



## Antioxidant and antiasthmatic effects of saucerneol D in a mouse model of airway inflammation

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### ABSTRACT

Chronic airway inflammation is a hallmark of asthma, which is an immune-based disease. We evaluated the ability of saucerneol D, a tetrahydrofuran-type sesquillignan isolated from *Saururus chinensis*, to regulate airway inflammation in an ovalbumin (OVA)-induced airway inflammation model. Furthermore, we determined whether heme oxygenase (HO)-1 was required for the protective activity of saucerneol D. The airways of OVA-sensitized mice exposed to an OVA challenge developed eosinophilia and mucus hypersecretion and exhibited increased cytokine levels. Mice were administered saucerneol D orally at doses of 20 and 40 mg/kg once daily on days 26–30. Saucerneol D administered orally significantly inhibited the number of OVA-induced inflammatory cells and the production of immunoglobulin E as well as Th2-type cytokines. Histopathology studies revealed a marked decrease in lung inflammation and goblet cell hyperplasia after saucerneol D treatment. In addition, saucerneol D induced HO-1 and led to a marked decrease in OVA-induced reactive oxygen species and malondialdehyde and an increase in superoxide dismutase and glutathione in lung tissues. These antioxidant effects were correlated with HO-1 induction. In our experiments, saucerneol D treatment reduced airway inflammation and suppressed oxidative stress in an OVA-induced asthma model.

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### 1. Introduction

Chronic inflammation is recognized as a central component of the pathophysiology of asthma. Invading inflammatory cells in lung tissue release a wide variety of mediators and cytokines that contribute to the clinical characteristic of asthma [1]. Bronchial asthma is a chronic inflammatory disease of the airways characterized by airway eosinophilia and goblet cell hyperplasia with mucus hypersecretion to inhaled allergens and nonspecific stimuli [2,3]. The inflammatory process in asthma is dominated by T helper-2 (Th2) cells, which produce interleukin (IL)-4, IL-5, and IL-13 [4]. In particular, eotaxin, IL-4, IL-5, and IL-13, which are produced by Th2 cells, are all related to inflammatory changes in the airway via the activation of eosinophils and the production of immunoglobulin E (IgE) by B cells [5–7]. Recently, heme oxygenase (HO)-1 was shown to be induced in the airways of patients with asthma and chronic

obstructive pulmonary disease (COPD) [8,9]. Moreover, overexpression of HO-1 decreases airway inflammation and mucus secretion in rodents [10], suggesting that HO-1 plays a critical role in protecting the host during airway inflammation. The induction of HO-1 expression by various stress stimuli, such as lipopolysaccharides and oxidants, is thought to be an adaptive mechanism that protects cells from oxidative injury [11]. Oxidative stress plays an important role in the pathogenesis of most airway diseases, particularly when inflammation is prominent [12]. There is increasing evidence that inflammation, which is characteristic of asthma, results in increased oxidative stress in the airways [13]. Reactive oxygen species (ROS) can be generated either endogenously by metabolic reactions, such as from mitochondrial electron transport during respiration or during activation of circulating inflammatory cells or phagocytes, or exogenously from air pollutants or cigarette smoke. As a result, increased levels of ROS have been shown to affect the extracellular environment, impacting on a variety of physiological processes [14,15]. Eosinophils, alveolar macrophages, and neutrophils from asthmatic patients produce more ROS than do those from normal subjects [16,17]. The overproduction of ROS or depression of protective mechanisms also results in bronchial hyperreactivity, which is characteristic of asthma [18,19]. It is proposed that ROS produced by phagocytes that have been

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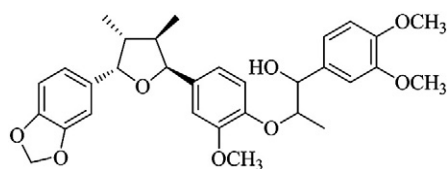


Fig. 1. Chemical structure of saucerneol D.

recruited to sites of inflammation is a major cause of the cell and tissue damage associated with many chronic inflammatory lung diseases, including asthma and COPD [20–24]. Although it is considered as one of the most important protective mechanisms against superoxide-anion-mediated injury, superoxide dismutase (SOD) activity produces an oxidant–hydrogen peroxide ( $H_2O_2$ ). Alterations in the level of these enzymes have been detected in asthmatic patients [25–27]. Malondialdehyde (MDA), a major product of lipid peroxidation (LP), is generally used as the indicator of oxidative stress [21]. Cell membrane homeostasis is destroyed by the increased production of LP. Damaged and dysfunctional membranes cause loss of calcium and of other transport systems, such as a reduction in intercellular gap junction communication [28]. Proteins such as SOD and glutathione (GSH) are major enzymes in the antioxidative defense system.

*Saururus chinensis* (Saururaceae) is used widely as a traditional medicine for the treatment of edema, jaundice, gonorrhea, pneumonia, and several inflammatory diseases [29]. Previous chemical studies of *S. chinensis* reported the presence of a large number of lignans [30], flavonoids, anthraquinones, and furanoditerpenes [31,32]. Herbal medicine is one of the main lines of complementary and alternative therapy of bronchial asthma, as it is the third most popular choice of adults and children suffering from bronchial asthma [33]. Previous investigations of saucerneol D revealed its anti-inflammatory activity in HeLa cells transfected with an NF- $\kappa$ B reporter [34]. However, there are no *in vivo* studies of the antiasthmatic properties of saucerneol D. Despite the wide variety of steroid and nonsteroid medications employed, all anti-inflammatory or antiasthma drugs available currently cause undesired, and possibly serious, side effects. Thus, development of new and more powerful drugs is required. Therefore, in this study, we evaluated a bronchial asthma model to examine the ability of saucerneol D to control Th2-type cytokines, IgE, oxidative stress, eosinophil infiltration, and other factors that play important roles in allergic inflammation. We hypothesized that saucerneol D would have an antiasthmatic effect on airway inflammation in a murine model of allergic asthma.

## 2. Materials and methods

### 2.1. Extraction and isolation of saucerneol D

Fresh *S. chinensis* (Saururaceae) was washed three times with tap water to remove salts, epiphytes, and sand and was stored at  $-20^\circ\text{C}$ . Frozen samples were lyophilized and homogenized in a grinder before extraction. Saucerneol D was isolated from the ethyl acetate extract of the roots of *S. chinensis*. The chemical structure of the isolated compound was established as saucerneol D (Fig. 1). The compound was obtained as an amorphous powder: mp,  $71\text{--}72^\circ\text{C}$ ;  $[\alpha]_D^{25}$ ,  $-75.7^\circ$  (c 0.65,  $\text{CHCl}_3$ );  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data were consistent with values from the literature [34]; FABMS  $m/z$ , 536  $[M]^+$ . The purity of this compound was above 99.5%, based on HPLC analysis.

