Alleviation effects of natural volatile organic compounds from *Pinus densiflora* and *Chamaecyparis obtusa* on systemic and pulmonary inflammation

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Abstract. *Chamaecyparis obtusa* (*C. obtusa*) and *Pinus densiflora* (*P. densiflora*) have been traditionally used as antibiotic, antinociceptive and anti-inflammatory agents in Asian folk medicine. Recent studies have demonstrated antioxidant, antiproliferative and anti-inflammatory effects of *C. obtusa* and *P. densiflora* extracts. In the present study, volatile organic compounds (VOCs) of *C. obtusa* and *P. densiflora* were examined to determine whether they have anti-inflammatory capabilities. To evaluate the anti-inflammatory effects of VOCs of *C. obtusa* and *P. densiflora*, lipopolysaccharide (LPS) was administered to the lung by nasal injection and to the whole body by intraperitoneal injection. Alterations in serum immunoglobulin E (IgE) levels and prostaglandin E2 (PgE2) were examined using ELISA. LPS-increased serum IgE and PgE2 levels were recovered by administration of dexamethasone and VOCs of *C. obtusa* and *P. densiflora*. Levels of mRNA expression of inflammatory cytokines were determined in an LPS-induced inflammation mouse model. Reverse transcription-quantitative polymerase chain reaction was used to determine the mRNA expression levels of cyclooxygenase 2, interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-13 in peripheral blood mononuclear cells. The expression of all examined cytokine mRNAs increased by LPS was suppressed by dexamethasone and VOCs of *C. obtusa* and *P. densiflora*. Similar tendencies were observed in lung tissues and cells obtained via bronchoalveolar lavage. The results of the present study suggested that VOCs of *C. obtusa* and *P. densiflora*, through their immunosuppressive activities, may have therapeutic potential in the treatment or prevention of inflammation.

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Key words: *Chamaecyparis obtusa*, *Pinus densiflora*, inflammation, cytokine, lung

Introduction

Inflammatory disease has been associated with cytokine and adhesion molecule expression levels. Inflammatory diseases, including asthma and chronic obstructive pulmonary disease are obstructive airway diseases that involve chronic inflammation of the respiratory tract, but the type of inflammation in these two diseases is markedly different with contrasting patterns of inflammatory cell and mediator involvement being reported (1). Allergic asthma is characterized by airway hyper-responsiveness to a variety of specific and non-specific stimuli, including chronic pulmonary eosinophilia, elevated serum immunoglobulin E (IgE) and excessive airway mucus production (2). IgE is an important mediator of allergic reactions, including allergic asthma, and serves a central role in asthma-related symptoms, airway inflammation and, possibly, airway remodeling (3). The pathophysiology of asthma is thought to be mediated by $CD4^+$ T lymphocytes producing a type 2 cytokine profile (4). When IgE molecules bind to the surface of an immune cell, that cell is simultaneously sensitized to the specific allergen. The sensitized immune cell immediately exhibits an inflammatory response, including the release of histamine, inducing the early phase of an allergic reaction. Following IgE release, immune cells synthesize other inflammatory molecules, including interleukins (ILs) (5,6).

Chamaecyparis obtusa (*C. obtusa*) is a species of cypress in the Cupressaceae family. *C. obtusa* is a slow-growing tree occurring in Japan and South Korea. Essential oil from *C. obtusa* contains several types of terpenes, including mono-, sesqui- and diterpenes (7). *C. obtusa* oil has been used as a folk remedy to reduce allergic reactions (8). Several previous studies have reported on the alleviatory effect of *C. obtusa* on allergic dermatitis in a mouse model (7,9,10). In addition, *C. obtusa* effectively suppresses the levels of serum IgE and pro-inflammatory cytokines, including ILs, and it affects mast cell appearance (2,11,12). *C. obtusa*, regarded as an effective

medicinal plant, contains active terpene compounds that include pharmacologically active molecules.

Pinus densiflora, also known as Korean red pine or Japanese pine, is a species of *Pinus* in the Pinaceae family. The majority of *Pinus* spp. occur in the northern hemisphere and certain *Pinus* spp. have been used in folk medicine for a long time. Pine pollen, bark and leaves are reported to have anti-inflammatory effects (13,14). Pine pollen has demonstrated antioxidant and anti-inflammatory effects (14), while pine bark and its essential oil have been reported to have anti-nociception and anti-inflammatory effects (13).

Volatile organic compounds (VOCs) have been reported to be associated with the risk of asthmatic and immune responses (15). However, to the best of our knowledge, there have been no reports indicating that exposure to the VOCs of *C. obtusa* (VOCCo) or *P. densiflora* (VOCPd) affect asthmatic symptoms, and, in particular, whether they affect IgE and cytokine levels. In addition, the mechanisms underlying the possible hypoallergic effects of VOCCo and VOCPd have not been elucidated. The aim of the present study was to determine whether VOCCo or VOCPd exposure may decrease inflammation and whether one or the two products may be suitable for consideration as a pharmaceutical candidate.

Materials and methods

Animal experiments. A total of 35 male BALB/c mice (7 mice per group; 7 weeks old; body weight, 30 g) were purchased from Koatech Technology Corporation (Pyeongtaek, Republic of Korea), housed in polycarbonate cages with *C. obtusa* or *P. densiflora* wood panels and corncob bedding, and acclimated in an environmentally controlled room (temperature, 23±2˚C; relative humidity, 50±10%; frequent ventilation; and a 12:12 h light:dark cycle). Animal diet were purchased from Purina Petcare (St. Louis, MO, USA), and mice had *ad libitum* access to animal diet and drinking water. The animal experiments were approved by the Institutional Animal Care and Use Committee of Chungbuk National University (Cheongju, Republic of Korea), and all procedures were performed in accordance with the guide for the care and use of laboratory animals published by the National Institutes of Health (Bethesda, MD, USA).

