Anti-inflammatory effects of natural volatile organic compounds from *Pinus koraiensis* and *Larix kaempferi* in mouse model

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Abstract

Natural volatile organic compounds (VOCs) extracted from conifers such as *P. koraiensis* and *L. kaempferi* have long been studied for their anti-oxidant, anti-proliferative, and anti-inflammatory effects. To evaluate the anti-inflammatory effects of VOCs from *P. koraiensis* and *L. kaempferi*, lipopolysaccharide (LPS) was administered to generate a mouse model for inflammation by the nasal route to the lungs and intraperitoneally to the whole body. VOCs of *P. koraiensis* and *L. kaempferi* were exposed to the mice by standardized wood panels with closed system. Increased levels of serum IgE and PGE2 were observed after exposure to dexamethasone and VOCs. We further determined the expression levels of inflammatory cytokine mRNA in the LPS-induced inflammation model by the reverse transcription quantitative polymerase chain reaction. Furthermore, the levels of cyclooxygenase-2, tumor necrosis factor-α, interleukin-1β, and interleukin-13 were determined in peripheral blood mononuclear cells. Those inflammatory cytokines and the key enzyme for inflammation cyclooxygenase-2 expression in PBMCs were strongly reversed by dexamethasone and VOCs. Lung tissues after nasal LPS exposure showed increased cytokine mRNA expressions which were suppressed by treatment with dexamethasone and VOCs. Furthermore, the damage induced by LPS was attenuated by dexamethasone and VOCs. In conclusion, the results from the present study indicate that VOCs of *P. koraiensis* and *L. kaempferi* have a therapeutic potential in the treatment or prevention of local and systemic inflammation due to their immunosuppressive effects.

Keywords: *Pinus koraiensis*, *Larix kaempferi*, inflammation, cytokine, lungs

Introduction

Inflammatory diseases are associated with cytokine and adhesion molecule expression levels[1–2]. Asthma and chronic obstructive pulmonary disease are obstructive airway diseases involving chronic inflammation of the respiratory tract, but having two distinct modes of action with different patterns of inflammatory cell and mediator involvement being observed[3]. Allergic asthma is characterized by airway inflammation and associated with increased levels of inflammatory cytokines and chemokines. The authors reported no conflict of interests.

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hyper-responsiveness to a variety of specific and nonspecific stimuli including chronic pulmonary eosinophilia, elevated serum immunoglobulin E (IgE), and excessive airway mucus production. IgE is an important mediator of allergic reactions including allergic asthma and plays a central role in asthma-related symptoms, airway inflammation, and possibly airway remodeling. The pathophysiology of asthma is thought to be mediated by CD4+ T lymphocytes producing a type 2 cytokine profile. Binding of IgE molecules to the surface of an immune cell sensitizes the cell to the specific allergen. The sensitized immune cell immediately expresses an inflammatory response, including the release of histamine, which induces the early phase of an allergic reaction. After IgE release, the immune cells synthesize other inflammatory molecules such as interleukins (ILs) and prostaglandins.

Immunosuppressive drugs are currently being used to control undesired immune responses, such as autoimmune diseases, allergies, and allograft rejection. FK506, cyclophosphamide (CTX) and prednisone are typical immunosuppressive drugs that are being used in the clinical treatment for many years.

Pinus koraiensis (P. koraiensis) also known as the Korean pine, is a species of Pinus in the Pinaceae family. Most Pinus species grow in the northern hemisphere and some have been used in folk medicine for a long time. Previous studies have reported the anti-oxidant and anti-inflammatory activities of the pine pollen and the anti-nociception and anti-inflammatory effects of the pine bark and its essential oil. Larix kaempferi (L. kaempferi) belongs to the family Pinaceae in the Pinopsida class. It is a medium-sized to large deciduous coniferous tree reaching 20–40 meters in height, with a trunk up to 1 meter in diameter. The extract oil from L. kaempferi has been used in folk remedies to reduce allergic reactions. Several previous studies have examined the alleviating effect of P. koraiensis on allergic dermatitis in a mouse model. In addition, L. kaempferi effectively suppresses the levels of serum IgE and proinflammatory cytokines such as ILs, and affects mast cell appearance. L. kaempferi, regarded as an effective medicinal plant, contains active terpene compounds with effective pharmacological molecules.