### 2.2. Animals

Specific 7-week-old, pathogen-free, inbred female BALB/c mice screened routinely 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 the performance of experiments, and were 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.

### 2.3. Sensitization and airway challenge

The mice were divided into four groups and airway inflammation was induced by ovalbumin (OVA) (grade III; Sigma–Aldrich, USA) in three groups using the method described by Oh and colleagues [35]. Briefly, mice were immunized via intraperitoneal injection of  $20\text{ }\mu\text{g}$  chicken OVA and 2 mg aluminum hydroxide in  $200\text{ }\mu\text{L}$  PBS buffer (pH 7.4) on days 0 and 14. Mice were exposed to a 1% (w/v in PBS) OVA solution for 20 min using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan) on days 28, 29, and 30 after the initial sensitization. Saucerneol D (20 or 40 mg/kg) was administered orally once daily on days 28–30. Negative and positive control mice were treated orally with PBS and dexamethasone (Dex; 3 mg/kg), respectively, once daily on days 28–30. Animals were sacrificed 48 h after the last challenge (i.e., on day 32) to characterize the suppressive effects of saucerneol D. A schematic diagram of the treatment schedule is shown in Fig. 2.

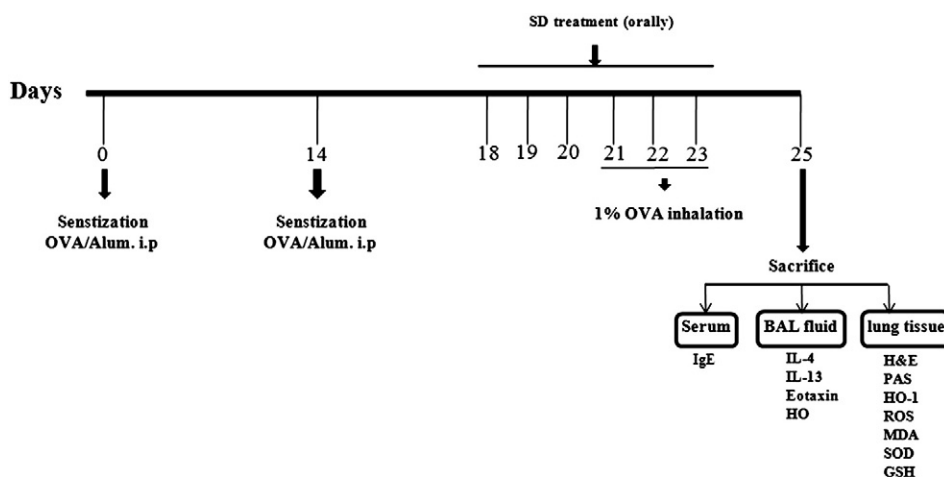


Fig. 2. Mouse model of airway inflammation and effects of treatment with saucerneol D.

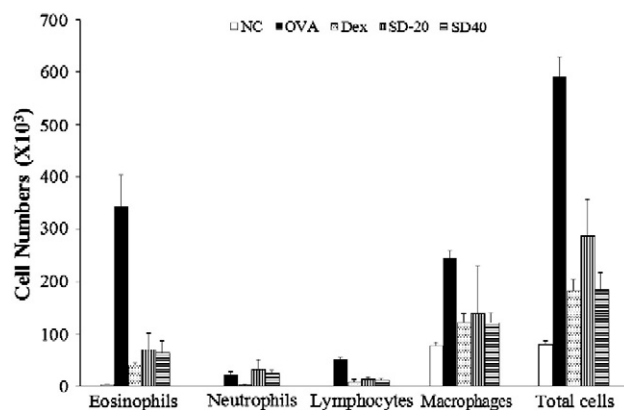


**Fig. 3.** Effects of saucerneol D on the recruitment of inflammatory cells to bronchoalveolar lavage fluid (BALF) of mice 48 h after the final OVA challenge. Cells were isolated by centrifugation and stained with Diff-Quik® Stain reagent. Cell numbers were determined by counting within at least five squares of a hemocytometer using a light microscope. Dead cells, which were stained with trypan blue, were excluded from the total cell counts. NC, negative control (PBS only); OVA, OVA-sensitized/-challenged mice; Dex, dexamethasone (3 mg/kg) + OVA-sensitized/-challenged mice; SD-20, saucerneol D (20 mg/kg) + OVA-sensitized/-challenged mice; SD-40, saucerneol D (40 mg/kg) + OVA-sensitized/-challenged mice. Saucerneol D or dexamethasone treatment was performed 1 h before challenge. \*Significantly different from NC,  $P < 0.05$ ; #significantly different from OVA,  $P < 0.05$ .

#### 2.4. Inflammatory cell counts in bronchoalveolar lavage fluid (BALF)

Mice were sacrificed with an overdose (50 mg/kg) of pentobarbital 48 h after the final challenge and tracheostomy was performed. After the instillation of ice-cold PBS (0.6 mL) into the lungs, BALF was obtained using three aspirations (total volume of 1.8 mL) via tracheal cannulation. BALF was centrifuged and the supernatant fractions were collected and stored at  $-70^{\circ}\text{C}$ . The total number of inflammatory cells was assessed by counting cells in at least five squares of a hemocytometer after exclusion of dead cells via trypan blue staining. BALF (100  $\mu\text{L}$ ) was pipetted onto a slide and centrifuged ( $200\times g$ ,  $4^{\circ}\text{C}$ , 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  $\mu\text{L}$  of each solution was spun onto a slide. After slides were dried, cells were fixed and stained using Diff-Quik® Staining reagent (B4132-1A; Dade Behring Inc., Deerfield, IL), according to the manufacturer's instructions.

#### 2.5. Measurement of total and OVA-specific IgE levels in BALF

BALF was collected via centrifugation (3000 rpm, 10 min) and stored at  $-70^{\circ}\text{C}$ . Total and OVA-specific IgE levels were measured using an enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with 100  $\mu\text{L}$ /well IgE (10  $\mu\text{g}/\text{mL}$ ; Serotec, Oxford, UK) in PBS-Tween 20. Antibodies in the serum were detected using isotype-specific secondary antibodies (anti-mouse IgE; Serotec). After washing four times, 200  $\mu\text{L}$  Lo-phenylene diamine dihydrochloride (Sigma, St Louis, MO) was added to each well. The plate was incubated for 10 min in the dark and absorbance was measured at 450 nm. Total and OVA-specific IgE concentrations were calculated from a standard curve generated using 250 ng/mL recombinant IgE (Serotec).

