied the successful establishment of model of inflammation. Meanwhile, the levels of those mediators of cells by Dexamethasone intervention was significantly lower than those of single LPS stimulation ($P < 0.01$). The levels of TNF-α, IL-1β and IL-6 of cells by APS intervention were significantly higher than those of single LPS stimulation ($P < 0.05$ or 0.01). The effects of Dexamethasone and APS intervention demonstrated the experimental procedure was proper.

n-Butanol Extract from *Melilotus Suaveolens Ledeb* on Pro-inflammatory Cytokines Release

As shown in Fig. 3A–C, after LPS stimulation with the extract intervention for 24 h, secretion of TNF-α, IL-1β and IL-6 were significantly decreased than that in single LPS stimulation ($P < 0.01$). Furthermore, it could be observed that the more the dosage of the extract increased, the better the effects of antagonizing pro-inflammatory cytokines would be ($P < 0.01$ at 10 times concentration).

Protein of COX-2 expression

The extract displayed strikingly decreased level of COX-2 protein as shown in Fig. 4 ($P < 0.01$). It was suggested that *n*-butanol extract from *M. suaveolens Ledeb* could control pro-inflammatory mediator production at protein levels.

Gene Expression of Pro-inflammatory Mediators

The extract displayed strong inhibition effect on expression of TNF-α and COX-2 mRNA as shown in Fig. 5A–B ($P < 0.01$ or 0.05). Furthermore, the more the dosage of the extract increased, the better the effects of antagonizing pro-inflammatory gene expression would be ($P < 0.01$ at 10 times concentration). It suggested that *n*-butanol extract from *M. suaveolens Ledeb* might control pro-inflammatory cytokine production at gene levels.

NO Release and iNOS Expression

As shown in Fig. 3D, after LPS stimulation with *n*-butanol extract from *M. suaveolens Ledeb* intervention, secretion of NO was significantly decreased than that by single LPS stimulation ($P < 0.01$). Furthermore, the more the dosage of the extract increased, the better the effects of antagonizing NO releasing would be ($P < 0.01$ at 10 times concentration). Also, as shown in Fig. 5C, the effect of *n*-butanol extract from *M. suaveolens Ledeb* on mRNA expression of iNOS was coinciding with that of NO ($P < 0.01$).
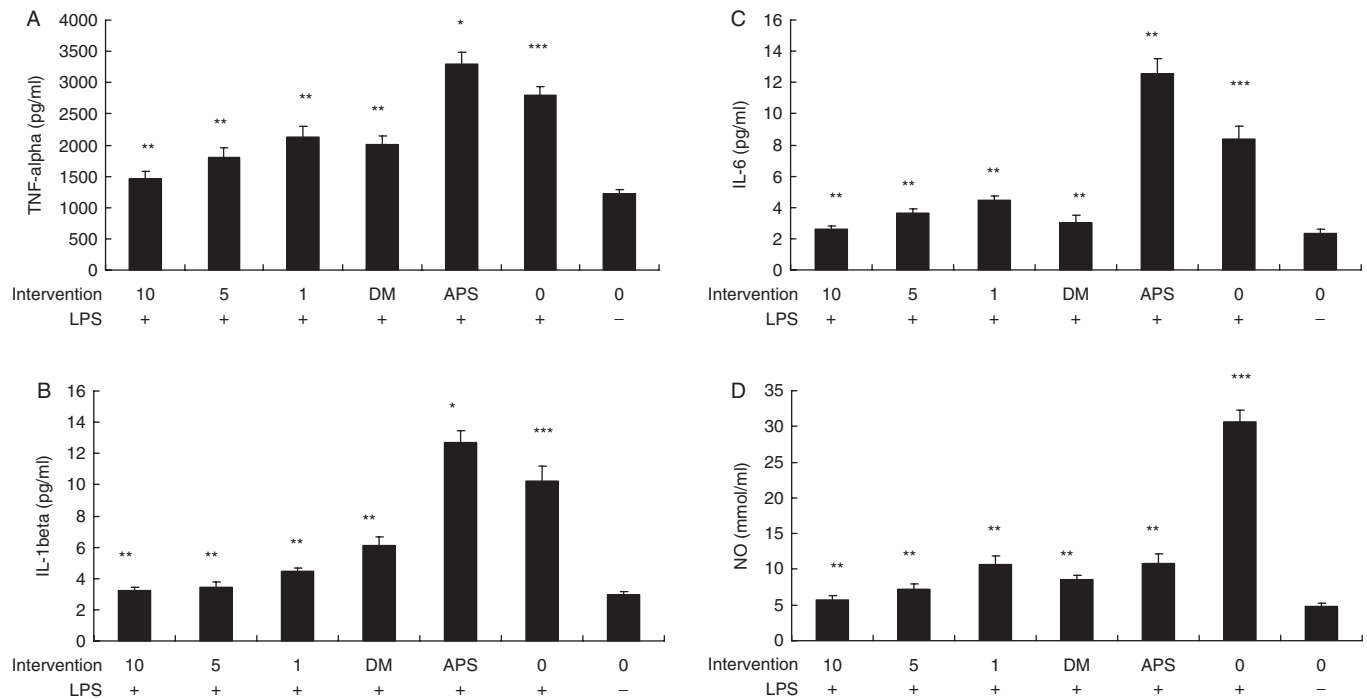
Figure 3. Effects of *n*-butanol extract from *M. suaveolens Ledeb* on production of pro-inflammatory cytokines and mediators. RAW264.7 cells were treated with LPS (10 ng/ml) in the presence of different concentrations of *n*-butanol extract from *M. suaveolens Ledeb* for 24 h. Cytokine or mediator levels were measured by ELISA or Griess method. Data were shown as mean ± SD (*n* = 3). *$P < 0.05$ compared to LPS alone; **$P < 0.01$ compared to LPS alone; ***$P < 0.01$ compared to normal cell. (**A**) Effect on TNF-α production; (**B**) effect on IL-1β production; (**C**) effect on IL-6 production and (**D**) effect on NO production.
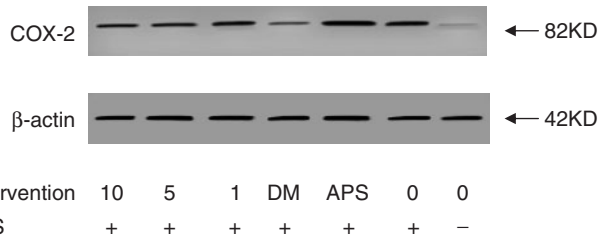
Figure 4. Effects of *n*-butanol extract from *M. suaveolens Ledeb* on COX-2 protein expression assayed by western blot analysis. RAW264.7 cells were treated with LPS (10 ng ml^{-1}) in the presence of different concentrations of *n*-butanol extract from *M. suaveolens Ledeb* for 24 h.

