Natural Product Sciences
16(4) : 259-264 (2010)

Effects of the Essential Oil Components from *Ligusticum chuanxiong* on Proinflammatory Mediators of RAW264.7 Macrophage Cells

Lim Hyerim and Seungwon Shin*

College of Pharmacy, Duksung Women's University, 132-714, Korea

Abstract – The essential oil fraction was obtained from the underground parts o of *Ligusticum chuanxiong* (Umbelliferae) by steam distillation, and its main components, *Z*-ligustilide and butylidene phthalide, were isolated by column chromatography. Its essential oil fraction and the isolated main components were examined for effects on their anti-inflammatory properties in RAW 264.7 macrophage cells to develop a new natural anti-inflammatory drug. The results showed that the *L. chuanxiong* essential oil fraction and its main components, *Z*-ligustilide and butylidene phthalide, inhibited the production of nitric oxide significantly in lipopolysaccharide (LPS)-treated RAW 264.7 cells. LPS-induced interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) production was also decreased in a dose-dependent manner. In addition, western blot analysis revealed that the *L. chuanxiong* essential oil fraction and also its main components, *Z*-ligustilide, and butylidene phthalide reduced the expression levels of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS).

Keywords – *Ligusticum chuanxiong,* essential oil, ligustilide, butylidene phthalide, anti-inflammatory effect, nitric oxide, IL-1β, IL-6, TNF-α, COX-2, iNOS

Introduction

Ligusticum chuanxiong Hort. (Umbelliferae) is a perennial herb cultivated mainly in Korea and China. The plant is one of the main sources for Cnidii Rhizoma, a traditional drug used for the treatment of headaches, abdominal pain, and general gynecological diseases including menstrual disorders, contusion, as well as many other ailments. The essential oil of *L. chuanxiong* is an important component for the clinical effects of this medicine (Su *et al.,* 2008; Sim and Shin, 2010) It has been reported to exhibit cardiovascular, antiplatelet, anti-inflammatory, antimicrobial, antioxidant, and insecticidal effects (Packer *et al.,* 2004; Liu *et al.,* 2005; Zhang *et al.,* 2007; Sim and Shin, 2008; Jeong *et al.,* 2009; Wang *et al.,* 2010).

In this study, the essential oil fraction was obtained from the underground parts of *L. chuanxiong* by steam distillation, which was then fractionated by column chromatography to afford its main components, *Z*-ligustilide and butylidene phthalide. The effects of these components on mediators of inflammation were assayed to evaluate their potential as a source of clinically useful anti-inflammatory agents.

Nitric oxide (NO) and various cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), are present in macrophages and function as mediators of inflammatory processes (Won *et al.,* 2005; Tewtrakul *et al.*, 2009; Zhao *et al.*, 2009). Nitric oxide synthase (NOS) and cyclooxygenase (COX) catalyze the formation of NO and prostaglandins (PG), respectively. Especially, COX-2 and iNOS (inducible NOS) are induced by cytokines or other immune stimulating factors, including lipopolysaccharide (LPS).

To investigate the anti-inflammatory properties of *L. chuanxiong* oil and its main components, we examined their effects on the survival and immune status of RAW 264.7 murine macrophage cells. Cell viability was determined by an MTT assay after treatment with various concentrations of the compounds. Inhibition of NO production in cells treated with LPS was evaluated by reaction with Griess reagent. The effects of the *L. chuanxiong* essential oil fraction, *Z*-ligustilide, and butylidene phthalide on immune stimulation were examined and compared using multiple assays. Levels of TNF-α, IL-6, and IL-1β released from the cells were measured by ELISA, and the enzyme expression levels of iNOS and COX-2 were investigated by Western blot analysis.

