



Protective effects of *Ulmus davidiana* var. *japonica* against OVA-induced murine asthma model via upregulation of heme oxygenase-1

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ABSTRACT

Aim of the study: Traditionally, the stem and root bark of *Ulmus davidiana* var. *japonica* (Ulmaceae) are Korean herbal medicines used for anti-inflammatory and anticancer therapy. In this study, we investigated the protective effects of *Ulmus davidiana* var. *japonica* ethanolic extract (UD) in a murine asthma model. Furthermore, we determined whether heme oxygenase (HO)-1 is required for the protective activity of UD.

Materials and methods: Airways of ovalbumin (OVA)-sensitized mice exposed to OVA challenge developed eosinophilia, mucus hypersecretion and increased cytokine levels. UD was applied 1 h prior to OVA challenge. Mice were administered UD orally at doses of 100 and 200 mg/kg once daily on days 18–23. Bronchoalveolar lavage fluid (BALF) was collected 48 h after the final OVA challenge. Levels of interleukin (IL)-4 and IL-5 in BALF were measured using enzyme-linked immunosorbent assays (ELISAs). Lung tissue sections 4 µm in thickness were stained with Mayer's hematoxylin and eosin for assessment of cell infiltration and mucus production with PAS (periodic acid shift reagent) staining, in conjunction with ELISA, immunohistochemistry and Western blot analyses for HO-1 protein expression.

Results and conclusion: Orally administered UD significantly inhibited the number of OVA-induced inflammatory cells and IgE production, along with reduced T-helper (Th)2 cytokine levels, such as IL-4 and IL-5, in BALF and lung tissue. In addition, UD induced a marked decrease in OVA-induced reactive oxygen species (ROS), inflammatory cell infiltration and mucus production in lung tissue. These effects were correlated with HO-1 mRNA and protein induction. Our results indicate that UD protects against OVA-induced airway inflammation, at least in part, via HO-1 upregulation.

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1. Introduction

Allergic asthma is a chronic airway disorder characterized by airway inflammation, mucus hypersecretion, and airway hyper-responsiveness (AHR) (Busse and Lemanske, 2001). Cumulative evidence shows that these inflammatory responses are attributed to T-helper 2 (Th2) cells, together with other inflammatory factors, including mast cells, B cells, eosinophils, cytokines and chemokines (Herrick and Bottomly, 2003). Upon activation, Th2 cells produce cytokines, including interleukin (IL)-4, IL-5, and IL-13. IL-4 is essential for B cell maturation and immunoglobulin (Ig) E synthesis, and participates in the initiation of Th2 inflammatory responses. IL-5 is pivotal for growth, differentiation, recruitment, and survival of eosinophils. IL-13 plays a significant role in the effector phase of Th2

responses, such as eosinophilic inflammation, mucus secretion, and AHR (Wynn, 2003). Asthma, a chronic airway disease, is characterized by inflammation, AHR, and altered tracheal responsiveness to relaxing agents. These effects are mediated largely by the release of histamine, prostaglandins, cytokines, adenosine and reactive oxygen species (ROS) from various cells, including inflammatory leukocytes, epithelial cells, and airway smooth muscle cells (Busse and Lemanske, 2001; Barnes and Drazen, 2002). ROS production is not only associated with intracellular signaling, but also reproduces several pathophysiologic features associated with asthma by altering the organization and function of cell membranes and increasing airway reactivity, airway secretion, promoting vascular permeability, and chemoattractant release (Nadeem et al., 2008; Van der Vliet et al., 2008). Heme oxygenase (HO) is the rate-limiting enzyme in the process of heme degradation, converting heme to biliverdin IX, carbon monoxide (CO) and iron. HO-1 is induced by a variety of stimuli, including hemin and oxidative stress, in various cell types (Pae et al., 2003). Recent basic and clinical research data indicate that HO-1 is a protective gene, and its overexpression

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has significant biological consequences, such as antioxidative, anti-inflammatory and antiproliferative effects as well as modulation of cell-cycle activities (Ryter and Choi, 2005; Song et al., 2002; Almolki et al., 2004). Recently, Wen et al. (2005) highlighted the value of anti-asthma herbal medicine for treatment of adult patients with moderate-to-severe allergic asthma. Therefore, a new approach to asthma therapy has been developed within the framework of herbal medicines as complementary or alternative therapy.

Ulmus davidiana var. *japonica*, a widely used herb in traditional Korean medicine, has established anticancer, antiviral, antibacterial, and anti-inflammatory properties (Lee et al., 2006; Jun et al., 1998; Jin et al., 2006, 2007; Kang et al., 2006; Suh et al., 2007). The bark of the stem and root of this plant are used in traditional Oriental medicine for the treatment of edema, mastitis, gastric cancer, and inflammation (Jun et al., 1998). Until recently, several flavonoids (Lee et al., 2008) and terpenoids (Shin et al., 2000) have been identified in the stem and root barks of *Ulmus davidiana* var. *japonica*, and their pharmacological actions were evaluated. This emphasizes the urgent need for development of effective anti-asthma drugs with fewer side effects. Novel therapeutic materials, such as *Ulmus davidiana* var. *japonica*, are under investigation, with a view to identifying safe lead compounds from natural sources with fewer side effects. However, the efficacy of *Ulmus davidiana* var. *japonica* is yet to be determined in patients with allergic diseases. In this investigation, we used a bronchial asthma model, specifically, an ovalbumin (OVA)-induced asthmatic mouse, to examine the anti-allergic and anti-inflammatory properties of *Ulmus davidiana* var. *japonica*. To our knowledge, this is the first study to provide experimental evidence that *Ulmus davidiana* var. *japonica* inhibits OVA-induced oxidative stress and inflammation in a murine model of asthma.