IL-10 Release

As shown in Fig. 6A, the level of IL-10 decreased the most under APS condition, which illustrated the effect of APS on reinforcing cellular immunity and inhibiting humoral immunity. Increase of IL-10 on single LPS stimulation displayed the regulatory action on cells after inflammatory reaction. The levels of IL-10 interfered by the extracts were similar to that by Dexamethasone.

HO-1 mRNA and Protein Expression

As shown in Fig. 6B and Fig. 7, the levels of HO-1 mRNA and protein of single LPS stimulated cells and Dexamethasone interfered cells were not different to the

levels of normal incubated cell. The levels of HO-1 mRNA and protein by the extracts and APS intervention were significantly higher than those by single LPS stimulation and normal incubation ($P < 0.01$), which suggested that *n*-butanol extract from *M. suaveolens Ledeb* could promote regression of inflammation. Furthermore, the higher concentration of the extract, the stronger expression of HO-1 mRNA and protein ($P < 0.01$ at 10 times concentration), which showed dose-dependent relation between effect of promoting anti-inflammatory mediators and concentration of *n*-butanol extract from *M. suaveolens Ledeb*.

Inhibition of NF-κB

The results of immunocytochemistry in Fig. 8 showed NF-κB activation was significantly blocked by *n*-butanol extract from *M. suaveolens Ledeb* ($P < 0.01$). It could be inferred that suppression of IL-1β, TNF-α, iNOS and COX-2 expression by *n*-butanol extract from *M. suaveolens Ledeb* might be due to the blocking of NF-κB activation.
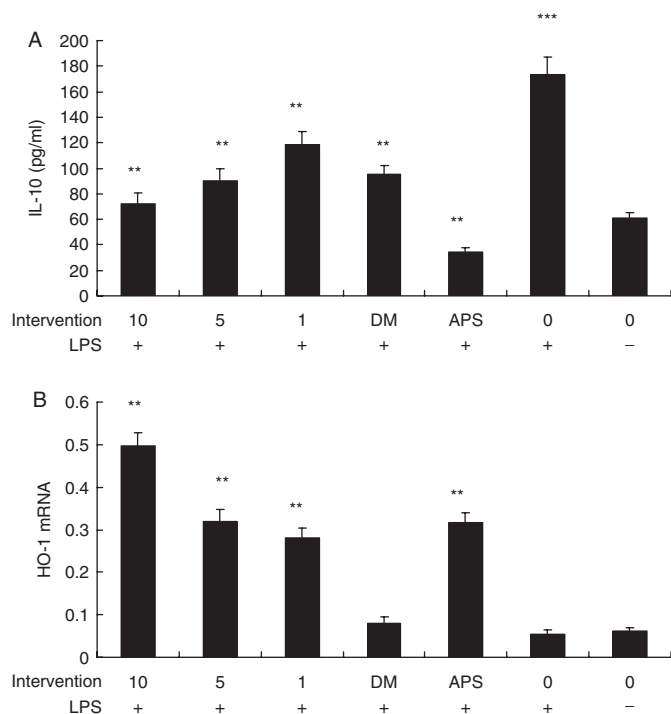
Discussion

