

Anti-inflammatory effects of essential oils from *Chamaecyparis obtusa* via the cyclooxygenase-2 pathway in rats

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Abstract. Essential oils are concentrated hydrophobic liquids containing volatile aromatic compounds from plants. In the present study, the essential oil of *Chamaecyparis obtusa* (*C. obtusa*), which is commercially used in soap, toothpaste and cosmetics, was extracted. Essential oil extracted from *C. obtusa* contains several types of terpenes, which have been shown to have anti-oxidative and anti-inflammatory effects. In the present study, we examined the anti-inflammatory effects of *C. obtusa* essential oil *in vivo* and *in vitro* following the induction of inflammation by lipopolysaccharides (LPS) in rats. While LPS induced an inflammatory response through the production of prostaglandin E₂ (PGE₂) in the blood and peripheral blood mononuclear cells (PMNCs), these levels were reduced when essential oil was pre-administered. Additionally, the mechanism of action underlying the anti-inflammatory effects of *C. obtusa* essential oil was investigated by measuring the mRNA expression of inflammation-associated genes. LPS treatment significantly induced the expression of transforming growth factor α (TNF α) and

cyclooxygenase-2 (COX-2) in rats, while *C. obtusa* essential oil inhibited this effect. Taken together, our results demonstrate that *C. obtusa* essential oil exerts anti-inflammatory effects by regulating the production of PGE₂ and TNF α gene expression through the COX-2 pathway. These findings suggest that *C. obtusa* essential oil may constitute a novel source of anti-inflammatory drugs.

Introduction

Inflammation is the most common cause of clinical pain resulting from tissue injury. The primary function of pain is to protect the organism from potential tissue-damaging stimuli. The aim of drug treatment for inflammation is to normalize pain sensitivity; however, drugs that are currently available are associated with critical side-effects and low efficacy. Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to be effective in the treatment of various disorders with inflammation and have fewer side-effects compared with steroids (1). NSAIDs function through binding to the cyclooxygenase (COX) enzymes to inhibit the production of prostaglandins from arachidonic acid. Two isoforms of the COX protein, COX-1 and -2, exist; COX-1 is constitutively expressed in the majority of organs and cells, whereas COX-2 is generally absent and is induced upon stimulation by inflammatory stimuli, including endotoxin lipopolysaccharides (LPS). This suggests that COX-2 is an inflammation-specific isoform (1-3). NSAIDs and COX-2 selective inhibitors are currently used to treat several inflammatory disorders (4-6). However, selective inhibitors of COX-2 have been associated with a risk of myocardial infarction and stroke. The recent withdrawal of conventional NSAIDs and selective COX-2 inhibitors due to their adverse cardiovascular and gastric side-effects has led to the development of alternative anti-inflammatory agents (7-9). Therefore, plant-derived natural agents, such as essential oil, are considered to be useful sources for the next generation of anti-inflammatory drugs.

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Abbreviations: *C. obtusa*, *Chamaecyparis obtusa*; PGE₂, prostaglandin E₂; TNF α , transforming growth factor α ; COX-2, cyclooxygenase-2; NSAIDs, nonsteroidal anti-inflammatory drugs; LPS, endotoxin lipopolysaccharides

Key words: *Chamaecyparis obtusa*, essential oil, anti-inflammation, PGE₂, TNF α , COX-2

Essential oils are concentrated hydrophobic liquids containing volatile aromatic compounds from plants. They are extracted by distillation, expression or solvent extracts, and are used in perfumes, cosmetics, soaps and other products, for adding scents to incense and household products and for flavoring food and drink. *Chamaecyparis obtusa* (*C. obtusa*), a tropical tree species found in Japan and the southern region of South Korea, is used for construction and furniture due to its advantage in structural properties and natural scent. Its essential oil is extracted from pruned leaves and twigs of the *C. obtusa* tree, and has been commercially used in soap, toothpaste and cosmetics as a functional additive. The *C. obtusa* essential oil contains several types of terpenes that have been shown to exert anti-oxidative and anti-inflammatory effects, including sabinene, limonene, bornyl acetate, borneol, α -terpineol and elemol, while essential oils from fruits contain myrcene, γ -terpinene, p-cymene, borneol, α -terpineol and β -caryophyllene (10,11).

In the present study, the anti-inflammatory property of the essential oil from *C. obtusa* was investigated *in vivo* and *in vitro*, and the ability of *C. obtusa* to serve as a source of novel pharmaceuticals was determined.

