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# In vitro Antiinflammatory Activity of the Essential oil Extracted from *Chrysanthemum sibiricum* in Murine Macrophage RAW 264.7 Cells

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**Abstract** – This research was undertaken to find the in vitro inflammatory action of the essenetial oil (CS-oil) extracted from *Chrysanthemum sibiricum* (Compositae) herbs. We investigated the effects of the CS-oil not only on the formation NO, $PGE_2$, and TNF-α but also on inducible nitric oxide synthase and cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-induced murine macrophage RAW 264.7 cells. The data obtained were consistent with the modulation of iNOS enzyme expression. A similar fashion was also observed when LPS-induced $PGE_2$ release and COX-2 expression were tested. The significant inhibitory effects were shown in concentration-dependent manners. In addition, CS-oil also mildly but significantly reduced the formation of TNF-a. These findings support the application of CS-oil as an antiinflammatory essential oil.
**Key words** – *Chrysanthemum sibiricum*, compositae, antiinflammatory, nitric oxide, prostaglandin $E_2$, COX-2, iNOS, and TNF-α

## Introduction

Nitric oxide (NO) is synthesized via the oxidation of L-arginine by a family of nitric oxide synthases (NOS) (Moncada *et al.*, 1993) which are either constitutive (cNOS) and inducible (iNOS). A cNOS is $Ca^{2+}$-dependent and releases small amounts of NO required for physiological functions (Bredt *et al.*, 1990) whereas the other form, iNOS is $Ca^{2+}$-independent (Lowenstein *et al.*, 1992) and is induced by lipopolysaccharide (LPS) or proinflammatory cytokines such as TNF-α, IL-1β, and IFN-γ. In practice, during inflammatory processes, large amounts of pro-inflammatory mediators, nitric oxide (NO) and prostaglandin $E_2$ ($PGE_2$) are generated by the iNOS and cyclooxygenase-2 (COX-2), respectively (Lee *et al.*, 1992). TNF-α, a primary mediator in the septic circulation, has been known to induce iNOS. Cyclooxygenase (COX) is the enzyme which converts arachidonic acid to prostaglandins (PGs). Like NOS, COX has been found in two isoforms, and COX-2 is an inducible form responsible for the production of large amounts of proinflammatory PGs at the inflammatory site (Weisz *et al.*, 1996). Therefore, an inhibitor of iNOS, COX and TNF-α may be effective as a therapeutic agent for inflammation.

In a course of searching for the antiinflammatory agent from the essential oils, we found the oil of *C. sibiricum* (CS-oil) showed a high potency on NO inhibition in LPS-activated macrophage cells. This whole plant and buds have been used to treat pneumonia, bronchitis, cough, and common cold in addition to cystitis and menstrual irregularity (Yun, 1995). Although several pharmacological effects of essential oil are reported, there have been very few reports on the effects of essential oils on the antiinflammatory role. Therefore, this communication will encourage an effort to search for new antiinflammatory essential oils.

## Materials and Methods

**Extraction of essential oil from *C. sibiricum*** – This plant material (1.5 kg) was extracted under steam distillation apparatus for 4 h. The distillated liquid was extracted with diethyl ether and dehydrated with anhydrous sodium sulfate followed by being evaporated on a rotatory evaporator at 40°C.

**Reagents** – DMEM medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tertazolium bromide (MTT), sulfanilamide,

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aprotinin, leupeptin, phenylmethylsulfonylfluride, dithiothreitol, N-mono-methyl-arginine (NMA) and *E.coli* lipopolysaccaride (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO). COX-2, iNOS monoclonal antibodies and the peroxidase conjugated secondary antibody were purchased from Santa Crutz Biotechnology, Inc. (Santa Cruz, CA). EIA kit for prostaglandin $E_2$ and TNF-α were obtained from Amersham Pharmacia Biotech. (Piscataway, NJ) and R&D systems (Minneapolis, MN), respectively.

**Cell Culture and sample treatment** – RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37°C in DMEM medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% $CO_2$. Cells were incubated with CS-oil at increasing concentrations and simulated with LPS 1 μg/ml for 24 h.

**MTT assay for cell viability** – Cytotoxicity studies were performed in a 96-well plate. RAW 264.7 cells were mechanically scraped and plated $2\times10^5$/well in 96-well plate containing 100 μl of DMEM medium with 10% FBS and incubated overnight. CS-oil was dissolved in dimethylsulfoxide (DMSO) for stock solution and this was attenuated for use. The DMSO concentrations in all assays did not exceed 0.1%. Twenty-four hours after seeding, 100 μl new media or CS-oil was added, and the plates were incubated for 24 h. Cells were washed once before adding 50 μl FBS-free medium containing 5 mg/ml MTT. After 4 h of incubation at 37°C, the medium was discarded and the formazan blue, which formed in the cells, was dissolved in 50 μl DMSO. The optical density was measured at 540 nm. The concentration required to reduce absorbance by 50% ($IC_{50}$) in comparison to control cells was determined.

