

Antiasthmatic Action of Dibenzylobutyrolactone Lignans from Fruits of *Forsythia viridissima* on Asthmatic Responses to Ovalbumin Challenge in Conscious Guinea-pigs

Jin Hee Lee, Ji Yun Lee, Tae Doo Kim and Chang Jong Kim*

Division of Pathophysiology, College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea

It was reported previously that dibenzylobutyrolactone lignans from *Forsythia viridissima* fruits, which are traditional medicines for the treatment of inflammatory diseases, have antiinflammatory effects. In this study, the effects on the immediate-phase response (IAR) and late-phase response (LAR) following aerosolized-ovalbumin challenge in ovalbumin-sensitized guinea-pigs were evaluated by measuring the specific airway resistance (sRaw), recruitment of leukocytes and chemical mediators in the bronchoalveolar lavage fluids (BALF) as well as a histopathological survey. Arctiin and matairesinol at 12.5 mg/kg significantly ($p < 0.05$) decreased sRaw by 51.83% and 43.15% in IAR and by 47.41% and 35.43% in LAR, respectively, whereas arctigenin at 25 mg/kg was significantly active, compared with the controls. Furthermore, arctiin and arctigenin dose-dependently inhibited histamine, and the activities of phospholipase A₂ (PLA₂) and eosinophil peroxidase (EPO) in BALF, respectively, whereas matairesinol inhibited EPO and PLA₂ at 12.5 mg/kg and histamine at 50 mg/kg, in addition, they moderately improved the infiltration of eosinophils, compared with controls. Dexamethasone, cromolyn and salbutamol significantly inhibited sRaw in both IAR and LAR, and the recruitment of leukocytes and chemical mediators, whereas salbutamol did not alter chemical mediators, in BALF. These results indicate the three lignans have antiasthmatic effects which were less active than those of the reference drugs. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: dibenzylobutyrolactone lignans; sRaw; histamine; PLA₂; EPO; antiasthmatic.

INTRODUCTION

Forsythiae Fructus, the dried fruits of *Forsythia viridissima*, *F. koreana* and *F. suspensa* (Oleaceae), has long been used in traditional herbal medicine as an antiinflammatory, diuretic, antidote and antibacterial agent (Jang *et al.*, 2001; Lee *et al.*, 1996). It contains many physiologically active dibenzylobutyrolactone lignans as secondary metabolites (Nishibe, 2001) that have been the source of widespread interest due to their various biological effects including antiinflammatory, antitumor, antioxidant, antiviral and neurodegenerative inhibitory activities (Jang *et al.*, 2001; Eich *et al.*, 1996; MacRae and Towers, 1984; Jang *et al.*, 2002). The biological activity of many kinds of dibenzylobutyrolactone lignans has been reported; matairesinol has demonstrated its inhibitory effects against the formation of proinflammatory mediators such as prostaglandin E₂, interleukin-1 α and -1 β , and tumor necrosis factor- α ; arctigenin has antitumor, antiviral and hepatoprotective properties as well as antioxidant and antiinflammatory effects (Kim *et al.*, 2006); arctiin, a glycoside of arctigenin, has antiinflammatory and environmental endocrine disrupting

activities (Zeng *et al.*, 2005). In previous studies, the methanol extract of *F. viridissima* had significant inhibitory effects on inflammation and asthma; in addition, arctigenin isolated from *F. viridissima* had antiinflammatory, antioxidant and antiallergy activities in arachidonic acid-induced ear edema, lipopolysaccharide-induced prostaglandin E₂ release and silica-induced intracellular reactive oxygen species production in animal models (Kang *et al.*, 2008; Kim *et al.*, 2000). However, to date, the effects of *F. viridissima* and its constituents on the asthmatic response has yet to be investigated.

In the present study, the dibenzylobutyrolactone lignans (Fig. 1), arctigenin, matairesinol and arctiin were isolated from the ethylacetate fraction of the methanol extract of *F. viridissima* by activity-guided separation and the effects of the lignans on the asthmatic responses in ovalbumin (OA)-sensitized conscious guinea-pigs were evaluated by measuring the specific airway resistance (sRaw) using double-chambered plethysmography during the immediate-phase response (IAR) and late-phase response (LAR) following an aerosolized-OA challenge, respectively (Jung *et al.*, 2007; Matsumoto *et al.*, 1994; Sanjar *et al.*, 1990; Huston *et al.*, 1988). Chemical mediators and leukocytes in bronchoalveolar lavage fluid (BALF) were measured and a histopathological survey of asthmatic lung tissues was conducted. The antiasthmatic activity of the lignans was then compared with that of the currently authorized antiasthmatic drugs (Cockcroft and Murdok, 1987).

* Correspondence to: Professor Chang Jong Kim, Division of Pathophysiology and Pharmacology, College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea.
E-mail: kimcj@cau.ac.kr

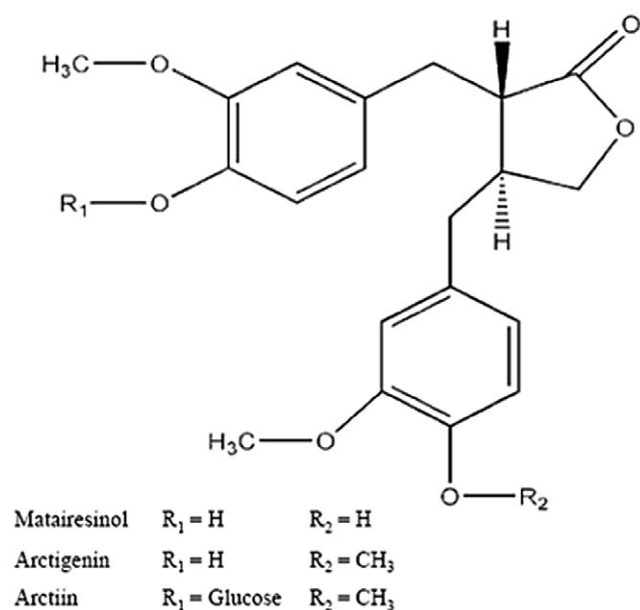


Figure 1. Chemical structure of dibenzylbutyrolactone lignans isolated from *Forsythia viridissima* fruits: matairesinol, arctigenin, arctiin.