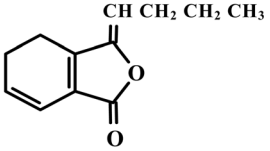
*Author for correspondence
Tel: +82-2-901-8384; E-mail: swshin@duksung.ac.kr

Experimental

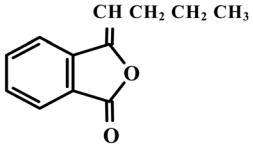
Reagents – Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin, streptomycin, fetal bovine serum (FBS), Tris-glycine-PAG precast gel containing 10% SDS, assay kits for cytokines, and secondary antibodies were purchased from Komabiotech (Seoul, Korea). Anti-COX-2, anti-iNOS, and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). LPS was purchased from Sigma-Aldrich Co. (St. Louis, MO).

Extraction and analysis of the essential oil fraction by gas chromatography and mass spectrometry – The essential oil fraction was obtained from the underground parts of *L. chuanxiong*, cultivated in Bongwha, Korea, by steam distillation for 5 h in a simultaneous steam distillation-extraction apparatus. The plant was identified by comparing its morphology with that of the authentic sample growing in the medicinal plant garden of the Korea Forest Service. A voucher specimen was deposited at the herbarium of Duksung Women's University (No. UMBLC2). The composition of the essential oil fraction was analyzed according to a previously described method (Sim and Shin, 2008).

Isolation of the main components of the *L. chuanxiong* essential oil fraction – The essential oil (20 mL) of *L. chuanxiong* was initially fractionated by silica gel column chromatography with hexane-dichloromethane (90:10). Fractions 2 - 8 were re-subjected to chromatography with hexane-ethyl acetate (95 : 5), yielding 65 mg of butylidene phthalide (98.7%) and 874 mg of *Z*-ligustilide (99.5%). The chemical structures were elucidated using UV, MS, ^{1}H-NMR, and ^{13}C-NMR and confirmed by comparison with previously reported spectral data (Zschocke *et al*., 2005; Sim and Shin, 2008).



Butylidene phthalide



Z-Ligustilide

Culture of RAW264.7 cells – RAW264.7 cells provided by the Korean Cell Line Bank were cultured in DMEM supplemented with penicillin (10 U/mL), streptomycin (10 μg/mL), and 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% CO_2. Cells were subcultured to generate cells for successive experiments.

Cell viability measurements using the MTT assay – A 100 μL amount of RAW264.7 cell suspension was seeded in each well of 96-well plates in triplicate at a density of 1×10^5 cells/well as determined using a hematocytometer. After 24 h incubation for cell adhesion, 5 μL of medium was removed from each well. Cells were treated with 5 μL of the *L. chuanxiong* essential oil fraction or either of its main components (dissolved in DMEM containing 2% Tween 80) at various concentrations to reach 6.25 - 200 μg/mL in each testing well. After incubation for 2 h, MTT solution (25 μL, 5 mg/mL in PBS) was added, and the cells were incubated for an additional 4 h at 37 °C in 5% CO_2. The medium was then discarded, and 2-propanol containing 0.04 M HCl was added to dissolve the produced formazan. The absorbance was measured at 570 nm with a microplate reader (BioTek, Winooski, VT). Results are expressed as the percentage of viable cells compared to the untreated control.

Assay for inhibition of NO production – The effects of the essential oil fraction of the *L. chuanxiong* and the isolated two compounds were determined in RAW 264.7 cells following LPS treatment on the production of NO. A 100 μL aliquot of the cell suspension was added to each well of 96-well plates in triplicate at a density of 10^5 cells/well. After 24 h incubation, the medium was discarded, and the cells were treated with 2.5 μL solutions of each sample at various concentrations, incubated for 1 h at 37 °C, treated with 2 μL of LPS (1 μg/mL), and then incubated for 18 h at 37 °C in 5% CO_2.

Assay for inhibition of TNF-α, IL-1β, and IL-6 production – To measure TNF-α, IL-1β and IL-6 production, 200 μL of the cell suspension (1×10^5/mL) was deposited in each well of a 96-well plate, treated with test components dissolved in DMSO, after 1 h, treated with LPS (1 μg/mL) and then incubated for 18 h at 37 °C in 5% CO_2. A 100 μL amount of the supernatant was then assayed using complete ELISA kits (Komabiotech, Seoul) according to the manufacturer's recommendations as previously reported (Chung and Shin, 2009).