Lipopolysaccharide (LPS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), dissolved in PBS, was used to induce inflammation in the BALB/c mice. LPS was regularly administered via intraperitoneal (i.p.) or intranasal (i.n.) routes for 7 days. The VOC-untreated groups included the vehicle (VE) group, the LPS (LPS 10 mg/kg) group and the LPS plus dexamethasone (LPS+DEX; LPS 10 mg/kg, DEX 2.5 mg/kg, i.p.)-treated group (7 mice/group). The VOC-treated groups included the LPS+VOCCo (LPS 10 mg/kg; *C. obtusa* wood panel, 1,026 cm^3) group and the LPS+VOCPd (LPS 10 mg/kg; *P. densiflora* wood panel, 1,026 cm^3) group. Following the completion of treatment, the mice were sacrificed by ether inhalation, and lung and blood samples were collected for analysis. The IACUC of Chungbuk National University approved all experimental procedures (approval no. CBNUA699-15-07).

Analysis of serum. At the end of the treatment period, blood samples were collected directly from the inferior vena cava using a 1-ml syringe. Serum was obtained by centrifugation at 3,000 x g for 10 min at 4˚C and was stored at -70˚C until use. Serum IgE and prostaglandin E2 (PgE2) levels were measured using mouse IgE Ready-Set-Go ELISA kits (cat. no. 50-112-5120; eBioscience; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the prostaglandin E2 Multispecies Competitive ELISA kit (cat. no. EHPGE2; Thermo Fisher Scientific, Inc.), respectively, according to the manufacturer's protocol.

Isolation of peripheral blood mononuclear cells (PBMCs). Whole blood from each treatment group (VE, LPS, LPS+DEX, LPS+VOCCo and LPS+VOCPd) was used for the extraction of PBMCs. Isolation of PBMCs was performed as previously described (16). In brief, heparinized peripheral blood was drawn from the jugular vein, immediately diluted with an equal volume of PBS without calcium and magnesium, and overlain 1:1 in a Percoll® solution. Following centrifugation at 400 x g for 45 min at room temperature, the cells at the interface between the blood plasma and the Percoll® solution were harvested and treated with 0.83% NH4Cl in a Tris-base buffer (pH 7.2) for 5 min to lyse the remaining erythrocytes. The resulting PBMCs were prepared for RNA isolation using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.).

Harvesting of bronchoalveolar fluid. Following euthanasia, bronchoalveolar (BAL) fluid was collected by lavaging lungs with 3 ml Hanks balanced salt solution (Thermo Fisher Scientific, Inc.) through an intratracheal tube. Subsequently, BAL fluid was centrifuged at 17,800 x g at (4˚C) for 5 min. Following centrifugation, total RNA from BAL fluid was extracted using TRIzol reagent.

Total RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR) amplification. Total RNA was extracted from mouse PBMCs, lung tissues and BAL fluid using TRIzol reagent, according to the manufacturer's protocol. RNA concentrations were measured using a microplate spectrophotometer (Epoch; BioTek Instruments, Inc., Winooski, VT, USA) at 260 nm. RNA quality was evaluated by performing electrophoresis on 1% agarose gel. Total RNA (1 μg) was reverse-transcribed into first-strand complementary DNA (cDNA) using Moloney murine leukemia virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) and random primers (9-mer; Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. Each cDNA sample (1 μl) was amplified with 10 μl 2X SYBR® Premix Ex Taq™ (Takara Bio, Inc.) and 10 pmol of each primer according to the manufacturer's protocol. qPCR-based amplification was performed using a 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following parameters: Denaturation at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 45 sec. Relative expression levels in each gene (normalized to that of 18S rRNA) were determined using RQ software (version 1.3; Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative expression (R) was calculated using the equation: $R = 2^{-(\Delta Cq\ sample - \Delta Cq\ control)}$ (17).

Histopathological analysis. Lung tissue was fixed using 10% formalin for 2 weeks at room temperature. The tissues were

embedded in paraffin, cut into sections (5-μm thick) and stained with hematoxylin and eosin (H&E). Tissue slides were deparaffinized at 65˚C for 5 min, put into xylene for 20 min. Deparaffinized slides was hydrated in a descending alcohol series (100, 90, 75 and 60% for 5 min) and tap water (10 min). Hydrated slides were stained with hematoxylin for 15 sec, washed with tap water for 10 min and stained with eosin for 30 sec at room temperature. Stained slides were dehydrated in an ascending alcohol series (60, 75, 80, 90 and 100%) and xylene for 5 min. Dehydrated slides were mounted with Canadian balsam. To investigate thickening of the bronchiolar wall, all tissue samples were examined and visualized using a light microscope (BX51; Olympus Corporation, Tokyo, Japan) at x200 magnification. Images was captured using an Olympus DP21 camera, DP controller and DP manager (Olympus Corporation).

Collection of volatile organic compounds in C. obtusa and *P. densiflora* panels. VOCCo and VOCPd were collected by trapping gas emitted from wood panels using a Sibata mini-pump (MP-Σ300; Sibata, Saitama, Japan). The composition of the obtained VOCs was analyzed by performing gas chromatography-mass spectrometry (GC-MS; Trace 1310/ISQ-LT; Thermo Fisher Scientific, Inc.). A gas chromatograph uses a column, through which different chemical constituents of a sample pass in a carrier gas at different rates depending on their various chemical and physical properties, and their interaction with a specific column filling, called the stationary phase. The function of the stationary phase in the column is to separate each component, causing each one to exit the column at a different time (retention time). A TR-5MS capillary column (30 cm x 0.25 mm x 0.25 μm; Thermo Fisher Scientific, Inc.) was used to entrap the gas, and He (1 ml/min; 25 psi) was used as the carrier gas. The analytes were desorbed in the injection port of the GC with an inlet temperature of 240˚C. The GC method was initiated with an initial oven temperature of 40˚C for 5 min. The temperature was then increased at 4˚C/min until it reached 200˚C, and was then held at 240˚C for 10 min (total run time, 90 min). The obtained VOC compounds were matched with a total ion chromatogram, and the NIST 11 (National Institute of Standards and Technology, Gaithersburg, MA, USA) and W9N08 mass spectral library (Wiley Publishing, Hoboken, NJ, USA). The quantity of each sample was analyzed with a calibration curve using standard chemicals.