Materials and methods

Animals and treatment. Male Sprague-Dawley (SD) rats (9 weeks old) were obtained from Koatech Co., Ltd. (Pyeongtaek-si, Korea). All the animals were housed in polycarbonate cages and acclimated in an environmentally controlled room (23±2°C; relative humidity, 50±10%; frequent ventilation; and a 12-h light/dark cycle) prior to use as previously described (12). *C. obtusa* essential oil used in this study was provided by Enbita Co., Ltd. (Seoul, Korea) and was produced through steam distillation of pruned twigs and leaves of the *C. obtusa* tree, according to previously described methods (13). The rats were treated with *C. obtusa* essential oil to investigate its effects. A total of 50 ml tap water or essential oil solution (25, 50 or 100%) was administered to the rats for 2 weeks. Rats in the control group were treated with a saline solution (0.9% NaCl), and the remaining rats (treatment group) were treated with 0, 25, 50 or 100% solutions of *C. obtusa* essential oil in the presence or absence of LPS (Sigma-Aldrich, St. Louis, MO, USA). Rats in the LPS-treated group (LPS-positive group) were administered 1 µg/kg LPS via an intraperitoneal (i.p.) injection 8 h prior to sacrifice; rats in the control group for LPS (LPS-negative group) were injected with an equivalent volume of saline solution at the same time-point with LPS. All the experimental procedures were approved by the Ethics Committee of the Chungbuk National University (Cheongju, Korea).

Peripheral blood mononuclear cell (PMNC) isolation. PMNCs were isolated from rat peripheral blood vessels using a double density gradient centrifugation method as previously described (14,15). Briefly, heparinized blood samples were overlaid on Histopaque-1077 solution (specific gravity, 1.077; Sigma-Aldrich) and Histopaque-1119 solution (specific gravity, 1.199; Sigma-Aldrich), centrifuged for 40 min at 700 x g and washed three times with cold phosphate-buffered saline (PBS). The viability of PMNCs, determined by trypan

blue dye exclusion, was >97%. The PMNCs were resuspended in RPMI-1640 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 0.02 mg/ml gentamicin and 5% heat-inactivated fetal bovine serum (FBS; Invitrogen, Grand Island, NY, USA) for use in subsequent experiments.

Prostaglandin E_2 (PGE₂) ELISA. For the *in vivo* experiment, rats were treated with various solutions of *C. obtusa* essential oil in the presence or absence of LPS. Following treatment, serum was collected from peripheral blood vessels and subjected to a PGE₂ ELISA assay. For the *in vitro* assay, rats were treated with the essential oil solutions for 2 weeks. PMNCs were harvested from rat peripheral blood vessels and plated on 24-well plates at a density of 0.5x10⁵. The PMNCs were then stimulated with 10 ng/ml LPS for the indicated times, and the supernatant was collected. For the PGE₂ ELISA assay, the collected serum and supernatant were analyzed using a monoclonal PGE₂ ELISA kit (Cayman Chemical, Ann Arbor, MI, USA).

Quantification of mRNA by RT-PCR. Total cellular RNA was extracted using TRIzol reagent (Invitrogen), and the concentration of RNA was determined at 260 nm. RT-PCR was performed as previously described (13). Briefly, total RNA (1 µg) was reverse transcribed into complementary DNA (cDNA) using M-MLV reverse transcriptase (Invitrogen) and a random primer (9-mer; Takara Bio, Inc., Shiga, Japan). The cDNA (1 µl) was used for PCR under standard conditions (13,16); denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. The primer sequences used were as follows: COX-2 sense, 5'-TACCCGGACTGGATTCTACG-3' and antisense, 5'-AAGTTGGTGGGCTGTCAATC-3'; TNF α sense, 5'-CTGAGCTCAAGCCCTGGTAT-3' and antisense, 5'-GGTCAGAGTAATGGGGGTCA-3'; and cytochrome c oxidase subunit 1 (1A) sense, 5'-CCAGGGTTTGGGAATTATTTTC-3' and antisense, 5'-GAAGATAAACCCTAAGGCTC-3', which was used as an internal control. The PCR products (10 µl) were separated on 2% agarose gel and stained with ethidium bromide (EtBr). The gel image was captured and analyzed using Quantity One software (Gel Doc EQ; Bio-Rad, Hercules, CA, USA).

Statistical analysis. Data were analyzed by non-parametric one-way analysis of variance using the Kruskal-Wallis test, followed by Dunnett's test for multiple comparisons. Data values were converted to ranks for these tests. All the statistical analyses were performed with SPSS for Windows (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of essential oil from *C. obtusa* on serum PGE₂ concentration. The regulation of serum PGE₂ by essential oils from *C. obtusa* was investigated. Rats were treated with various concentrations of the essential oil for 2 weeks and 1 µg/kg LPS was injected to induce an inflammatory reaction following treatment with the essential oil.