2. Methods and materials

2.1. Preparation of *Ulmus davidiana* var. *japonica* ethanolic extract (UD)

The bark of *Ulmus davidiana* var. *japonica* was purchased in October 2008 from HMAX (Chungbuk, Republic of Korea). These materials were confirmed taxonomically by professor Je-Hyun Lee of Dongguk University, Gyeongju, Republic of Korea. A voucher specimen has been deposited at the Korea Institute of Oriental Medicine (KIOM), Daejeon, Republic of Korea. Fresh *Ulmus davidiana* var. *japonica* was washed three times with tap water to remove salts, epiphytes and sand, and stored at -20°C . Frozen samples were lyophilized and homogenized in a grinder before extraction. Extracts were prepared from dried bark of *Ulmus davidiana* var. *japonica* (200 g) with 70% EtOH (2 L $3\times$) followed by sonication for 1 h. The extract solution was filtered through filter paper and evaporated to dryness (13.09 g). The yield of dried extract from starting crude material was 6.5%.

2.2. Chromatographic system and chemicals

Analysis was performed using a Shimadzu LC-20A HPLC system (Shimadzu Co., Kyoto, Japan), consisting of a solvent delivery unit, an on-line degasser, a column oven, an autosampler, and a PDA detector. The data processor employed LcSolution software (Version 1.24). The analytical column used was a Gemini C18 (250 mm $\times$ 4.6 mm; particle size 5 μm , Phenomenex, Torrance, CA, USA). The mobile phases were solvent A (1.0% acetic acid in H_2O) and solvent B (1.0% acetic acid in acetonitrile). The gradient flow was as follows: (A)/(B)=95/5 (0 min) $\rightarrow$ (A)/(B)=90/10 (10 min) $\rightarrow$ (A)/(B)=30/70 (40 min; hold for 5 min) $\rightarrow$ (A)/(B)=95/5 (50 min; hold for 10 min). The column temperature was maintained

at 40°C . The analysis was carried out at a flow-rate of 1.0 mL/min with PDA detection from 190 to 400 nm. The injection volume was 10 μL .

(–)-Catechin, catechin-7-O- α -L-rhamnopyranoside, catechin-7-O- β -D-apiofuranoside, catechin-3-O- α -L-rhamnopyranoside and procyanidin B₃ were isolated from *Ulmus davidiana* by a series of chromatography procedures. HPLC-grade reagents, methanol, acetonitrile, and water were obtained from J.T. Baker (Phillipsburg, NJ, USA). Other chemicals were of analytical grade.

2.3. Preparation of standard and sample solution

Standard stock solutions of (–)-catechin, catechin-7-O- α -L-rhamnopyranoside, catechin-7-O- β -D-apiofuranoside, catechin-3-O- α -L-rhamnopyranoside and procyanidin B₃ (all 500 $\mu\text{g/mL}$) were prepared in methanol and held below 4°C . Working standard solutions were prepared by serial dilution of stock solutions with methanol. The sample powder was weighed (1.0 g) into a 100 mL flask and methanol was added to the volumetric mark. The mixture was sonicated for 60 min at room temperature. After extraction, the mixture was filtered through a 0.2 μm membrane filter. The resulting solution was used HPLC analysis.

2.4. Animals

Specific 7-week-old pathogen-free (SPF) inbred female BALB/c mice routinely screened serologically for relevant respiratory pathogens were purchased from Daehan Biolink Co., Ltd. (Seoul, Korea). Mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to experiments, and provided water and standard chow *ad libitum*. All experimental procedures were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of Korea.

2.5. Sensitization and airway challenge

Mice were divided into groups ($n=7$) receiving the following treatments: (1) sham sensitization plus challenge with phosphate buffered saline (PBS; ipNeb), (2) sensitization plus challenge with OVA (Sigma A5503; Sigma, St. Louis, MO, USA) (ipNeb), and (3) sensitization with OVA (i.p.) plus challenge with OVA (Neb) and UD (100 or 200 mg/kg) or Montelukast (30 mg/kg) p.o. Briefly, mice were sensitized with an intraperitoneal injection of 20 μg OVA emulsified in 2 mg aluminum hydroxide in 200 μL PBS buffer (pH 7.4) on days 0 and 14. Concentrated UD was freshly dissolved in PBS prior to use. Mice were orally administered UD (100 or 200 mg/kg/day) each day from days 18 to 23 consecutively. Mice were challenged through the airway with OVA (1%, w/v, in PBS) for 30 min using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan) on days 21, 22, and 23 after initial sensitization. Animals were sacrificed 48 h after the final challenge (day 25) to characterize the suppressive effects of UD. A schematic diagram of the treatment schedule is shown in Fig. 1.

2.6. Inflammatory cell counts in bronchoalveolar lavage fluid (BALF)

Mice were sacrificed with an overdose (50 mg/kg) of pentobarbital 48 h after the last challenge, and tracheostomy performed. Following instillation of ice-cold PBS (0.6 mL) into the lungs, BALF was obtained with three aspirations (total volume 1.8 mL) via tracheal cannulation. BALF was centrifuged, and the supernatant fractions collected and stored at -70°C . The total inflammatory cell numbers were assessed by counting cells in at least five squares of a hemocytometer after exclusion of dead cells via trypan blue

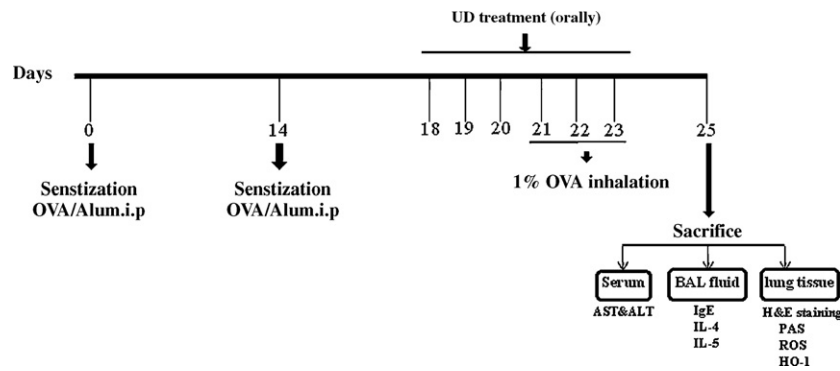


Fig. 1. Mouse model of airway inflammation and treatment with *Ulmus davidiana* var. *japonica* ethanolic extract (UD).

staining. BALF (100 μ L) was pipetted on a slide and centrifuged (200 $\times$ g, 4 $^{\circ}$ C, 10 min) to fix cells using a cytospin machine (Hanil Science Industrial, Seoul, Korea). Cell pellets were suspended in 0.5 mL PBS, and 100 μ L of each solution spun onto a slide. After slides were dried, cells were fixed and stained using Diff-Quik[®] Staining reagents (B4132-1A; Dade Behring Inc., Deerfield, IL, USA), according to the manufacturer's instructions.

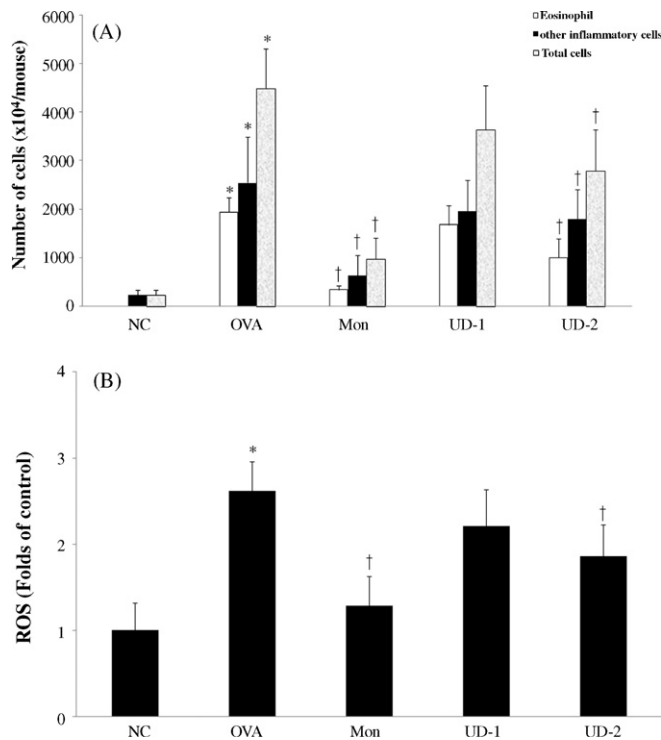


Fig. 2. Effects of UD on recruitment of inflammatory cells and reactive oxygen species (ROS) in bronchoalveolar lavage fluid (BALF) of mice 48 h after the final OVA challenge. (A) Cells were isolated by centrifugation, and stained with Diff-Quik[®] Stain reagent. Cell numbers were determined by counting within at least five squares of a hemocytometer under a light microscope. Dead cells, stained with trypan blue, were excluded from the total number. (B) Oxidative stress was quantified in BALF, based on DCF fluorescence. Values represent relative fluorescence units per milligram protein. NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; UD-1, UD (100 mg/kg)+OVA-sensitized/challenged mice; UD-2, UD (200 mg/kg)+OVA-sensitized/challenged mice. UD or montelukast treatment was performed 1 h before challenge. *Significant difference from NC, $P < 0.05$, †significant difference from OVA, $P < 0.05$.

2.7. The 2',7'-dichlorofluorescein (DCF) fluorescence assay

Induction of oxidative stress was monitored using 2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Eugene,

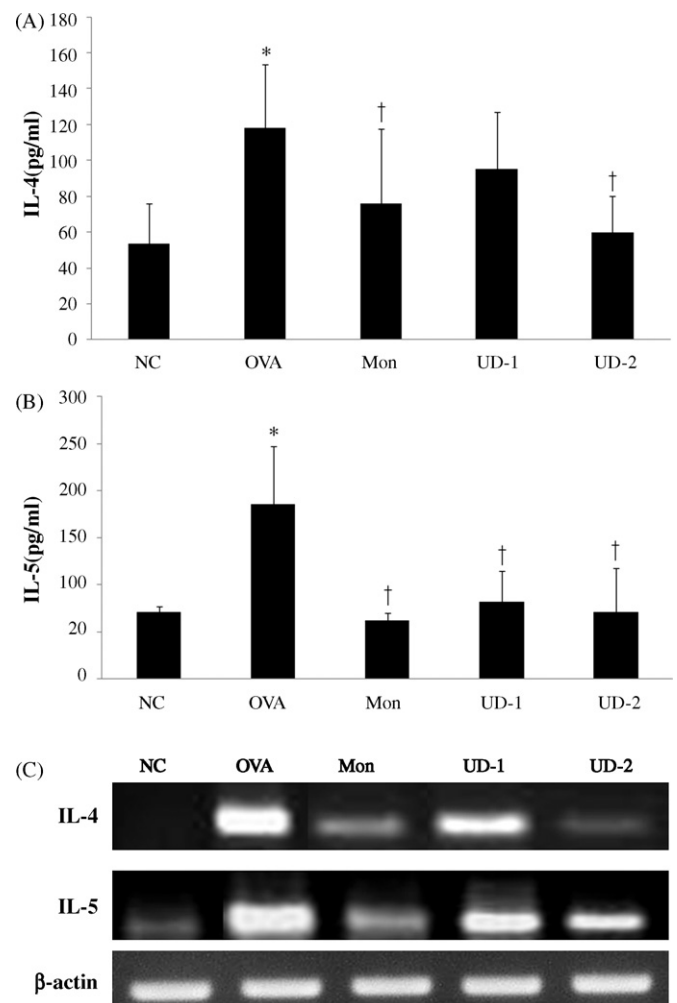


Fig. 3. Effects of UD on cytokine levels in BALF and lung tissue. BALF and lung tissue were collected 48 h after the last OVA challenge of mice. Individual samples were analyzed using ELISA. (A) IL-4 level in BALF; (B) IL-5 level in BALF; (C) interleukin IL-4 and IL-5 mRNA levels in lung tissue. NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; UD-1, UD (100 mg/kg)+OVA-sensitized/challenged mice; UD-2, UD (200 mg/kg)+OVA-sensitized/challenged mice. UD or montelukast treatment was performed 1 h before challenge. *Significant difference from NC, $P < 0.005$, and †significant difference from OVA, $P < 0.05$.

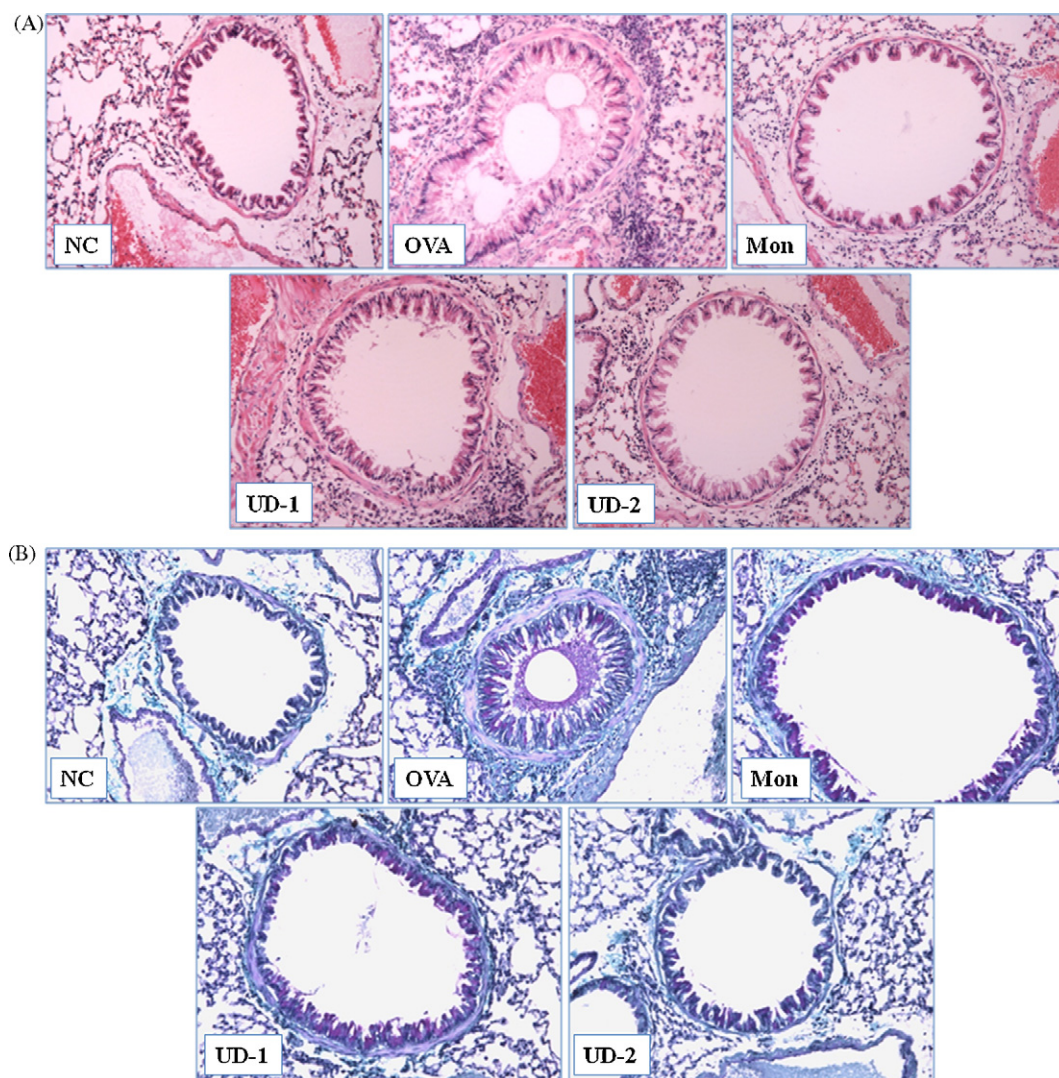


Fig. 4. Effects of UD on recruitment of leukocytes and mucus production in lung tissue. Histological examination of lung tissue was performed 48 h after the final OVA challenge. Lung tissues were fixed, sectioned at 4 μ m thickness, and stained with H&E solution (A) and periodic acid Schiff (PAS) (B). (Magnification 200 $\times$.) NC, normal control mice (PBS only); OVA, OVA-sensitized/challenged mice; UD-1, UD (100 mg/kg) + OVA-sensitized/challenged mice; UD-2, UD (200 mg/kg) + OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + OVA-sensitized/challenged mice. UD or montelukast treatment was performed 1 h before challenge.

OR, USA), which is converted into highly fluorescent DCF by cellular peroxides, including hydrogen peroxide. In brief, BALF was treated with 25 μ M DCF-DA for 10 min at 37 $^{\circ}$ C. Intracellular ROS activity was measured by determining fluorescence at 488 nm excitation and 525 nm emission using a fluorescence plate reader (Perkin-Elmer, Waltham, MA, USA).

2.8. Measurement of IgE levels in BALF

BALF cells were collected and stored at -70° C after centrifugation (300 $\times$ g, 10 min). Total and OVA-specific IgE levels were measured using Enzyme-linked immunosorbent assays (ELISA). Microtiter plates were coated with 100 μ L/well IgE (10 μ g/mL; Serotec, Oxford, UK) in PBS-Tween 20. Antibodies in BALF were detected using isotype-specific secondary antibodies (anti-mouse IgE; Serotec, Oxford, UK). After washing four times, 200 μ L O-phenylenediamine dihydrochloride (Sigma, St Louis, MO, USA) was added to each well. The plate was incubated for 10 min in the dark, and absorbance measured at 450 nm. Total and OVA-specific IgE concentrations were calculated from a standard curve generated using 250 ng/mL recombinant IgE (Serotec, Oxford, UK).

2.9. Measurement of Th2 cytokines in BALF

ELISA experiments were performed according to the manufacturer's instructions. IL-4 and IL-5 levels in BALF were measured using specific mouse IL-4 and IL-5 ELISA kits (BioSource International, Camarillo, CA, USA).

2.10. Semi-quantitative RT-PCR

RT-PCR was performed to estimate the relative quantities of IL-4 and IL-5 mRNA in the lung. Total RNA was extracted from lung tissue using TRIzol reagent, according to the manufacturer's instructions. To prepare cDNA, total RNA was incubated at 37 $^{\circ}$ C for 90 min using the first-strand cDNA synthesis kit (Promega, Madison, WI, USA). The primer sequences were as follows: mouse IL-4, 5'-TCT CTA GAT CAT GGG CAT TTT GAA CGA GGT C-3' and 5'-TGC ATG ATG CTC TTT AGG CTT TCC-3', and mouse IL-5, 5'-ATG ACT GTG CCT CTG TGC CTG GAG C-3' and 5'-CTG TTT TTC CTG GAG TAA ACT GGG G-3'. PCR was performed with initial denaturation at 94 $^{\circ}$ C for 5 min, followed by 30 cycles of denaturation at 94 $^{\circ}$ C for 1 min, annealing at 60 $^{\circ}$ C (IL-4, IL-5, and β -actin), extension

at 72 °C for 1 min, and final extension at 72 °C for 10 min. β -Actin was used as the internal control for individual amplification reactions. The final PCR products were separated on 1% agarose gels, and visualized with ethidium bromide staining. Densitometric analysis was performed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

2.11. Heme oxygenase (HO) enzyme activity

HO-1 enzyme activity in the mouse lung was quantified by assessing bilirubin generation using modified established techniques. Briefly, lungs were homogenized on ice in one volume of 100 mmol/L phosphate buffer with 2 mmol/L $MgCl_2$ and centrifuged for 15 min at $12,000 \times g$. The supernatant was used to measure HO-1 enzyme activity. ELISA assays were performed according to the manufacturer's instructions. The HO-1 level in lung tissue was measured using a specific mouse HO-1 ELISA kit (DAKARA, Japan). Values are expressed as per mg protein.

2.12. Lung tissue histopathology

After BALF was obtained, lung tissue was fixed in 10% (v/v) neutral buffered formalin for 24 h at 4 °C. Tissues were embedded in paraffin, sectioned at 4 μ m thickness, and stained with H&E solution (hematoxylin, Sigma MHS-16, and eosin, Sigma HT110-1-32) and periodic acid Schiff (PAS) (IMEB Inc., San Marcos, CA, USA) to measure mucus production. Tissues were subsequently mounted and coverslipped using Dako mounting medium (Dakocytomation, Denmark, CA, USA). For immunohistochemistry, paraffin sections were deparaffinized, dehydrated and washed in PBS with 0.3% Triton X-100, and preincubated for 10 min at room temperature with 10% goat serum to block non-specific staining. Subsequently, slides were incubated with primary mouse anti-rabbit HO-1 antibody (1/200; Abcam) overnight at 4 °C. After removal of primary antibodies, sections were washed and incubated with biotinylated secondary antibody at 37 °C for 1 h, followed by the avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Excess secondary antibody was removed, sections washed with PBS and incubated with 0.05% diaminobenzidine (1:200; Millipore, Billerica, MA, USA) for 10 min. Sections were counterstained, rinsed in PBS to terminate the reaction, and coverslipped for microscopic examination.

2.13. Measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum

ALT and AST levels were determined to assess liver and kidney function using commercial kits (BECKMAN coulter, Inc., Fullerton, CA, USA) and an auto-analyzer (BECKMAN CX4, CA, USA).

2.14. Image capture and photomicrography

Photomicrographs were obtained using a Photometric Quantix digital camera running a Windows program, and montages assembled in Adobe Photoshop 7.0. Images were cropped and corrected for brightness and contrast, but not otherwise manipulated.

2.15. Statistical analysis

Data are expressed as means $\pm$ standard deviations (SD). One-way ANOVA was used to compare mean responses among the treatments. A statistical probability of $P < 0.05$ was considered significant.

3. Results

3.1. Effects of UD on OVA-induced eosinophilia and ROS generation in BALF

To determine whether UD has a protective effect on airway inflammation, the total cell and eosinophil numbers in BALF were measured in OVA-challenged mice untreated or treated with UD. OVA-challenged mice displayed markedly increased total cell and eosinophil numbers in BALF. UD significantly attenuated cell influx to BALF (Fig. 2A).

ROS levels in BALF were markedly increased in OVA-challenged mice (2.62-fold) shown in Fig. 2B. However, pre-administration of UD led to a significant dose-dependent decrease (UD-1; 2.21- and UD-2; 1.86-fold, $p < 0.05$) in the ROS level in BALF (Fig. 2B).

3.2. Effects of UD on cytokine levels in BALF

The effects of UD on cytokine release in the OVA-induced asthma mouse model were assessed by measuring the levels of cytokines (IL-4 and IL-5) in BALF using ELISA 48 h after the final challenge. OVA challenge led to significant increases in the levels of IL-4 and IL-5 in BALF, compared with control. In the UD-treated group, cytokine elevation was significantly suppressed in a dose-dependent manner in relation to the OVA-challenged group. Montelukast (positive control) additionally induced significant decreases in IL-4 (Fig. 3A) and IL-5 levels (Fig. 3B) compared with OVA-challenged mice. In view of the finding that UD inhibits the number of inflammatory cells in BALF, we assessed the mRNA levels of associated molecules. As shown in Fig. 3C, UD significantly inhibited IL-4

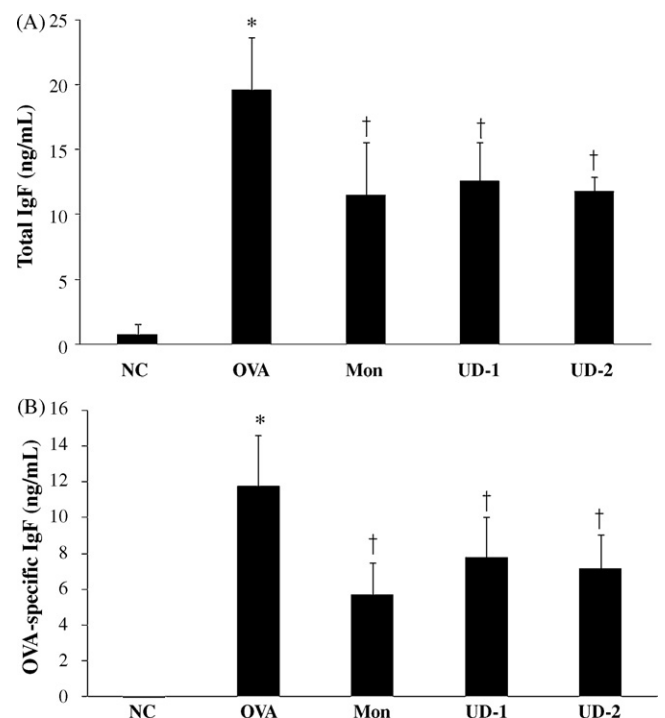


Fig. 5. Effects of UD on total and OVA-specific IgE levels in BALF. BALF was collected 48 h after the final OVA challenge in mice. Each sample was analyzed using ELISA. (A) Total IgE level; (B) OVA-specific IgE level; NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; UD-1, UD (100 mg/kg) + OVA-sensitized/challenged mice; UD-2, UD (200 mg/kg) + OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + OVA-sensitized/challenged mice. UD or montelukast treatment was performed 1 h before challenge. *Significant difference from NC, $P < 0.005$, †significant difference from OVA, $P < 0.05$.

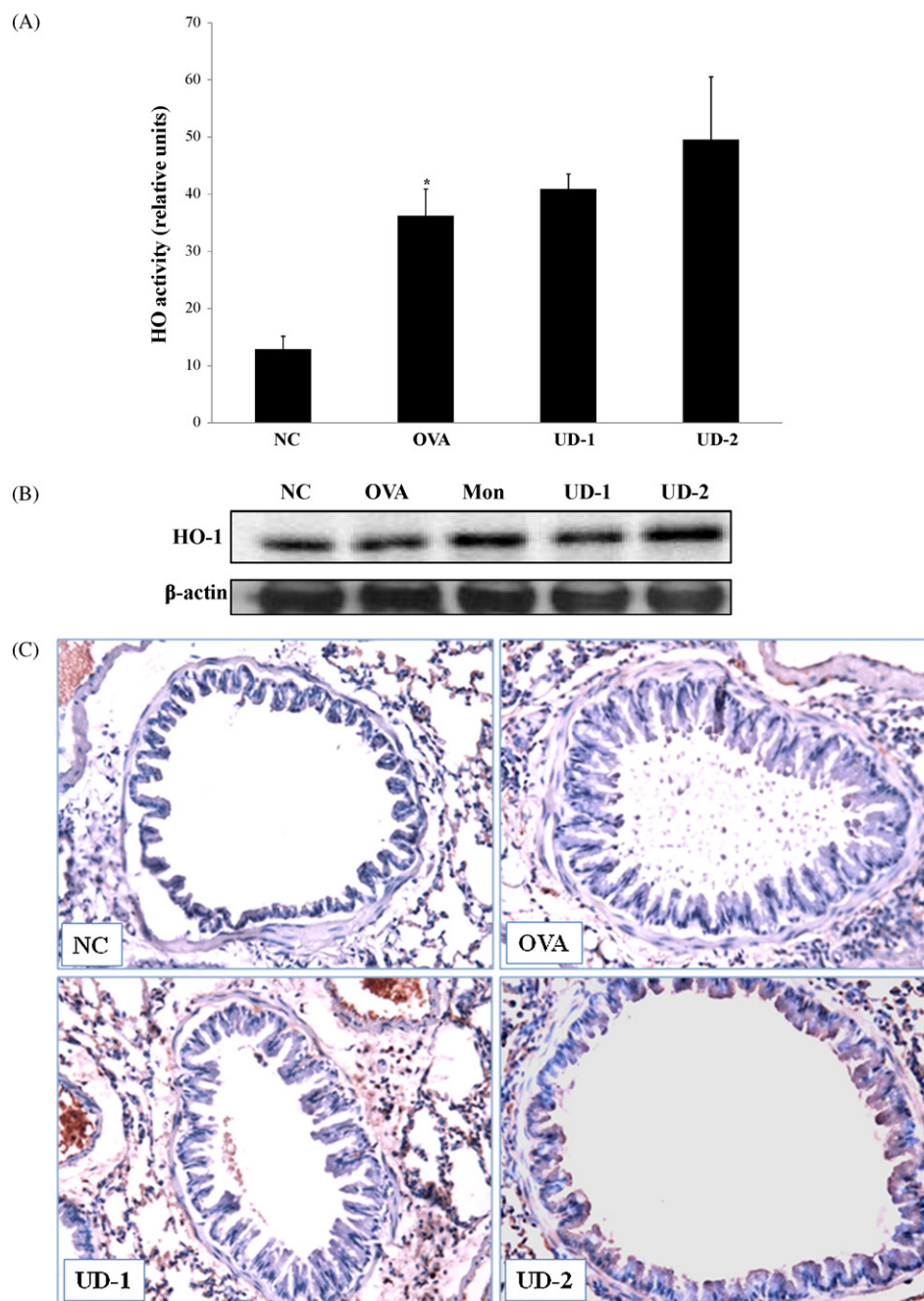


Fig. 6. Effects of UD on HO-1 activity and protein levels. Lung tissue was collected 48 h after the final OVA challenge in mice. Each sample was analyzed using ELISA, Western blotting and immunohistochemistry. (A) HO-1 enzyme activity in lung tissue and (B) HO-1 protein expression in the lung was determined using Western blot analysis after OVA challenge with or without UD. HO-1 protein level was normalized to that of β -actin. (C) HO-1 immunoreactivity in lung tissue; NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; UD-1, UD (100 mg/kg)+OVA-sensitized/challenged mice; UD-2, UD (200 mg/kg)+OVA-sensitized/challenged mice. UD or montelukast treatment was performed 1 h before challenge. *Significant difference from NC, $P < 0.05$.

and IL-5 mRNA expression following asthma induction. Our results clearly demonstrate that UD suppresses IL-4 and IL-5 expression in BALF and lung tissue of the asthmatic mouse model, similar to montelukast.