Western blots for COX-2 and iNOS – Subcultured RAW 264.7 cell suspensions (10 mL/plate) were adjusted to a density of 5×10^4 cells/mL and then transferred to seven petri dishes [1, cells only; 2, treated with LPS alone; 3 - 6, treated with LPS and 50 μg/mL or 5 μg/mL of the essential oil fraction of *L. chuanxiong* or *Z*-ligustilide in DMEM containing 2% Tween 20, respectively. One hour after treatment with test samples, LPS (μg/mL) was added to the cell culture media and the cells were incubated for 24 h. They were then washed with cold PBS and centrifuged.

Pro-PREP Protein Extraction Solution (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea) was used to lyse the cells according to the manufacturer's protocol. After the protein concentration for each aliquot was determined by the Bradford method, suspensions were boiled in SDS-PAGE loading buffer. A 20 μg amount of protein from each sample was subjected to gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in distilled water on a shaker at room temperature for 90 min. The membranes were then incubated in primary antibody (1 : 1000, anti-COX-2, anti-iNOS, or anti-actin antibodies) for 3 h at room temperature. After being washed three times with Tris-buffered saline containing 0.5% Tween 20 (TBS-T), the blots were incubated with HRP-conjugated secondary antibody solution (1 : 4000 for anti-COX-2 and anti-iNOS, and 1 : 2000 for anti-actin) for 90 min at room temperature and washed again three times. The bands on the gel were detected using enhanced chemiluminescence reagent (ECL) followed by exposure to photographic film for 1 min. The band intensities were quantified using a Gel Logic 200 imaging system (Kodak, New York).

Statistics – Reported values are the means ± SD of triplicate independent experiments. Statistical analysis was performed with Student's *t*-test.

Results and Discussion

Cnidii Rhizoma has been an important traditional medicine for the treatment of a variety of ailments, as previously described earlier in this report, and is often prescribed with other herbal drugs. The inhibitory activities of *L. chuanxiong* extracts on the production of NO and various cytokines have been the subject of previous study (Ryu *et al.*, 2003). *Z*-Ligustilide and butylidene phthalide, the main components of the *L. chunxiong* essential oil, are also present in many other plants of Umbelliferae (Beck and Chou, 2007). Wang *et al.* (2010) have reported that *Z*-ligustilide, isolated from *Angelica sinica,* attenuates the proinflammatory response in primary rat microglia. Du *et al.* (2007) reported that *Z*-ligustilide attenuates response to pain induced by acetic acid or formalin. In the present study, the anti-inflammatory activity of the essential oil fraction of *L. chunxiong* was evaluated by determining the effects on the production of NO and proinflammatory cytokines, IL-1β, IL-6, and TNF-α in RAW 264.7 cells. To determine the relative contribution of the main components to this activity, *Z*-ligustilide, and butylidene phthalide were also tested and the results compared with those of the essential oil fraction.

To exclude possible effects of the components on cell viability, we first treated RAW 264.7 cells with various concentrations of *L. chuanxiong* essential oil, *Z*-ligustilide, and butylidene phthalide, respectively and determined the survival rate using an MTT assay. As demonstrated in Fig. 1, treatment with the essential oil fraction or its main components at concentrations between 6.25 μg/mL and 100 μg/mL did not significantly reduce cell viability. Butylidene phthalide exhibited toxicity at the highest concentrations tested, 100 and 200 μg/mL, resulting in survival rates of 22.2 ± 7.2% and 17.1 ± 3.2%, respectively. However, at concentrations lower than 50 μg/mL, no significant effect on cell survival was observed. On the basis of these results, subsequent experiments were conducted at sample concentrations below 50 μg/mL.