To analyze the levels of serum PGE₂, ELISA with a specific antibody for PGE₂ was performed. The levels of LPS-induced

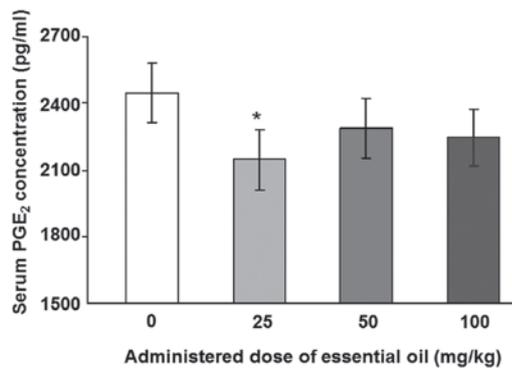


Figure 1. Effects of various doses of *Chamaecyparis obtusa* essential oil on lipopolysaccharide (LPS)-induced concentrations of prostaglandin E₂ (PGE₂) in the serum of rats. The rats were treated via oral administration of the essential oils for 2 weeks, and 1 μ g/kg LPS was intraperitoneally injected on the last day of treatment. Serum samples were collected from rat blood samples 8 h after the LPS injection. The serum samples were filtered with a 0.45- μ m pore size membrane and PGE₂ concentrations were determined using ELISA. *P=0.036 compared with control group, as determined by one-way ANOVA followed by a Dunnett's test (n=4).

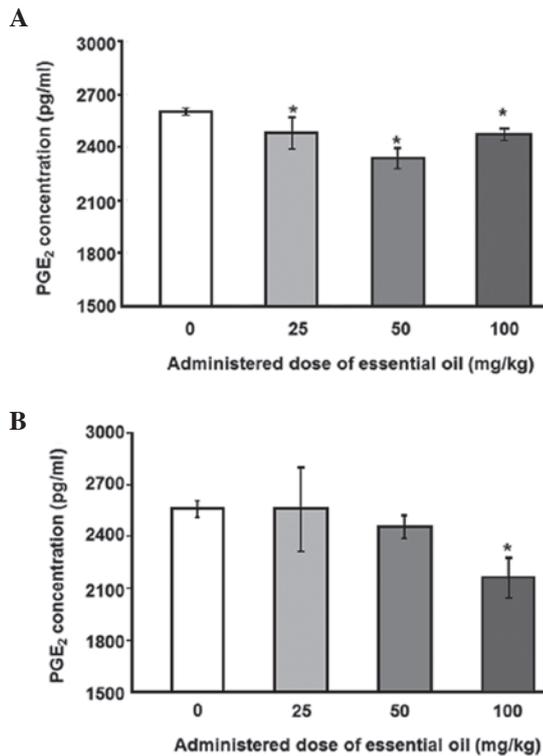


Figure 2. Effect of various doses of *Chamaecyparis obtusa* essential oil on lipopolysaccharide (LPS)-induced production of prostaglandin E₂ (PGE₂) using peripheral blood mononuclear cells (PMNCs). PMNCs were isolated from blood samples obtained from rats treated via oral administration of the essential oil for 2 weeks. Isolated PMNCs were stimulated by 10 ng/ml LPS for (A) 12 or (B) 24 h, and the culture supernatants were harvested and filtered with a 0.45- μ m pore size membrane. PGE₂ concentrations in the culture supernatant were determined using ELISA. *P \leq 0.001 compared with control group, as determined by one-way ANOVA followed by a Dunnett's test (n=4).

serum PGE₂ were significantly reduced in animals treated with essential oil at a dose of 25 mg/kg (Fig. 1). Other concentrations of *C. obtusa* essential oil (50 and 100 mg/kg) also

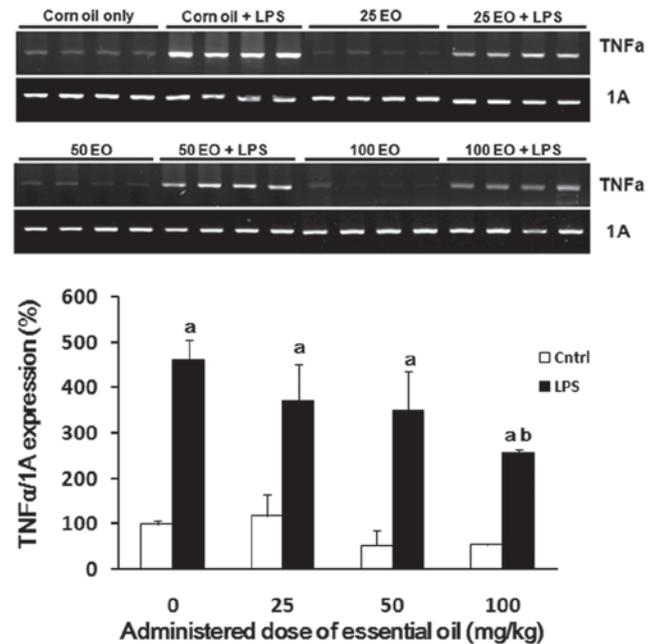


Figure 3. Effect of *Chamaecyparis obtusa* essential oil (EO) on lipopolysaccharide (LPS)-induced expression of tumor necrosis factor α (TNF α) mRNA. The rats were treated via oral administration of various doses of the essential oil for 2 weeks, and 1 μ g/kg LPS was intraperitoneally injected on the last day of treatment. The mRNA samples were extracted from the lungs of rats 8 h after the LPS injection and analyzed by RT-PCR with primers for TNF α as described in Material and methods. ^aP<0.05 compared with the LPS-negative group; ^bP<0.05 compared with the LPS-positive control group.

reduced the levels of serum PGE₂; however, these results were not statistically significant. Next, the effects of essential oil were evaluated, using PMNCs to verify the increase in serum PGE₂ in the rats. PMNCs were isolated following treatment with various concentrations of the essential oil for 2 weeks and administration of 10 ng/ml LPS for 12 (Fig. 2A) or 24 h (Fig. 2B). The essential oil was shown to have protective effects on LPS-induced inflammation by reducing PGE₂ secretion in the PMNCs. The 12-h LPS-induced PGE₂ secretion level was inhibited by treatment with the essential oil at all the doses examined, and the maximum inhibition was observed at a dose of 50 mg/kg. The inhibition of PGE₂ production induced by LPS treatment for 24 h was shown to be significant only when 100 mg/kg of the essential oil was applied.

Effect of essential oils from *C. obtusa* on expression of the inflammation-related genes TNF α and COX-2 in lung tissue.

To examine the possible mechanisms of action underlying the anti-inflammatory effects of the essential oil, we analyzed the expression of inflammation-associated genes in the lungs of rats following treatment with the essential oil in the presence or absence of LPS. As expected, LPS enhanced the expression of TNF α (Fig. 3). LPS-induced TNF α expression in the lung was reduced by administration of the essential oil in a dose-dependent manner, where the most potent effect was observed at a dose of 100 mg/kg (56% reduction).

COX-2 mRNA expression was also assessed to determine the mechanism via which the essential oil exerts its anti-inflam-

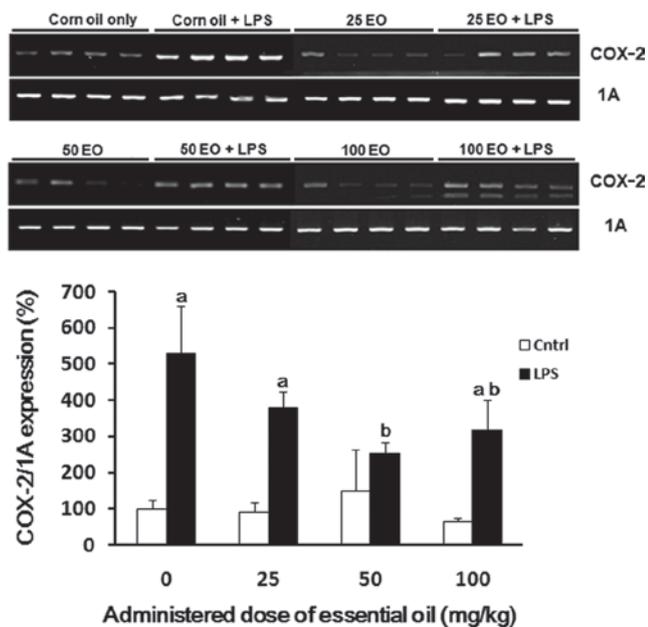


Figure 4. Effect of *Chamaecyparis obtusa* essential oil (EO) on lipopolysaccharide (LPS)-induced expression of cyclooxygenase-2 (COX-2) mRNA. The rats were treated via oral administration of various doses of the essential oil for 2 weeks, and 1 μ g/kg LPS was intraperitoneally injected on the last day of treatment. The mRNA samples were extracted from the lungs of rats 8 h after the LPS injection, and COX-2 expression was analyzed by RT-PCR. ^aP<0.05 compared with the LPS-negative group; ^bP<0.05 compared with the LPS-positive control group.

matory effects (Fig. 4). The expression of COX-2 was increased by LPS treatment (up to 5-fold) compared with the control group (Fig. 4). The induction of COX-2 expression by LPS treatment was inhibited by pre-administration of the essential oil; this inhibition was most marked at a dose of 50 mg/kg.