Statistical analysis. The results of all experiments are presented as the mean ± standard deviation. Data were analyzed using one-way analysis of variance and Tukey's post hoc test for multiple comparisons. Data were ranked according to the results of these tests. All statistical analyses were performed using Graphpad software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of VOCCo and VOCPd on serum inflammatory cytokines IgE and PgE2 in LPS-treated mice. For investigation of the anti-inflammatory effects of VOCCo and VOCPd in a BALB/c mouse inflammation model, each mouse was administered with LPS i.p. Compared with the VE group, marked inductions of serum IgE and PgE2 levels were observed in the LPS-treated group. The LPS-elevated serum IgE level was recovered by DEX treatment (Fig. 1). Treatment with VOCCo also resulted in a decrease in the LPS-induced serum IgE level. In addition, the VOCCo and VOCPd treatments reduced PgE2 levels from that in the LPS group. These anti-inflammatory effects on serum IgE and PgE2 levels indicated that exposure to VOCCo or VOCPd can relieve a systemic inflammatory condition, suggesting that VOCCo and VOCPd could be used for inflammatory relief.

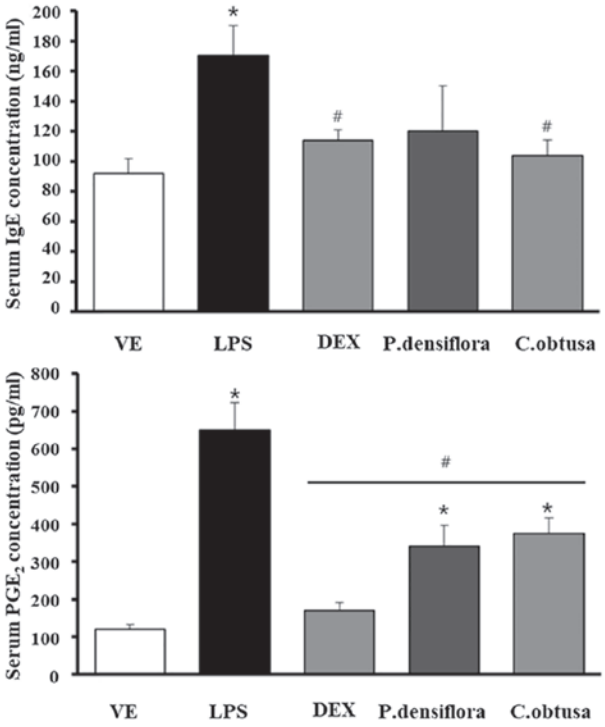

Figure 1. Effects of natural volatile organic compounds of *P. densiflora* and *C. obtusa* on serum concentrations of IgE and PgE2 in an LPS-induced (i.p.) inflammation model. The serum IgE and PgE2 levels in BALB/c mice were measured at the end of the treatment period using ELISA assays. Groups: VE, vehicle; LPS, negative control; LPS+DEX, positive control; *P. densiflora* and *C. obtusa* with LPS treatment, experimental groups. Results are expressed as the mean ± standard deviation. *P<0.05 vs. VE-treated group; #P<0.05 vs. LPS-treated group. IgE, immunoglobulin E; PgE_2, prostaglandin E2; VE, vehicle; LPS, lipopolysaccharide; DEX, dexamethasone.

Effects of VOCCo and VOCPd on the expression of inflammatory cytokines in PBMCs in LPS-treated mice. The present study investigated whether VOCCo and VOCPd can inhibit the expression of inflammatory cytokines in PBMCs of mice with LPS-induced inflammation. Expression levels of COX-2, TNF-α, IL-1β and IL-13 mRNA in PBMCs were examined by performing qPCR. Exposure to VOCCo or VOCPd recovered the COX-2, TNF-α, IL-1β and IL-13 mRNA expression levels from those in the LPS group (Fig. 2). The aforementioned effects on serum cytokines (Fig. 1) and the changes in the expression of inflammatory cytokines in PBMCs suggested that VOCCo and VOCPd have anti-inflammatory effects.

Effects of VOCCo and VOCPd on the expression of inflammatory cytokines in lung tissue in LPS-treated mice. The present