MATERIALS AND METHODS

Plant materials and chemicals. The fruits of *F. viridissima* Lindl. were collected at Sok Ri Mountain in Korea, and identified by Professor D. R. Han (Division of Pharmacognosy, College of Pharmacy, Chung-Ang University). A voucher specimen no. FVL/Fruit/01/2007 has been submitted at the College of Pharmacy, Chung-Ang University. Fetal bovine serum (FBS) was purchased from Invitrogen Co. (Grand Island, NY, USA). Ovalbumin (OA, type V), dexamethasone, salbutamol, disodium cromoglycate, bovine serum albumin (BSA) (fraction V), *o*-phthalaldehyde (OPA), *O*-phenylenediamine dihydrochloride (OPD), dimethylsulfoxide (DMSO) and Wright's stain were purchased from Sigma Chemical Co. (St Louis, MO, USA). Standard arctigenin, matairesinol and arctiin were purchased from Sigma Co. and were also supplied by Professor Jin-Woong Kim, College of Pharmacy, Seoul National University. Piprinhydrinate was purchased from Yungjin Pharm. Co., Ltd (Hwa Sung-Gun, Korea). Zoletil 50 was purchased from Virbac Laboratory (06516 Carros, France). A bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Co. (Rockford, IL, USA). 1-Palmitoyl-2-(10-pyrenyldecanoyl)-sn-glycero-3-phosphorylcholine (10-pyrene PC) was purchased from Molecular Probes (Leiden, Netherlands). Preparative thin liquid chromatography (TLC) was purchased from Merck & Co. Inc. (Darmstadt, Germany). Other reagents were of reagent grade. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid-basic salt solution/bovine serum albumin (HEPES-BSS/BSA) (pH 7.4) was made by 132 mM NaCl, 6 mM KCl, 20 mM HEPES, 1.2 mM KH₂PO₄, 1.1 mM CaCl₂·H₂O, 1.0 mM MgSO₄, 5.5 mM dextrose and 1% BSA.

Extraction and isolation. The dried and powdered *F. viridissima* (3.0 kg) underwent extraction with MeOH (3 × 6 L) to yield a solid dark extract (547.0 g). This was

successfully partitioned with *n*-hexane (3 × 3 L), EtOAc (3 × 3 L) and *n*-BuOH (3 × 3 L) to give fractions of *n*-hexane (57.5 g), EtOAc (119.0 g), *n*-BuOH (26.0 g) and water (22.0 g). The EtOAc fraction (119.0 g) was fractionated successfully by silica-gel column chromatography (6.5 × 85.0 cm) eluted with a CHCl₃-MeOH solvent system (100:0 to 0:100), to yield fraction **1** (46.7 g) and **2** (27.9 g), respectively. Fraction **1** was recrystallized by EtOAc to give compound **1** (9.38 g). Fractions **1** and **2** were partitioned by silica-gel column chromatography (3.5 × 41.5 cm) eluted with *n*-hexane-EtOAc solvent (4:6, v/v), to yield compound **2** (2.12 g) and compound **3** (2.37 g), respectively. High performance liquid chromatography (HPLC) analysis of the lignans was modified by the method from Nishibe (2002). The preparative HPLC system used a C₁₈ column (4.6 × 150 mm, i.d. 5 μm) consisting of a Gilson 305 (France) and a Gilson UV 116 detector. The mobile phase was MeOH-H₂O-acetic acid (35:45:3) with a flow rate of 0.5 mL/min. The isolated compounds were allowed to recrystallize in EtOAc and were subsequently purified by preparative thin-layer chromatography (TLC). The structures of compounds **1–3** were identified as arctigenin, matairesinol and arctiin by comparing their phytochemical and spectroscopic data (Jang *et al.*, 2001; Nishibe, 2002; Eich *et al.*, 1996; Shoeb *et al.*, 2006; Kim *et al.*, 2007) with those reported previously. The chemical purities used in this experiment were 98.8%, 96.8% and 99.4%, respectively. A voucher specimen no. FVL/Fruit/Lignan/01-001~003/2007 has been deposited at the College of Pharmacy, Chung-Ang University.

Animals. Pathogen-free male Dunkin-Hartley guinea-pigs (250–300 g) were purchased from HanLim Lab. Animal Co. (Hwa Sung-Gun, Korea). They were housed for over 1 week in a normal environmentally controlled-animal room (temperature 25°C, humidity 55%, aeration 10–18/h, illumination 300–500 Lux) with the usual laboratory animal food (Superfeed Co., Won Ju City, Korea) and water *ad libitum*. This present study was performed according to international, national and institutional rules regarding animal experiments, clinical studies and biodiversity rights. All procedures with experimental animals were approved by the Institutional Animal Care and Use Committee of the Institute for Molecular-Based New Drug Development at Chung-Ang University.

Sensitization. Guinea-pigs were actively sensitized to ovalbumin (OA) by injections of 50 mg of the protein twice on the same day [one intraperitoneal (i.p.) and one subcutaneous (s.c.)], each in 1 mL of 0.9% saline (Sanjar *et al.*, 1990). Then 21 days after sensitization, 1% OA (0.1 mL per site) was injected intradermally and the skin thickness was measured 1 h later. A doubling of skin thickness indicated successful sensitization (Sanjar *et al.*, 1990). Animals that did not respond to the intradermal OA were excluded from the study.

Drug treatment and aerosolized-OA challenge. After measuring the baseline airway function before the aerosolized-OA challenge, each animal was challenged with 1% OA inhalation or vehicle for 5 min in the double-chambered plethysmograph (HSE type 855, Germany). Sensitized animals were fasted overnight

and treated with dibenzylbutyrolactone lignans, reference drugs (salbutamol, dexamethasone and cromolyn sodium) or vehicle (saline) 1 h prior to allergen challenge, 6 and 12 h, and again at 20 h following the challenge. Then 30 min before the OA challenge, the animals were pretreated with the antihistamine, piprinhydrate (5 mg/kg), to prevent sudden death secondary to systemic hypersensitivity. Twenty-five minutes later, each animal was placed in the double-chambered plethysmograph and permitted to acclimatize for 5 min. The animal was then challenged with 10 mL of OA (1.0 mg/mL in saline) or saline aerosol by the jet nebulizer for 5 min (Matsumoto *et al.*, 1994).

Measurement of airway function. Airway function in conscious guinea-pigs was measured with the double-chambered plethysmograph (Jung *et al.*, 2007; Matsumoto *et al.*, 1994; Sanjar *et al.*, 1990). A conscious animal was positioned with its neck extending through the partition of a head and body chamber. The tidal volume, respiratory rate and specific airway resistance (sRaw) were measured by a volumetric differential pressure transducer (PT₅, Grass Co. USA), noninvasive respiratory analyser and ink-writing oscilloscope with a polygraph (7E, Grass Co. USA) before the challenge (baseline) and at 5 min (IAR) and 24 h (LAR) following the challenge. The phase displacement was measured at the end of inspiration on the steep changeover to the expiration phase. The evaluation unit was linked to a computer for continuous data capture. The sRaw, expressed in mmHg × s, was analysed by the Pennock Program 89 (Pennock *et al.*, 1979) following the formula:

$\tan\theta = \omega \times R \times C$; where θ = phase displacement, $\omega = 2\pi \times$ respiration rate (f), $R \times C$ = time constant of the respiratory system, $C = V/P$ = thoracic gas volume (V)/($P_{\text{atm}} - 47$ mmHg). The sRaw is therefore obtained as: $R \times V = (P_{\text{atm}} - 47) \times \tan\theta / (2\pi \times f)$ [mmHg × s], $R \times V = (P_{\text{atm}} - 47) \times 13.6 \times \tan\theta / \omega$ [mmHg × s].

The increase in sRaw was expressed as a percentage of the baseline value before drug treatment and compared with controls (OA): sRaw increase (%) = [(sRaw after challenge/sRaw before challenge) - 1] × 100. Furthermore, inhibition in sRaw was expressed as the percentage of the value of the drug-treated group against that of the OA-challenged control group using the following equation: Inhibition of sRaw (%) = {1 - (sRaw of drug-treated group/sRaw of OA-challenged control)} × 100.

