Original Article

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

Lei Zhao¹, Jun-Yan Tao², Shu-Ling Zhang¹, Feng Jin³, Ran Pang¹ and Ji-Hua Dong⁴

¹Department of Hepatology & Infectious Disease, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, ²College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, 430072, ³Department of Neurosurgery and ⁴Central Lab, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430022, P.R. China

*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**


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 immunocorrecting, 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). Cyclooxygenase-2 (COX-2) is the key enzyme regulating the production of prostaglandins.
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 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). Fifty grams air-dried aerial parts of M. suaveolens Ledeb were powdered and extracted by 70% ethanol for three times at 85°C (3 x 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 μg ml⁻¹, 5 μg ml⁻¹ and 1 μg 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, Chromelone management system and UV–VIS (UVD 170U) model detector. The column was Kromasil C-18 (250 x 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% H₃PO₄ solution. The procedure was as follows: 0–8 min

**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 x 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 μg ml⁻¹, 5 μg ml⁻¹ and 1 μg ml⁻¹ for interfering in inflammation cellular model.
bovine serum and was cultured at 37\degree C and 10 ng ml\(^{-1}\) at the bottom of well, cell supernatants were disposed 24 hours later, when the cells were observed for adherence from China Center for Typical Culture Collection (ATCC), cytotoxic effect of the extract was evaluated by MTT assay. At 4 h prior to culture termination, 20\(\mu\)l MTT solution (5 mg ml\(^{-1}\) in a phosphate-buffered saline, pH 7.4) was added. When cell culture was terminated, 150\(\mu\)l dimethyl sulfoxide was added into each well for solubilization. The optical density at 490 nm was measured by a Spectramax 250 microplate reader.

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\(\mu\)l MTT solution (5 mg ml\(^{-1}\) in a phosphate-buffered saline, pH 7.4) was added. When cell culture was terminated, 150\(\mu\)l 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-\(\alpha\), IL-1\(\beta\) and IL-6 in Supernatant

Inhibitory effects of 10\(\mu\)g ml\(^{-1}\), 5\(\mu\)g ml\(^{-1}\) and 1\(\mu\)g ml\(^{-1}\) extract on the cytokine TNF-\(\alpha\), IL-1\(\beta\) 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-\(\alpha\), IL-1\(\beta\) 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\(\mu\)l of medium or standard NaNO\(_2\) was mixed with 100\(\mu\)l 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-\(\alpha\), COX-2, iNOS and HO-1

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

**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\(\mu\)g ml\(^{-1}\) of streptomycin and 10% fetal bovine serum and was cultured at 37\degree 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 glucocorticosteroid drug widely used in clinical practice,
Mus-COX-2: primer was as below: the method was performed to analyze the results (18). The reverse transcription reaction system included: 5.5 μl H2O, 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) MgCl2, 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⁻ΔΔCT method was performed to analyze the results (18). The primer was as below:

Mus-COX-2:
- Forward: 5’-GAAGTCTTTGCTGGTGCGCTG-3’
- Reverse: 5’-GTCTGCTGGTTTGGAAATATTG-3’

Mus-iNos:
- Forward: 5’-GGAGCGAGTTGGATGGAGTC-3’
- Reverse: 5’-GTAGAGGGGTTCCTGAGTG-3’

Mus-TNF-α:
- Forward: 5’-GTTGGAACGTGGCAAGAGGC-3’
- Reverse: 5’-AGACAGAGAGCGTGTTGGG-3’

Mus-HO-1:
- Forward: 5’-CACAGATGGCCGTACCTCGTC-3’
- Reverse: 5’-GTGAGGCCACTGGAGGAG-3’

Mus-β-actin:
- Forward: 5’-GCTACAGCTTCAACCACACAG-3’
- Reverse: 5’-GGTCTTTTACGGATGTCAACGTC-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% glycine, 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 transferred 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% H2O2–methanol solution for 20 min, in order to block endogenous peroxidase. 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-horseradish peroxidase 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.
Results

HPLC Fingerprint of \(n\)-butanolExtract 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 substances 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

![HPLC fingerprint of the \(n\)-butanol extract from *Melilotus suaveolens* Ledeb](image-url)
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 24h 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 24h, 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).
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.

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.

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
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 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.
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-\( \alpha \), 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-\( \kappa B \) 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-\( \kappa B \) 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-\( \kappa B \).

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-\( \gamma \), TNF-\( \alpha \) 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 Dexamethasone 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-\( \kappa B \), on anti-inflammatory mediators and on comparison to Dexamethasone 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

Figure 8. Effects of \( n \)-butanol extract from \( M. \) suaveolens Ledeb on NF-\( \kappa B \) translocation. RAW264.7 cells were treated with LPS (10 ng ml\(^{-1}\)) in the presence of different concentrations of the extract for 2 h. Cell nucleus stained to brown presented NF-\( \kappa B \) activation and cytoplasm stained to brown presented NF-\( \kappa B \) inactivation. (A) Extract plus LPS of 10 \( \mu \)g ml\(^{-1}\); (B) 5 \( \mu \)g ml\(^{-1}\) extract plus LPS; (C) 1 \( \mu \)g ml\(^{-1}\) extract plus LPS; (D) Dexamethasone plus LPS; (E) APS plus LPS; (F) simple LPS and (G) normal medium.
ingredients in *M. suaveolens* Ledeb and further application of this medicinal plant product on certain inflammation-related disease, such as encephalitis, hepato-pathy and systemic inflammatory response syndrome.

References

Submit your manuscripts at http://www.hindawi.com