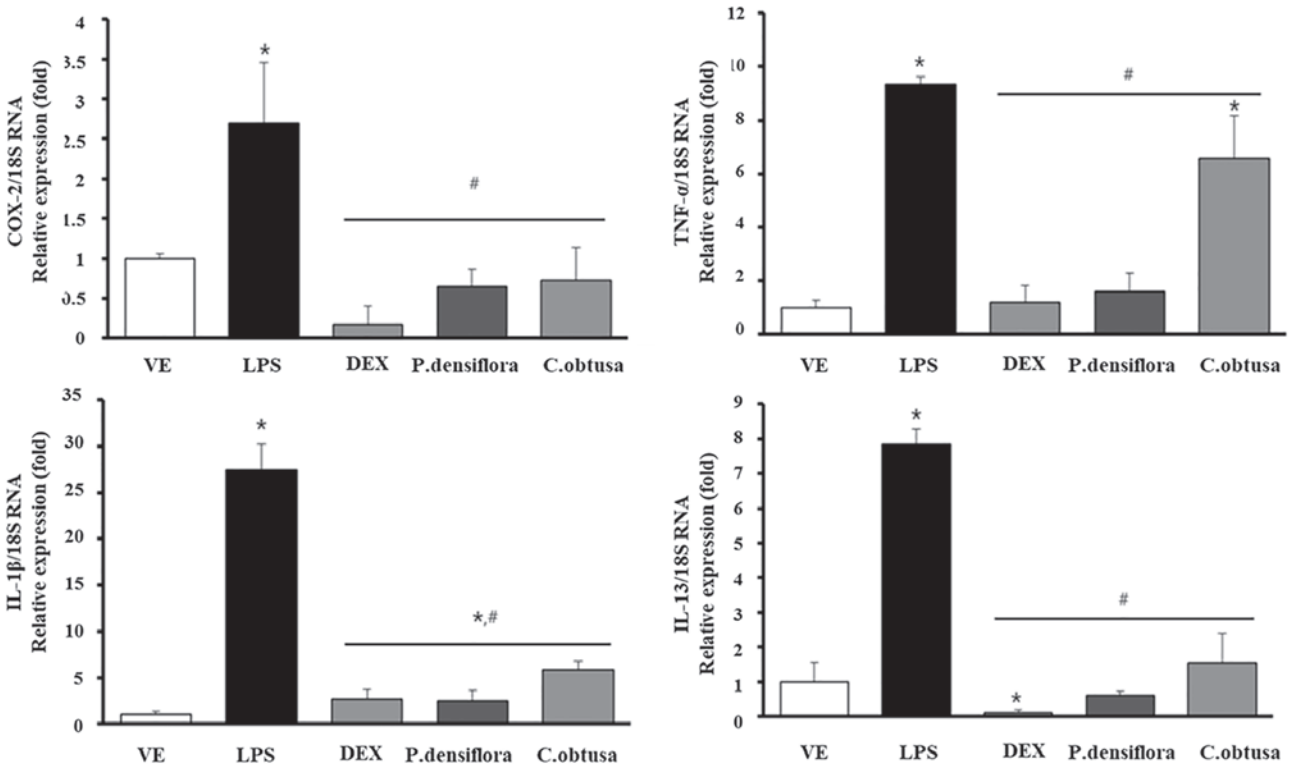

Figure 2. Effects of natural volatile organic compounds of *P. densiflora* and *C. obtusa* on expression of inflammatory cytokines (COX-2, TNF-α, IL-1β and IL-13) in peripheral blood mononuclear cells of LPS-treated mice (i.p.). Groups: VE, vehicle; LPS, negative control; LPS+DEX, positive control; *P. densiflora* and *C. obtusa* with LPS treatment, experimental groups. Results are expressed as the mean ± standard deviation. *P<0.05 vs. VE-treated group; #P<0.05 vs. LPS-treated group. COX-2, cyclooxygenase 2; TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide; DEX, dexamethasone; VE, vehicle.

study investigated whether VOCCo and VOCPd can inhibit the expression of pulmonary inflammatory cytokines in lung tissues of mice intranasally treated with LPS. Expression levels of COX-2, TNF-α and NF-κB mRNA in lung tissue were examined by performing qPCR. Exposure to VOCCo and VOCPd recovered the COX-2, TNF-α and NF-κB mRNA expression levels from those in the LPS-treated group (Fig. 3). The changes in expression of inflammatory cytokines in lung tissue suggested that VOCCo and VOCPd have anti-inflammatory effects in the lung (Fig. 3).

Effects of VOCCo and VOCPd on the expression of inflammatory cytokines in cells in BAL fluid. The present study investigated whether VOCCo and VOCPd treatment would reduce the expression of pulmonary inflammatory cytokines in cells in the BAL fluid of mice intranasally treated with LPS. The number of cells in the BAL fluid was increased by LPS treatment, but treatment with DEX, VOCPd and VOCCo recovered BAL fluid cell numbers (Fig. 4A). In addition, expression levels of COX-2, NF-κB, TNF-α, IL-1β and IL-13 mRNA in cells in the BAL fluid were examined by performing qPCR. Exposure to VOCCo and VOCPd recovered the COX-2, TNF-α and NF-κB mRNA expression levels from those in the LPS group (Fig. 4). Based on the changes in expression of inflammatory cytokines in cells in the BAL fluid, VOCCo and VOCPd have anti-inflammatory effects on the lung (Fig. 4B-F).

Effects of VOCCo and VOCPd on bronchial thickness in mice with LPS-induced airway inflammation. For observation of changes in bronchial thickness, lung tissues with airway inflammation resulting from i.n. LPS treatment were collected and stained with H&E. LPS treatment induced acute inflammation of lung tissue and increased bronchial wall thickness, compared with that in the VE group. Bronchial wall thickness was lower in the VOCCo and VOCPd groups than that in the LPS group (Fig. 5). The inflammation alleviatory effects of VOCCo and VOCPd on the lung were similar to that of the positive control, DEX, treatment. These results indicated that VOCCo and VOCPd can reduce LPS-induced airway inflammation in a mouse model.

Contents of natural VOCs from C. obtusa and *P. densiflora*. In order to identify the effective molecules in natural VOCs from wood for alleviation of systemic/local inflammation, VOCs in the *C. obtusa* and *P. densiflora* panels were collected and the quantity was analyzed (Tables I and II) and composition (Tables III and IV) of natural VOCs with GC-MS. *P. densiflora* emit VOCs 1.92-fold more than *C. obtusa* (2,068.29 ng/l for *C. obtusa* and 3,975.87 ng/l for *P. densiflora* for 4 weeks). The total mass of VOCs differ due to the species of wood. *P. densiflora* emitted more VOCs than *C. obtusa*. *P. densiflora* and *C. obtusa* emitted α-pinene and limonene which has anti-inflammatory effects. These results demonstrated that emitted VOCs from wood are able to alleviate systemic and local airway inflammation.