Over the past, it has been confirmed that *Melilotus* extract has anti-inflammatory properties similar to those
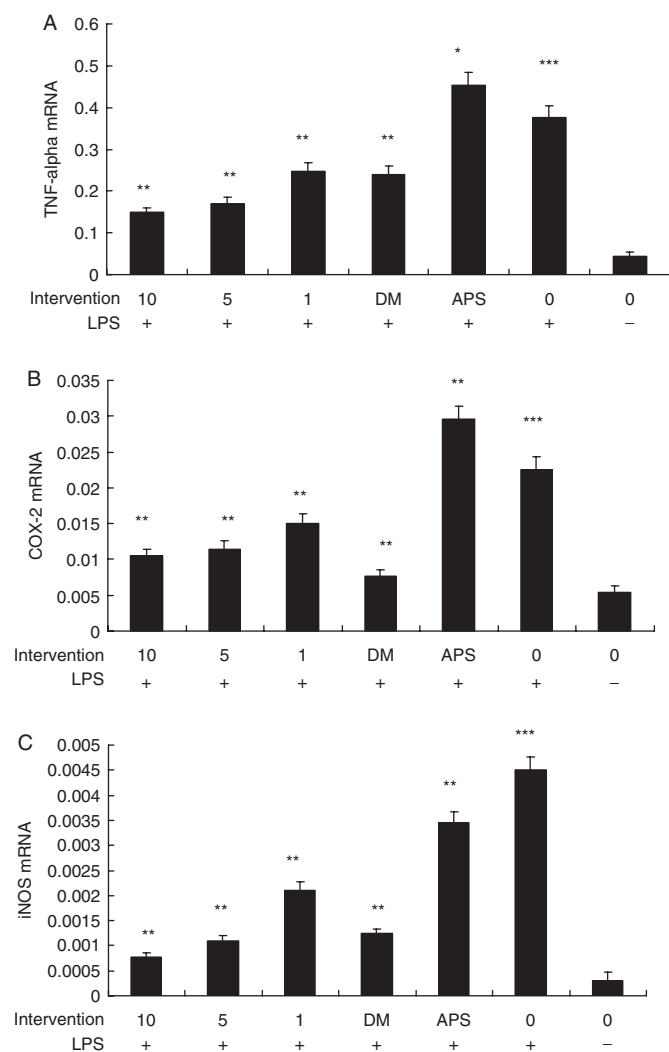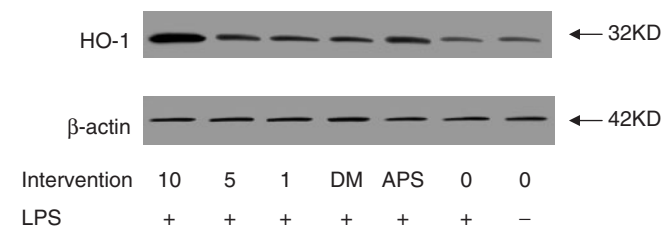
Figure 5. Effects of *n*-butanol extract from *M. suaveolens Ledeb* on mRNA expression of pro-inflammatory cytokines and mediators. RAW264.7 cells were treated with LPS (10 ng ml^{-1}) in the presence of different concentrations of *n*-butanol extract from *M. suaveolens Ledeb* for 4 h. The mRNA levels were measured by real-time quantitative PCR. Data were shown as mean ± SD ($n = 3$). *$P < 0.05$ compared to LPS alone; **$P < 0.01$ compared to LPS alone; ***$P < 0.01$ compared to normal cell. (**A**) Effect on TNF-α mRNA expression; (**B**) effect on COX-2 mRNA expression and (**C**) effect on iNOS mRNA expression.