Volatile organic compounds (VOCs) are reported to be associated with asthma and immune responses. However, to the best of our knowledge, there are no reports indicating that exposure to VOCs of P. koraiensis or L. kaempferi reduces inflammatory symptoms and, in particular, affects specific IgE and cytokine release. In addition, the mechanisms underlying the alleviative effects of VOCs have not been elucidated. The aim of the current study was to determine whether exposure to VOCs improves the inflammation in a mouse LPS-induced inflammatory model and is a suitable candidate for use as a pharmaceutical and functional material.

Materials and methods

Animal experiments

BALB/c mice (7 weeks old) were purchased from Koatech (Pyeongtaek, Republic of Korea) and housed in polycarbonate cages with P. koraiensis or L. kaempferi panels and corn cob bedding, in an environmentally controlled room (temperature, (23±2) °C; relative humidity, (50±10)%; frequent ventilation; and a 12:12 hours light-dark cycle). The animal experiments were approved by the Chungbuk National University Animal Care and Use Committee (Cheongju, 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, St. Louis, MO, USA) dissolved in phosphate buffered saline (PBS) was used for the induction of inflammation in BALB/c mice. LPS was regularly administered (100 μg/kg i.p. with 100 μL of PBS, 1 mg/kg i.n. with 50 μL of PBS) via the intraperitoneal and intranasal route for 7 days. The VOC-untreated groups included the vehicle-treated (VE) group, the LPS-treated (LPS) group, and the LPS with anti-inflammatory drug dexamethasone-treated (DEX) group. The VOC-treated groups included the LPS+VOC of P. koraiensis group (P. koraiensis panel, 1 026 cm²) and the LPS+VOC of L. kaempferi group (L. kaempferi panel, 1 026 cm²). After completion of treatments, the mice were sacrificed by ether inhalation, and the lungs and blood were collected for analysis.

Serological analysis of serum

At the end of the experiment, blood samples were collected directly from the inferior vena cava using a 1 mL syringe. Serum was obtained by centrifuging the blood at 3 000 g for 10 minutes at 4 °C and stored at −70 °C for further use. Serum IgE and prostaglandin E2 (PGE2) levels were measured using the Mouse IgE Ready-Set-Go ELISA kits (eBioscience, San Diego, CA, USA) and Mouse PGE2 ELISA Ready-Set-Go kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were
isolated from whole blood of each treatment group (VE, LPS, DEX, LPS+VOC of P. koraiensis, and LPS+VOC of L. kaempferi) as previously described[17]. Briefly, peripheral blood drawn from the inferior vena cava was collected in heparin vials, immediately diluted with an equal volume of PBS without calcium and magnesium and overlaid 1:1 on a Percoll® solution. After centrifugation at 400 g for 45 minutes at room temperature, the cells at the interface between the plasma and Percoll solution were harvested and treated with 0.83% NH₄Cl in a tris-base buffer (pH7.2) for 5 minutes to lyse the remaining erythrocytes. The resulting PBMCs were prepared for RNA isolation with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA).

Total RNA extraction and real-time polymerase chain reaction amplification

Total RNA was extracted from mouse skin using TRIzol reagent according to the manufacturer's instructions. RNA concentrations were assessed using a microplate spectrophotometer (Epoch; BioTek Instruments, 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 the Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) and random primers (9-mer; Takara Bio, Otsu, Shiga, Japan). Each cDNA sample (1 μL) was amplified with 10 μL of 2 × SYBR Premix Ex Taq (Takara Bio) and 10 pmol/L of each primer. Quantitative polymerase chain reaction (PCR)-based amplification was performed using a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) with the following parameters: denaturation at 95 °C for 5 minutes followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 45 seconds. Relative expression levels in each gene (normalized to that of 18S rRNA) were determined using the RQ software (version 1.3; Applied Biosystems).

Statistical analysis

The results of all experiments were presented as mean±standard deviation (SD) values. Data were analyzed with a nonparametric one-way analysis of variance (ANOVA) and Tukey's test for multiple comparisons, and then ranked accordingly. All statistical analyses were performed using the Graphpad™ software. P value <0.05 was considered statistically significant.

Results

Effects of VOCs of P. koraiensis and L. kaempferi on serum inflammatory cytokines IgE and PGE₂ in LPS-treated mice

To investigate the anti-inflammatory effects of VOCs of P. koraiensis in the inflammatory BALB/c mouse model, each mouse was administered LPS via the intraperitoneal route. Compared to the VE group, marked induction of serum IgE and PGE₂ was observed in the LPS-treated group. The elevated serum IgE levels were recovered in the DEX group (Fig. 1). Treatment with VOCs of P. koraiensis also resulted in a decrease in serum IgE levels. In addition, treatment with VOCs of P. koraiensis and L. kaempferi reduced the PGE₂ levels as compared to those in the LPS-treated group. These anti-inflammatory effects on serum IgE and PGE₂ levels indicated that exposure to VOCs of P. koraiensis and L. kaempferi relieved the systemic inflammatory response.
condition, suggesting that VOCs of both *P. koraiensis* and *L. kaempferi* can be used for their continuous inflammation-relieving effect.