Discussion

Various medications, including corticosteroids, calcineurin inhibitors and immune-suppressants, can be used to control

Table I. Total mass of natural volatile organic compounds obtained from a closed system containing a wood panel (ng/l).

	C. obtusa				*P. densiflora*			
	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week
1	161.20	396.18	640.58	410.62	952.54	1150.97	633.45	822.63
2	186.84	966.62	247.61	536.52	1046.3	1063.94	976.08	1073.81
3	306.60	1004.80	578.40	768.92	1073.40	953.90	1261.95	918.628
Average	218.21	789.20	488.86	572.02	1,024.08	1,056.27	957.16	938.36

C. obtusa, *Chamaecyparis obtusa*; *P. densiflora*, *Pinus densiflora*.

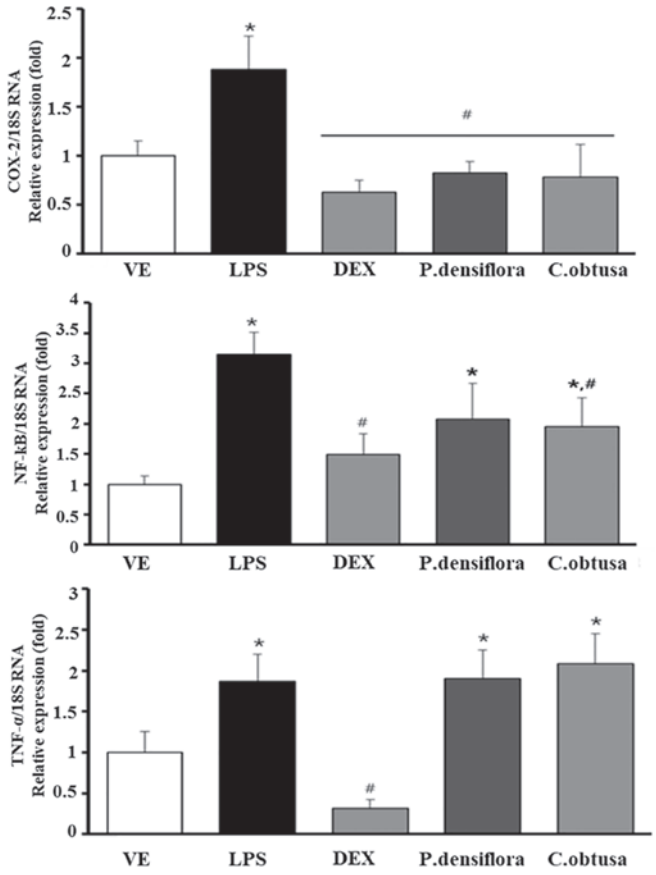

Figure 3. Effects of natural volatile organic compounds of *P. densiflora* and *C. obtusa* on expression of inflammatory cytokines (COX-2, NF-κB and TNF-α) in lung tissue of LPS-treated mice (i.n.). Groups: VE, vehicle; LPS, negative control; LPS+DEX, positive control; *P. densiflora* and *C. obtusa* with LPS treatment, experimental groups. Values are expressed as the mean ± standard deviation. *P<0.05 vs. VE-treated group; #P<0.05 vs. LPS-treated group. COX-2, cyclooxygenase 2; NF, nuclear factor; TNF, tumor necrosis factor; LPS, lipopolysaccharide; DEX, dexamethasone; VE, vehicle.

systemic or local inflammation (18). Corticosteroids are the most commonly used medications for the control of inflammation (18,19). However, long-term use of corticosteroids is associated with side effects and drug tolerance within the endocrine system (19,20). To avoid such side effects, prescriptions may indicate a short course of corticosteroid treatment (21). Numerous natural products from plants are being investigated to determine whether they can be used for the treatment of inflammation (22). Recently, herbal-based therapy has been a treatment focus in Asia and Europe due to its efficacy and safety. A wide variety of phenolic substances derived from plants have been reported to have marked antioxidant and anti-inflammatory activities, which may indicate their chemopreventive potential (23). In 2015, Tu Youyou won a Nobel prize for research into the anti-malaria drug artemisinin, which was derived from *Artemisia annua* (24). Recently, elemol, a compound derived from *C. obtusa*, was reported to function as an anti-inflammatory reagent, and elemol treatment is expected to prevent the onset of allergic diseases and to ameliorate allergic symptoms (9,10,25,26). In addition, certain VOCs from plants have been reported to have antioxidant and anti-inflammatory activities, and some can directly act as anti-inflammatory reagents (7,27). Although the mechanisms behind certain herbal remedy effects have not been fully elucidated, many such remedies have been reported to have scientific merit and clinical benefit in treating patients (7,8,25,28-30).

The present study investigated the effects of VOCCo and VOCPd in an LPS-induced mouse model of systemic and local inflammation. The LPS-increased serum IgE and PgE2 levels were significantly decreased by application of VOCCo and VOCPd in the LPS-induced systemic inflammation mouse model (Fig. 1). Exposure to VOCCo and VOCPd were revealed to result in successful alleviation of the effect of LPS on inflammation-related cytokines in the blood. Inflammatory cytokine mRNAs (COX-2, TNF-α, IL-1b and IL-13) from PBMCs of systemic LPS-treated mice were significantly inhibited by VOCCo and VOCPd treatment (Fig. 2), suggesting that the two VOCs may contribute toward suppression of LPS-stimulated cytotoxic- and helper-T-cell-mediated cytokine expression. Th1 and Th2 responses regulate several immune signaling cascades. Therefore, balancing the Th1/Th2 cytokine responses may be fundamental to the treatment of inflammation (31).

In addition to examining the systemic anti-inflammatory effect of VOCCo and VOCPd treatments, the alleviatory effects of VOCCo and VOCPd were investigated in a local inflammation mouse model created by i.n. LPS spray treatment. The LPS-increased level of the inflammatory T-cell-mediated cytokine COX-2 in lung tissue was significantly decreased by VOCCo and VOCPd treatments. Furthermore, the level of transcription factor for inflammatory cytokine NF-κB, increased by i.n. LPS, was also decreased by treatment with VOCCo and VOCPd. However, the LPS-increased TNF-α level was not decreased by treatment with VOCCo or VOCPd (Fig. 3). In

Table II. Natural volatile organic compound emissions per unit area (ng/cm^2/l).

	C. obtusa				*P. densiflora*			
	1st week	2nd week	3rd week	4th week	1st week	2nd week	3rd week	4th week
1	0.11	0.26	0.43	0.30	0.47	0.56	0.31	0.40
2	0.12	0.65	0.17	0.39	0.51	0.52	0.47	0.52
3	0.20	0.67	0.39	0.56	0.53	0.46	0.61	0.44
Average	0.14	0.53	0.33	0.42	0.50	0.51	0.46	0.45

C. obtusa, *Chamaecyparis obtusa*; *P. densiflora*, *Pinus densiflora*.

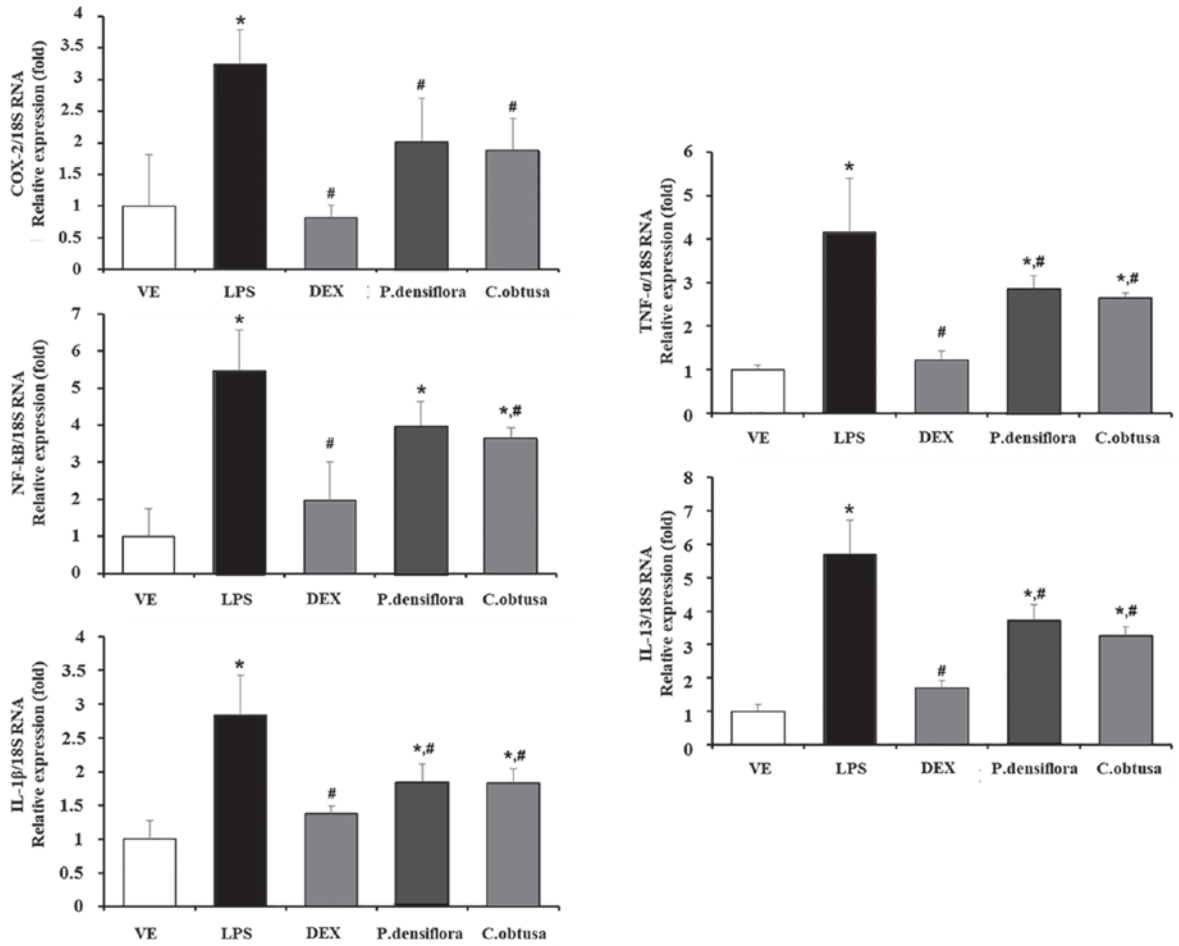

Figure 4. Effects of natural volatile organic compounds of *P. densiflora* and *C. obtusa* on cell numbers in BAL fluid and expression of cytokines (COX-2, NF-κB, TNF-α, IL-1β and IL-13) in immune cells in BAL fluid of LPS-treated mice (i.n.). Groups: VE, vehicle; LPS, negative control; LPS+DEX, positive control; *P. densiflora* and *C. obtusa* with LPS treatment, experimental groups. Values are expressed as the mean ± standard deviation. *P<0.05 vs. VE-treated group; #P<0.05 vs. LPS-treated group. BAL, bronchoalveolar; COX-2, cyclooxygenase 2; NF, nuclear factor; TNF, tumor necrosis factor; LPS, lipopolysaccharide; DEX, dexamethasone; VE, vehicle.

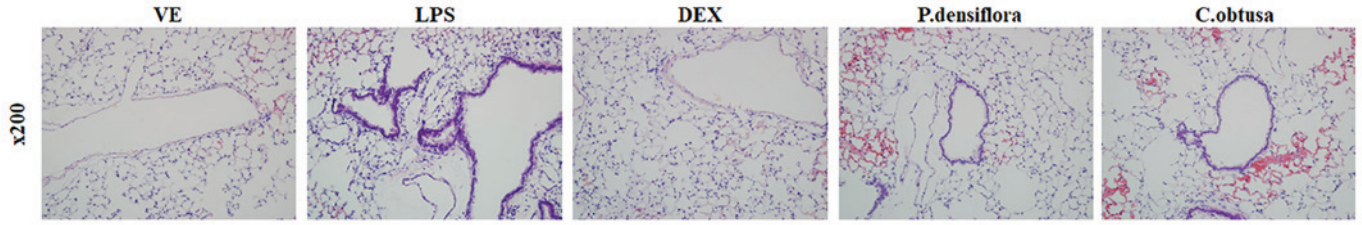

Figure 5. Effects of natural volatile organic compounds of *P. densiflora* and *C. obtusa* on airway inflammation. Bronchiole and lung tissues were stained with H&E. Histological examination of bronchial wall thickening in the LPS-treated group and the LPS plus volatile organic compounds of *P. densiflora* or *C. obtusa* treatment groups. (magnification, x200). LPS, lipopolysaccharide; H&E, hematoxylin and eosin.

Table III. Natural volatile organic compounds contents in *P. densiflora* wood panel (gas chromatography-mass spectrometry, ng/l).

RT	Chemical	1st week	2nd week	3rd week	4th week	Average
26.35	N-carproaldehyde	117.73	173.51	280.77	188.23	190.06
29.31	1-hexanol	9.58	31.51	47.89	40.70	32.42
29.31	2hexen-1-ol	0.00	3.13	1.38	0.71	1.30
29.74	2hexenal	3.06	10.84	7.25	0.00	5.29
31.38	heptanal	15.54	64.01	60.88	39.83	45.07
32.37	α-pinene	137.82	119.08	96.04	111.66	116.15
33.58	DL-camphene	3.86	7.85	6.18	10.40	7.07
33.58	Camphene	5.34	8.93	7.44	11.35	8.26
34.04	1-Heptanol	10.41	51.73	55.09	50.02	41.81
34.51	sabinene	35.18	13.50	1.20	3.28	13.29
34.52	β-Myrcene	55.04	24.37	6.72	9.54	23.92
35.06	β-pinene	86.76	56.49	38.50	44.70	56.61
36.01	α-phellandrene	16.72	13.59	1.75	5.34	9.35
36.04	3-hexenylester	0.60	0.14	0.27	0.10	0.27
36.10	octanal	2.88	29.99	36.44	16.36	21.42
36.51	α-terpinene	0.59	0.00	0.00	0.00	0.15
36.62	benzaldehyde	11.93	21.95	9.82	34.45	19.54
37.09	limonene	51.29	55.07	30.25	46.73	45.84
37.09	ocimene	49.72	22.29	24.14	53.72	37.47
37.40	Meta-cymene	0.00	29.61	4.68	44.12	19.60
37.40	Para-cymene	15.79	45.67	34.01	36.55	33.00
37.47	β-phellandrene	224.82	144.78	80.22	62.50	128.08
37.95	1,8-cineole	0.00	0.12	1.42	2.02	0.89
38.52	γ-terpinene	0.59	0.00	0.00	0.00	0.15
39.61	benzyl alcohol	0.06	0.00	0.00	0.00	0.01
40.01	terpinolene	12.59	7.38	2.68	4.81	6.87
40.39	nonanal	3.69	13.74	24.52	16.61	14.64
41.43	acetophenone	0.09	0.00	0.10	0.06	0.06
43.53	isopulegol	1.48	1.38	1.57	1.14	1.39
43.69	pinocarveol	1.60	6.01	5.74	9.88	5.81
44.65	4-Terpineol	1.88	2.67	1.47	1.84	1.97
44.65	terpinene-4-ol	1.14	0.43	0.00	0.00	0.39
44.83	borneol	1.05	0.00	0.00	1.38	0.61
45.21	α-terpineol	8.50	8.30	2.75	6.53	6.52
46.43	cryptone	4.92	13.47	10.82	7.95	9.29
46.61	carveol	0.00	0.53	0.00	0.00	0.13
47.55	verbenone	7.36	18.48	23.21	19.94	17.25
50.25	Isothymol (carvacrol)	0.96	0.00	0.00	0.00	0.24
52.10	α-Longipinene	12.73	4.99	4.94	4.76	6.85
52.71	geranyl acetate	0.05	0.00	0.01	0.00	0.01
53.65	β-ELEMENE	0.00	0.00	1.10	2.65	0.94
54.68	Azulene	0.02	0.00	0.00	0.00	0.00
55.80	β-farnesene	4.41	2.44	1.24	0.28	2.09
56.71	caryophyllene	85.96	38.41	36.85	39.74	50.24
56.75	α-cedrene	20.29	9.88	7.86	8.51	11.63
58.97	Humulene (a-caryophyllene)	0.08	0.00	0.00	0.00	0.02
	Total	1024.08	1056.27	957.16	938.36	993.96

RT, retention time; *C. obtusa*, *Chamaecyparis obtusa*; *P. densiflora*, *Pinus densiflora*.

Table IV. Natural volatile organic compound contents in *Chamaecyparis obtusa* wood panel (gas chromatography-mass spectrometry, ng/l).

RT	Chemical	1st week	2nd week	3rd week	4th week	Average
26.35	N-carproaldehyde	5.09	9.63	6.41	9.04	7.54
29.31	1-hexanol	0.00	0.00	3.91	0.25	1.04
29.31	2hexen-1-ol	0.00	0.10	0.63	0.00	0.18
29.74	2hexenal	0.00	9.79	0.00	3.27	3.26
31.38	heptanal	0.00	1.36	0.08	0.81	0.56
32.37	α-pinene	114.56	115.39	93.31	97.83	105.27
33.58	DL-camphene	0.36	3.48	1.86	3.61	2.33
33.58	Camphene	1.99	4.94	3.17	5.15	3.81
34.04	1-Heptanol	0.16	0.22	0.36	0.46	0.30
34.51	sabinene	1.37	15.25	1.29	2.85	5.19
34.52	β-Myrcene	6.20	26.85	5.64	8.60	11.82
35.06	β-pinene	3.50	4.32	2.99	3.11	3.48
36.62	benzaldehyde	0.00	0.00	0.34	0.00	0.08
37.09	limonene	18.08	118.87	56.40	67.05	65.10
37.09	ocimene	15.43	23.17	76.54	89.41	51.14
37.40	Meta-cymene	8.70	76.21	35.51	39.87	40.07
37.40	Para-cymene	7.93	85.26	37.99	43.05	43.56
37.47	β-phellandrene	2.04	8.95	5.65	9.62	6.57
38.52	γ-terpinene	0.00	0.16	0.00	0.00	0.04
39.99	linalool	0.46	0.91	0.00	0.00	0.34
40.01	terpinolene	1.76	15.45	4.91	7.90	7.51
40.39	nonanal	0.00	0.77	0.00	0.00	0.19
41.43	acetophenone	0.00	0.45	0.01	0.07	0.13
43.53	isopulegol	0.70	0.51	0.69	0.92	0.71
43.69	pinocarveol	0.00	3.39	3.50	2.70	2.40
44.65	4-Terpineol	4.03	29.65	17.24	20.41	17.83
44.65	terpinene-4-ol	3.28	48.99	24.64	32.31	27.30
44.83	borneol	3.39	38.80	19.27	25.60	21.76
45.21	α-terpineol	8.14	75.30	32.32	44.60	40.09
45.93	myrtenol	0.00	0.00	0.00	0.36	0.09
46.61	carveol	0.00	0.81	0.00	0.00	0.20
47.55	verbenone	0.95	9.69	5.45	4.76	5.21
49.19	bornyl acetate	1.23	18.34	10.36	9.85	9.94
49.19	isobornyl acetate	0.00	18.89	7.23	10.48	9.15
52.10	α-Longipinene	0.04	0.06	0.43	0.59	0.28
52.71	geranyl acetate	0.14	0.00	0.06	0.02	0.06
53.65	β-ELEMENE	8.44	23.00	29.89	24.26	21.40
55.80	β-farnesene	0.00	0.00	0.00	2.27	0.57
56.71	caryophyllene	0.00	0.00	0.41	0.67	0.27
57.81	aromadendrene	0.04	0.25	0.36	0.25	0.22
58.97	Humulene (a-caryophyllene)	0.19	0.00	0.00	0.00	0.05
	Total	218.22	789.20	488.86	572.02	517.07

RT, retention time.

order to provide a more detailed assessment of inflammatory changes associated with local inflammation of mouse lung, cells were collected from the BAL fluid. The number of cells in the BAL fluid was increased by i.n. LPS treatment, but the numbers were decreased following DEX, VOCPd and VOCCo treatments (Fig. 4A). The expression of the cytotoxic- and helper-T-cell-mediated cytokines, COX-2, NF-κB, TNF-α, IL-1b and IL-13, was also assessed (Fig. 4B-F). VOCCo and

VOCPd successfully inhibited expression of all examined T-cell-mediated inflammatory cytokines. Furthermore, VOCCo and VOCPd decreased the expression levels of IL-1b and IL-13 more than those of COX-2 and TNF-α. The results imply that VOCCo and VOCPd may have local inflammation alleviatory effects that can inhibit helper-T-cell-mediated pathways to a greater extent than their alleviatory effects on cytotoxic-T-cell-mediated pathways. Histopathological assessment of lung tissue via H&E staining indicated that LPS-induced airway inflammation was alleviated by VOCCo and VOCPd treatment, and that those anti-inflammatory effects were similar to those obtained by DEX treatment, suggesting that the two tested VOCs can reduce airway inflammation.

To initiate elucidation of the two VOCs, gases discharged by wood planks of *C. obtusa* and *P. densiflora* were collected using a Sibata minipump. The quantity of VOCs from the two wood planks were different. Despite the difference in the quantity of VOCs from the wood planks, the effect of the VOCs did not differ as much as the quantity of VOCs. The GC-MS analysis results revealed that VOCCo contains 41 volatile compounds and VOCPd contains 46 volatile compounds. Each of these VOCs contained monoterpenes and sesquiterpenes, with limonene and α-pinene being particularly abundant. The main constituents of VOCCo and VOCPd were also similar. Terpenes and sesquiterpenes are well-known anti-inflammatory compounds (32,33), but other compounds, including α-terpineol and borneol also have anti-inflammatory effects (11,12). Among the pinene family, *P. densiflora* and *C. obtusa* emitted α-pinene and limonene, which have anti-inflammation effect (10,34). These results demonstrated that emitted VOCs from wood may alleviate systemic and local airway inflammation.

In conclusion, the results of the present study have demonstrated anti-inflammatory effects of VOCCo and VOCPd in systemic and local inflammation mouse models. Based on the results of the present study, VOCCo and VOCPd may contain potent therapeutic compounds that may be beneficial in the treatment of systemic and local inflammations, as well as in the regulation of serum IgE and PgE2 levels, and COX-2, TNF-α, IL-1b and IL-13 T-cell-derived cytokine levels in inflammatory lesions.

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Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CA, JHL and EBJ were responsible for study design. CA and JHL conducted the study. CA, JHL and EBJ analyzed the animal experiment data. JWK, MJP and SSL analyzed and interpreted the GC/MS data. CA and EBJ drafted the manuscript. EBJ reviewed the integrity of the data analysis. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the IACUC of Chungbuk National University for all experimental procedures (approval no. CBNUA699-15-07).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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