Measurement of BALF parameters including WBC count. At 24 h following antigen challenge, the animals were terminally anesthetized with Zoletil 50, a mixture of zolazepam and tiletamine (1:1) (20 mg/kg, i.p.), after measurement of lung function parameters, and killed with an overdose of Zoletil 50. The trachea was exposed and cannulated with a polyethylene tube connected to a syringe, and the lungs were lavaged with four 5 mL aliquots of Ca²⁺- and Mg²⁺-free Hank's balanced salt solution (HBSS) containing ethylenediaminetetraacetic acid (EDTA) (10 mM) and HEPES (10 mM), before the BALF was collected. The BALF was centrifuged (200 × g for 10 min at 4°C) to remove the supernatant, the cells were resuspended in 1 mL of HBSS, and the total leukocytes were counted manually with a hemocytometer. Cell counts were performed on centrifuged prepara-

tions (Cytospin II Shandon Southern Instruments, Pittsburgh, PA) stained with Wright's stain. A minimum of 300 cells was counted and classified as macrophages, eosinophils, neutrophils, lymphocytes, or monocytes based on normal morphological criteria.

Histamine assay. Histamine in BALF was measured using the fluorometric method (Shore *et al.*, 1959). Briefly, BALF (1 mL) was transferred to a test tube and H₂O (1 mL) added. Then 4 min after adding 0.4 mL 1 N NaOH followed by 0.1 mL of OPA reagent, 0.2 mL 3 N HCl was added. The mixture was stirred thoroughly after each addition, transferred to a 96-well plate, and the fluorescence was measured at excitation 350 nm, emission 650 nm with a spectrophotometer (FL600 Microplate Fluorescence Reader, Bio-Tek, USA).

PLA₂ assay. Using 10-pyrene PC, the phospholipase A₂ (PLA₂) activity was measured in the presence of serum albumin with a spectrophotometer at excitation 345 nm, emission 398 nm. Spectrofluorometric analysis of pyrene phospholipids and fatty acids was performed in reaction solution (Radvanyi *et al.*, 1989). The 10-pyrene PC was dried under nitrogen and suspended in ethanol at 0.2 mM. The reaction solution was prepared by sequentially adding 1 mL of buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA; 10 µL of substrate (2 µM, final concentration); 10 µL of a 10% BSA solution (0.1%, final concentration) and 6 µL of 1 M CaCl₂ (6 mM, final concentration). The fluorescence of the reaction medium (blank) was recorded and the reaction was initiated by the addition of BALF. The specific activity in nanomoles per minute per milligram protein (nmol/min/mg) was obtained by dividing the activity by the amount of protein in milligrams. To calculate the specific activity, the protein content was analysed with a BCA protein assay kit (Pierce Co., USA).

EPO assay. The level of free eosinophil peroxidase (EPO) in the supernatant of the BALF was determined as an index of eosinophil activation according to the method described by Strath and colleagues (Strath *et al.*, 1985). For the determination of free EPO, an aliquot of BALF was centrifuged at 200 × g for 10 min at 4°C. The substrate solution consisted of 0.1 mM OPD in 0.05 M Tris-HCl containing Triton X-100 and 1 mM hydrogen peroxide. The OPD was prepared as a 5 mM stock solution in water, stored at -70°C and diluted 1:50 into buffer immediately before use. Except where otherwise stated, all incubations were carried out at pH 8.0 in the presence of 0.1% Triton X-100. One hundred microliters of substrate was added to 100 µL of samples in microtiter plate wells which were then left at 37°C for 30 min before stopping the reaction by the addition of 50 µL of 4 M sulfuric acid. The absorbance was measured at 492 nm with a fluorospectrophotometer (FL600 Microplate Fluorescence Reader, Bio-Tek). Samples containing contaminating hemoglobin in the supernatant were discarded. The specific activity in nanomoles per minute per milligram protein (nmol/min/mg) was obtained by dividing the activity by the amount of protein in milligrams. To calculate the specific activity, the protein content was analysed with a BCA protein assay kit.

Histological staining. Lung tissues were fixed by inflating the lungs with phosphate-buffered formalin saline

via a tracheal cannula to a pressure of 15 cmH₂O. They were then removed and prepared for staining with hematoxylin and eosin (H & E) before mounting in Canada balsam. The predominant eosinophil/neutrophil infiltration into bronchiolar and bronchial tissue was assessed according to the method of Richard *et al.* (1989). The intensity of the 'infiltrating' eosinophil response was graded on an arbitrary scale of 0–4 (0, no response; 1, mild response; 2, moderate response; 3, strong response; 4, severe response, as judged by the density of leukocytes infiltrating the peribronchial and peribronchiolar tissues). Leukocytes were negligible in the bronchi and bronchiolar tissues after saline inhalation in antigen-sensitized animals.

Data analysis. All values are represented as mean $\pm$ SEM of six animals. Statistical significance was calculated by analysis of variance (ANOVA) followed by the Student–Newman–Keuls method. Differences were considered significant when *p* values were less than 0.05. All statistics were performed with SigmaStat plot.

RESULTS

Decrease of sRaw by dibenzylbutyrolactone lignans

The OA inhalation significantly ($p < 0.01$) increased sRaw by $264.85 \pm 31.49\%$ (3.30 ± 0.41 to 8.74 ± 0.36 mmHg $\times$ s) in the IAR and by $158.35 \pm 20.33\%$ (3.30 ± 0.41 to 5.21 ± 0.35 mmHg $\times$ s) in the LAR compared with the saline-inhaled controls in OA-sensitized guinea-pigs (Fig. 2).

Arctiin and matairesinol (12.5 mg/kg) significantly ($p < 0.05$) decreased sRaw by $51.83 \pm 4.11\%$ and $43.15 \pm 5.75\%$ in the IAR and by $47.41 \pm 4.78\%$ and $35.43 \pm 5.12\%$ in the LAR compared with OA controls, respectively. Arctigenin (25 mg/kg) significantly ($p < 0.05$) decreased sRaw by $29.21 \pm 3.15\%$ in the IAR and by $38.69 \pm 3.57\%$ in the LAR compared with OA controls (Fig. 3). Dexamethasone (5 mg/kg) significantly ($p < 0.01$) decreased sRaw by $83.55 \pm 6.24\%$ in the IAR and by $73.72 \pm 4.78\%$ in the LAR. Cromolyn sodium (10 mg/kg) significantly ($p < 0.01$) decreased sRaw by $83.16 \pm 6.22\%$ in the IAR and by $76.10 \pm 5.26\%$ in the LAR. Salbutamol (5 mg/kg) was found significantly to decrease sRaw by $79.40 \pm 4.72\%$ in the IAR ($p < 0.01$) and by $46.73 \pm 4.43\%$ in the LAR ($p < 0.05$) (Fig. 3). These results showed that the inhibitory effect of dibenzylbutyrolactone lignans on sRaw was lower than that of the reference drugs.