Figure 6. Effects of *n*-butanol extract from *M. suaveolens Ledeb* on expression of anti-inflammatory cytokines and mediators. RAW264.7 cells were treated with LPS (10 ng ml^{-1}) in the presence of different concentrations of *n*-butanol extract from *M. suaveolens Ledeb* for 24 h (IL-10) or 18 h (HO-1 mRNA). The IL-10 levels were measured by ELISA and HO-1 mRNA levels were measured by real-time quantitative PCR. Data were shown as mean ± SD ($n = 3$). *$P < 0.05$ compared to LPS alone; **$P < 0.01$ compared to LPS alone; ***$P < 0.01$ compared to normal cell. (**A**) Effect on IL-10 production and (**B**) effect on HO-1 mRNA expression.
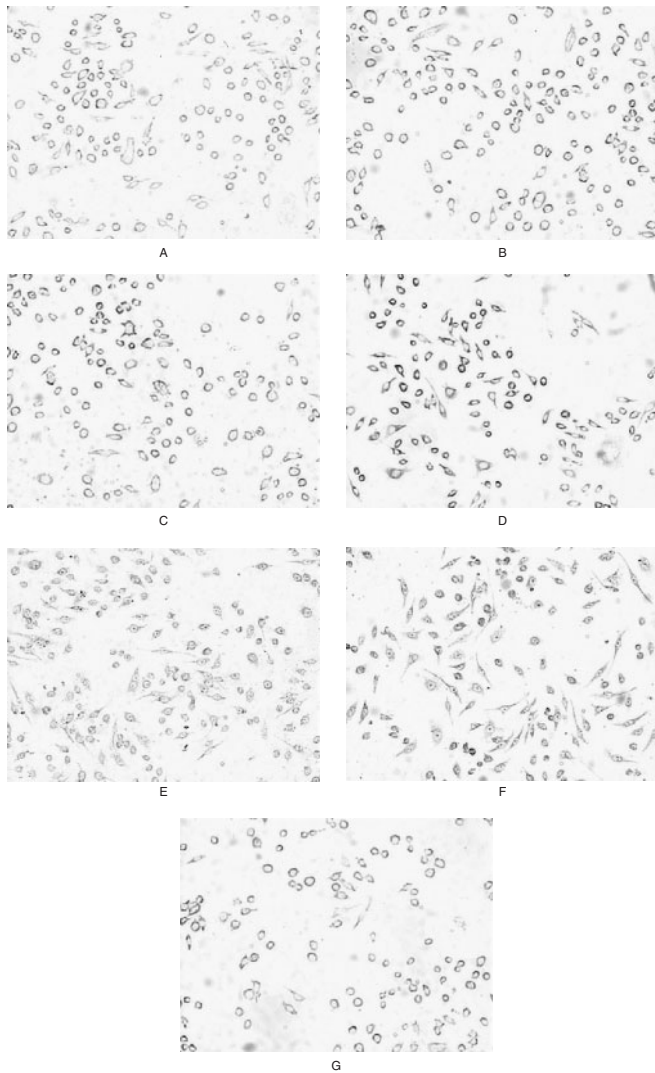

Figure 7. Effects of *n*-butanol extract from *M. suaveolens Ledeb* on HO-1 protein expression assayed by western blot analysis. RAW264.7 cells were treated with LPS (10 ng ml^{-1}) in the presence of different concentrations of *n*-butanol extract from *M. suaveolens Ledeb* for 24 h.

of hydrocortisone sodium hemisuccinate and coumarin, and in bone marrow acute phase response, *Melilotus* has an inhibitory action that is lower than that of hydrocortisone sodium hemisuccinate and similar to coumarin (5). However, *Melilotus* on anti-inflammation has not been well explored in detail so far, and anti-inflammatory effect on *n*-butanol extract from *M. suaveolens Ledeb* was not investigated in literature.

As many active ingredients in a medicinal plant are organic, and the contents of *M. officinalis* were preliminarily studied in the past, we chose *n*-butanol extraction to simplify the components of *M. suaveolens Ledeb*. HPLC fingerprint of the herbal extract showed

that the main content of *n*-butanol extract from *M. suaveolens Ledeb* was coumarin. Although it was reported that hyperoside was also an effective ingredient of *Melilotus* on anti-inflammation, there was no hyperoside detected in the *n*-butanol extract from *M. suaveolens Ledeb*. In addition, another two ingredients with higher polarity than that of rutin, hyperoside and coumarin were discovered. It is our next investigation, whether and how the ingredients play an interaction role with coumarin for anti-inflammation in the extract.