Increases in the levels of NO, an important inflammatory

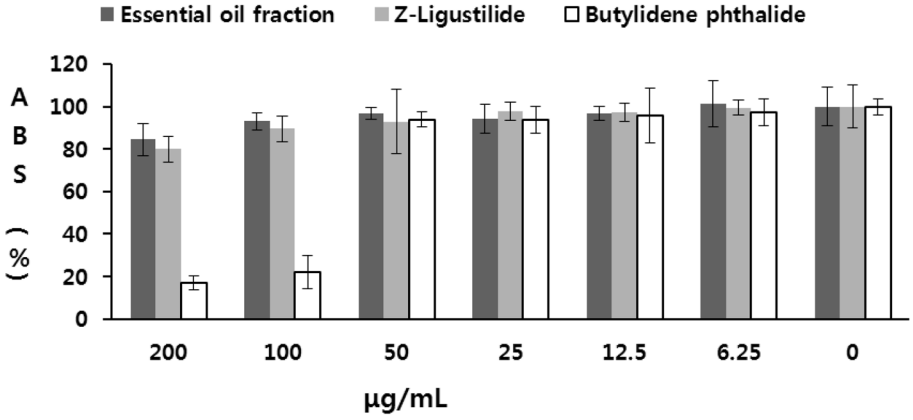


Fig. 1. The survival rate (%) of RAW 264.7 cells following treatment with the *L. chuanxiong* essential oil fraction, *Z*-ligustilide, and butylidene phthalide compared to the control (0) as determined by MTT assay. Values are the means ± SD of triplicate independent experiments.

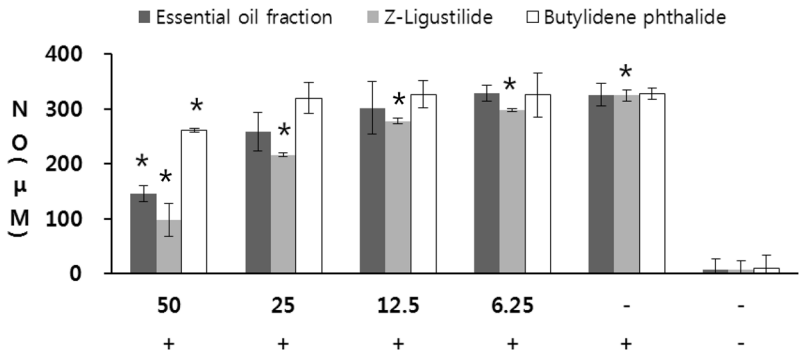


Fig. 2. Inhibitory effects of *L. chuanxiong* essential oil fraction, *Z*-ligustilide, and butylidene phthalide at selected concentrations (0 - 50 μg/mL) on the production of nitric oxide (NO) in RAW 264.7 cells following LPS treatment (1 μg/mL). Values are the means ± SD of triplicate independent experiments. *significantly different ($p < 0.05$) compared to the control (sample: –, LPS: +).