**Effects of VOCs of *P. koraiensis* and *L. kaempferi* on the expression of inflammatory cytokines in peripheral blood mononuclear cells in LPS-treated mice**

We investigated whether the VOCs of *P. koraiensis* and *L. kaempferi* inhibited the expression of inflammatory cytokines in PBMCs of LPS-treated mice. Expression levels of COX-2, TNF-α, IL-1β, and IL-13 mRNA in PBMCs were examined by performing real-time PCR (*Fig. 2*). Exposure to VOCs of both *P. koraiensis* and *L. kaempferi* recovered the COX-2, TNF-α, IL-1β, and IL-13 mRNA expression levels as compared to the LPS-treated group. Based on the observed effects on serum cytokines (*Fig. 1*) and the changes in expression of inflammatory cytokines in PBMCs, the VOCs of both *P. koraiensis* and *L. kaempferi* showed anti-inflammatory properties.

**Effects of VOCs of *P. koraiensis* and *L. kaempferi* on reducing expression of inflammatory cytokines in lungs in nasal-LPS treated mice**

We investigated whether the VOCs of *P. koraiensis* and *L. kaempferi* inhibited the expression of pulmonary inflammatory cytokines in PBMCs of nasal-LPS treated mice. We examined the expression levels of COX-2, TNF-α, and NF-κB mRNA in lung cells by performing real-time PCR (*Fig. 3*). Exposure to VOCs of both *P. koraiensis* and *L. kaempferi* recovered the COX-2, TNF-α, and NF-κB mRNA expression levels compared to that in the LPS-treated group. These results indicated that VOCs of both *P.
**koraiensis** and *L. kaempferi* exerted an in vivo anti-inflammatory effect in the lungs.

**Effects of VOCs of *P. koraiensis* and *L. kaempferi* on attenuating lung damage of nasal-LPS treated mice**

For evaluation of lung damage, especially bronchus thickness and mucus secretion, lung (bronchial) tissues damaged by LPS treatment were stained with H&E. LPS induced an increase in bronchial wall thickness compared to that in the vehicle-treated mice, while the increased bronchial wall thickness was recovered by dexamethasone and VOCs of *P. koraiensis* and *L. kaempferi* (*Fig. 4*). Therefore, anti-inflammatory effects of the VOCs of *P. koraiensis* and *L. kaempferi* on nasal LPS induced lung inflammation provided a promising potential for inflammation recovery similar to the positive control, dexamethasone.

**VOC contents of *P. koraiensis* and *L. kaempferi***

For one month, the terpene contents in VOCs were analyzed every other day by entrapping the gas. Quantitative analysis of the components of VOCs of *P. koraiensis* and *L. kaempferi* were shown in *Table 1*. The concentrations and amount of the components that diffused from *L. kaempferi* were higher than that from *P. koraiensis*. Both VOCs contained alpha-pinene, beta-pinene, carproaldehyde, limonene, terpinolene, alpha-terpineol, borneol, and camphor. In addition, *P. koraiensis* emited 3-carene, alpha-pinene, 1, 8-cineole, isopulegol, pinocarveol, 4-terpineol, verbenone, caryophyllene, and alphacedrene, whereas *L. kaempferi* emited beta-farnesene. The concentrations of VOCs also differed in a closed system; *P. koraiensis* contained 564.37 ng/L VOC and *L. kaempferi* contained 80.91 ng/L VOC.

**Discussion**

Various medications are used to control systemic or local inflammation, such as corticosteroids, calcineurin inhibitor, and immune-suppressants [19], of which corticosteroids are the most widely used [19–20]. However, long term use of corticosteroids is...
Table 1 Contents in VOCs in P. koraiensis and L. kaempferi panel (ng/L)

<table>
<thead>
<tr>
<th>Retention time</th>
<th>Compound</th>
<th>Pinus koraiensis</th>
<th>Larix kaempferi</th>
</tr>
</thead>
<tbody>
<tr>
<td>26.3</td>
<td>N-carproaldehyde</td>
<td>131.24</td>
<td>50.57</td>
</tr>
<tr>
<td>29.1</td>
<td>1-hexanol</td>
<td>16.05</td>
<td>7.55</td>
</tr>
<tr>
<td>29.68</td>
<td>2-hexenal</td>
<td>0.42</td>
<td>0.17</td>
</tr>
<tr>
<td>31.4</td>
<td>Heptanal</td>
<td>5.29</td>
<td>4.00</td>
</tr>
<tr>
<td>32.31</td>
<td>α-pinene</td>
<td>74.19</td>
<td>85.64</td>
</tr>
<tr>
<td>33.51</td>
<td>Camphene</td>
<td>9.06</td>
<td>2.97</td>
</tr>
<tr>
<td>33.95</td>
<td>1-Heptanol</td>
<td>0.98</td>
<td>0.11</td>
</tr>
<tr>
<td>34.99</td>
<td>β-pinene</td>
<td>39.48</td>
<td>29.86</td>
</tr>
<tr>
<td>36.17</td>
<td>3-carene</td>
<td>54.38</td>
<td>-</td>
</tr>
<tr>
<td>36.43</td>
<td>α-terpineene</td>
<td>0.06</td>
<td>-</td>
</tr>
<tr>
<td>36.53</td>
<td>Benzaldehyde</td>
<td>9.88</td>
<td>0.96</td>
</tr>
<tr>
<td>36.6</td>
<td>2-ethyl-1-hexanol</td>
<td>-</td>
<td>0.23</td>
</tr>
<tr>
<td>37.02</td>
<td>Limonene</td>
<td>60.11</td>
<td>13.64</td>
</tr>
<tr>
<td>37.02</td>
<td>Ocimene</td>
<td>57.96</td>
<td>trace</td>
</tr>
<tr>
<td>37.33</td>
<td>Meta-cymene</td>
<td>23.32</td>
<td>-</td>
</tr>
<tr>
<td>37.33</td>
<td>Para-cymene</td>
<td>26.09</td>
<td>trace</td>
</tr>
<tr>
<td>37.4</td>
<td>β-phellandrene</td>
<td>1.29</td>
<td>11.99</td>
</tr>
<tr>
<td>37.84</td>
<td>1, 8-cineole</td>
<td>3.05</td>
<td>-</td>
</tr>
<tr>
<td>39.93</td>
<td>Terpinolene</td>
<td>10.41</td>
<td>1.93</td>
</tr>
<tr>
<td>40.3</td>
<td>Nonanal</td>
<td>12.16</td>
<td>16.29</td>
</tr>
<tr>
<td>43.38</td>
<td>Isopulegol</td>
<td>0.50</td>
<td>-</td>
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<tr>
<td>43.56</td>
<td>Pinocarveol</td>
<td>1.27</td>
<td>-</td>
</tr>
<tr>
<td>44.65</td>
<td>Borneol</td>
<td>4.98</td>
<td>0.66</td>
</tr>
<tr>
<td>44.65</td>
<td>Camphor</td>
<td>5.08</td>
<td>0.04</td>
</tr>
<tr>
<td>44.65</td>
<td>4-Terpineol</td>
<td>0.53</td>
<td>-</td>
</tr>
<tr>
<td>45.1</td>
<td>α-terpineol</td>
<td>16.52</td>
<td>3.27</td>
</tr>
<tr>
<td>47.44</td>
<td>Verbenone</td>
<td>0.69</td>
<td>-</td>
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<tr>
<td>49.38</td>
<td>Bornyl acetate</td>
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<td>0.10</td>
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<tr>
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<td>Isobornyl acetate</td>
<td>2.47</td>
<td>0.45</td>
</tr>
<tr>
<td>51.98</td>
<td>α-Longipinene</td>
<td>9.76</td>
<td>0.18</td>
</tr>
<tr>
<td>52.76</td>
<td>Geranyl acetate</td>
<td>0.52</td>
<td>-</td>
</tr>
<tr>
<td>55.64</td>
<td>β-farnesene</td>
<td>-</td>
<td>1.85</td>
</tr>
<tr>
<td>56.55</td>
<td>Caryophyllene</td>
<td>20.76</td>
<td>-</td>
</tr>
<tr>
<td>56.55</td>
<td>α-cedrene</td>
<td>4.42</td>
<td>-</td>
</tr>
<tr>
<td>66.33</td>
<td>Globulol</td>
<td>-</td>
<td>0.19</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>604.87</td>
<td>232.65</td>
</tr>
</tbody>
</table>

The current study investigated the effects of VOCs of P. koraiensis and L. kaempferi in an LPS-induced systemic and local inflammation mouse model. IgE is an immunoglobulin that plays a role in acute and chronic inflammatory allergic diseases[30]. Lowered IgE implicates for alleviation of inflammation. The treatment with P. koraiensis showed decreased tendency of IgE which meant certain alleviation of inflammatory response induced by LPS. The induced serum PGE$_2$ levels significantly decreased after exposure to the VOCs in the LPS-induced systemic inflammation mice model. Exposure to VOCs of both P. koraiensis and L. kaempferi resulted in successful alleviation of the inflammatory cytokines in the bloodstream suggesting that they may contribute to the suppressed stimulation of T cell-mediated cytokines. Similar to the systemic concentrations of inflammatory cytokines, the mRNA expressions of inflammatory cytokines (COX-2, TNF-α, IL-1β and IL-13) from the PBMCs of systemic LPS-treated mice were also significantly inhibited. COX-2 and pro-inflammatory cytokines such as TNF-α, IL-1β and IL-13 are expressed by many cells including macrophages, NK cells, monocytes, and neutrophils. These enzyme and cytokines are involved in the proliferation, differentiation, and apoptosis of cells as well as immune cell migration toward inflammatory sites; hence, inhibition of these cells suggested that the VOCs of P. koraiensis and L. kaempferi may contribute to the suppressed stimulation of cytotoxic- and helper T cell-mediated cytokines. Since Th1 and...
Th2 types of reactions mutually regulate several immune signaling cascades, balancing the Th1/Th2 types of reactions may be fundamental to the treatment of inflammation[31]. Prostaglandins are lipid autacoids derived from arachidonic acid, involving the inflammatory response. They are generated from arachidonate by the action of cyclooxygenase (COX) isoenzymes[32]. The decreased expression of COX-2 resulted in the decreased serum concentration of its enzymatic product PGE$_2$ as presented in Fig. 1B.

Following the systemic anti-inflammatory effects of VOCs of both $P$. koraiensis and L. kaempferi, we investigated their effect on the alleviation of local inflammation in a mouse model by inoculating LPS as an intranasal spray. In the lung tissue, the inflammatory T-cell mediated cytokine COX-2 significantly decreased after exposure to the VOCs. Also, exposure to the VOCs resulted in decreased levels of the transcription factor for inflammatory cytokine NF-$\kappa$B. However, no decrease was observed in the levels of TNF-$\alpha$. Release of TNF-$\alpha$ from human PBMC are correlated with documented inflammatory activity. But both P. koraiensis and L. kaempferi does not decrease TNF-$\alpha$ in both PBMCs and lung tissues[33]. The levels of NF-$\kappa$B and IKK$\alpha$, which initiate the transcription of inflammatory cytokines, decreased after exposure to the VOCs of L. kaempferi and P. koraiensis, thereby indicating that the VOCs were capable of blocking the inflammatory cascades at the transcriptional level. Finally, treatment with dexamethasone and VOCs of P. koraiensis and L. kaempferi resulted in the recovery of bronchial wall thickness from nasal LPS treatment.

The VOCs of $P$. koraiensis and L. kaempferi were collected by trapping the gases using a SHIBATA minipump. The GC-MS analysis revealed that the VOCs of P. koraiensis contained 41 volatile compounds, whereas the VOCs of L. kaempferi contained 46 volatile compounds. The main contents of both VOCs were similar[16–17] and consisted of monoterpenes and sesquiterpenes, especially abundant quantities of limonene and $\alpha$-pinene. Terpenes and sesquiterpenes are well-known anti-inflammatory compounds[34–35]. Both VOCs contained alpha-pinene, beta-pinene, carpopaldehyde, limonene, terpinolene, $\alpha$-terpineol, borneol, and camphor. All these compounds are reported to exert anti-inflammatory effects[36–41].

In conclusion, this study demonstrates the anti-inflammatory effects of VOCs from both P. koraiensis and L. kaempferi in a systemic and local inflammation mouse model. The VOCs decrease the expression of inflammatory cytokines by dissociating NF-$\kappa$B from IxB via IKK$\alpha$ expression. Our results further suggest that the VOCs could be potent therapeutic compounds for treating inflammation, by regulating the serum IgE and PGE$_2$ levels and T cell-derived cytokines such as TNF-$\alpha$, IL-1$\beta$, and IL-13 and isoenzyme COX-2 in inflammatory lesions of systemic and local inflammation mouse model.

Acknowledgments

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References