Discussion

Since ancient times, plants have been used for their medicinal properties, which are partially attributed to essential oils (17). Essential oils are natural, complex and multi-component oils composed mainly of terpenes with additional components (18). Essential oils are widely used to prevent and treat human diseases, including cancer and cardiovascular diseases. Furthermore, their bioactivity as antibacterial, antiviral, antioxidant and antidiabetic agents has previously been reported (18). Recent studies have demonstrated the anti-inflammatory properties of essential oils from several plants (19-21). In the present study, we demonstrated that the essential oil from *C. obtusa* has an anti-inflammatory effect on LPS-induced inflammation in rats. The serum concentration of PGE₂ was reduced when the rats were treated with the essential oil. In addition, the synthesis of PGE₂ from blood PMNCs showed concomitant results with that of serum. These results suggest that PGE₂ is synthesized by blood monocytes following stimulation by LPS, and that this inflammatory process is ameliorated by *C. obtusa* essential oil.

PGE₂ is produced in the body and accumulates with LPS treatment through stimulation of the COX pathway. The quantitative analysis of PGE₂ represents inflammatory activities

in vivo and *in vitro* (22). In the present study, LPS-induced PGE₂ production was reduced in the serum and PMNCs of rats by treatment with *C. obtusa* essential oil. Similar results have been previously reported; *Hyptis pectinata* essential oil has been shown to have anti-inflammatory effects by inhibiting the production of PGE₂ and nitric oxide (23). *Cryptomeria japonica* essential oil has been demonstrated to inhibit the LPS-induced production of PGE₂ and cytokines in macrophages, including interleukin (IL)-1 β and IL-6 (24); furthermore, the chemical composition of *Cryptomeria japonica* essential oil was analyzed and its major components were revealed to be elemol and sabinene. Elemol and sabinene are also known to be terpenes of *C. obtusa* essential oil (10,11).

In the present study, we investigated the effects of *C. obtusa* essential oil on the regulation of TNF α mRNA expression following LPS treatment. As expected, the expression of TNF α was increased in the lung tissues of rats following LPS treatment, which implied induction of the immune response by LPS. Furthermore, the induced expression of TNF α was abrogated by treatment with *C. obtusa* essential oil. To verify the mechanism of action underlying the anti-inflammatory effects of *C. obtusa* essential oil, we also examined the regulation of COX-2 expression levels. Pathogen-associated immune responses are antigenic, and are able to activate monocytes or macrophages to synthesize various inflammatory cytokines, including IL-1, IL-6 and TNF, which have been reported to be mediated via COX expression (24-26). The expression of COX-2 was increased following treatment with LPS, and this upregulation was inhibited by *C. obtusa* essential oil in a dose-dependent manner. These results suggest that the anti-inflammatory effects of this essential oil occur via COX-2-mediated regulation of PGE₂ and TNF α signaling pathways.

The anti-inflammatory effects of essential oils through the COX-2 signaling pathway have been previously demonstrated; the essential oil from *Houttuynia cordata* has been shown to possess anti-inflammatory properties through inhibiting the LPS-induced production of PGE₂ (9). In this study, the anti-inflammatory effects of the essential oil were mediated by the inhibition of COX-2 gene expression, while COX-1, the non-inflammation-specific isoform, was not involved. Katsuwawa *et al* (27) also demonstrated that the essential oil of lemongrass suppresses COX-2 promoter activity in human macrophage-like U937 cells.

In conclusion, our results demonstrate that *C. obtusa* essential oil exerts its anti-inflammatory effects by regulating the production of PGE₂ in the blood and the gene expression of TNF α in rats. The anti-inflammatory effects of *C. obtusa* essential oil are mediated by inhibiting the expression of the inflammation-specific COX-2 enzyme. Our results suggest that *C. obtusa* essential oil is a novel source of inflammation-specific pharmacological drugs. However, further studies are required to investigate the specific mechanisms of action and potential side-effects of *C. obtusa* essential oil.

Acknowledgements

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