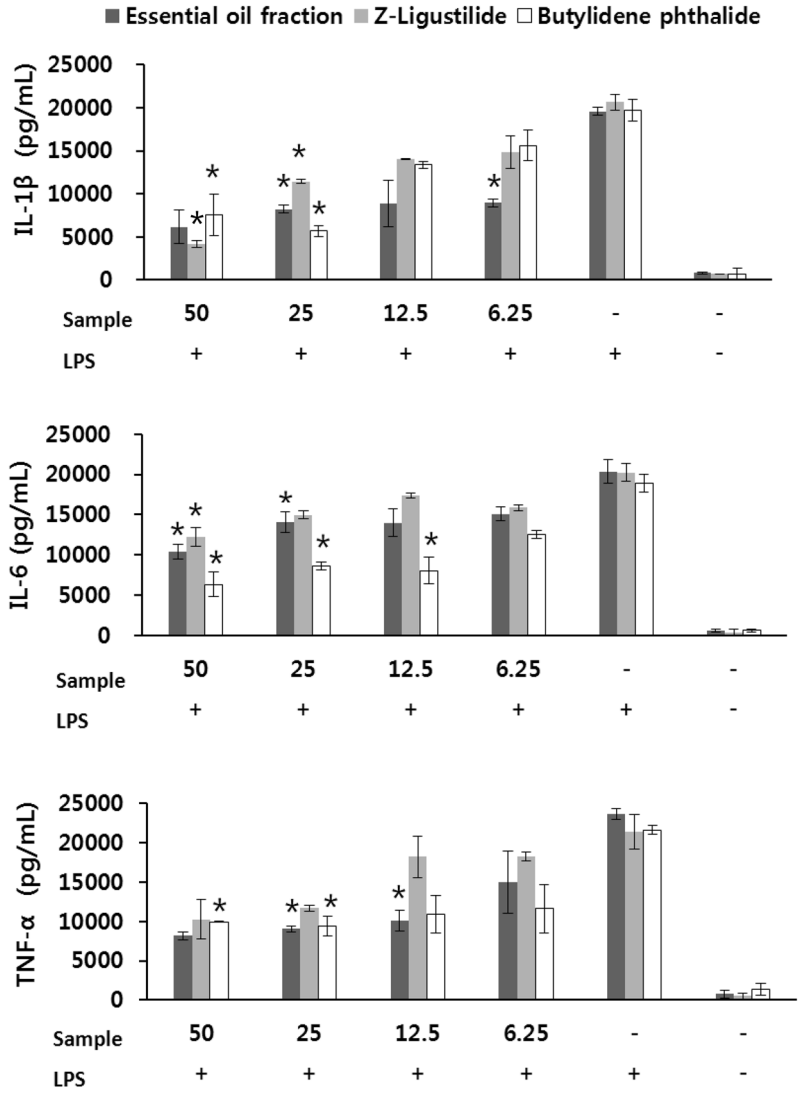


Fig. 3. Effects of *L.chuanxiong* essential oils (50~0 μg/ml) on IL-1β, IL-6, and TNF-α release following LPS treatment (1 μg/ml) as measured using ELISA. Values are the means ± SD of triplicate independent experiments. *significantly different ($p < 0.05$) compared to the control (sample: –, LPS: +).

mediator released by activated macrophages, have been observed in many inflammatory diseases. To evaluate the effects of the essential oil fraction and its main components on the release of NO in activated macrophages, changes in NO production at selected concentrations of test samples were measured in the RAW 264.7 cell culture system. As shown in Fig. 2, the release of NO increased ca. 21-fold after 24-h exposure to 1 μg/mL of LPS, in the absence of test samples. All three samples suppressed NO release significantly, with *Z*-ligustilide demonstrating the highest activity. *Z*-Ligustilide decreased NO levels from 324.9 μM (control) to 103.6 μM and 216.6 μM, corresponding to 68.2% and 33.3% suppression, at concentrations of 50 μg/mL and 25 μg/mL. At 50 μg/mL of the essential oil fraction and butylidene phthalide reduced the NO level to 153.5 μM and 260.8 μM, respectively.

The highly increased production of cytokines such as IL-1β, IL-6 and TNF-α, results from inflammatory macrophage stimulation. To estimate the effects of the *L. chuanxiong* essential oil fraction, *Z*-ligustilide, and butylidene phthalide on the production of these inflammatory mediators, the levels of IL-1β, IL-6 and TNF-α were measured by ELISA assay following LPS treatment. Treatment with each test sample at concentrations ranging from 6.25 μg/mL to 50 μg/mL significantly reduced the production of the three cytokines, mostly in a dose-dependent manner (Fig. 3). At a concentration of 50 μg/mL for essential oil fraction, *Z*-ligustilide, and butylidene phthalide, the suppression of IL-1β release was 67.8%, 79.7%, and 60.0%, respectively. The essential oil fraction showed activity (> 50% inhibition of IL-1β release) higher than that of *Z*-ligustilide at sample concentrations between 25 μg/mL and 6.25 μg/mL. Similarly, the essential oil fraction exhibited a higher suppression of IL-6 and TNF-α release compared with *Z*-ligustilide at concentrations between 50 μg/mL and 12.5 μg/mL. Butylidene phthalide showed generally showed a higher suppression of cytokine release compared to *Z*-ligustilide.