#### 2.6. Measurement of cytokine and chemokine levels in BALF

Cytokine and chemokine (eotaxin) levels in the BALF were measured using ELISA. The BALF concentrations of IL-4, IL-13, and eotaxin were measured using ELISA kits, according to the manufacturer's instructions (BioSource International, Camarillo, CA).

#### 2.7. Western blotting

Lung tissue was homogenized in lysis buffer containing protease inhibitors (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.1% SDS, 1 mM EGTA, 100  $\mu\text{g}/\text{mL}$  PMSF, 10  $\mu\text{g}/\text{mL}$  pepstatin A, and 100  $\mu\text{M}$   $\text{Na}_3\text{VO}_3$ ). Homogenates were centrifuged at  $12,000\times g$  for 25 min at  $48^{\circ}\text{C}$  and the concentration of proteins in the supernatant fractions was determined using the Bradford reagent (Bio-Rad, Hercules, CA). Equal amounts of protein (25  $\mu\text{g}$ ) were subjected to 10% SDS-PAGE. Separated proteins were transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ) after electrophoresis at 100 V for 90 min. Membranes were blocked with 5% nonfat dry milk dissolved in TBST buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20) overnight at  $4^{\circ}\text{C}$  and incubated with mouse anti-HO-1 and anti-actin antibodies (1:1000 dilution; Abcam Inc., Cambridge, MA) overnight at  $4^{\circ}\text{C}$ . After removal of the primary antibody, membranes were washed three times with TBST buffer at room temperature and were incubated

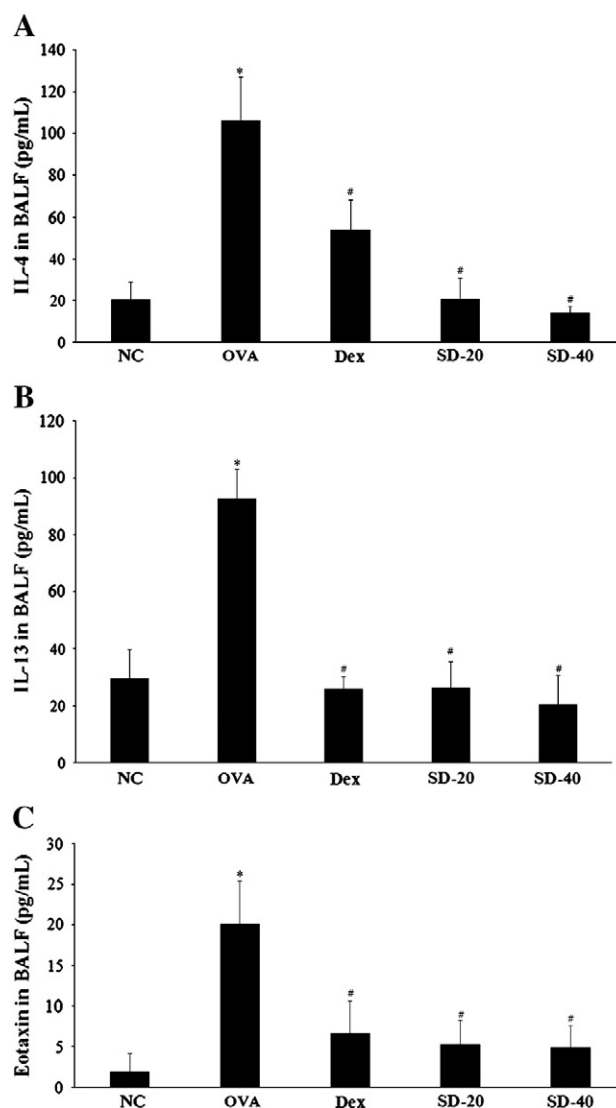


**Fig. 4.** Effects of saucerneol D on cytokine and chemokine levels in the BALF. BALF was collected from mice 48 h after the last OVA challenge. Individual samples were analyzed using ELISA (A) IL-4; (B) IL-13; (C) eotaxin. NC, negative control (PBS only); OVA, OVA-sensitized/-challenged mice; Dex, dexamethasone (3 mg/kg) + OVA-sensitized/-challenged mice; SD-20, saucerneol D (20 mg/kg) + OVA-sensitized/-challenged mice; SD-40, saucerneol D (40 mg/kg) + OVA-sensitized/-challenged mice. Saucerneol D or dexamethasone treatment was performed 1 h before challenge. \*Significantly different from NC,  $P < 0.05$ ; #significantly different from OVA,  $P < 0.05$ .

further with a horseradish peroxidase-conjugated secondary antibody (1:2,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Subsequently, membranes were rewashed with TBST buffer and immunoreactive bands were visualized using ECL reagent (Amersham Biosciences). Densitometric values of the bands were determined using Bio-Rad Gel Doc 2000 gel documentation system (Bio-Rad, Hercules, CA) and statistically analyzed.

## 2.8. 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 µm 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), to assess mucus production. Tissues were then mounted and cover slipped using Dako mounting medium (Dakocytomation, Glostrup, Denmark).

## 2.9. 2',7'-Dichlorofluorescein (DCF) fluorescence assay

Induction of oxidative stress was monitored using 2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Eugene, OR), which is converted into the highly fluorescent DCF by cellular peroxides including hydrogen peroxide. In brief, lung tissue was washed with PBS and next treated with 25 µM DCF-DA for 10 min at 37 °C. To measure intracellular ROS activity, fluorescence was determined at 488 nm excitation and 525 nm emission using a fluorescence plate reader (Perkin-Elmer, Waltham, MA).