TNF-α, IL-1β, IL-6 are classic pro-inflammatory cytokines, which rise in most inflammatory stimulations.

Figure 8. Effects of *n*-butanol extract from *M. suaveolens Ledeb* on NF-κB translocation. RAW264.7 cells were treated with LPS ($10\,\mathrm{ng\ ml^{-1}}$) in the presence of different concentrations of the extract for 2 h. Cell nucleus stained to brown presented NF-κB activation and cytoplasm stained to brown presented NF-κB inactivation. (**A**) Extract plus LPS of $10\,\mathrm{\mu g\ ml^{-1}}$; (**B**) $5\,\mathrm{\mu g\ ml^{-1}}$ extract plus LPS; (**C**): $1\,\mathrm{\mu g\ ml^{-1}}$ extract plus LPS; (**D**) Dexamethasone plus LPS; (**E**) APS plus LPS; (**F**) simple LPS and (**G**) normal medium.

In our research, we examined the effect of *n*-butanol extract from *M. suaveolens Ledeb* on those three cytokines and the inhibiting effect inferred the anti-inflammatory properties of that medicinal plant.

Subsequently, we detected the intra-cellular inflammatory regulatory factors, in order to make clear the anti-inflammatory mechanism of *n*-butanol extract from *M. suaveolens Ledeb*. As COX-2 is a key protein to regulate evoke of inflammation, we examined protein expression of COX-2. Then, we further assayed the mRNA expression of COX-2 and the most important cytokine, TNF-α, to ascertain the influence of the extract at gene level.

NO is a multi-function mediator to deliver signals in many reactions. Inflammation is one of the most occasions for NO to play a central role. It has been affirmed that including COX-2, many pro-inflammatory cytokines and proteins are regulated or influenced by NO and on inflammatory condition both NO and its enzyme, iNOS evaluate. Thereby, we chose NO and iNOS as an index to measure the anti-inflammatory effect.

NF-κB is a central factor to control nuclear transcriptional signals by which many cellular functions, including inflammation, are activated. After detecting the main inflammatory cytokines and mediators, we explored the conditions of nuclear transcription by assaying whether NF-κB was activated.

In this research, the pathway of activating inflammation was examined carefully, and it could be proved that *n*-butanol extract from *M. suaveolens Ledeb* has suppressive effects on inflammation. Furthermore, we compared the anti-inflammatory effect between *n*-butanol extract from *M. suaveolens Ledeb* and Dexamethasone. It showed that *n*-butanol extract from *M. suaveolens Ledeb* has a similar efficacy on antagonizing the producing, releasing and activating of pro-inflammatory mediators, cytokines and NF-κB.

Apart from pro-inflammatory factors, we also detected effect of *n*-butanol extract from *M. suaveolens Ledeb* on anti-inflammatory factors. IL-10 is a typical anti-inflammatory cytokine after inflammatory stimulation that is capable of inhibiting synthesis of pro-inflammatory cytokines like IF-γ, TNF-α and GM-CSF made by cells such as macrophages and the Type 1 T helper cells. HO-1 is an inducible enzyme in response to suppress stress such as inflammation, oxidative stress, hypoxia, etc. Both the factors are considered as important roles on anti-inflammation. As reported, Dexamethasone can inhibit the release of IL-10 at high dosage (20), while *n*-butanol extract from *M. suaveolens Ledeb* had a similar effect on IL-10 in our research. However, *n*-butanol extract from *M. suaveolens Ledeb* had an ability on inducing production of HO-1 but Dexamethesone not, which showed the difference between the extract and Dexamethasone. This feature might be a possible application of *M. suaveolens Ledeb* in clinical practice in the future.

Although *M. suaveolens Ledeb* has been reported for its anti-inflammation properties in clinical practice, the inner molecular biological mechanisms have not been well investigated. The effect of *n*-butanol extract from *M. suaveolens Ledeb* on pro-inflammatory cytokines and mediators, on blocking translocation of NF-κB, on anti-inflammatory mediators and on comparison to Dexamethesone are first explored. Additionally, two high polar ingredients in *M. suaveolens Ledeb* are discovered in the extract. Based on our past study on herbal research (21,22) and recent advance on herbal medicine (23,24), the next exploration is on analyzing more activities of the

ingredients in *M. suaveolens Ledeb* and further application of this medicinal plant product on certain inflammation-related disease, such as encephalitis, hepatopathy and systemic inflammatory response syndrome.

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