3.3. Effects of UD on airway goblet cell hyperplasia and mucus production in lung tissue

In OVA-challenged lung tissue, leukocytes infiltrated peribronchiole and perivascular connective tissue; most infiltrating

inflammatory cells were eosinophils (Fig. 4A; OVA). UD (100 and 200 mg/kg) markedly attenuated eosinophil-rich leukocyte infiltration compared with OVA-challenged mice (Fig. 4A; UD-1 and UD-2).

Mucus hypersecretion is characteristic of airway remodeling. To assess the possible UD-induced suppression of mucus overproduction caused by goblet cell hyperplasia, we stained lung sections with PAS. In OVA-challenged mice, mucus overproduction was clearly observed as a violet color in the bronchial airways, compared with PBS-treated mice (Fig. 4B; OVA). In contrast, the mucus

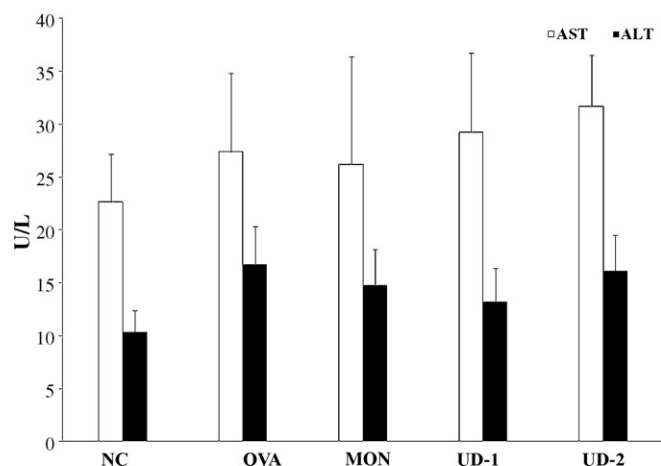


Fig. 7. Levels of AST and ALT in sera. Serum was collected 48 h after the final OVA challenge of mice. Each sample was analyzed using commercial kits (Beckman Coulter, Inc., Fullerton, CA, USA). UD or montelukast treatment was performed 1 h before challenge.

stain was markedly diminished in UD+OVA-challenged mice in a dose-dependent manner (Fig. 4B; UD-1 and UD-2).

3.4. Effects of UD on release of total and OVA-specific IgE into BALF

Levels of total and OVA-specific IgE were evaluated using ELISA in each experimental group 48 h after the final OVA challenge. Total and OVA-specific IgE levels in BALF were markedly increased in OVA-challenged mice (total IgE: 19.59 ± 4.09 ng/mL, $P < 0.005$ and OVA-specific IgE: 11.79 ± 2.81 ng/mL, $P < 0.005$) compared with negative control mice. Notably, UD treatment suppressed the levels of total (UD-1; 13.9 ± 2.96 and UD-2; 12.78 ± 1.10 ng/mL,

$P < 0.05$) and OVA-specific IgE (UD-1; 7.81 ± 2.25 and UD-2; 7.19 ± 1.88 ng/mL, $P < 0.05$) in OVA-challenged mice (Fig. 5A and B).

3.5. Effects of UD on HO-1 expression

Next, we investigated whether UD upregulates HO-1 expression and protects against OVA-challenged lung inflammation. To determine whether the inhibitory effects of UD on airway inflammation are related to HO-1 induction, expression was assessed by ELISA, Western blot, ELISAs and immunohistochemistry analyses. As shown in Fig. 6A, UD treatment led to increased HO-1 activity in a dose-dependent manner. UD induced the most significant increase in activity at a dose of 200 mg/kg, compared with OVA-challenged mice. Consistent with this increased activity, HO-1 protein levels were significantly increased in UD-treated mice (Fig. 6B). Moreover, immunohistochemistry analyses revealed higher levels of dose-dependent HO-1 expression in both OVA-challenged and UD mice compared with negative controls (Fig. 6C).

3.6. Cytotoxicity of UD in a murine model of allergic asthma

AST and ALT kits were used for the measurement of AST and ALT, respectively, in serum, according to the manufacturer's manual. UD did not affect the activities of serum toxicity marker enzymes (ALT, AST), indicating normal liver function (Fig. 7).

3.7. HPLC analysis

Three-dimensional chromatogram was obtained using HPLC-PDA detector. Using optimized chromatography conditions, five compounds were eluted before 30 min in sample analysis by using mobile phases consisting of solvent A (1.0% acetic acid in H_2O) and solvent B (1.0% acetic acid in acetonitrile). The three-dimensional HPLC chromatogram of *Ulmus davidiana* extract was shown in Fig. 8.

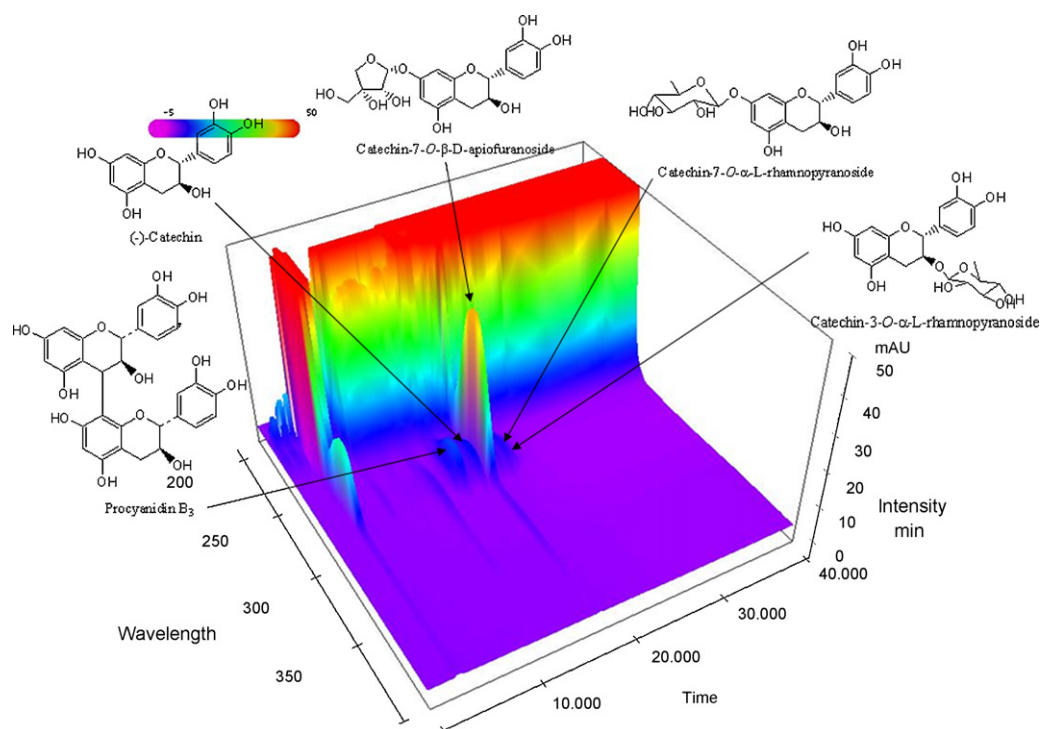


Fig. 8. Three-dimensional chromatogram of *Ulmus davidiana* extract by HPLC-PDA.

4. Discussion and conclusions

Asthma is a chronic inflammatory disorder characterized by reversible airway obstruction, bronchial hyperresponsiveness and airway inflammation. *Ulmus davidiana* var. *japonica* is traditionally used for the treatment of inflammatory diseases. However, there have been no reports on the anti-asthmatic effects of UD and possible involvement of HO-1. In the present study, we show for the first time that the *Ulmus davidiana* var. *japonica* ethanolic extract (UD) plays a protective role in OVA-induced allergic airway inflammation in mice, characterized by infiltration of inflammatory cells in both BALF and lung tissue. UD exerts its protective effects by inhibiting allergen-specific production of reactive oxygen species (ROS), Th2-type cytokines and IgE. OVA sensitization and challenge led to a significant increase in the number of inflammatory cells containing eosinophils and macrophages in BALF, compared with the negative control. However, UD induced marked inhibition of OVA-induced eosinophilia in a dose-dependent manner. Previous studies have indicated that eosinophils contribute to several clinical features of allergic asthma, including tissue damage and AHR (Lotvall et al., 1998).

Initiation of allergic response occurs with allergen presentation by antigen-presenting cells to CD4⁺T cells. Antigen-activated CD4⁺T cells induce several characteristic features of asthma, including secretion of cytokines such as IL-4 and IL-5, which regulate mucus production, inflammation, and adhesion molecules (Hogan et al., 2003; Taube et al., 2004). Th2-derived cytokines, such as IL-4 and IL-13, are produced by resident cells in the airways, including alveolar macrophages, mast cells and epithelial cells, as well as recruited eosinophils and lymphocytes (Barnes et al., 1998). In the knockout murine model, these cytokines are implicated in sensitization and allergic responsiveness to increases in the IgE level (Hamelmann et al., 2000) and eosinophilia in the airways (Webb et al., 2000). Because IgE levels in BALF are dependent on IL-4 and IL-5 are considered an additional index of Th2 cytokine secretion in the pathogenesis of asthma, we measured the IgE levels in BALF. UD induced significant decreases in the IgE, IL-4 and IL-5 levels in BALF in the OVA-induced mouse model. Our results support the use of UD as an effective agent in suppressing the early stages of allergic asthma.

In our experiments, UD induced a significant reduction in goblet cell hyperplasia and mucus production in an OVA-induced murine asthma model. Eosinophils infiltrating the airway also affected mucus secretion of epithelial goblet cells (Shimizu et al., 2003). These results indicate that mucus hypersecretion is attenuated owing to the protective effects of UD on cytokine production and eosinophilia, and that UD is highly capable of inhibiting the development of allergic status in the OVA-induced asthma model. We additionally examined induction of HO activity in lung homogenates, together with local inflammation and oxidative stress upon UD treatment. HO-1 expression and activity are induced in experimental models of different pulmonary diseases, including hyperoxia (Otterbein et al., 1999), influenza viral infections (Hashiba et al., 2001), and ozone-induced lung injury (Hisada et al., 2000). A protective effect of HO is evident in allergic lung disease models. Kitada et al. (2001) reported increased HO-1 immunostaining in alveolar macrophages in a murine model of asthma, whereas Jia et al. (1999) showed that HO induction with hemoglobin prevented bronchial plasma extravasation after intravenous OVA challenge in sensitized rats. Indeed, in our study, HO activity was increased in lung homogenates following UD administration in the OVA-induced murine asthma model.

We analyzed the five flavonoids such as (–)-catechin, catechin-7-O- α -L-rhamnopyranoside, catechin-7-O- β -D-apiofuranoside, catechin-3-O- α -L-rhamnopyranoside and procyanidin B₃ using HPLC in UD. Our result showed good separation in UD. On the

other hand, we must be preformed to activity test for major compounds.

In conclusion, our data provide evidence that activated HO decreases lung inflammation, mucus hypersecretion and oxidative stress, which contribute to its protective effects. These findings indicate that UD may be an effective therapeutic agent for airway inflammation via HO-1 upregulation.

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