Decrease of leukocytes in BALF by dibenzylbutyrolactone lignans

The OA-sensitized guinea-pigs significantly ($p < 0.01$) revealed a 4-fold increase of total leukocytes in BALF compared with the saline animals ($7.72 \pm 0.32 \times 10^6$ to $31.28 \pm 0.85 \times 10^6$ cells) (Fig. 4). The OA challenge increased neutrophils and eosinophils in BALF by $296.22 \pm 10.31\%$ and $1086.96 \pm 19.40\%$ ($2.91 \pm 0.31 \times$

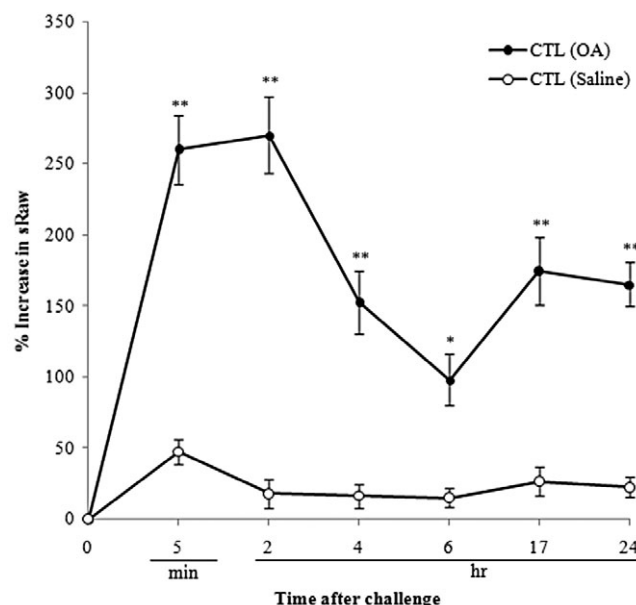


Figure 2. Time-dependent changes of sRaw in saline- or OA-inhaled controls. Animals were actively sensitized by OA, and challenged with saline- or OA-inhalation on day 21. sRaw was determined following the challenge. Time-dependent changes of sRaw shows the IAR at 5 min and the LAR at 24 h following OA challenge. Percent increase in sRaw = (sRaw after challenge/sRaw before challenge – 1) $\times$ 100. Each value represents the mean $\pm$ SEM ($n = 6$). Significantly different from saline-inhaled controls, * $p < 0.05$ and ** $p < 0.01$. CTL, saline- or OA-inhaled control.

10^6 to $8.62 \pm 0.82 \times 10^6$ and $0.69 \pm 0.17 \times 10^6$ to $7.50 \pm 0.33 \times 10^6$ cells), respectively (Fig. 4).

The dibenzylbutyrolactone lignans inhibited recruitment of total leukocytes, especially neutrophils and eosinophils, into BALF. Arctigenin, matairesinol and arctiin (50 mg/kg) significantly ($p < 0.05$) inhibited leukocyte recruitment ($31.78 \pm 0.85 \times 10^5$ cells to $24.93 \pm 2.09 \times 10^5$, $24.95 \pm 1.00 \times 10^5$, and $25.91 \pm 1.29 \times 10^5$ cells, respectively (Fig. 4).

Matairesinol, arctigenin and arctiin (50 mg/kg) significantly ($p < 0.05$) inhibited neutrophil recruitment compared with the OA controls ($8.62 \pm 0.82 \times 10^5$ cells to $7.04 \pm 0.86 \times 10^5$, $7.41 \pm 0.96 \times 10^5$, $7.67 \pm 0.95 \times 10^5$ cells, respectively) (Fig. 4). Arctiin, matairesinol and arctigenin (50 mg/kg) significantly ($p < 0.05$) inhibited eosinophil recruitment compared with the OA controls ($7.50 \pm 0.33 \times 10^5$ cells to $5.52 \pm 0.46 \times 10^5$, $5.75 \pm 0.15 \times 10^5$, $6.06 \pm 0.62 \times 10^5$ cells, respectively) (Fig. 4). Dexamethasone (5 mg/kg) and cromolyn sodium (10 mg/kg) also significantly decreased, not only total leukocytes, but also the recruitment of all different subtypes of leukocytes, particularly all subtypes of leukocytes, whereas salbutamol (5 mg/kg) significantly inhibited neutrophils and lymphocytes in BALF compared with the OA controls (Fig. 4).

Improvement of the histopathological changes by dibenzylbutyrolactone lignans

The lungs of OA-inhaled guinea-pigs showed recruitment of eosinophils and neutrophils in the alveolar sac, peripheral vasculature and terminal bronchioles, and a pavement of leukocytes in venules and arterioles (Fig.

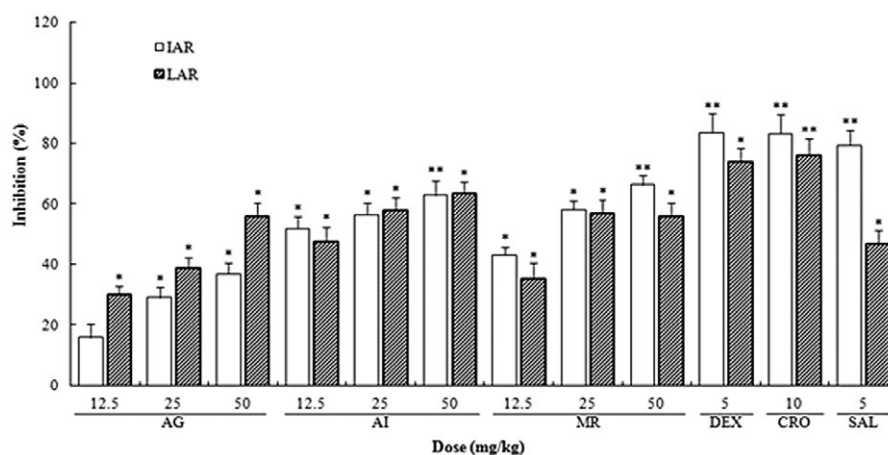


Figure 3. Effects of dibenzylbutyrolactone lignans on sRaw in the IAR and LAR. Animals were actively sensitized by injection of OA, and challenged with OA inhalation on day 21. Data show that inhibitory effect (%) of drugs on the sRaw of OA-challenged control in the IAR (8.74 ± 0.36 mmHg $\times$ s) at 5 min and in the LAR (5.21 ± 0.35 mmHg $\times$ s) at 24 days following the OA challenge. Inhibition of sRaw (%) = $\{1 - (\text{sRaw of drug-treated group} / \text{sRaw of OA-challenged control})\} \times 100$. Each value represents the mean $\pm$ SEM ($n = 6$). Significantly different from OA-inhaled controls, * $p < 0.05$ and ** $p < 0.01$. CTL, saline- or OA-inhaled control; AG, arctigenin; AI, arctiin; MR, matairesinol; DEX, dexamethasone; CRO, cromolyn sodium; SAL, salbutamol.

5B), compared with the normal architecture in the saline-inhaled control (Fig. 5A) (Sanjar *et al.*, 1990; Toward and Broadley, 2004). The lungs of naive controls at 24 h showed normal structural architecture without inflammatory cell infiltration around the terminal bronchioles (Fig. 5A). In contrast, lungs of naive OA-controls at 24 h following exposure to aerosolized-OA showed recruitment of neutrophils and eosinophils (Fig. 5B). Lungs from the arctigenin (50 mg/kg)-treated group at 24 h post-exposure to aerosolized-OA exhibited lower recruitment of leukocytes in alveolar sacs (Fig. 5C). Lungs from the arctiin (50 mg/kg)-treated group 24 h following exposure to aerosolized-OA revealed improvement in the pathological changes compared with OA-inhaled controls (Fig. 5D). Lungs from the matairesinol (50 mg/kg)-treated groups 24 h post-exposure to aerosolized-OA exhibited mild peribronchiolar leukocyte infiltration (Fig. 5E). The intensity of the 'infiltrating' eosinophil responses according to method of Richard *et al.* (1989) showed that lung of arctiin, arctigenin and matairesinol (50 mg/kg)-treated guinea-pigs significantly ($p < 0.05$) inhibited not only the infiltration of inflammatory cells, particularly, eosinophils and neutrophils in the alveolar sac and the peripheral vasculature by about 30%, respectively (Table 1).

Decrease of histamine content, and PLA₂ and EPO activities in BALF by dibenzylbutyrolactone lignans

The histamine content in BALF of saline- and OA-inhaled guinea-pigs was 147.90 ± 10.30 and 478.10 ± 15.30 ng/mL, respectively, indicating an increase of $323.20 \pm 25.89\%$ in OA-inhaled guinea-pigs compared with the saline controls (Fig. 6). In addition, the PLA₂ activity in the BALF of OA-inhaled guinea-pigs was significantly ($p < 0.001$) increased by $319.20 \pm 26.22\%$ compared with saline controls (2.40 ± 0.32 to 7.80 ± 0.25 nmol/min/mg) (Fig. 6). Furthermore, EPO, a marker of eosinophil activation in OA-inhaled guinea-pigs, was significantly ($p < 0.001$) increased by $335.30 \pm 11.84\%$ (12.10 ± 3.55 to 40.50 ± 2.24 mU/mL) (Fig. 6).

Arctiin inhibited the activities of EPO and PLA₂ in the BALF in a dose-dependent manner (Fig. 6), whereas arctigenin and arctiin (12.5 mg/kg) significantly ($p < 0.05$) decreased the histamine content in BALF ($29.96 \pm 8.33\%$ and $31.98 \pm 5.18\%$, respectively); however, their activity was lower than that of dexamethasone (5 mg/kg) and cromolyn sodium (10 mg/kg) (61.69 ± 7.21 and $70.28 \pm 8.33\%$, $p < 0.01$, respectively) and was more effective than that of salbutamol (5 mg/kg) ($10.18 \pm 2.16\%$).

Arctiin and matairesinol (12.5 mg/kg) significantly ($p < 0.05$) decreased EPO activity in BALF ($41.36 \pm 10.26\%$; $39.55 \pm 1.36\%$, respectively), but again, their activity was lower than that of dexamethasone (5 mg/kg) and cromolyn sodium (10 mg/kg) (82.79 ± 4.03 and $66.42 \pm 5.34\%$, $p < 0.01$, respectively), and more effective than that of salbutamol (5 mg/kg) ($31.49 \pm 10.82\%$).

Matairesinol (12.5 mg/kg) significantly ($p < 0.05$) decreased PLA₂-specific activity in BALF by $49.30 \pm 6.15\%$, and AI (25 mg/kg) and arctigenin (50 mg/kg) significantly ($p < 0.05$) decreased PLA₂-specific activity in BALF by $58.84 \pm 8.99\%$ and $30.50 \pm 9.42\%$, respectively. However, their activity was again lower than that of cromolyn sodium (10 mg/kg) and dexamethasone (5 mg/kg) ($70.28 \pm 8.33\%$ and $61.69 \pm 7.21\%$, respectively), and more effective than that of salbutamol (5 mg/kg).

DISCUSSION

A guinea-pig model of IAR and LAR was developed through the inhalation of OA in OA-sensitized guinea-pigs pretreated with an antihistamine, piprinhydrinate (5 mg/kg), without using an adjuvant to prevent sudden death due to systemic hypersensitivity (Jung *et al.*, 2007; Matsumoto *et al.*, 1994; Sanjar *et al.*, 1990; Huston *et al.*, 1988). This asthma model, produced by inhalation of aerosolized antigen, is similar to the antigen delivery route in human asthma (Kane *et al.*, 1996). In the current study, aerosolized-OA inhalation revealed 2.65-fold and 1.58-fold increases of sRaw in the IAR and

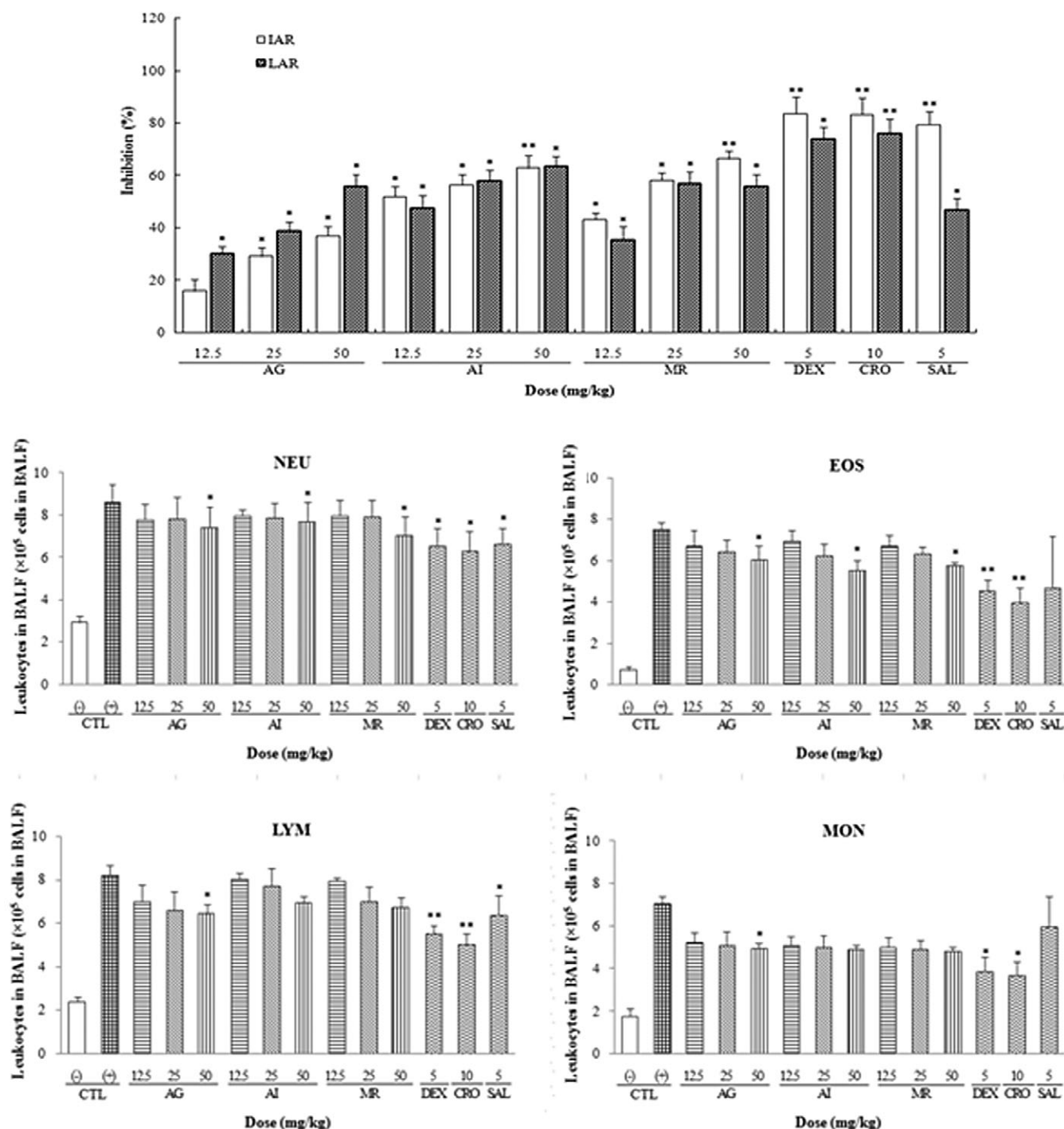


Figure 4. Effects of dibenzylbutyrolactone lignans on total leukocytes and different subtypes of leukocytes in BALF. NEU, neutrophils; EOS, eosinophils; LYM, lymphocytes; MON, monocytes. Each value represents the mean $\pm$ SEM ($n = 6$). Significantly different from OA-inhaled controls, * $p < 0.05$ and ** $p < 0.01$. CTL, saline- or OA-inhaled control; AG, arctigenin; AI, arctiin; MR, matairesinol; DEX, dexamethasone; CRO, cromoglycol sodium; SAL, salbutamol.

LAR compared with naive controls, respectively (Fig. 3). Furthermore, these asthmatic animals in the BAL technique revealed a 4-fold increase in the recruitment of leukocytes into the lung compared with the saline control, in particular a 10.9-fold increase in eosinophils, and a 3.0-fold increase in neutrophils, respectively (Fig. 4), which is similar to a segmental challenge in humans (Kane *et al.*, 1996). Subsequent histopathological studies showed the recruitment of leukocytes in the alveolar sac and lung tissues (Fig. 5 and Table 1). OA inhalation also significantly ($p < 0.001$) increased chemical mediators,

such as histamine, and EPO and PLA₂ activities during the LAR compared with the control (Fig. 6).

Dibenzylbutyrolactone lignans, occurring in *F. viridis-sima*, *Saussurea medusa*, *Arctium lappa*, *Torreya nucifera* and *Ipomea cairica*, had been reported to have antioxidant and antiinflammatory properties. Previously, it was demonstrated that arctigenin also has potent antiinflammatory action (Kang *et al.*, 2008), and also in our preliminary study, the methanol extract of *F. viridis-sima* showed antiasthmatic effects that significantly inhibited sRaw in the IAR and LAR. Thus,

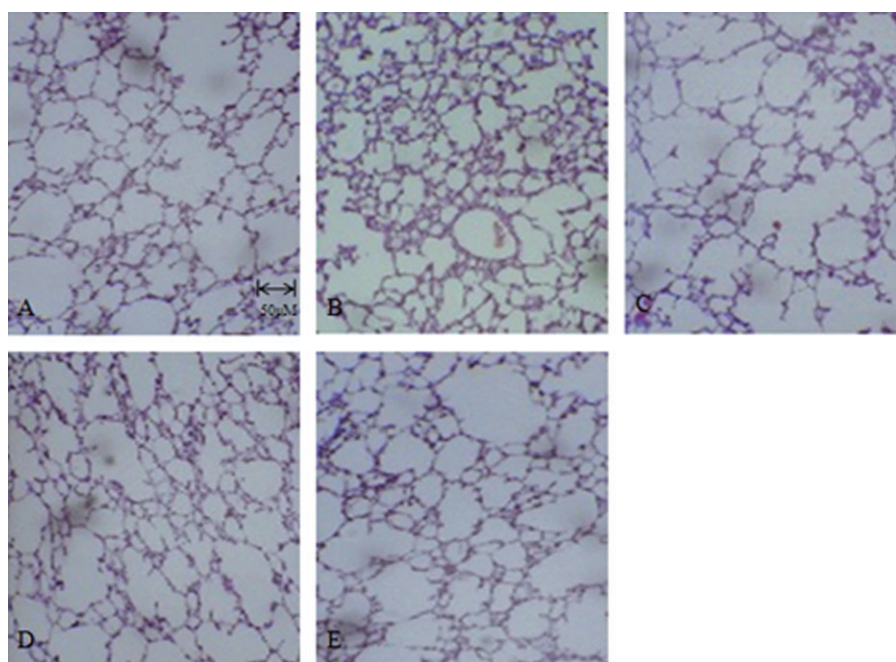


Figure 5. Effects of dibenzylbutyrolactone lignans on the lung by pathological survey. Lung of an OA-control at 24 h after exposure to aerosolized-OA showing recruitment of neutrophils and eosinophils (B) compared with a saline-inhaled control with normal structural architecture, and no inflammatory cell infiltration in the alveolar sacs (A). Lung of a guinea-pig treated with AG (50 mg/kg) at 24 h following exposure to aerosolized-OA exhibited lower recruitment of leukocytes in alveolar sacs (C). Lung of a guinea-pig treated with AI (50 mg/kg) at 24 h post-exposure to aerosolized-OA demonstrated improvement in the pathological changes compared with OA-inhaled controls (D). Lung of guinea-pig treated with MR (50 mg/kg) at 24 h after exposure to aerosolized-OA exhibited mild peri-bronchiolar leukocyte infiltration (E) (H & E stain, original magnitude $\times 100$). This figure is available in colour online at <http://www.interscience.wiley.com/journal/ptr>

Table 1. Histological assessment of eosinophil infiltration in lung of saline- and dibenzylbutyrolactone lignans-treated guinea-pigs 24 h after antigen inhalation

Drug	Dose (mg/kg)	Infiltration of eosinophil (% inhibition) ^a
Control (saline)	–	– (–)
Control (OA)	–	3.12 ± 0.28 (–)
Matairesinol	12.5	2.81 ± 0.18 (9.94)
	25	2.47 ± 0.18 (20.93)
	50	2.15 ± 0.11 (31.09) ^b
Arctigenin	12.5	2.69 ± 0.19 (16.35)
	25	2.48 ± 0.16 (20.51)
	50	2.12 ± 0.13 (32.05) ^b
Arctiin	12.5	2.48 ± 0.19 (20.51)
	25	2.36 ± 0.12 (24.36)
	50	2.08 ± 0.12 (33.34) ^b
Dexamethasone	5	1.81 ± 0.06 (41.99) ^b
Cromolyn sodium	10	1.94 ± 0.07 (37.82) ^b
Salbutamol	5	2.78 ± 0.25 (10.99)

^a Graded 0 to 4 (see method).

^b $p < 0.05$.

further study was conducted to isolate the antiasthmatic constituents from the ethylacetate fraction of the methanol extract of *F. viridissima* by chromatography, yielding the three dibenzylbutyrolactone lignans, matairesinol, arctigenin and arctiin. All dibenzylbutyrolactone lignans significantly inhibited sRaw in the IAR and LAR in a dose-dependent manner. Arctiin and matairesinol at 12.5 mg/kg significantly ($p < 0.05$) revealed a decrease of sRaw in IAR and LAR, respec-

tively, whereas arctigenin at 25 mg/kg significantly decreased sRaw in the IAR and LAR, compared with the controls (Fig. 3).

In addition, dibenzylbutyrolactone lignans inhibited the recruitment of total leukocytes, especially neutrophils and eosinophils into BALF. Arctigenin, matairesinol and arctiin (50 mg/kg) significantly ($p < 0.05$) inhibited leukocyte recruitment, respectively, and also cromolyn sodium (10 mg/kg), dexamethasone (5 mg/kg) and salbutamol (5 mg/kg) also significantly inhibited the recruitment of total leukocytes in BALF (Fig. 4). Matairesinol, arctigenin and arctiin (50 mg/kg) significantly ($p < 0.05$) inhibited the recruitment of leukocytes, particularly neutrophils and eosinophils, compared with OA controls. Furthermore, dexamethasone (5 mg/kg) and cromolyn sodium (10 mg/kg) also significantly decreased the recruitment of neutrophils, eosinophils and lymphocytes, whereas salbutamol (5 mg/kg) significantly decreased the recruitment of neutrophils and lymphocytes, in BALF compared with OA controls (Fig. 4). These decreases of the recruitment of total leukocytes, especially neutrophils and eosinophils, were consistent with the histological survey showing that dibenzylbutyrolactone lignans decreased the infiltration of leukocytes in the lung tissues (Fig. 5) and the infiltration of eosinophils in the alveolar sac (Table 1).

Furthermore, arctigenin and arctiin dose-dependently inhibited the histamine content, and the activities of PLA₂ and EPO, respectively, whereas matairesinol inhibited EPO and PLA₂ at 12.5 mg/kg and histamine at 50 mg/kg, compared with the OA controls. In addition, dexamethasone, cromolyn and salbutamol significantly inhibited the recruitment of leukocytes and chemical mediators in BALF (Toward and Broadley,

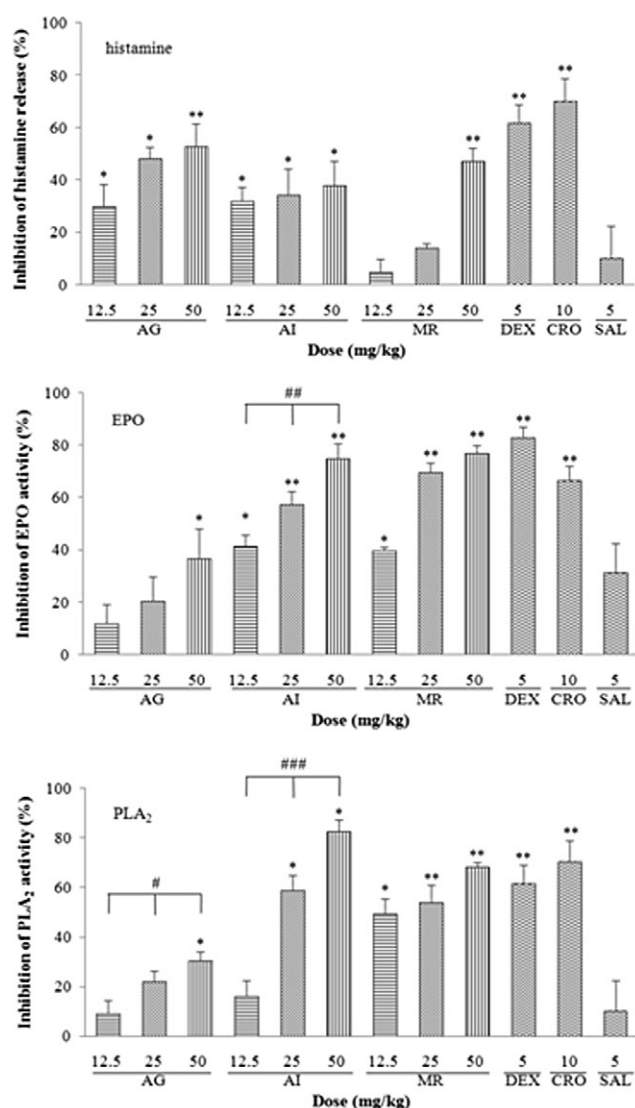


Figure 6. Effects of dibenzylbutyrolactone lignans on histamine, and EPO and PLA₂ activities in BALF. Inhibition (%) = $(a - c)/(a - b) \times 100$, a and b; OA- and saline-inhaled control, c; dibenzylbutyrolactone lignan-treated group. Each value represents the mean $\pm$ SEM ($n = 6$). Significantly different from OA-inhaled controls, * $p < 0.05$ and ** $p < 0.01$. Significantly dose-dependent, # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$. AG, arctigenin; AI, arctiin; MR, matairesinol; DEX, dexamethasone; CRO, cromolyn sodium; SAL, salbutamol.

2004; Cockcroft and Murdok, 1987), whereas salbutamol did not alter chemical mediators in BALF.

These results indicate three dibenzylbutyrolactone lignans have antiasthmatic effects which were less active than those of reference drugs, cromolyn sodium (mast cell stabilizer), steroid and salbutamol (β_2 -agonist), and

which might be same as steroid and mast cell stabilizer. Arctiin and matairesinol were the most active in the decreasing sRaw and of EPO and PLA₂ activities in BALF, whereas arctigenin was the most active in decreasing histamine content in BALF. The effects of the reference drugs were similar to the effects on human asthma, but salbutamol was less active than the other reference drugs during LAR. Salbutamol was observed to inhibit sRaw and the recruitment of leukocytes, but not the histamine content and activities of PLA₂ and EPO in BALF compared with the OA control. These results suggest that the contraction of airway smooth muscle characteristic of LAR development may be involved along with other factors, and that the oral administration of salbutamol, in spite of inhalation treatment, may explain the lesser effects of salbutamol on LAR observed in this model compared with human asthma. Cromolyn sodium (10 mg/kg, p.o.) had a significantly antiasthmatic effect in both IAR and LAR in this asthmatic animal model, whereas its absorption from the gastrointestinal tract is very poor in humans and a dose of 2 mg inhaled four times daily has an antiasthmatic effect in humans (Svendsen *et al.*, 1987). So therefore its gastrointestinal absorption in guinea-pigs may be different to that in humans, and a low blood level of cromolyn sodium may inhibit the chemical mediator release from bronchial mast cells and the activation of leukocytes. Thus, the dibenzylbutyrolactone lignans have antiasthmatic properties in a dose-dependent manner, suggesting that their mechanism of action may be similar to that of dexamethasone and cromolyn sodium in both the IAR and LAR.

In conclusion, the dibenzylbutyrolactone lignans significantly reduced sRaw and eosinophil accumulation in asthma via the inhibition of mast cell degranulation in the IAR as well as recruitment of eosinophils and neutrophils, and pro-inflammatory enzymes, PLA₂, in the LAR. These findings indicate that they may be useful therapeutics against asthma in clinical practice, and that their antiasthmatic activity in the IAR and LAR may be due to the inhibition of leukocyte recruitment, particularly eosinophils and neutrophils, histamine release and PLA₂ activity.

Acknowledgement

We thank Professor Jin-Woong Kim, College of Pharmacy, Seoul National University for giving us standard lignans. This research was supported by a Chung-Ang University Research Grant in 2009.

Conflict of Interest

The authors have declared that there is no conflict of interest.

REFERENCES

- Cockcroft DW, Murdok KY. 1987. Comparative effect of inhaled salbutamol, sodium cromoglycate and beclomethasone dipropionate on allergen-induced early asthmatic responses, late asthmatic responses and increased bronchial hyperreactivity to histamine. *J Allergy Clin Immunol* **79**: 734–740.
- Eich E, Pertz H, Kaloga M *et al.* 1996. (-)-Arctigenin as a lead structure for inhibitors of human immunodeficiency virus type-1 integrase. *J Med Chem* **39**: 86–95.
- Huston PA, Church MK, Clay TP, Miller P, Holgate SP. 1988. Early and late-phase bronchoconstriction after allergen challenge of nonanesthetized guinea pigs. I. The association of disordered airway physiology to leukocyte infiltration. *Am Rev Respir Dis* **137**: 548–557.
- Jang YP, Kim SR, Choi YH *et al.* 2002. Arctigenin protects cultured cortical neurons from glutamate-induced neurodegeneration by binding to kainite receptor. *J Neurosci Res* **68**: 233–240.

- Jang YP, Kim SR, Kim YC. 2001. Neuroprotective dibenzylbutyrolactone lignans of *Torreya nucifera*. *Planta Med* **67**: 470–472.
- Jung CH, Lee JY, Cho CH, Kim CJ. 2007. Anti-asthmatic action of quercetin and rutin in conscious guinea-pigs challenged with aerosolized ovalbumin. *Arch Pharm Res* **30**: 1599–1607.
- Kane GC, Pollice M, Kim CJ *et al.* 1996. A controlled trial of the effect of the 5-lipoxygenase inhibitor, zileuton, on lung inflammation produced by segmental antigen challenge in human beings. *J Allergy Clin Immunol* **97**: 646–654.
- Kang HS, Lee JY, Kim CJ. 2008. Anti-inflammatory activity of arctigenin from *Forsythiae Fructus*. *J Ethnopharmacol* **116**: 305–312.
- Kim MR, Moon HT, Lee DG, Woo ER. 2007. A new lignin glycoside from the stem bark of *Styrax japonica* S. et Z. *Arch Pharm Res* **30**: 425–430.
- Kim NY, Kang TH, Song EK, Pae HO, Chung HT, Kim YC. 2000. Inhibitory effect of butanol fraction of the aqueous extract of *Forsythia koreana* on the nitric oxide production by murine macrophage-like RAW 264.7 cells. *J Ethnopharmacol* **73**: 323–327.
- Kim SH, Jang YP, Sung SH, Kim CJ, Kim JW, Kim YC. 2006. Hepatoprotective dibenzylbutyrolactone lignans of *Torreya nucifera* against CCl₄-induced toxicity in primary cultured rat hepatocytes. *Biol Pharm Bull* **26**: 1202–1205.
- Lee JS, Min BS, Bae KW. 1996. Effects of *Forsythiae fructus* on L1210 and HL-60 cells. *Yakhak Hoeji* **40**: 462–467.
- MacRae WD, Towers GH. 1984. An ethnopharmacological examination of *Virola elongata* bark: a South American arrow poison. *J Ethnopharmacol* **12**: 75–92.
- Matsumoto T, Ashida Y, Tsukuda R. 1994. Pharmacological modulation of immediate and late airway response and leukocyte infiltration in the guinea pig. *J Pharmacol Exp Ther* **269**: 1236–1244.
- Nishibe S, Kawamura T, Tanka T, Adlercreutz H. 2001. On the contents of lignans in *Forsythia* Fruit and in *Forsythia* Leaf. *Nat Med* **55**: 300–303.
- Nishibe S. 2002. The plant origins of herbal medicines and their quality evaluation. *Yakugaku Zasshi* **122**: 363–379.
- Pennock BE, Pennock CP, Rogers RM, Cain WA, Wells JH. 1979. A noninvasive technique for measurement of changes in specific airway resistance. *J Appl Physiol* **46**: 399–406.
- Radvanyi T, Jordan L, Russo-Marie F, Bon C. 1989. A sensitive and continuous fluorometric assay for phospholipase A₂ using pyrene-labeled phospholipids in the presence of serum albumin. *Anal Biochem* **177**: 103–121.
- Richard VN, Griffin RL, Oosteen JA, Morris J, Wiska DG, Dunn CJ. 1989. Effect of the selective leukotriene B₄ antagonist U-75302 on antigen-induced bronchopulmonary eosinophilia in sensitized guinea pigs. *Am Rev Respir Dis* **140**: 1712–1718.
- Sanjar S, Aoki S, Kristersson SD, Moley J. 1990. Antigen challenge induces pulmonary airway eosinophil accumulation and airway hyperreactivity in sensitized guinea-pigs: the effect of anti-asthma drugs. *Br J Pharmacol* **99**: 679–686.
- Shoeb M, MacManus SM, Kumarasamy Y *et al.* 2006. Americanin, a bioactive dibenzylbutyrolactone lignin, from the seeds of *Centaurea America*. *Phytochemistry* **67**: 2370–2375.
- Shore PA, Burkhalter A, Cohn VC. 1959. A method for the fluorometric assay of histamine in tissue. *Am Soc Pharmacol Exp Ther* **127**: 8233–8245.
- Strath M, Warren DJ, Sanderson CJ. 1985. Detection of eosinophils using an eosinophil peroxidase assay. Its use as an assay for eosinophil differentiation factors. *J Immunol Methods* **83**: 209–215.
- Svensden UG, Frølund L, Madsen F, Nielsen NH, Holstein-Rathlou NH, Weeke BJ. 1987. A comparison of the effects of sodium cromoglycate and beclomethasone dipropionate on pulmonary function and bronchial hyperreactivity in subjects with asthma. *Allergy Clin Immunol* **80**: 68–74.
- Toward TJ, Broadley KJ. 2004. Early and late bronchoconstrictions, airway hyper-reactivity, leucocyte influx and lung histamine and nitric oxide after inhaled antigen: effects of dexamethasone and rolapram. *Clin Exp Allergy* **34**: 91–102.
- Zeng Y, Yokohira M, Takeuchi H. 2005. Lack of significant modifying effect of arctiin on prostate carcinogenesis in probasin/SV40 T antigen transgenic rats. *Cancer Lett* **222**: 145–151.