Nitrite assay – Nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium by Griess reaction (Green *et al.*, 1982). Briefly, 100 μl of cell culture medium were mixed with 100 μl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl] and incubated at room temperature for 10 min, and then the absorbance at 550 nm was measured in a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was calculated from a sodium nitrite standard curve freshly prepared in culture medium.

**$PGE_2$ and TNF- assay** – $PGE_2$ and TNF-α level in macrophage culture medium were quantified by EIA kits according to the manufactures instructions.

**Western blot assay** – Cellular proteins were extracted from control and CS-oil-treated RAW 264.7 cells. The washed cell pellets were resuspended in lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM NaF, 0.5 mM Na orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin and incubated with 15 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. Protein concentration was determined by Bio-Rad protein assay reagent according to the manufactures instruction. 40-50 μg of cellular proteins from treated and untreated cell extracts were electroblotted onto nitrocellulose membrane following separation on a 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4°C, followed by incubation for 4 h with a 1:500 dilution of monoclonal anti-iNOS and COX-2 antibody (Santacruz, CA, U.S.A.). Blots were washed 2 times with PBS and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Santacruz, CA, U.S.A.) for 1 h at room temperature. Blots were again washed three times in Tween 20/Tris-buffered saline (TTBS) and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.).

**Data analysis** – Data are reported as mean SEM values of three independent determinations. All experiments were done at least three times, each time with three or more independent observations. Statistical analysis was performed by Students t test.

## Results and Discussion

We have studied physiological roles of the essential oils for the development of biologically active substances (Choi *et al.*, 2001, Choi *et al.*, 2002). Although it is believed that the major biological activities may depend on the electrophilic ingredients of the oil, the antiinflammatory action *in vitro* has ramained ambiguous. Lee *et al.* (1999) have reported a sesquiterpene lactone, dehydrocostuslactone with inhibitory action on iNOS and TNF-α. We selected CS-oil with a potent NO inhibitory action from 10 essential oils.

It is well known that macrophages play a crucial role in both non-specific and acquired immune responses, and macrophage activation by LPS leads to a functionally diverse series of responses including the production of proinflammatory cytokines (IL-1, TNF-α and IL-6), the activation of phospholipase $A_2$ producing lipid metabolites of arachidonic acid such as PGs, and NO production.

As shown in Fig. 1, the LPS (1 μg/ml) highly induced $NO_2^-$ production in murine macrophage RAW 264.7 cells. Treatment of CS-oil (0.2-1.0 μg/ml) prevented NO production

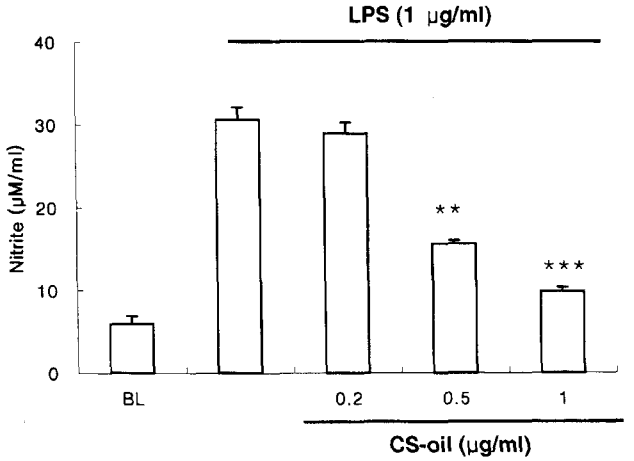


**Fig. 1.** Evaluation of nitrite production by RAW 264.7 cells stimulated for 24 h with LPS alone or in combination with increasing concentrations (0.2-1.0 μg/ml) of essential oil (CS-oil) extracted from *C. sibiricum*. The values are the means of at least 3 determinations±S.E.M. Probability levels (Student's t-test): *** $p<0.001$ vs. LPS-treated group. BL (blank).

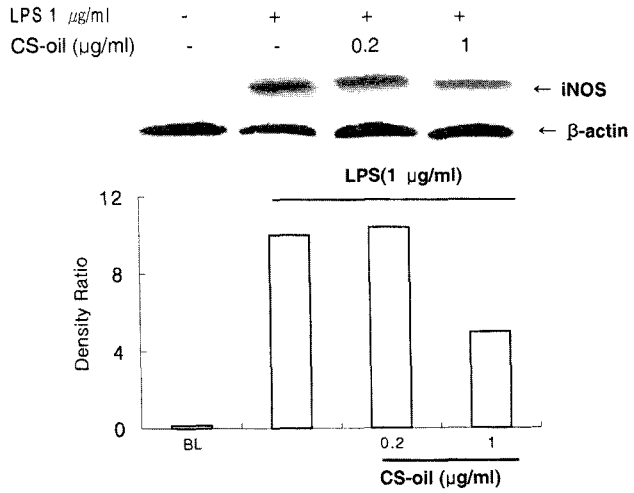


**Fig. 2.** Effect of essential oil (CS-oil) extracted from *C. sibiricum* on iNOS production by LPS-induced RAW 264.7 macrophage for 24 h.