## 2.10. Measurement of glutathione peroxidase (GSH), malondialdehyde (MDA), and superoxide dismutase (SOD) levels in lung tissue

The homogenates of lung tissues were centrifuged at 12,000×g (4 °C) for 20 min to collect supernatants for determination of GSH, SOD, MDA concentrations. GSH, MDA, and SOD activity was assayed

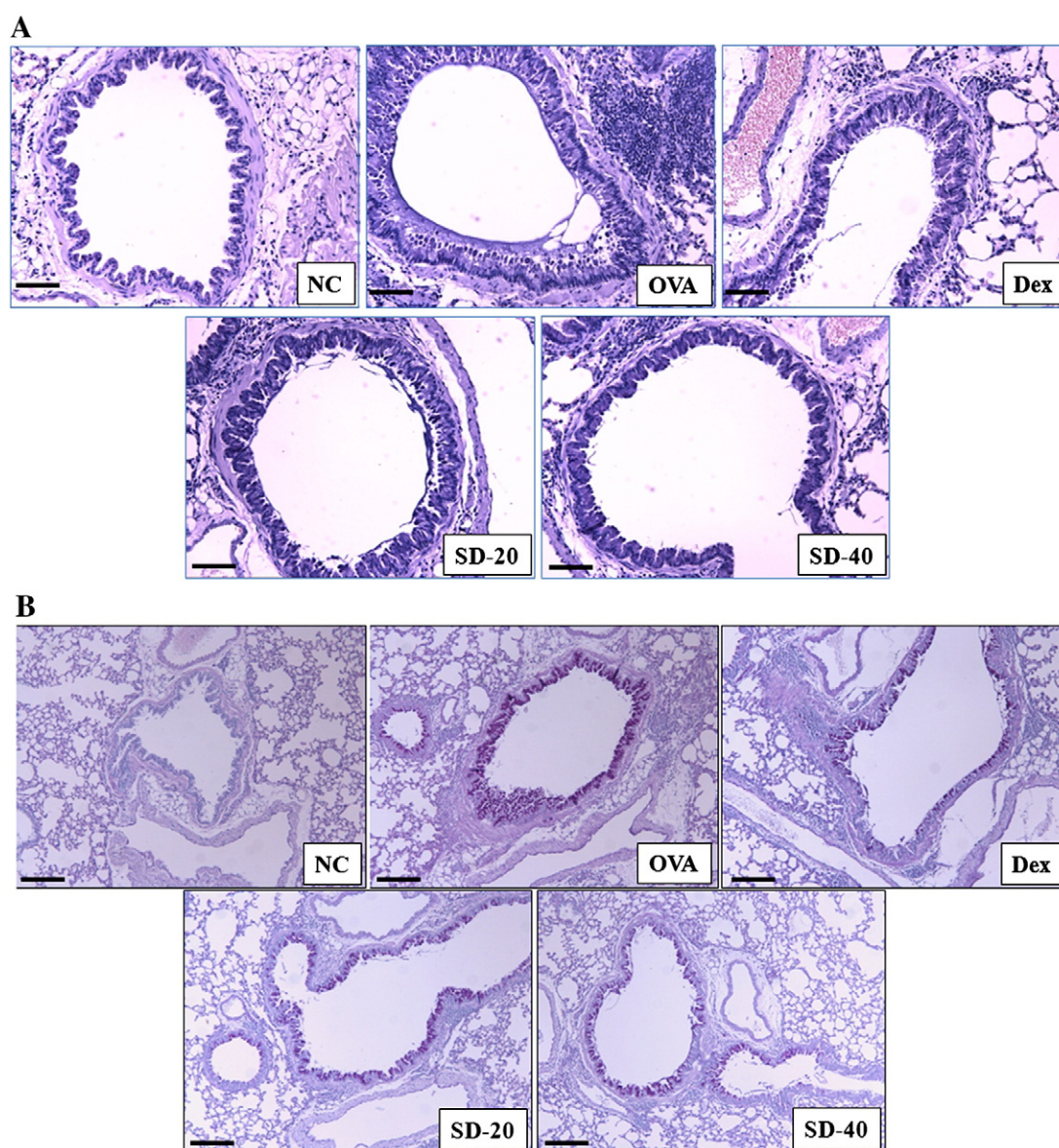


**Fig. 5.** Effects of saucerneol D on the recruitment of leukocytes and mucus production in lung tissue. Histological examination of lung tissues was performed 48 h after the final OVA challenge. Lung tissues were fixed, sectioned at 4 µm thickness, and stained with H&E solution (A) and periodic acid-Schiff (PAS) (B). NC, negative control (PBS only); OVA, OVA-sensitized/-challenged mice; Dex, dexamethasone (3 mg/kg) + OVA-sensitized/-challenged mice; SD-20, saucerneol D (20 mg/kg) + OVA-sensitized/-challenged mice; SD-40, saucerneol D (40 mg/kg) + OVA-sensitized/-challenged mice. Saucerneol D or dexamethasone treatment was performed 1 h before challenge. Scale bars: A, 20 µm; B, 40 µm.

according to the manufacturer's instructions (Cayman, Michigan, CA). GSH based on the reaction between glutathione remaining after the action of GSH that absorbs maximally at 412 nm by a spectrophotometer. SOD activity was determined using the modified method of NADH-phenazinemetosulphate-nitroblue tetrazolium formazan inhibition reaction, which was measured spectrophotometrically at 560. MDA was measured as the production of lipid peroxide (LPO), which in combination with thiobarbituric acid (TBA) forms pink chromogen compound whose absorbance at 530 nm was recorded.

### 2.11. Image capture and statistical analysis

Photomicrographs were obtained using a Photometric Quantix digital camera running a Windows program, and montages were assembled using Adobe Photoshop 7.0. Images were cropped and corrected for brightness and contrast, but were not manipulated otherwise. Data were expressed as the mean  $\pm$  standard deviation. Statistical comparisons were performed using one-way analysis of variance, with significance set at  $P < 0.05$ .

## 3. Results

### 3.1. Effects of saucerneol D on OVA-induced eosinophilia in BALF

To evaluate the suppression of inflammatory cells by saucerneol D in OVA-challenged mice, we counted the number of cells that were recruited to the BALF. The number of eosinophils, neutrophils, lymphocytes, macrophages, and total cells in the BALF was increased significantly at 48 h after the OVA challenge compared with their number in PBS-challenged mice. Oral administration of saucerneol D at doses of 20 and 40 mg/kg 1 h before OVA challenge inhibited eosinophils, neutrophils, lymphocytes, macrophages, and total cells significantly compared with OVA-induced mice that were not exposed to saucerneol D. In addition, the decrease in the number of infiltrated leukocytes was due mainly to the decrease in the levels of infiltrated macrophages and eosinophils. These results suggest that saucerneol D has an inhibitory effect on lung inflammation in our asthma model. A positive control showed a similar decrease in the number of cells in the BALF after administration of 40 mg/kg of saucerneol D (Fig. 3).

### 3.2. Effects of saucerneol D on the levels of cytokines and chemokines in the BALF

To determine whether saucerneol D influenced cytokine and chemokine secretion in the BALF, the levels of IL-4, IL-13, and eotaxin in this fluid were measured using ELISA 48 h after the final challenge. As shown in Fig. 4, OVA challenge triggered a significant increase in cytokine levels in the BALF compared with those observed in control group. The increase in the level of cytokines in OVA-induced mice treated with saucerneol D was suppressed significantly compared with the OVA-induced group. Our results demonstrate clearly that saucerneol D reduces IL-4, IL-13, and eotaxin concentrations in the BALF of asthmatic mice.

### 3.3. Effects of saucerneol D on airway goblet cell hyperplasia and mucus production in lung tissue

As saucerneol D inhibited inflammatory cell recruitment into the BALF, we examined its antiasthmatic effects. Lung tissues were collected 48 h after the final OVA challenge. We observed a marked infiltration of inflammatory cells into perivascular and peribronchial connective tissues of OVA-induced asthmatic lungs compared with normal tissue. Moreover, the majority of leukocytes were eosinophils. The NC group and the Dex control mice displayed no changes in inflammatory cell infiltration. Eosinophilia in OVA-induced mice

treated with saucerneol D was significantly attenuated compared with that observed in OVA-induced mice (Fig. 5A).

Mucus hypersecretion is a characteristic of airway remodeling. Goblet cells containing mucus were increased in OVA-induced mice. To determine whether saucerneol D suppressed the mucus overproduction caused by goblet cell hyperplasia, we stained lung sections with PAS. Mucus overproduction was observed clearly as a violet color in the bronchial airways of OVA-challenged mice compared with the NC group. In contrast, the mucus stain was diminished markedly in OVA-sensitized and -challenged mice treated with saucerneol D (Fig. 5B). Our data confirm that saucerneol D significantly reduces goblet cell hyperplasia and the mucus hypersecretion resulting from the airway remodeling process.

### 3.4. Effects of saucerneol D on total and OVA-specific IgE levels

Total and OVA-specific IgE levels in the BALF were increased markedly in OVA-induced mice compared with the levels detected in the NC group. Saucerneol D pretreatment led to a significant reduction in the levels of total and OVA-specific IgE, in a dose-dependent manner (Fig. 6).

### 3.5. Effects of saucerneol D on OVA-induced ROS, MDA, SOD, and GSH levels in lung tissue

Preadministration of saucerneol D led to a significant decrease in the generation of ROS and in the level of MDA in lung tissue. In addition, the

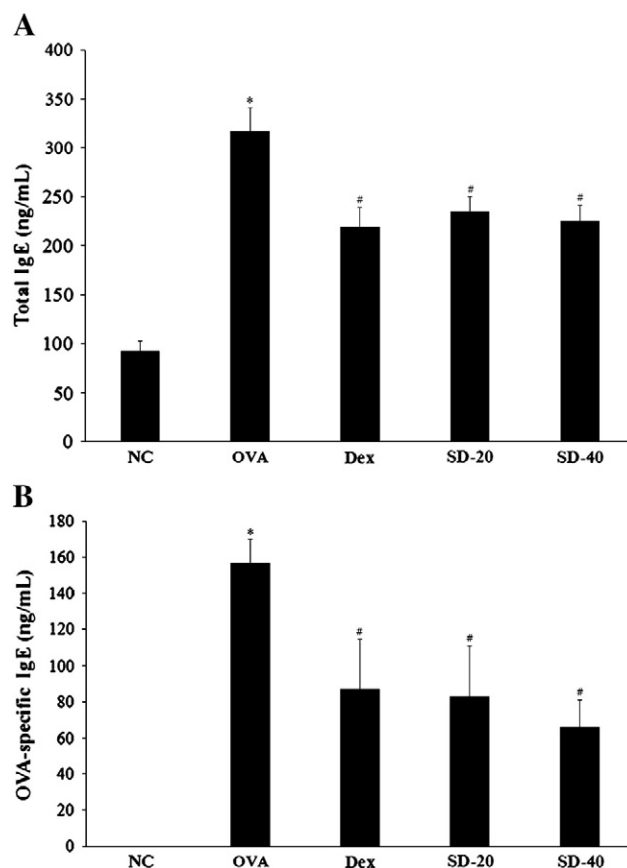


**Fig. 6.** Effects of saucerneol D on the levels of total and OVA-specific IgE in the BALF. BALF was collected from mice 48 h after the final OVA challenge. Each sample was analyzed using ELISA. (A) Total IgE level. (B) OVA-specific IgE level. NC, negative control (PBS only); OVA, OVA-sensitized/-challenged mice; Dex, dexamethasone (3 mg/kg) + OVA-sensitized/-challenged mice; SD-20, saucerneol D (20 mg/kg) + OVA-sensitized/-challenged mice; SD-40, saucerneol D (40 mg/kg) + OVA-sensitized/-challenged mice. Saucerneol D or dexamethasone treatment was performed 1 h before challenge. \*Significantly different from NC,  $P < 0.05$ ; #significantly different from OVA,  $P < 0.05$ .

levels of oxidative stress markers, such as SOD and GSH, were increased markedly in OVA-challenged mice, as shown in Fig. 7.

### 3.6. Effects of saucerneol D on HO-1 expression

Next, we investigated whether saucerneol D upregulated the expression of HO-1 as part of the mechanism underlying its protective effect against OVA-induced lung inflammation. To determine whether the inhibitory effects of saucerneol D on airway inflammation were related to HO-1 induction, its expression was assessed using ELISA, Western blotting, and immunohistochemical analyses. As shown in Fig. 8A, saucerneol D treatment led to increased HO-1 activity in lung tissue. The induction of HO-1 activity was similar after treatment with saucerneol D at 20 and 40 mg/kg.

Compared with OVA-challenged mice, saucerneol D induced HO-1 activity at doses of 20 and 40 mg/kg. Consistent with this increase in activity, HO-1 protein levels were increased markedly in saucerneol D-treated mice (Fig. 8B). However, treatment with saucerneol D (20 or 40 mg/kg) led to a higher level of expression of HO-1.

## 4. Discussion

The present study used an OVA-induced model of allergic airway inflammation to demonstrate that ROS production was increased in lung tissues and that the administration of saucerneol D significantly attenuated ROS generation, MDA activity, Th2 cytokine expression, and bronchial inflammatory cell infiltration and increased SOD and GSH levels in OVA-challenged mice.

The pathogenesis of asthma is associated with increased infiltration of inflammatory cells and excessive mucus secretion into the airway [36]. OVA-induced asthma is recognized as a disease that results from

chronic airway inflammation characteristically associated with the infiltration of lymphocytes, eosinophils, macrophages, and neutrophils into the bronchial lumen. Eosinophils are markers of allergic airway inflammation in asthma and play a central role in the pathogenesis of this disease [37–39]. Before investigating such effects in detail, using saucerneol D at 20 and 40 mg/kg, we made a preliminary study of the effects of saucerneol D (given at 10, 20, 40, and 80 mg/kg) on serum IgE levels, to ascertain an optimal dosage (data not shown). Doses of both 20 and 40 mg/kg were most effective. In the OVA-induced model of allergic asthma, saucerneol D resulted in a significant decrease in the number of eosinophils and macrophage counts in the BALF. Moreover, histopathological analysis revealed that saucerneol D treatment suppressed the infiltration of inflammatory cells and the hyperplasia of goblet cells. Many clinical studies also documented a correlation between pulmonary eosinophilia and asthma, as well as a correlation with the level of eosinophils in the BALF [40]. Thus, the suppression of pulmonary eosinophilia by saucerneol D could be explained by the inhibition of the differentiation and migration of eosinophils. The current consensus regarding the etiology of allergic asthma is that it is an aberrant Th2-type response to environmental allergens characterized by overproduction of IL-4, IL-5, and IL-13, which are critical for the maintenance of ongoing IgE-mediated eosinophilic inflammation [38]. IL-4 is the Th2 cytokine that is most important for the induction of isotype switching to IgE in B lymphocytes [41]. IL-13 can also regulate the recruitment of eosinophils to the airways, via its various effects on epithelial and smooth-muscle cells [42]. We showed here that administration of saucerneol D opposed the effects of OVA challenge on IL-4, IL-13, and eotaxin, reducing both the levels of secreted cytokines and chemokines in the BALF. These results indicate that saucerneol D provides relief against airway inflammation by reducing the levels of Th2 cytokines, such as IL-4 and IL-13. Consistent with the reduction of the levels of these cytokines, the recruitment of

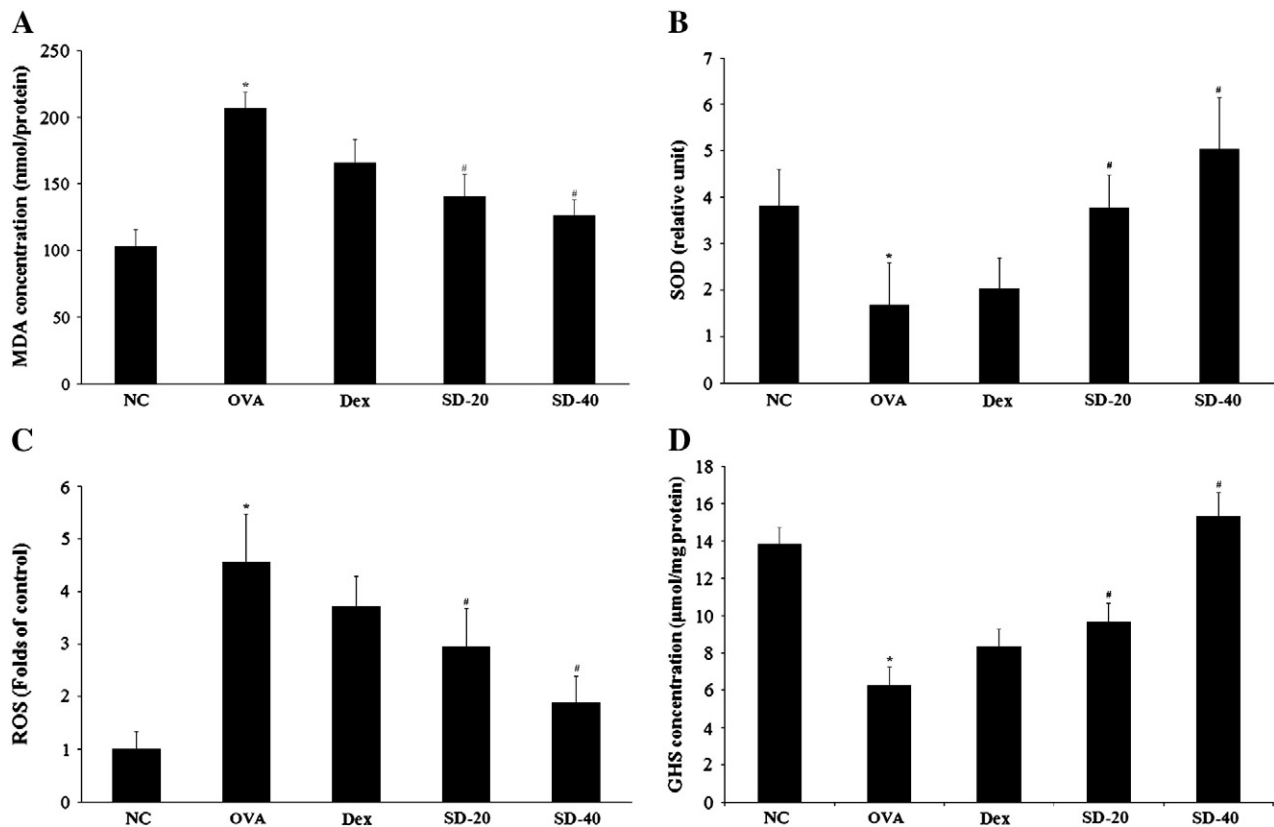


**Fig. 7.** Effects of saucerneol D on MDA, ROS, SOD, and GSH levels in lung tissue. Lung tissues were collected from mice 48 h after the final OVA challenge. Each sample was analyzed using ELISA. Enzymatic activity of (A) MDA, (B) ROS, (C) SOD, and (D) GSH in lung tissue. NC, negative control (PBS only); OVA, OVA-sensitized/-challenged mice; Dex, dexamethasone (3 mg/kg) + OVA-sensitized/-challenged mice; SD-20, saucerneol D (20 mg/kg) + OVA-sensitized/-challenged mice; SD-40, saucerneol D (40 mg/kg) + OVA-sensitized/-challenged mice. Saucerneol D treatment was performed 1 h before challenge. \*Significantly different from NC,  $P < 0.05$ ; #significantly different from OVA,  $P < 0.05$ .

