

Effects of Hydroxy Pentacyclic Triterpene Acids from *Forsythia viridissima* on Asthmatic Responses to Ovalbumin Challenge in Conscious Guinea Pigs

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For the identification of anti-inflammatory ingredients from *Forsythiae fructus* (FF), we isolated three hydroxyl pentacyclic triterpene acids (HTAs), namely, oleanolic acid, ursolic acid, and betulinic acid, from an ethylacetate fraction of FF, and evaluated the effect of these triterpene acids on asthmatic guinea pigs by measuring specific airway resistance (sRaw) during both immediate-phase response (IAR) and late-phase response (LAR) following ovalbumin challenge using a double-chambered plethysmograph. Evaluation of leukocytes and chemical mediators in bronchoalveolar lavage fluid (BALF), in addition to a histopathological survey, was also performed. Ursolic, oleanolic and betulinic acids dosed at 12.5 mg/kg significantly ($p < 0.05$) decreased sRaw by 46.80%, 46.54% and 44.27% during in IAR, respectively. And ursolic acid (25 mg/kg), and oleanolic and betulinic acids (50 mg/kg) significantly ($p < 0.05$) decreased sRaw by 38.19%, 38.15% and 35.55% in LAR, respectively. Histamine and phospholipase A₂ activity in BALF were significantly decreased by HTAs at 12.5 mg/kg, whereas eosinophil peroxidase (EPO) activity in BALF and recruitment of eosinophils were significantly decreased by HTAs at 25 mg/kg, as well as improvement of pathological changes. However, betulinic acid at 12.5 mg/kg, and ursolic and oleanolic acids at 25 mg/kg significantly inhibited leukocytes in BALF, especially eosinophils and neutrophils. Three HTAs were found to have dose-dependent anti-asthmatic effects and ursolic acid is the most active, but their activities were less than those of sodium cromoglycate, salbutamol, and dexamethasone. These results indicate HTAs had anti-asthmatic activity by decreasing of sRaw, and eosinophil recruitment and release of inflammatory mediators into the lungs.

Key words *Forsythia viridissima*; triterpene acid; anti-asthmatic; specific airway resistance; histamine; phospholipase A₂

Bronchial asthma is considered to be a chronic inflammatory disease of the airways. Thus, anti-inflammatory therapy is considered central for its long-term asthma management.¹⁾ Inhaled corticosteroids are highly effective chronic treatments, and are capable of suppressing the underlying inflammatory process. Furthermore, combination inhalers containing corticosteroids and long-acting β_2 -agonists are the principle approach to asthma therapy, and are being increasingly used in patients with persistent symptoms.^{2–3)} However, concern persists regarding the use of inhaled corticosteroids, because the fear is irrational long-term side effects, such as, osteoporosis and growth stunting in children.³⁾ Although these effective medications are delivered by inhalation, compliance is surprisingly poor.^{3,4)} But dichromone flavonoid derivative, cromolyn sodium (CRO), which is a similar chemical structure to flavonoids, inhibits the release of chemical mediators, and has been used as anti-asthmatic agent, especially the prophylactic treatment of both allergic and exercise-induced asthma via the first dry-powder inhaler.⁵⁾

Forsythiae fructus (FF), the dried fruits of *Forsythia viridissima* LINDLE (Oleaceae), which are known as Yeon Kyo in Korea, had been reported to have the anti-inflammatory ingredients such as triterpenoids, lignans, flavonoids, and their glycosides,^{6,7)} and so are used as a herbal medicine due to their anti-inflammatory, diuretic, antidote, extrusion of pus, and antibacterial effects in Korea, China, and Japan.⁸⁾ In a previous study, we found that the natural lignans and flavonoids of FF inhibit inflammatory and asthmatic responses.^{9,10)} In the present study, we isolated three hydroxyl pentacyclic triterpene acids (HTAs), namely, oleanolic acid (OA), ursolic acid (UA), and betulinic acid (BA), from an ethylacetate fraction of FF, and sought to determine their ef-

fects and the underlying mechanisms involved on immunoglobulin E (IgE)-mediated asthmatic response induced by an aerosolized ovalbumin (AOVA) challenge in ovalbumin-sensitized guinea pigs. Their anti-asthmatic effects, which were investigated by measuring sRaw during immediate-phase response (IAR) and late-phase response (LAR) in a double-chambered plethysmograph,^{10–12)} were compared with those elicited by the authorized anti-asthmatic drugs, salbutamol (SAL), dexamethasone (DEX), and CRO. In addition, the chemical mediators of inflammation, histamine, eosinophil peroxidase (EPO), phospholipase A₂ (PLA₂), and leukocytosis, were quantified in bronchoalveolar lavage fluid (BALF), and accompanying histopathological changes in lung tissues were monitored.

MATERIALS AND METHODS

Plant Materials and Chemicals *Forsythiae Fructus*, *Forsythiae viridissima* fruits were collected near Sokri Mountain in the Republic of Korea. Dexamethasone (DEX), salbutamol (SAL), disodium cromoglycate (CRO), bovine serum albumin (Fraction V), ovalbumin (OVA), *o*-phthalaldehyde (OPA), *o*-phenylenediamine dihydrochloride (OPD), dimethylsulfoxide (DMSO), Wright's stain, and standard triterpene acids (oleanolic acid, ursolic acid, betulinic acid) were purchased from Sigma (St. Louis, MO, U.S.A.). Piprinhydrinate was obtained from Yungjin Pharm. Co., Ltd. (Hwa-Sung, Kyung Ki, Korea). Zoletil 50 from Virbac Lab. (Carros, France), bichoninic acid (BCA) protein assay kits from Pierce (Rockford, IL, U.S.A.), 1-palmitoyl-2-(10-pyrenyl)decanyl-*sn*-glycero-3-phosphorylcholine (10-pyrene PC) from Molecular Probes (Leiden, Netherlands),

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phospholipase A₂ (PLA₂) and eosinophil peroxidase (EPO) from Amersham (Piscataway, NJ, U.S.A.). All other reagents used were of reagent grade. 4-(2-Hydroxyethyl)-1-piperazine-ethane-sulfonic acid (HEPES)-balanced salt solution (BSS)/bovine serum albumin (BSA) (pH 7.4) contained 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 bovine serum 1% albumin. HTAs and reference drugs were dissolved in saline containing 10% DMSO.

Extraction and Isolation Dried and powdered FF (6.0 kg) was extracted with MeOH (3×6 l) to yield a solid dark extract (1094.0 g). This was suspended in 10 l of H₂O for 24 h to produce a precipitate (390.0 g), which (200 g) was subjected to column chromatography on a silica gel column (6.5×85.0 cm) using benzene–EtOAc (9 : 1) as eluant to give fraction 1 (No. 40–54, 33.4 g) and fraction 2 (No. 55–110, 29.8 g). Fraction 1 was also chromatographed on a silica gel column (3.5×41.5 cm) using *n*-hexane–EtOAc solvent (4 : 6) as eluant to give compound **1** (620.34 mg; yield 0.0103%). Fraction 2 was subjected to silica gel column chromatography (3.5×41.5 cm) using CHCl₃–MeOH–benzene (4 : 1 : 1) as eluant followed by preparative TLC (MeOH : H₂O–HAc, 7 : 3 : 0.5) to give compounds **2** (948.24 mg; yield 0.0158%) and **3** (501.48 mg; yield 0.0084%). Compounds **1**–**3** were recrystallized in EtOH, and their structures were identified as oleanolic acid, ursolic acid, and betulinic acid by comparing their phytochemical and spectroscopic data with previously reported values.^{13–17} HPLC analysis of compounds **1**–**3** was performed using a Gilson 305 unit (Villiers Le Bel, France) and a Gilson UV 116 detector equipped with a C₁₈ column (4.6×150 mm, i.d. 5 μm) and acetonitrile : 1.25% phosphate solution (86 : 14 v/v) as a mobile phase at a flow rate of 0.5 ml/min. The purities of compounds **1**–**3** were; 94.7, 93.9 and 95.7%, respectively.

Animals Pathogen-free male Dunkin–Hartley guinea pigs (250–300 g) were purchased from the HanLim Laboratory Animal Co. (Hwa Sung Gun, Republic of Korea). Animals were housed acclimated for one week in an environmentally controlled-animal facility (25 °C, RH 55%, aeration 10–18/h, illumination 300–500 lux). Food (Superfeed Co., Won Ju City, Korea) and water were available *ad libitum*. This study was performed according to international, national and institutional rules regarding animal experiments, and all procedures were approved by the Animal Care and Use Committee at the Institute for Molecular-Based New Drug Development at Chung-Ang University.

Sensitization Guinea-pigs were actively sensitized to ovalbumin (OVA) by injecting 50 mg in 1 ml of 0.9% saline twice during day 1 (i.p. and s.c., respectively).¹⁰ Twenty-one days after sensitization, 1% OVA (0.1 ml per site) was injected intradermally and skin thicknesses were measured 1 h later. A doubling of skin thickness and the presence of eosinophilia at the injection site was defined as adequate sensitization.^{9,10,12} Non-sensitized animals were not used.^{9,10,12}

Drug Treatment and Aerosolized-OVA Challenge After measuring baseline airway function before treatment, animals were challenged with 1% OVA inhalation or saline (naive) for 5 min in a double-chambered plethysmograph (HSE type 855, Germany). Sensitized animals were fasted overnight and treated with test drugs, reference drugs, or vehicle 1 h prior to allergen challenge, which repeated twice at

12 and 20 h after the allergen challenge. Thirty minutes before OVA challenge, the animals were pretreated with antihistamine, piprinhydrinate (5 mg/kg) to prevent sudden death due 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 OVA (1.0 mg/ml in saline) or saline aerosol delivered using a jet nebulizer for 5 min.^{10,12}

Measurement of Airway Function The airway functions of conscious guinea pigs were measured using a double-chambered plethysmograph.^{9–11,14} Briefly, an animal was positioned with its neck extending through the partition between the head and body chamber. Tidal volume (TV), respiratory rate (RR) and specific airway resistance (sRaw) were measured using a volumetric differential pressure transducer (PT5, Grass Co., U.S.A.), a noninvasive respiratory analyzer, and an oscilloscope with polygraph (7E, Grass Co., U.S.A.), before challenge (baseline) and at 5 min (IAR) and 24 h (LAR) after challenge, respectively. Phase displacement was measured at end inspiration during the change to the inspiration and expiration phase. The plethysmograph was linked to a computer for continuous data capture. sRaw was expressed as mmHg×s and analyzed using the Pennock Program¹¹ which utilizes: $\tan \theta = \omega \times R \times C$ (θ =phase displacement; $\omega = 2\pi \times$ respiration rate; $R \times C$ is a time constant of the respiratory system; $C = V/P$, thoracic gas volume (V)/($P_{\text{atm}} - 47$ mmHg).

sRaw was calculated using:

$$R \times V = (P_{\text{atm}} - 47) \times \tan \theta / (2\pi \times f) \quad [\text{mmHg} \times \text{s}]$$

$$R \times V = (P_{\text{atm}} - 47) \times 13.6 \times \tan \theta / \omega \quad [\text{mmHg} \times \text{s}]$$

The phase displacement was measured at the end of inspiration during the change to the expiration phase. The plethysmograph linked to a computer for continuous data capture. sRaw was expressed as a percentage of the baseline value before drug treatment. Increases in sRaw were expressed as percentage of baseline values before drug treatment and were compared with control values: Percentage increase in sRaw = [(sRaw after challenge/sRaw before challenge) – 1] × 100. Furthermore, inhibition in sRaw were expressed as percentage of value of drug-treated group against that of OVA-challenged control group using the following equation:

$$\text{inhibition of sRaw (\%)} = [1 - (\text{sRaw of drug-treated group} / \text{sRaw of OVA-challenged control})] \times 100$$

Measurements of Leukocyte and Chemical Mediators in BALF. Leukocyte Count At 24 h after antigen challenge, animals were sacrificed with Zoletil 50 (a mixture of zolazepam and tiletamine (1 : 1) (20 mg/kg, i.p.)) after measuring lung function parameters. The trachea was exposed and cannulated with a polyethylene tube connected to a syringe, and the lungs were lavaged four times with 5-ml aliquots of Ca²⁺ and Mg²⁺ free Hank's balanced salt solution (HBSS) containing ethylenediaminetetraacetic acid (EDTA) (10 mM) and HEPES (10 mM), and bronchoalveolar lavage fluid (BALF) was collected. BALF was centrifuged at 200×*g* for 10 min at 4 °C to remove supernatant, and the cells obtained were resuspended in 1 ml of HBSS. Total leukocytes in BALF were determined using a hemocytometer. Cell counts

were performed on centrifuged preparations (Cytospin II Shandon Southern Instruments, Pittsburgh, PA, U.S.A.) stained with Wright's stain. A minimum of 300 cells per sample were counted, and were classified as macrophages, eosinophils, neutrophils, lymphocytes, or monocytes based on normal morphologic criteria.¹¹⁾

Histamine Assays Histamine levels in BALF were measured using the fluorometric method.¹⁸⁾ Briefly, BALF (1 ml) was transferred to a test tube and H₂O (1 ml) added. Four minutes 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 transferred to a 96-well plate, and the fluorescence was measured at 650 nm (λ_{excit} 350 nm) using a spectrofluorophotometer (FL600 Microplate Fluorescence Reader, Bio-Tek, U.S.A.).

PLA₂ Assays Using pyrene-labeled phospholipids (10-pyrene PC), PLA₂ activity was measured in the presence of serum albumin using a spectrophotometer at 398 nm (λ_{excit} 345 nm). Spectrofluorometric analyses of pyrene phospholipids and fatty acids were performed in reaction solution.¹⁹⁾ 10-pyrene PC was dried under nitrogen and suspended in ethanol at 0.2 mM. The reaction solution was prepared by adding 1 ml of buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 10 μ l of substrate; 10 μ l of a 10% bovine serum albumin solution and 6 μ l of 1 M CaCl₂ to substrate. The fluorescence of the reaction medium (blank) was recorded and the reaction was initiated by adding BALF. Specific activity was measured in nanomoles per minute per milligram of protein (nmol/min/mg). To calculate specific activities, protein contents were analyzed using BCA protein assay kits (Pierce Co., Minneapolis).

EPO Assay The level of free EPO in supernatant from BALF was used as an index of eosinophil activation, according to the method described by Strath and co-workers.²⁰⁾ To determine free EPO levels an aliquot of BALF was centrifuged at 200 $\times$ g for 10 min at 4°C, and added to the substrate solution of 0.1 mM OPD in 0.05 M Tris-HCl containing Triton X-100 and 1 mM hydrogen peroxide. OPD was made up as a 5 mM stock solution in water, stored at -70°C and diluted 1:50 with Tris buffer immediately before use. Except when stated otherwise, all incubations were carried out at pH 8.0 in the presence of 0.1% Triton X-100. One hundred microliters of substrate were added to 100 μ l of the samples in microtiter plate wells, the plates were then left at 37°C for 30 min before stopping the reaction by adding 50 μ l of 4 M sulfuric acid. Absorbances were read at 492 nm using a fluorospectrophotometer (FL600 Microplate Fluorescence Reader, Bio-Tek). Samples containing contaminating haemoglobin in supernatant were discarded.²⁰⁾

Histological Staining Lung tissues were fixed by inflating the lungs with phosphate-buffered formalin saline using a tracheal cannula to a pressure of 15 cm H₂O, and 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 method of Richard and co-workers.²¹⁾ The intensity of the "infiltrating" eosinophil response was graded on an arbitrary scale of 0 to 4 (0=no response; 1=mild response; 2=moderate response; 3=strong response; 4=sever response, as judged by the density of leukocytes infiltrating to the peribronchial and peri-

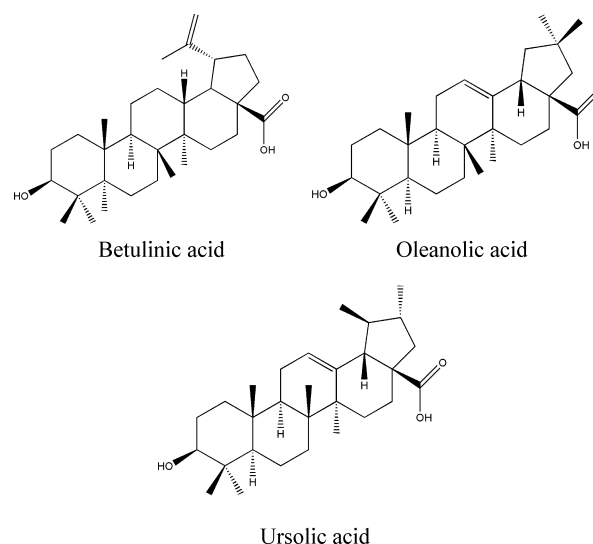


Fig. 1. Chemical Structure of the Triterpenic Acids Isolated from *Forsythiae Fructus*

bronchiolar tissues). Leukocytes were negligible in the bronchi and bronchiolar tissues after saline inhalation in antigen-sensitized animals.

Data Analysis All values are presented as means $\pm$ S.E.M.s of 6 animals. Statistical significance was determined using the Student's *t*-test and analysis of variance (ANOVA) followed by the Student-Newman-Keuls method. Significance was accepted for *p* values of <0.05. All statistical calculations were performed using SigmaStat plot.

RESULTS

Triterpenic Acids Reduced sRaw sRaw markedly increased at 5 min (IAR) and 24 h (LAR) after aerosolized-ovalbumin (AOVA) challenge in sensitized guinea pigs. sRaw was significantly ($p<0.01$) increased in OVA-sensitized guinea pigs; IAR by $287.64 \pm 20.71\%$ from 3.69 ± 0.22 to 10.59 ± 0.36 mmHg $\times$ s, and LAR by $158.69 \pm 10.00\%$ from 3.69 ± 0.22 to 5.83 ± 0.35 mmHg $\times$ s as compared with vehicle controls (Fig. 2).

HTAs showed significant inhibitory activities on IAR increases by OVA challenge in OVA-sensitized guinea pigs in a dose-dependent manner (Fig. 3; OA; $F_{(5,20)}=7.948$, $p=0.003$; BA; $F_{(5,20)}=7.275$, $p=0.005$, UA; $F_{(5,20)}=4.542$, $p=0.024$), and on LAR increases (Fig. 3; BA; $F_{(5,20)}=28.065$, $p<0.001$, OA; $F_{(5,20)}=27.930$, $p<0.001$, UA; $F_{(5,20)}=24.855$, $p<0.001$).

UA (12.5 mg/kg) significantly ($p<0.05$) decreased sRaw by $46.80 \pm 5.15\%$ during IAR as compared with the AOVA (positive) control, and UA (25 mg/kg) significantly ($p<0.05$) decreased sRaw by $38.19 \pm 6.90\%$ during LAR compared to the AOVA control (Fig. 3). OA (12.5 mg/kg) significantly ($p<0.05$) decreased sRaw by $46.54 \pm 5.90\%$ during IAR as compared with the AOVA control, and OA (50 mg/kg) significantly ($p<0.05$) decreased sRaw by $38.15 \pm 6.99\%$ during LAR as compared with the AOVA control (Fig. 3). BA (12.5 mg/kg) significantly ($p<0.05$) decreased sRaw by $44.27 \pm 2.80\%$ during IAR as compared with the AOVA control, and BA (50 mg/kg) significantly ($p<0.05$) decreased sRaw by $35.55 \pm 6.90\%$ during LAR as compared with the AOVA control (Fig. 3). Furthermore, CRO (10 mg/kg) signif-

icantly ($p<0.01$) decreased sRaw by $80.26\pm5.72\%$ during IAR and by $73.36\pm4.45\%$ during LAR; DEX (5 mg/kg) significantly ($p<0.01$) decreased sRaw by $80.40\pm7.76\%$ during IAR and by $68.93\pm5.69\%$ during LAR; and salbutamol (5 mg/kg) significantly decreased sRaw by $78.80\pm8.75\%$ during IAR ($p<0.01$) and by $31.52\pm6.39\%$ during LAR ($p<0.05$) (Fig. 3).

Thus, HTAs were shown to have a dose-dependent anti-asthmatic effect, like similar the AOVA control. Furthermore, DEX and CRO also significantly ($p<0.01$) inhibited sRaw during IAR and LAR.

Decreases in Leukocyte Levels in BALF by Triterpenic Acids AOVA-inhaled guinea pigs during LAR had significantly ($p<0.01$) more total leukocytes in BALF than naive animals ($31.28\pm1.09\times10^6$ to $8.18\pm1.11\times10^6$ cells). AOVA-

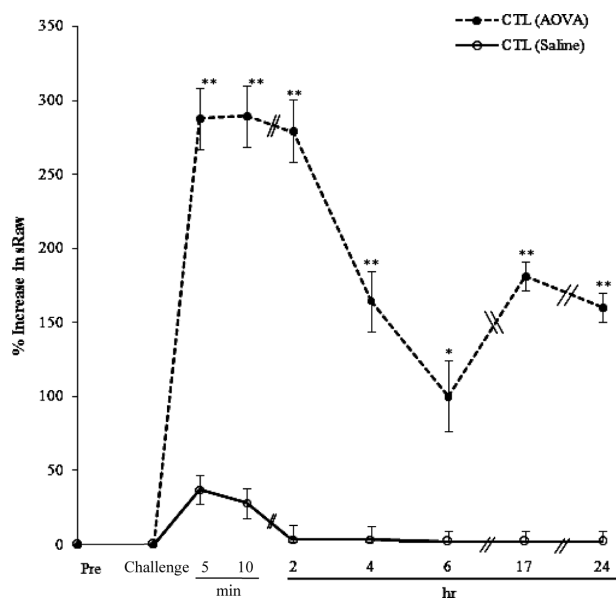


Fig. 2. Time-Dependent Changes of sRaw in Saline (Negative) and AOVA (Positive) Controls

Animals were actively sensitized using OVA (by s.c. and i.p. injections), and challenged by AOVA inhalation on Day 21. sRaw was determined after AOVA challenge. Time-dependent changes in sRaw showed IAR and LAR after AOVA challenge. % Increase in sRaw = $(\text{sRaw after challenge} / \text{sRaw before challenge} - 1) \times 100$. sRaw before challenge was 3.35 ± 0.29 mmHg/s, and increased values represent means $\pm$ S.E.M.s ($n=6$). Significantly different from saline (negative) controls, * $p<0.05$ and ** $p<0.01$. CTL: saline- or AOVA-inhaled control.

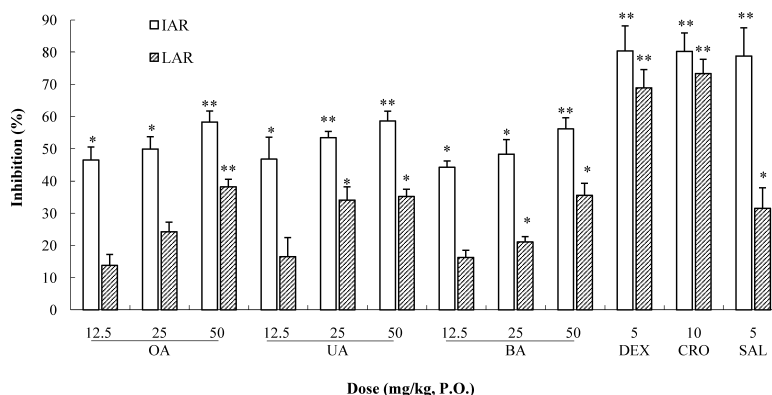


Fig. 3. Inhibitory Activities of Triterpenic Acids on sRaw during IAR and LAR

Test drugs were orally administered 1 h prior to AOVA challenge in IAR, and 12 and 23 h after challenge. The data showed IAR at 5 min and LAR at 24 h after AOVA challenge. Inhibition of sRaw (%) = $[1 - (\text{sRaw of drug-treated group} / \text{sRaw of OA-challenged control})] \times 100$. Value represent means $\pm$ S.E.M. ($n=6$). Significantly different from AOVA-inhaled controls, * $p<0.05$ and ** $p<0.01$. CTL: vehicle- or AOVA-inhaled controls, OA: oleanolic acid, UA: ursolic acid, BA: betulinic acid, DEX: dexamethasone, CRO: cromolyn sodium, SAL: salbutamol.

challenge caused leukocytosis, and neutrophil and eosinophil levels in BALF increased significantly by $253.01\pm4.27\%$ and $843.18\pm6.71\%$ ($9.26\pm0.54\times10^6$ to $3.66\pm0.79\times10^6$ and $7.42\pm0.54\times10^6$ to $0.88\pm0.15\times10^6$ cells), respectively (Fig. 4).

HTAs inhibited the recruitment of total leukocytes into BALF in a dose dependent manner (Fig. 4; BA: $F_{(5,20)}=7.907$, $p=0.004$, UA: $F_{(5,20)}=4.473$, $p=0.025$, OA: $F_{(5,20)}=4.019$, $p=0.034$), and inhibited the recruitment of eosinophils (Fig. 4; OA: $F_{(5,20)}=4.382$, $p=0.027$, UA: $F_{(5,20)}=4.169$, $p=0.031$). DEX (5 mg/kg) and SAL (5 mg/kg) also significantly inhibited total leukocytes in BALF (Fig. 4).

HTAs inhibited the recruitment of leukocytes, neutrophils, and eosinophils into BALF in a dose dependent manner (Fig. 4). BA (12.5 mg/kg) significantly ($p<0.05$) reduced leukocyte recruitment as compared with positive control ($31.28\pm0.39\times10^5$ to $27.32\pm0.22\times10^5$ cells (Fig. 4). UA and OA (25 mg/kg) also significantly ($p<0.05$) reduced leukocyte recruitment by 16.72 and 15.51% as compared with positive control, respectively (Fig. 4). Furthermore, UA and OA at a dose of 25 mg/kg also inhibited neutrophil and eosinophil levels in BALF ($p<0.05$), as did BA at 50 mg/kg ($p<0.05$). DEX (5 mg/kg) and CRO (10 mg/kg) also significantly ($p<0.01$) reduced the recruitments of neutrophils, eosinophils, and lymphocytes by BALF, whereas SAL (5 mg/kg) did not (Fig. 4).

Reductions in Inflammatory Chemical Mediators and Enzymes in BALF by HTAs AOVA-challenged guinea pigs during LAR showed significant increases in the activities of inflammatory chemical mediators, histamine, EPO and PLA₂ (Fig. 5). Histamine contents in the BALF samples of vehicle- and AOVA-challenged guinea pigs were 102.08 ± 3.65 and 406.53 ± 20.30 ng/ml during LAR, respectively, which was equivalent to an increase of $323.20\pm25.89\%$ in AOVA-challenged guinea pigs as compared with vehicle controls (Fig. 5).

EPO, a marker of eosinophil activation in AOVA-challenged guinea pigs increased significantly increased by $335.32\pm12.11\%$ (12.09 ± 3.55 to 40.54 ± 4.24 mU/ml/mg) (Fig. 5), and PLA₂ activity increased by $319.20\pm26.22\%$ as compared with vehicle-controls (2.14 ± 0.27 to 7.17 ± 0.61 nmol/min/mg) during LAR (Fig. 5).

HTAs inhibited the releases of chemical mediators and

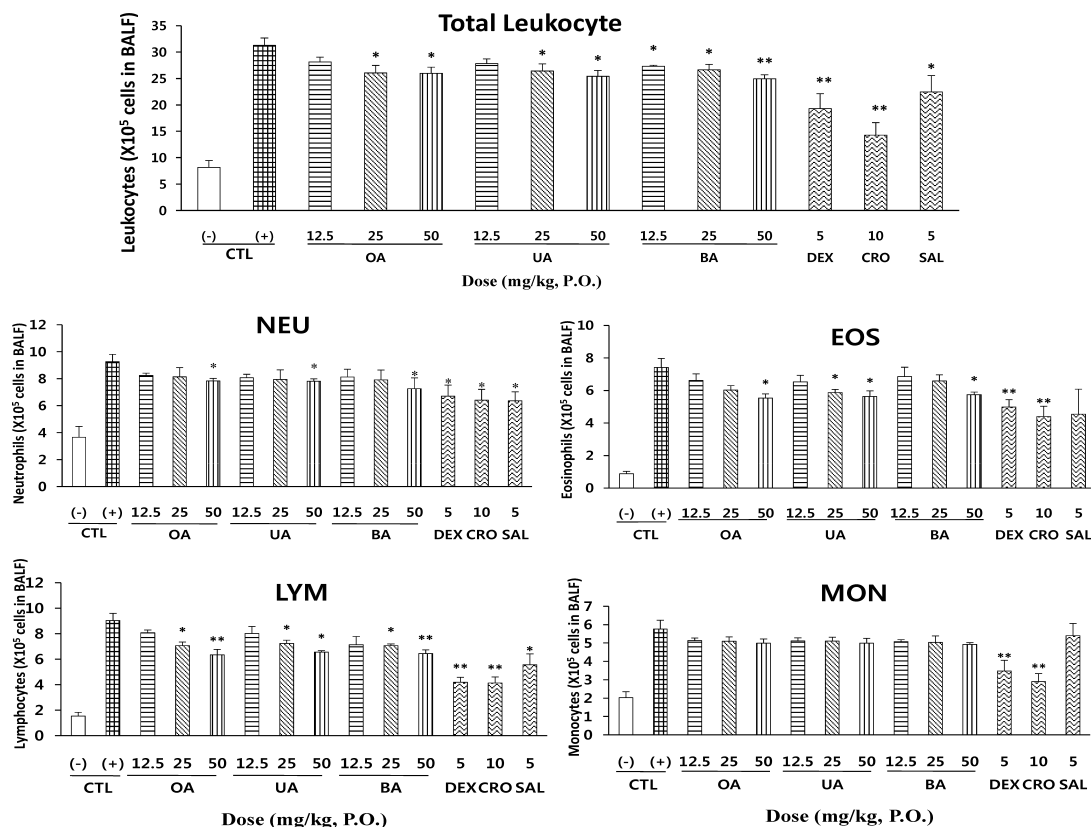


Fig. 4. Effects of Triterpenic Acids on Total Leukocytes and Their Differential Count in BALF in LAR

Test drugs were orally administered 1 h prior to AOVA challenge, and 12 and 23 h after challenge. Value represent means $\pm$ S.E.M. ($n=6$). Significantly different from the AOVA-treated controls, * $p<0.05$ and ** $p<0.01$. CTL: vehicle- or AOVA-inhaled control, NEU; neutrophils, EOS; eosinophils, LYM; lymphocytes, MON; monocytes. OA: oleanolic acid, UA: ursolic acid, BA: betulinic acid, DEX: dexamethasone, CRO: cromolyn sodium, SAL: salbutamol.

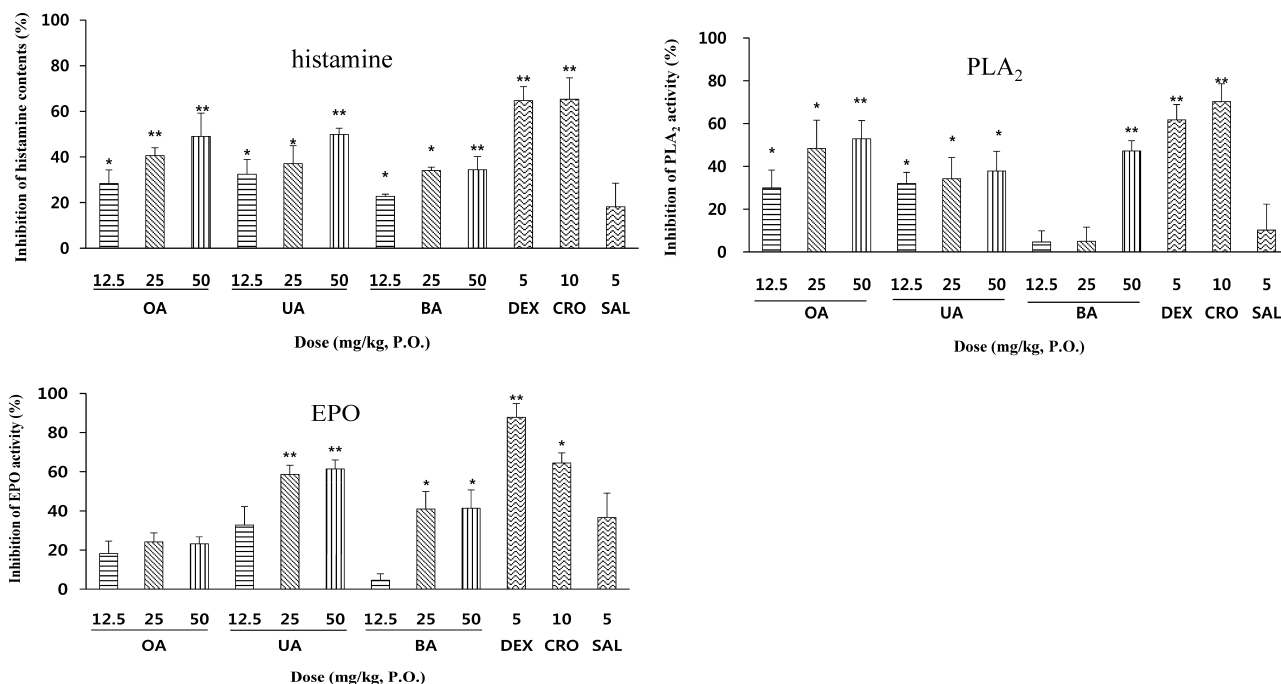


Fig. 5. Effects of Triterpenic Acids on Histamine, Specific PLA₂ and EPO Activities in BALF

Test drugs were orally administered 1 h prior to AOVA challenge, and 12 and 20 h after AOVA challenge. Inhibition (%) = $\frac{(a-c)/(a-b)}{100} \times 100$, a and b ; OVA- and vehicle-inhaled control values, c ; triterpenic acids-treated group value. histamine, specific PLA₂ and EPO activities. Challenge of AOVA to guinea pigs increased histamine content (102.08 ± 3.65 to 406.53 ± 20.30 ng/ml), specific PLA₂ (2.14 ± 0.27 to 7.17 ± 0.61 nmol/min/mg) and specific EPO (12.09 ± 3.55 to 40.54 ± 4.24 mU/ml/mg) during LAR, as compared with saline-challenged control, respectively. Inhibition (%) exhibited a decrease in chemical mediators as compared with AOVA-inhaled control, respectively. Values represent means $\pm$ S.E.M. ($n=6$). Significantly different from the AOVA-inhaled control, * $p<0.05$ and ** $p<0.01$. CTL: saline- or AOVA-inhaled control, OA: oleanolic acid, UA: ursolic acid, BA: betulinic acid, DEX: dexamethasone, CRO: cromolyn sodium, SAL: salbutamol.

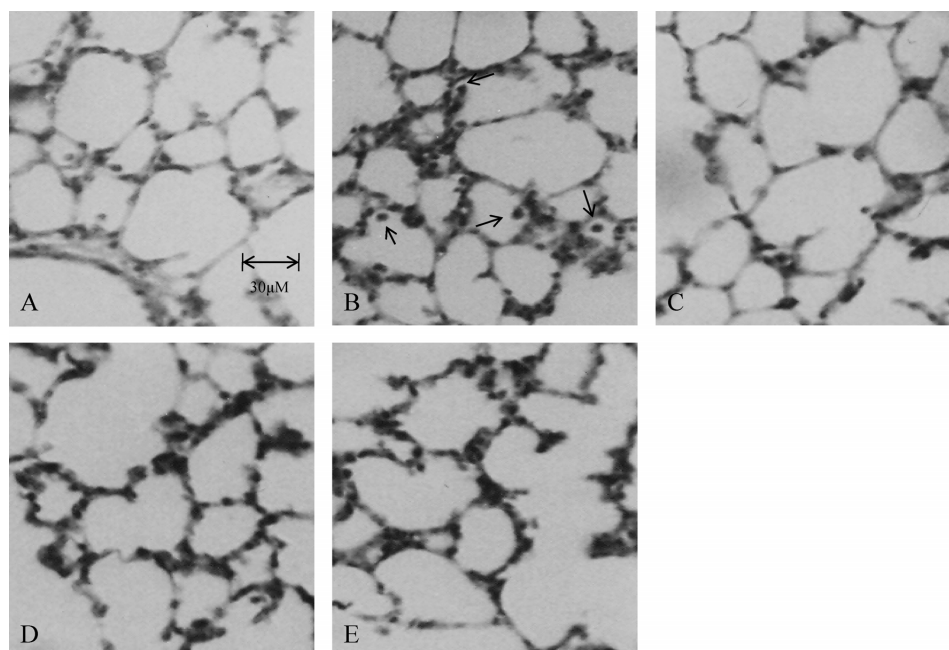


Fig. 6. Pathologic Effects of Triterpenic Acids on Lungs

The lungs of the OVA-controls at 24 h after exposure to AOVA showed neutrophil and eosinophil recruitment (B) compared to negative controls which have normal architecture and no inflammatory cell infiltration in terminal bronchioles (A). Lungs of guinea pigs treated with oleanolic acid (50 mg/kg) at 24 h after exposure to AOVA exhibited less recruitment of leukocytes in alveolar sacs (C). Lungs of guinea pigs treated with ursolic acid (50 mg/kg) at 24 h after exposure to AOVA seemed to be improved with respect to inflammatory changes compared to AOVA controls (D). Lungs of guinea pigs treated with betulinic acid (50 mg/kg) at 24 h after exposure to AOVA exhibited mild leukocyte infiltration around bronchioles (E) (H & E, original magnification: $\times 100$).

histamine in a dose dependent manner (Fig. 5; BA; $F_{(5,20)}=12.755$, $p<0.001$; UA; $F_{(5,20)}=11.614$, $p<0.001$; OA; $F_{(5,20)}=9.378$, $p=0.002$), EPO (Fig. 5; UA; $F_{(5,20)}=15.108$, $p<0.001$; BA; $F_{(5,20)}=7.350$, $p=0.005$, OA; $F_{(5,20)}=4.287$, $p=0.028$), and PLA₂ activity (Fig. 5; UA; $F_{(5,20)}=22.577$, $p<0.001$; BA; $F_{(5,20)}=10.701$, $p<0.001$; OA; $F_{(5,20)}=5.750$, $p=0.011$) in BALF were decreased.

UA, OA, and BA at a dose of 12.5 mg/kg significantly ($p<0.05$) inhibited histamine release into BALF (32.43 ± 6.46 , 28.37 ± 5.97 , and $22.74 \pm 0.96\%$, respectively). However, their activities were lower than those of DEX (5 mg/kg) and CRO (10 mg/kg) (64.69 ± 6.11 and $65.28 \pm 9.33\%$, $p<0.01$, respectively).

UA and BA at a dose of 25 mg/kg significantly ($p<0.05$) reduced EPO activity (58.64 ± 4.64 and $40.91 \pm 8.94\%$, respectively). However, their activities were less than those of DEX (5 mg/kg) and CRO (10 mg/kg) (activity reductions of $87.79 \pm 7.07\%$, $p<0.01$ and $64.42 \pm 5.22\%$, $p<0.05$, respectively).

UA and OA at 12.5 mg/kg significantly decreased PLA₂ activity (by $31.98 \pm 5.18\%$ and $29.96 \pm 8.33\%$, $p<0.05$, respectively), but their activities were lower than those of CRO (10 mg/kg) and DEX (5 mg/kg) (activity reductions of $70.28 \pm 8.33\%$, $p<0.01$ and $61.69 \pm 7.21\%$, $p<0.05$, respectively).

Histopathological Change Attenuations by HTAs The lungs of AOVA-challenged guinea pigs showed eosinophil and neutrophil recruitment in the alveolar sacs, peripheral vasculature, and terminal bronchioles, and the pavement of leukocytes in venules and arterioles (Fig. 6B), compared to vehicle-treated controls (Fig. 6A), which is consistent with previous reports.^{12,22,23} Lung of vehicle controls at 24 h after saline challenge showed a normal structural architecture, and

no inflammatory cell infiltration around terminal bronchioles (Fig. 6A). In contrast to naïve animals, AOVA-controls showed recruitment of neutrophils and eosinophils at 24 h after exposure of aerosolized-OVA (Fig. 6B). UA administered at 50 mg/kg reduced leukocyte recruitment in alveolar sacs at 24 h after exposure of aerosolized-OVA (Fig. 6D). OA at 50 mg/kg ameliorated inflammatory changes at 24 h after exposure to aerosolized-OVA (Fig. 6C), and BA at 50 mg/kg ameliorated leukocyte infiltration into alveolar sacs at 24 h after exposure (Fig. 6E). Lung of UA, OA and BA (50 mg/kg)-treated guinea pigs significantly ($p<0.05$) inhibited not only the infiltration of inflammatory cell, particularly, eosinophils and neutrophils in the alveolar sac and the peripheral vasculature by about 30%, respectively (Table 1).

DISCUSSION

We developed a guinea pig model of IAR and LAR through the inhalation of AOVA in OVA-sensitized guinea pigs. We pretreated all guinea pigs with an antihistamine, piprinhydrinate (5 mg/kg), without using an adjuvant such as Al(OH)₃, to prevent sudden death due to systemic hypersensitivity.^{9,12,22} This asthma model, produced by inhalation of aerosolized antigen, is similar to the antigen delivery route in human asthma.²³

The values we obtained through measurement of sRaw via two-chambered plethysmography encompassed both pulmonary and nasal resistance, and indicated significant narrowing of bronchi and bronchioles during LAR in this model compared to saline-challenged controls.²² The AOVA challenge in OVA-sensitized guinea pigs increased sRaw during IAR and LAR by 2.87 and 1.58 times, respectively, versus saline control ($p<0.001$) as compared with sRaw before

Table 1. Histological Assessment of Eosinophil Infiltration in Lung of Saline- and Dibenzybutyrolactone Lignans-Treated-Guinea Pigs 24 h after Antigen Inhalation

Drugs	Dose (mg/kg, <i>p.o.</i>)	Infiltration of eosinophil (% inhibition) ^{a)}
Control (OA)	—	3.12±0.28 (—)
OA	12.5	2.81±0.18 (9.94)
	25	2.47±0.18 (20.93)
	50	2.15±0.11 (31.09)*
UA	12.5	2.69±0.19 (16.35)
	25	2.48±0.16 (20.51)
	50	2.12±0.13 (32.05)*
BA	12.5	2.48±0.19 (20.51)
	25	2.36±0.12 (24.36)
	50	2.08±0.12 (33.34)*
DEX	5	1.81±0.06 (41.99)*
CRO	10	1.94±0.07 (37.82)*
SAL	5	2.78±0.25 (10.99)

a) Graded 0 to 4 (see Materials and Methods). **p*<0.05. Control (OA): OA-inhaled control, OA: oleanolic acid, UA: ursolic acid, BA: betulinic acid, DEX: dexamethasone, CRO: cromolyn sodium, SAL: salbutamol.

challenge (3.35 ± 0.29 mmHg/s) (Fig. 2). Furthermore, BAL and subsequent histopathological studies of control animals showed that the recruitment of leukocytes, particularly eosinophils and neutrophils, into the lung increased by 2.53 and 8.43 times (Fig. 4), respectively, similar to the results of studies examining segmental challenge in the human lung.²³⁾ This asthmatic model was used to evaluate the comparative effects of oral administration of HPAs and reference drugs on sRaw, and recruitment of leukocytes and chemical mediators in BALF during LAR. HPAs orally administered at a doubling dose were used to determine the standard dose for humans, in spite of inhalation treatment using the reference drugs in humans.

During preliminary studies, the MeOH extract of FF was seen to exert anti-asthmatic effects that significantly inhibited sRaw during IAR and LAR (data not shown). Thus, a further study was conducted to isolate the anti-asthmatic constituents of FF by activity-guided fractionation. This yielded three HPAs, OA, UR, and BA. In the present study, all three triterpenic acids significantly inhibited sRaw during IAR and LAR in a dose dependent manner. All HPAs administered at 12.5 mg/kg significantly inhibited sRaw during IAR, whereas HPAs administered at 25 mg/kg significantly inhibited sRaw during LAR (Fig. 3). Furthermore, all HPAs administered at 12.5–25 mg/kg significantly inhibited total leukocyte recruitment by inhibiting eosinophil and neutrophil recruitment in BALF. However, their activities were lower than those of CRO, SAL, and DEX (Fig. 4). In addition, all HPAs significantly inhibited the histamine content and activities of PLA₂ and EPO in BALF, whereas BA had fewer effects on the activities of PLA₂ and EPO than on those of OA and UA (Fig. 5). These results indicate that HPAs exert significant anti-asthmatic activity during IAR and LAR in this asthma model, probably due to the inhibition of leukocyte recruitment, particularly of eosinophils and neutrophils, and histamine content and PLA₂ activity in BALF. These may act as mast cell stabilizers and bronchodilators in FF, indicating a potential herbal medicine for treatment of inflammatory diseases through the downmodulation of mast cell activation.²⁴⁾ However, all HPAs were less active than reference drugs in

this asthma model, and further study is required to evaluate the associated rates of gastrointestinal absorption.

The reference drugs, CRO (mast cell stabilizer), steroid, and SAL (β_2 -agonist), were observed to significantly inhibit sRaw during both IAR and LAR in our guinea pig asthma model. These effects are similar to their effects in human asthma, but SAL was less active than the other reference drugs during LAR. SAL was observed to inhibit the recruitment of leukocytes, but not the histamine content and activities of PLA₂ and EPO in BALF. 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 SAL, in spite of inhalation treatment, may explain the lesser effects of SAL on LAR observed in this model compared to human asthma.^{22,25)} CRO (10 mg/kg, *p.o.*) significantly (*p*<0.05) inhibited sRaw in both IAR and LAR in this asthmatic animal model, whereas absorption of CRO from the gastrointestinal tract is very poor in human and CRO at 2 mg inhaled four times daily has anti-asthmatic effect in human.²⁶⁾ So therefore we believe that the gastrointestinal absorption of CRO in guinea pig can be different from that in human, and at the low blood level of CRO may inhibit the chemical mediator release from bronchial mast cells and the activation of leukocytes.²⁶⁾ Furthermore, BA significantly inhibited sRaw during both of IAR and LAR. BA administered at 12.5 mg/kg significantly inhibited the recruitment of leukocytes, histamine content and EPO activity in BALF, but BA administered at 50 mg/kg hardly inhibited PLA₂ activity in BALF.

In conclusion, we observed anti-asthmatic effects of HPAs in a guinea pig asthma model. Many plants contain HPAs, a large class of plant secondary metabolites that exhibit various inhibitory activities on inflammation. Further study of the structure–activity relationships of HPAs are warranted.

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REFERENCES

- 1) Hanania N. A., *Chest*, **133**, 989–998 (2008).
- 2) Banner K. H., Marchini F., Buschi A., Moriggi E., Semeraro C., Page C. P., *Pulm. Pharmacol.*, **8**, 37–42 (1995).
- 3) Barnes P. J., *Trends Mol. Med.*, **12**, 515–520 (2006).
- 4) