

Antiasthmatic Activity of Luteolin-7-*O*-glucoside from *Ailanthus altissima* through the Downregulation of T Helper 2 Cytokine Expression and Inhibition of Prostaglandin E₂ Production in an Ovalbumin-Induced Asthma Model

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Previously, we reported that an ethanol extract of *Ailanthus altissima* has antiinflammatory activity in an ovalbumin (OVA)-sensitized murine asthmatic model. To determine the biological compounds from this plant, luteolin-7-*O*-glucoside (L7G) was isolated and its antiasthmatic activity was evaluated in an *in vivo* murine asthmatic model. L7G (10 to 100 mg/kg, *per os* (*p.o.*)) reduced the amount of eosinophil infiltration in bronchoalveolar lavage (BAL) fluid in a dose-dependent manner. In comparison, dexamethasone (5 mg/kg, *p.o.*), which was used as a positive control, also strongly inhibited the number of infiltrating eosinophils. L7G inhibited both the prostaglandin E₂ (PGE₂) and serum immunoglobulin E level in BAL fluid in a dose-dependent manner. In addition, L7G inhibited the transcript profiles of interleukin (IL)-4, IL-5, and IL-13 mRNA expression levels in the murine asthma model, as determined using reverse transcription-polymerase chain reaction (RT-PCR). These results suggest that the antiasthmatic activity of L7G in OVA-induced lung inflammation may occur in part *via* the downregulation of T helper 2 cytokine transcripts as well as the inhibition of PGE₂ production.

Key words luteolin-7-glucoside; bronchoalveolar lavage; asthma; prostaglandin E₂; T helper 2 cytokine

Allergen-induced bronchial asthma is a complex inflammatory disorder characterized by airway hyperresponsiveness (AHR), eosinophilia, and mucus hypersecretion by goblet cells. Numerous eosinophil- and T helper 2 (Th2) lymphocyte-produced cytokines, such as interleukin (IL)-4, IL-5, and IL-13, *via* Th2 cell production play key roles in AHR and serum immunoglobulin E (IgE) in animal models.^{1,2)} In addition to its role in the activation of mature eosinophils, IL-5 is the principal Th2 cytokine involved in terminal differentiation and proliferation of eosinophil precursors.^{3,4)} Further exposure to allergens leads to mast cell activation and release of histamine and lipid mediators such as prostaglandins and leukotrienes, which are important in an inflammatory response.^{5,6)}

Flavonoids are widely distributed polyphenol compounds in the plant kingdom. Some flavonoid derivatives were previously reported to possess various biological functions such as antiinflammatory, antiallergic and antiasthmatic activities *in vitro* and *in vivo*.⁷⁾ Numerous studies have demonstrated that the antiinflammatory activity of certain flavonoids may be due to inhibition of enzyme activity involved in the arachidonic acid cascade, enzymes such as phospholipase A₂ (PLA₂), cyclooxygenase (COX), and lipoxygenases (LOXs).⁸⁾ Previous reports found that luteolin-7-*O*-glucoside (3,4,5-hydroxyl-flavone-7-*O*-glucoside, L7G) from dandelion flowers suppresses inducible nitric oxide synthase (iNOS) and COX-2 protein expression in RAW264.7 cells,⁹⁾ COX-2 inhibitory activity,¹⁰⁾ matrix metalloproteinase-2 and -9 inhibitory activity,¹¹⁾ and *in vivo* antiinflammatory¹²⁾ and gastroprotective activities.¹³⁾ Although many biological activities of L7G have been reported, the anti-asthmatic activity of L7G has not been reported to date. As part of an ongoing investigation of new biologically active compounds from me-

dicinal herbs, the present study was designed to determine the mechanism underlying the antiasthmatic effects of L7G isolated from *Ailanthus altissima*.

MATERIALS AND METHODS

Plant Material L7G was isolated from the leaves and branches of *A. altissima*. The chemical structure of the isolated compound was established to be luteolin-7-*O*-glucoside (Fig. 1) by comparison of ¹H- and ¹³C-NMR data with those reported previously.¹⁴⁾ The purity of this compound is greater than 99.5% based on HPLC analysis.

Animals Female BALB/c mice (16–20 g) were obtained from Hyochang Science (Daegu, Korea) and fed with laboratory chow (Purina, Seoul, Korea) and water *ad libitum*. Animals were acclimatized in a specific pathogen-free animal facility under the conditions of 20–22 °C, 40–60% relative humidity, and a 12h/12h (light/dark) cycle at least for 7 d.

Preparation of Allergic Airway Asthmatic Model and L7G Treatment Six-week-old female BALB/c were sensitized by intraperitoneal (i.p.) administration on days 0, 7, and 14 with 100 µg/ml of ovalbumin (OVA) in phosphate buffered saline (PBS) mixed with equal volumes of alum (Pierce Biotechnology, Rockford, IL, U.S.A.) as an adjuvant

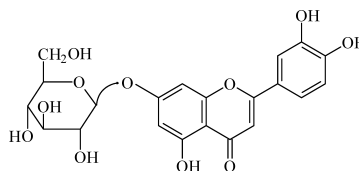


Fig. 1. Chemical Structure of Luteolin-7-*O*-glucoside (L7G)

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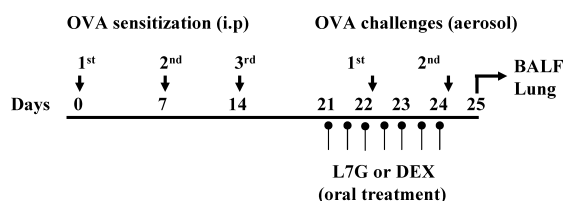


Fig. 2. Immunization Protocol

All mice were sensitized intraperitoneally (i.p.) with 200 μ l (100 μ l of OVA and 100 μ l of alum) on days 0, 7, and 14. The mice were then exposed to aerosolized OVA (1% OVA in PBS) or PBS for 1 h on days 22 and 24. The mice were orally administered L7G or dexamethasone (DEX) seven times at 12-h intervals from days 21 to 23. On day 24, 18 h after the last aerosol exposure, BALF and lungs were obtained.

in a total volume of 200 μ l. On days 22 and 24, mice were exposed to aerosolized OVA (1% OVA in PBS) or PBS for 1 h. The mice were administered with L7G or dexamethasone (DEX, Sigma) 7 times orally every 12 h from 1 d before the first challenge to 1 h before the second challenge. The challenge and treatment protocol are shown in Fig. 2.

Bronchoalveolar Lavage Procedure Eighteen hours after the last aerosol challenge, the mice were killed. To obtain bronchoalveolar lavage fluid (BALF), the mice were anesthetized and a tracheal cannula was inserted in each mouse *via* a midcervical incision. The airway of each mouse was lavaged three times with 1.5 ml of PBS. BALF was immediately centrifuged (2 min, 4°C, 160 $\times$ *g*). After removing the supernatant, the cells were resuspended in 0.5 ml of PBS. Ten microliters of the BALF as used to determine total leukocytes with a hemocytometer. The remaining samples were cytopun for routine Diff-Quick staining (Sysmex, Kobe, Japan), and the number of eosinophils was determined by counting more than 200 cells in a microscopic field. The percentage of eosinophils was expressed as a percentage of the total leukocytes. The data are represented as arithmetic mean $\pm$ S.D. ($n=6$).

Measurement of Serum IgE Level Blood was collected from the mice while under ether anesthesia. Serum samples were obtained by centrifugation and stored at -80°C until assay. The serum IgE level was measured using an enzyme-linked immunosorbent assay antibody (BD Biosciences, Franklin Lakes, NJ, U.S.A.). The optical density (OD) value was determined using a microplate reader.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA from mouse lung was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. RT-PCR was carried out using an RNA PCR kit (Takara, Shiga, Japan) in a thermal cycler (GeneAmp PCR system 2400, Perkin-Elmer, Norwalk, CT, U.S.A.). Total RNA (1 μ g) from each sample was used as a template for the RT reaction using 2.5 pmol of oligo(dT)₂₀ primers and 5 U of avian myeloblastosis virus (AMV) reverse transcriptase in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, RNase inhibitor 1 U/ μ l, and 1 mM dNTP mixture. The RT product (2 μ l) was used for PCR. PCR was carried out in a final volume of 20 μ l containing 1 mM MgCl₂, 1X PCR buffer, 1 mM dNTP mixture, 1 U Takara Taq DNA polymerase, and 0.2 μ M of each primer. Amplifications were carried out using a thermal cycler with the following profile: 5 min at 94°C before the first cycle, 30 s for denaturation at 94°C, 30 s for primer annealing, 30 s for extension at 72°C, and 5 min at 72°C after the last cycle. Primer sequences and

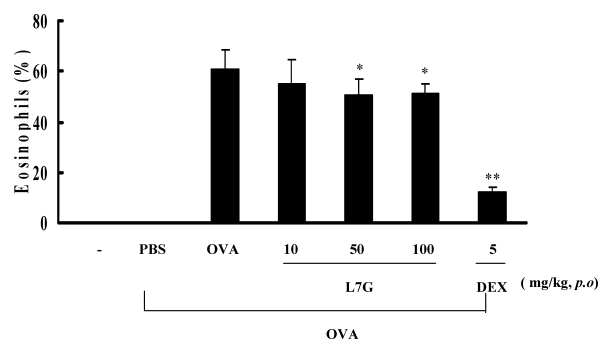


Fig. 3. Inhibition of Eosinophil Recruitment to BALF and Lungs by L7G

The OVA-challenged mice showed a marked increase in the number of eosinophils, whereas the number of eosinophils in BALF from the L7G-treated and DEX-treated mice was almost undetectable (Diff-Quick staining). The percentage of eosinophil was expressed as the percentage in total leukocytes. Data are expressed as arithmetic means $\pm$ S.D. ($n=6$). * $p<0.05$, ** $p<0.01$ in a comparison of L7G or DEX with the controls (OVA).

PCR product sizes were IL-4, 5-ACG GCA CAG AGC TAT TGA TG-3 and 5-ATG GTG GCT CAG TAC TAC GA-3, 454 bp, 35 cycles; IL-5, 5-GCA CAG TGG TGA AAG AGA CC-3 and 5-GCC CAC TCT GTA CTC ATC AC-3, 313 bp, 35 cycles; IL-13, 5-ACA GCT CCC TGG TTC TCT CA-3 and 5-CCA GCA AAG TCT GAT GTG AG-3, 500 bp, 35 cycles; and β -actin-specific primers, 5-CAC CCG CCA CCA GTT CGC CA-3 and 5-CAG GTC CCG GCC AGC CAG GT-3, 574 bp, 25 cycles were used as the positive comparative controls. After amplification, 10 μ l of each reaction mixture was analyzed with 1.2% agarose gel electrophoresis, and the bands were visualized with ethidium bromide staining.

Statistical Analysis All values are expressed as arithmetic mean $\pm$ S.D. One-way ANOVA was used to determine statistical significance.

RESULTS

Inhibition of Eosinophil Infiltration into the Lung by L7G Eighteen hours after the final intranasal OVA or PBS challenge, BALF from the lungs was collected to determine the level of eosinophil recruitment. The number of total leukocytes in the BALF obtained from the PBS-challenged group was $1.4 \pm 0.1 \times 10^5$ cells, indicating that few eosinophils were detected in this group. On the other hand, the total number of leukocytes ($5.9 \pm 1.5 \times 10^5$) and eosinophils ($3.6 \pm 0.4 \times 10^5$) in the BALF of the OVA-challenged was significantly higher than that in the PBS-challenged group (data not shown). When the mice were orally administered L7G (10, 50, 100 mg/kg) seven times, the number of eosinophils was slightly reduced compared with the OVA-challenged group mice (Fig. 3). Under the same conditions, DEX (5 mg/kg) strongly inhibited the recruitment of eosinophils.

Effects of L7G on the Level of PGE₂ in BALF and Total Serum IgE Level Eighteen hours after the final intranasal OVA or PBS challenge, the BALF prostaglandin E₂ (PGE₂) level from the lungs and total serum IgE levels were determined. As shown in Fig. 4, the PGE₂ level in BALF of naive and OVA-challenged mice were 542.5 ± 150 and 1442.3 ± 200 pg/ml, respectively (Fig. 4A). Total IgE levels in the serum of naive and sensitized animals were 0.44 ± 0.03 and 2.08 ± 0.11 ng/ml, respectively (Fig. 4B). Both PGE₂ and

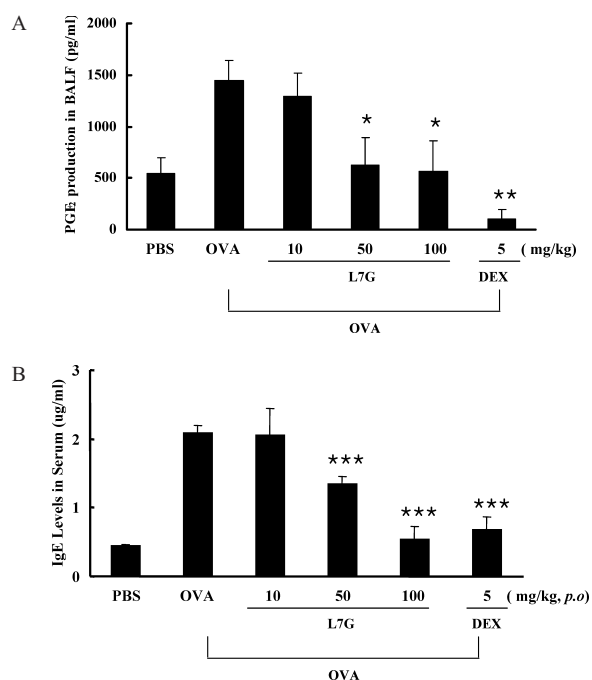


Fig. 4. Effects of L7G on Prostaglandin E₂ Production in BALF (A) and Total Serum IgE Level (B)

BALF was immediately centrifuged (2 min, 4 °C, 160×g). After removing the cells, the supernatant was obtained. Serum samples were obtained by centrifugation and stored at -80 °C until assay. Aliquots of supernatant and serum were used for the determination of PGE₂ and IgE levels. Data are represented as arithmetic means ± S.D. (n=6). *p<0.05, **p<0.01, and ***p<0.001 in a comparison of L7G or DEX with the controls (OVA).

total serum IgE levels were significantly suppressed in L7G 50 mg/kg-treated mice.

Effects of L7G on the Transcription Levels in the Asthmatic Lung Total RNA was extracted from the lungs 18 h after RT-PCR analysis to verify the effects of L7G on the mRNA expression level of Th2 cytokines because L7G reduced the number of infiltrating eosinophils in BALF. As shown in Fig. 5, the expression of Th2 cytokine mRNA was very weak and almost undetectable in normal lung tissue, whereas it was clearly induced in the OVA-challenged mice. However, pretreatment of OVA-challenged mice with L7G reduced the transcription levels of IL-4, IL-5, and IL-13. DEX also inhibited the mRNA expression of those genes in the OVA-challenged lung.

DISCUSSION

Previously, we reported the antiinflammatory activity of ethanol extracts of *A. altissima* in an OVA-induced lung inflammation animal model.¹⁵ Recently, our group has reported on the antiinflammatory compounds from *A. altissima*, such as scopoletin, quercetin, and luteolin. These compounds show strong COX-2 and 5-LOX dual inhibitory activity.¹⁶ Furthermore, we successfully isolated L7G from this plant and showed that it inhibited both prostaglandin D₂ (PGD₂) and LTC₄ production in bone marrow-derived mast cells (submitted for publication). However, the underlying mechanisms of the antiasthmatic activity of L7G have not been elucidated sufficiently. Therefore, the results of our studies and others led us to determine the biological activity

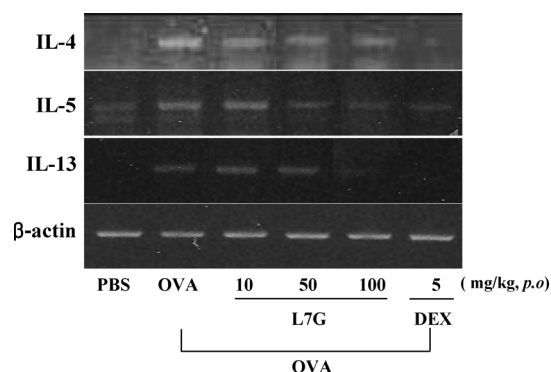


Fig. 5. Effects of L7G on Th2 Cytokine mRNA Expression in Lung Tissues

The mice were intraperitoneally sensitized with OVA three times in the presence of adjuvant alum and were subsequently challenged twice with PBS (lane 1), OVA (lane 2), OVA challenge plus L7G pretreatment (lane 3; 10 mg/kg, lane 4; 50 mg/kg, lane 5; 100 mg/kg), or OVA plus DEX pretreatment (lane 6). Eighteen hours after the final challenge, the total RNA from the lungs of the mice was subjected to RT-PCR.

of L7G in an *in vivo* animal model. This study was the first to provide experimental evidence demonstrating that L7G inhibits OVA-induced airway inflammation in a murine model of asthma.

It was reported that repeated challenges with allergens induce increase in the COX-2 protein level and a corresponding increase in the PGE₂ level in BALF in murine lungs.^{17,18} Among the eicosanoid producing enzymes, COX-2 was found to be essential for the production of PGs in various inflammatory sites.¹⁹ Therefore the inhibitory activity of L7G against the production of PGE₂ in BALF in murine lungs was examined. As shown in Fig. 4A, L7G inhibited PGE₂ generation in a dose-dependent manner. Our previous results showed that L7G inhibits the COX-2-dependent phases of PGD₂ production in cytokine-stimulated mouse bone marrow-derived mast cells (BMMCs) in a concentration-dependent manner (data not shown). Therefore the reduced level of PGE₂ in BALF may be due to the inhibition of COX-2 protein expression in infiltrating inflammatory cells. Under the same conditions, DEX (5 mg/kg) inhibited most PGE₂ production. Figure 3 demonstrates that when L7G 50–100 mg/kg was administered orally to the mice, the number of eosinophils was slightly reduced in a dose-dependent manner, indicating that L7G has preventive effects in the allergic airway inflammation model. Clinical and experimental investigations suggested a strong correlation between the presence of Th2 cells and disease severity, further suggesting an important role of these cells in asthmatic diseases.^{20,21} Among the Th2 cytokines, high levels of IL-4, IL-5, and IL-13 are produced in the asthmatic lung and are believed to be the key regulators of many of the hallmark features of the disease.^{22–24} The effects of L7G on the mRNA expression level of Th2 cytokines, such as IL-4, IL-5, and IL-13 were examined because L7G reduces the number of recruited eosinophils in BALF. As shown in Fig. 5, IL-4, IL-5, and IL-13 mRNA levels were undetectable in normal lung tissue and induced in the OVA-challenged mice. However, pretreatment of the OVA-challenged mice with L7G (50–100 mg/kg) reduced mRNA transcription levels of the Th2 cytokines. DEX (5 mg/kg) also inhibited mRNA expression of these genes in the OVA-challenged lung. This result

is consistent with the antiinflammatory activity of *A. altissima* in OVA-induced lung inflammation. Next, we examined the effects of total serum IgE level in an experimental animal model. In general, IgE synthesis by B cells is primarily regulated by Th2 cytokines such as IL-4 and IL-13 which play a key role in the hyperproduction of IgE. As shown in Fig. 4B, OVA-sensitized mice showed an increase in total serum IgE level after OVA challenge, as described previously.²⁵ L7G inhibited this rise in serum total IgE level, probably as result of the inhibition of IL-4 and IL-13 mRNA transcription. Elevated levels of IgE are associated with bronchial asthma, a disease characterized by eosinophilic inflammation of the airways. Activation of antigen-specific Th2 cells in the lung with the subsequent release of IL-4 and IL-5 is believed to play an important role in the pathogenesis of this disease. To determine the risk of acute toxicity, L7G was given orally to mice at dose up to 500 mg/kg. No apparent toxicity including mortality and changes in body weight was noted during the 14-d observation period (data not shown). Therefore the oral administration of L7G may be nontoxic and used safely in humans at moderate doses. To the best of our knowledge, this paper is the first to show that L7G isolated from *A. altissima* has antiasthmatic activity in an *in vivo* animal model. In conclusion, the antiinflammatory activity of *A. altissima* could be attributed at least in part to a biological compound such as L7G and may take part in the inhibition of PGE₂ generation and transcription of Th2 cytokine mRNA expression. These findings could also serve as an additional rationale for the use of *A. altissima* in inflammatory disorders.

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