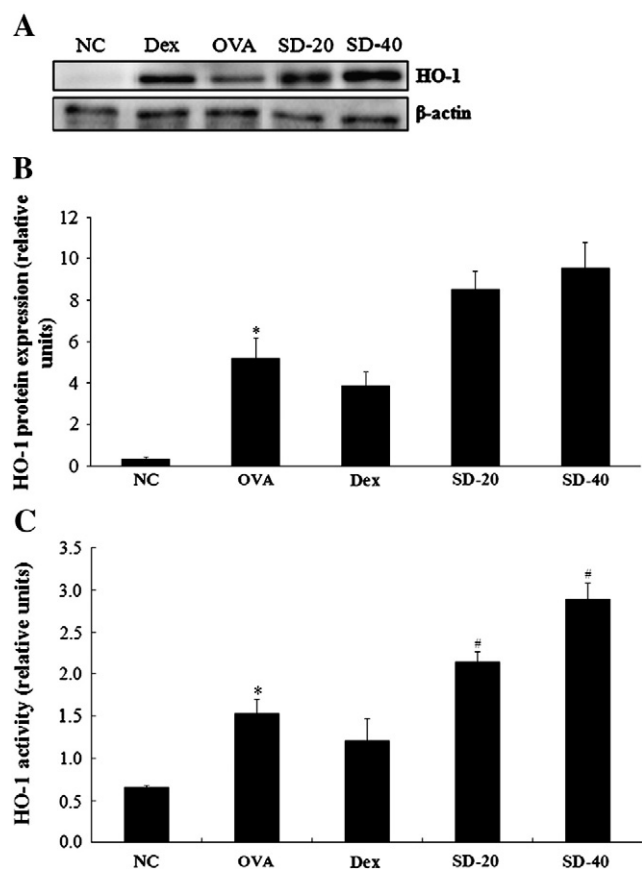


**Fig. 8.** Effects of saucerneol D on HO-1 activity and protein level. Lung tissues were collected from mice 48 h after the final OVA challenge. Each sample was analyzed using ELISA, Western blotting, and immunohistochemistry. (A) The level of expression of the HO-1 protein in the lung was determined using Western blotting analysis after OVA challenge in the presence or absence of AD. The level of HO-1 protein was normalized to that of  $\beta$ -actin. (B) Quantification of band intensities of HO-1. Three independent experiments were determined by densitometry. (C) Enzymatic activity of HO-1 in lung tissue. NC, negative control (PBS only); OVA, OVA-sensitized/-challenged mice; Dex, dexamethasone (3 mg/kg) + OVA-sensitized/-challenged mice; SD-20, saucerneol D (20 mg/kg) + OVA-sensitized/-challenged mice; SD-40, saucerneol D (40 mg/kg) + OVA-sensitized/-challenged mice. Saucerneol D treatment was performed 1 h before challenge. \*Significantly different from NC,  $P < 0.05$ ; #significantly different from OVA,  $P < 0.05$ .

inflammatory cells, especially of eosinophils and macrophages, was suppressed markedly in the BALF after saucerneol D treatment.

Oxidative stress due to oxidant–antioxidant imbalance and to environmental oxidants is an important component of inflammation and respiratory diseases. The lungs are endowed with a battery of endogenous agents called antioxidants. GSH is the main enzyme of the enzymatic antioxidant defense system that is responsible for the protection against the increase in the production of ROS [43]. The level of MDA is a measure of LP and its measurement provides an estimate of free radical activity [44]. SOD is present essentially in every cell of the body and plays an important role in protecting cells and tissues against oxidative stress. In the present study, saucerneol D attenuated ROS and MDA levels in an OVA-induced airway inflammation murine model. Our findings demonstrated that saucerneol D inhibits the OVA-induced reduction of SOD and GSH levels in a dose-dependent manner, suggesting that the protective effect of saucerneol D is related to its reduction of oxidative stress. HO-1 is induced in a mouse model of asthma; conversely, its deficiency leads to an increase in chronic inflammation and leukocyte recruitment [45,46]. This interpretation is also consistent with the observation that HO-1 overexpression reduces ocular inflammation by downregulating proinflammatory cytokines [47]. Based on these previous reports,

we examined whether HO-1 induction played a role in the saucerneol D-mediated attenuation of airway inflammation.

In conclusion, our results indicated clearly that saucerneol D treatment reduces the accumulation of eosinophils and other inflammatory cells (neutrophils, lymphocytes, and macrophages) in the BALF and lung tissues of an OVA-induced murine asthma model; that IgE, chemokine (eotaxin), IL-4, and IL-13 levels were upregulated; and that saucerneol D reduced significantly the severity of airway inflammation and the accompanying oxidative stress. Furthermore, our data provided evidence that the induction of HO-1 by saucerneol D may be responsible for the protective effects of this agent against airway inflammation and that it may be a promising candidate in herbal medicine for the treatment of asthma.

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## References

- [1] Salvi S, Krishna MT, Sampson A, Holgate ST. The anti-inflammatory effects of leukotriene-modifying drugs and their use in asthma. *Chest* 2001;119:1533–46.
- [2] Kay AB. Asthma and inflammation. *J Allergy Clin Immunol* 1991;87:893–910.
- [3] Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000;161:1720–45.
- [4] Wegmann M. Th2 cells as targets for therapeutic intervention in allergic bronchial asthma. *Expert Rev Mol Diagn* 2009;9:85–100.
- [5] Sanderson CJ, Warren DJ, Strath M. Identification of a lymphokine that stimulates eosinophil differentiation in vitro. Its relationship to interleukin-3, and functional properties of eosinophils produced in cultures. *J Exp Med* 1985;162:60.
- [6] Lopez AF, Begley CG, Williamson DJ, Warren DJ, Vadas MA, Sanderson CJ. Murine eosinophil differentiation factor. An eosinophil-specific colony-stimulating factor with activity for human cells. *J Exp Med* 1986;163:1085.
- [7] Azuma C, Tanabe T, Konishi M, Kinashi T, Noma T, Matsuda F, et al. Cloning of cDNA for human T-cell replacing factor (interleukin-5) and comparison with the murine homologue. *Nucleic Acids Res* 1986;14:9149.
- [8] Cho HY, Reddy SP, Kleeberger SR. Nrf2 defends the lung from oxidative stress. *Antioxid Redox Signal* 2006;8:76–87.
- [9] Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1–Nrf2–ARE pathway. *Annu Rev Pharmacol Toxicol* 2007;47:89–116.
- [10] Almolki A, Guenegou S, Golda L, Boyer M, Benallaoua N, Amara R, et al. Heme oxygenase-1 prevents airway mucus hypersecretion induced by cigarette smoke in rodents and humans. *Am J Pathol* 2008;173:981–92.
- [11] Rushworth SA, MacEwan DJ, O’Connell MA. Lipopolysaccharide-induced expression of NAD(P)H:quinone oxidoreductase 1 and heme oxygenase-1 protects against excessive inflammatory responses in human monocytes. *J Immunol* 2008;181:6730–7.
- [12] Paredi P, Kharitonov SA, Barnes PJ. Analysis of expired air for oxidation products. *Am J Respir Crit Care Med* 2002;166:31–7.
- [13] Baker JC, Tunnicliffe WS, Duncanson RC, Ayres JG. Dietary antioxidants and magnesium in type 1 brittle asthma: a case control study. *Thorax* 1999;54:115–8.
- [14] Richter C, Gogvadze V, Laffranchi R, Schlappbach R, Schweizer M, Suter M, et al. Oxidants in mitochondria: from physiology to diseases. *Biochim Biophys Acta* 1995;1271:67–74.
- [15] Gutteridge JM, Halliwell B. Free radicals and antioxidants in the year 2000. A historical look to the future. *Ann NY Acad Sci* 2000;899:136–47.
- [16] Calhoun WJ, Reed HE, Moest DR, Stevens CA. Enhanced superoxide production by alveolar macrophages and air-space cells, airway inflammation, and alveolar macrophage density changes after segmental antigen bronchoprovocation in allergic subjects. *Am Rev Respir Dis* 1992;145:317–25.
- [17] Teramoto S, Shu CY, Ouchi Y, Fukuchi Y. Increased spontaneous production and generation of superoxide anion by blood neutrophils in patients with asthma. *J Asthma* 1996;33:149–55.
- [18] Hulsman AR, Raatgeep HR, den Hollander JC, Stijnen T, Saxena PR, Kerrebijn KF, et al. Oxidative epithelial damage produces hyperresponsiveness of human peripheral airways. *Am J Respir Crit Care Med* 1994;149:519–25.
- [19] Sadeghi-Hashjin G, Folkerts G, Henricks PA, Verheyen AK, van der Linde HJ, van Ark I, et al. Peroxynitrite induces airway hyperresponsiveness in guinea pigs in vitro and in vivo. *Am J Respir Crit Care Med* 1996;153:1697–701.
- [20] Rahman I, MacNee W. Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. *Am J Physiol* 1999;277:1067–88.
- [21] Hatch GE. Asthma, inhaled oxidants, and dietary antioxidants. *Am J Clin Nutr* 1995;61:625S–30S.