To test the effects of *L. chuanxiong* essential oil on expression of iNOS and COX-2 that occurs during inflammatory responses, Western blot analysis was performed and the intensities of the resulting bands were calculated using an image analyzing system. As demonstrated in Fig. 4, the intensities of the COX-2 and iNOS bands were mostly significantly and dose-dependently reduced by treatment with 50 μg/mL and 6.25 μg/mL of the essential oil fraction or Z-ligustilde. Both of the samples significantly decreased the band intensity of iNOS in a dose-dependent manner. The band intensities of iNOS were decreased to 39% and 50 % of control level by 50 μg/mL of the essential oil fraction or Z-ligustilde, respectively. At the same concentration the essential oil fraction caused relatively mild changes in the levels of COX-2, while Z-ligustilde decreased the band intensity to 15% of control.

The results from this study demonstrated that the essential oil fraction of *L. chuanxiong* and its two main components, *Z*-ligustilide and butylidene phthalide, possess significant anti-inflammatory effects. The activity of the essential oil fraction may be due not only to *Z*-ligustilide,

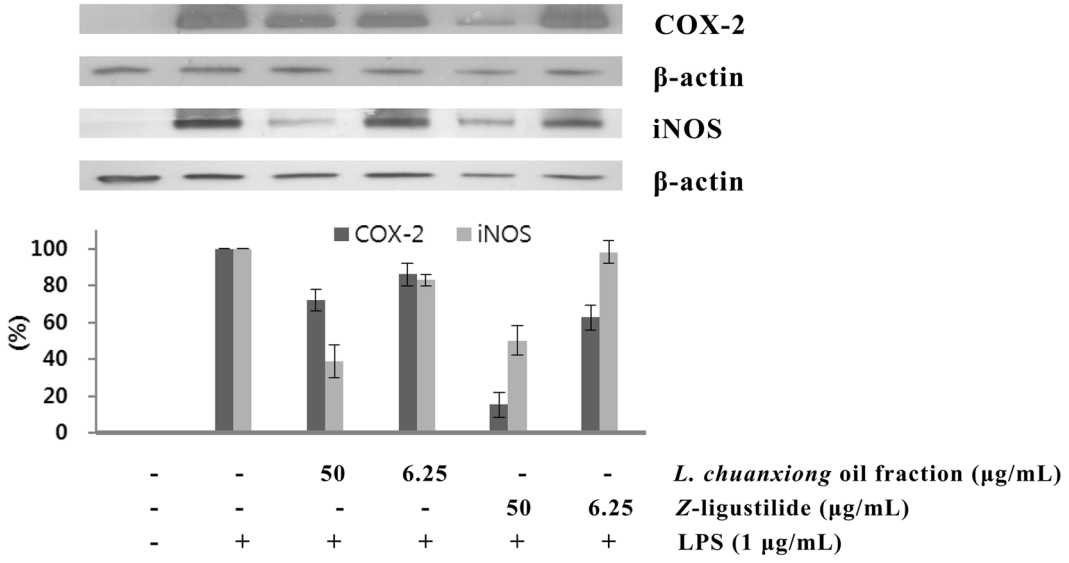


Fig. 4. Western blot analysis of the effects of the *L. chuanxiong* essential oil fraction and Z-ligustilide on the levels of iNOS and COX-2. Relative intensities (%) of the bands to the control were expressed on the graph.

which comprises over 40% of the fraction, but also to other components, and in particular, butylidene phthalide. Further studies are required to fully clarify its anti-inflammatory mechanism.

Acknowledgements

This study was supported by the Duksung Women's University Research Grants (2010).

References

Beck, J.J. and Chou, S.C., The Structural diversity of phthalides from the apiaceae *J. Nat. Prod.,* **70**, 891-900 (2007).

Chung, E.Y. and Shin, S., In Vitro anti-inflammatory effects of essential oil of *Artemisia iwayomogi* and its main component, vulgarone B. *Nat. Prod. Sci*. **15**, 229-233 (2009).