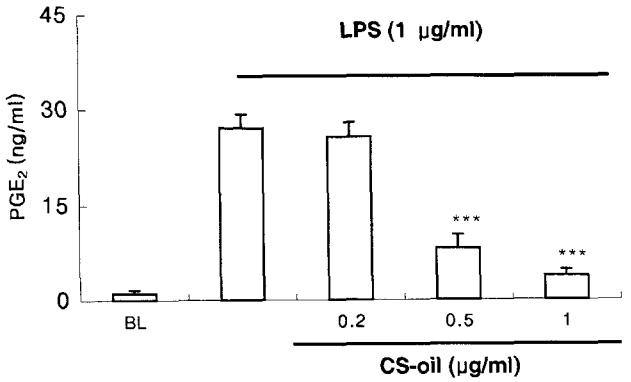


**Fig. 3.** Effect of CS-oil on $PGE_2$ production in LPS-induced RAW 264.7 macrophage for 24 h. The values are the means of at least 3 determinations±S.E.M. The values are the means of at least 3 determinations±S.E.M. Probability level (Student's t-test): *** $p<0.001$ vs. LPS-treated group.

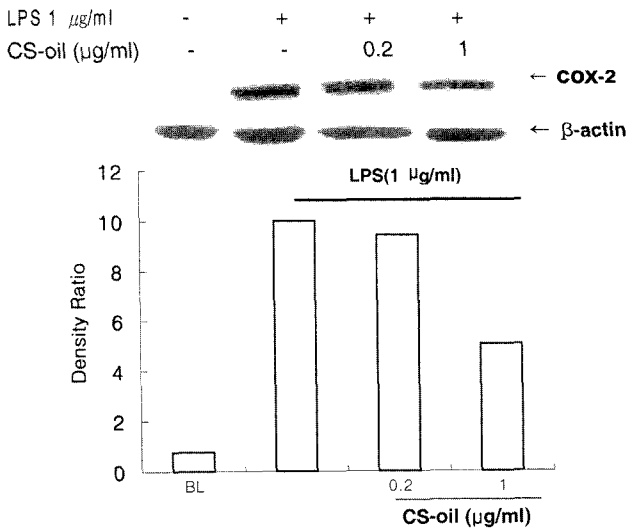


**Fig. 4.** Modulation by CS-oil of LPS-induced COX-2 expression in RAW 264.7 cells.

in a dose-dependent fashion and cell viability was observed to be over 92% by the MTT assay. Results from western blotting analysis further indicated that LPS-induced expression in RAW 264.7 macrophages was significantly reduced (Fig. 2). The enzyme iNOS is responsible for long-lasting NO production and it is strikingly induced by LPS as shown in this study. Therefore, these results suggest that inhibition of LPS-induced NO production is dne to iNOS gene expression.

Mechanism of various antiinflammatory action is at least shared by the inhibition of prostaglandin synthesis, which is mediated by COX. This exists in two isoforms, COX-1 and 2, each with distinct expression pattern in various cell types. COX-1 has been suggested to provide a physiologic level of prostaglandins for normal platelet, stomach, and kidney function. In contrast, COX-2 has been found to be highly induced at inflammatory sites in animals as well as patients with inflammatory diseases (Seibert *et al.*, 1994; Masferrer *et al.*, 1994), and it is considered to be responsible for pro-inflammatory prostaglandin formation. In addition to inhibition of NO release and iNOS induction, CS-oil also significantly inhibited $PGE_2$ production and COX-2 gene expression in LPS-treated RAW 264.7 macrophages (Fig. 3 and 4).

Proinflammatory cytokines such as TNF-α, IL-1 and IL-6 have been shown to control inflammation *in vitro* as well as *in vivo* (Feldmann *et al.*, 1996; Harada *et al.*, 1994), and these cytokines are thought to be interlinked in a

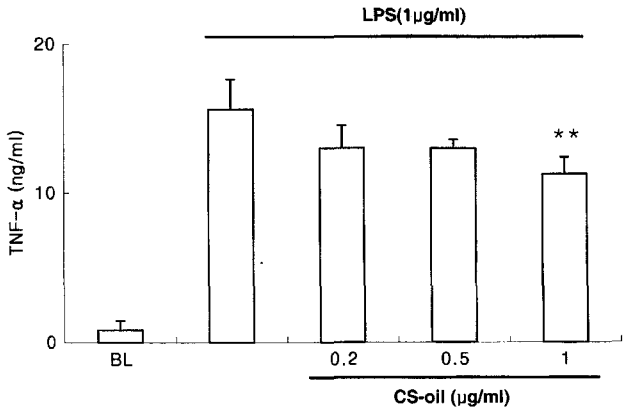


**Fig. 5.** Effect of CS-oil on LPS-induced TNF-α production by RAW 264.7 cells. The values are the means of at least 3 determinations±S.E.M. Probability level (Student's t-test) : $p<0.01$ vs. LPS-treated group.

cascade, being produced serially by macrophages during an inflammatory response. Furthermore, the development of hyperalgesic states during inflammation is thought to be mediated by proinflammatory cytokines (Watkins *et al.*, 1995). Therefore, we investigated whether CS-oil would possibly influence the formation of TNF-α in an *in vitro* model. CS-oil mildly affected the production of TNF-α (Fig. 5).

## Acknowledgements

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