- [22] Rahman I, Macnee W. Oxidant/antioxidant imbalance in smokers and chronic obstructive pulmonary diseases. *Thorax* 1996;51:348–51.
- [23] Rao KV, Rao NS. Chemistry of *Saururus cernuus*, VI: three new neolignans. *J Nat Prod* 1990;53:212.
- [24] Dworski R. Oxidant stress in asthma. *Thorax* 2000;55(Suppl. 2):S51–3.
- [25] Shanmugasundaram KR, Kumar SS, Rajasee S. Excessive free radical production in the blood of children suffering from asthma. *Clin Chim Acta* 2001;305:107–14.
- [26] Kinnula VL, Carpo JD. Superoxide dismutases in the lung and human lung diseases. *Am J Respir Crit Care Med* 2003;167:1600–19.
- [27] Ghosh S, Janocha AJ, Aronica MA, Swaidani S, Comhair SA, Xu W, et al. Nitrotyrosine proteome survey in asthma identifies oxidative mechanism of catalase inactivation. *J Immunol* 2006;176:5587–97.
- [28] Bachowski S, Kolař KL, Xu Y, Ketcham CA, Stevenson DE, Walborg Jr EF, et al. Role of oxidative stress in the mechanism of dieldrin's hepatotoxicity. *Ann Clin Lab Sci* 1997;27:196–209.
- [29] Chung BS, Shin MG. Dictionary of Korean folk medicine. Seoul: Young Lim Publishing Co; 1990.
- [30] Rao KV, Rao NS. Chemistry of *Saururus cernuus*, VI: three new neolignans. *J Nat Prod* 1990;53:212.
- [31] Rao KV, Reddy GC. Chemistry of *Saururus cernuus*, V. sauristolactam and other nitrogenous constituents. *J Nat Prod* 1990;53:309.
- [32] Sung SH, Huh MS, Kim YC. New tetrahydrofuran-type sesquiterpenes of *Saururus chinensis* root. *Chem Pharm Bull (Tokyo)* 2001;49:1192.
- [33] Ernst E, Huntley A. Herbal medicines for asthma: a systematic review. *Thorax* 2000;55:925–9.
- [34] Hwang BY, Lee JH, Nam JB, Hong YS, Lee JJ. Lignans from *Saururus chinensis* inhibiting the transcription factor NF- $\kappa$ B. *Phytochemistry* 2003;64:765.
- [35] Oh SR, Lee MY, Ahn K, Park BY, Kwon OK, Joung H, et al. Suppressive effect of verproside isolated from *Pseudotsuga longifolia* on airway inflammation in a mouse model of allergic asthma. *Int Immunopharmacol* 2006;6:978–86.
- [36] Busse WW, Rosenwasser LJ. Mechanisms of asthma. *J Allergy Clin Immunol* 2003;111:S799–804.
- [37] Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature* 2008;454:445–54.
- [38] Busse WW, Lemanske Jr RF. Asthma. *N Engl J Med* 2001;344:350–62.
- [39] Pearlman DS. Pathophysiology of the inflammatory response. *J Allergy Clin Immunol* 1999;104:S132–7.
- [40] Barrett NA, Austen KF. Innate cells and T helper 2 cell immunity in airway inflammation. *Immunity* 2009;31:425–37.
- [41] Finkelman FD, Holmes J, Katona IM, Urban Jr JF, Beckmann MP, Park LS, et al. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu Rev Immunol* 1990;8:303–33.
- [42] Wills-Karp M. Interleukin-13 in asthma pathogenesis. *Immunol Rev* 2004;202:175–90.
- [43] Rahman I, Biswas SK, Kode A. Oxidant and, antioxidant balance in the airways and airway diseases. *Eur J Pharmacol* 2006;533:222–39.
- [44] Tracz MJ, Alam J, Nath KA. Physiology and pathophysiology of heme: implications for kidney disease. *J Am Soc Nephrol* 2007;18:414–20.
- [45] Vogt BA, Shanley TP, Croatt A, Alam J, Johnson KJ, Nath KA. Glomerular inflammation induces resistance to tubular injury in the rat. A novel form of acquired, heme oxygenase-dependent resistance to renal injury. *J Clin Invest* 1996;98:2139–45.
- [46] Willis D, Moore AR, Frederick R, Willoughby DA. Heme oxygenase: a novel target for the modulation of the inflammatory response. *Nat Med* 1996;2:87–90.
- [47] Ohta K, Kikuchi T, Arai S, Yoshida N, Sato A, Yoshimura N. Protective role of heme oxygenase-1 against endotoxin-induced uveitis in rats. *Exp Eye Res* 2003;77:665–73.