Du, J., Yu, Y., Ke, Y., Wang, C., Zhu, L., and Qian, Z.M., Ligustilide attenuates pain behavior induced by acetic acid or formalin. *J. Ethnopharmacol*. **112**, 211-214 (2007).

Jeong, J.B., Ju, S.Y., Park, J.H., Lee, J.R., Yun, K.W., Kwon, S.T., Lim, J.-H., Chung, G.Y., and Jeong, H.J. Antioxidant activity in essential oils of *Cnidium officinale* makino and *Ligusticum chuanxiong* Hort and their inhibitory effects on DNA damage and apoptosis induced by ultraviolet B in mammalian cell. *Cancer Epidemiol*. **33**, 41-46 (2009).

Liu, L., Ning Z.Q., Shan S., Zhang, K, Deng, T., Lu, X.P., and Cheng, Y.Y., Phthalide lactones from *Ligusticum* chuanxiong inhibit lipopolysaccharide induced TNF-α production and TNF-α-mediated NF-êB activation. *Planta Med*. **71**, 808-813 (2005).

Packer, L., Ong, C.N., and Halliwell, B., Herbal and traditional medicine, Marcel Dekker, New York, pp. 247-254 (2004).

Ryu, J.-H., Ahn, H., Kim, J.Y., and Kim, Y.-K., Inhibitory activity of plant extracts on nitric oxide synthesis in LPS-activated macrophages. *Phytother. Res*. **17**, 485-489 (2003).

Sim, Y. and Shin, S., Combinatorial anti-*Trichophyton* effects of *Ligusticum chuanxiong* essential oil components with antibiotics. *Arch. Pharm. Res*. **31**, 497-502 (2008).

Sim, Y. and Shin, S., Anti-*Aspergillus* activities of the *Ligusticum chuanxiong* essential oil alone and in combination with antibiotics. *Nat. Prod. Sci*. **16**, 175-179 (2010).

Su, S., Hua, Y., Duan, J.-A., Shang, E., Tang, Y., Bao, X., Lu, Y., and Ding, A., Hypothesis of active components in volatile oil from a Chinese herb formulation, 'Shao-Fu-Zhu-Yu decoction', using GC-MS and chemometrics., *J. Sep. Sci*. 31, 1085-1091 (2008).

Tewtrakul, S., Wattanapiromsakul, C., and Mahabusarakam, W., Effects of compounds from *Garcinia mangostana* on inflammatory mediators in RAW264.7 macrophage cells. *J. Ethnopharmacol.* **121**, 379-382 (2009).

Wang. J., Du, J., Wang, Y., Kuang, X., and Wang, C., *Z*-ligustilide attenuates lipopolysaccharide-induced proinflammatory response via inhibiting NF-κB pathway in primary rat microglia. *Acta Pharmacol. Sin*. **31**, 791-797 (2010).

Won, J.H., Im, H.-T., Kim, Y.-H., Yun, K.I., Park, H.-J., Choi, J.W., and Lee, K.-T., Anti-inflammatory effect of buddlejasaponin IV through the inhibition of iNOS and COX-2 expression in RAW264.7 macrophages via the NFκB inactivation. *Brit. J. Pharmacol*. **148**, 216-225 (2006).

Zhang, C., Qi, M., Shao, Q., Zhou, S., and Fu, R., Analysis of the volatile compounds in *Ligusticum chuanxiong* Hort. using HS-SPME-GC-MS. *J. Pharmaceut. Biomed.,* **44**, 464-470 (2007).

Zhao, F., Wang, L., and Liu, K,. *In vitro* anti-inflammatory effects of arctigenin, a lignan from *Arctium lappa* L., through inhibition on iNOS pathway. *J. Ethnopharmacol.* **122**, 457-462 (2009).

Zschocke, S., Klaiber, I., Bauer, R., and Vogler, B., HPLC-coupled spectroscopic techniques (UV, MS, NMR) for the structure elucidation of phthalides in *Ligusticum chuanxiong*. *Molecular Diversity*, **9**, 33-39 (2005).

Received October 13, 2010
Revised November 22, 2010
Accepted December 10, 2010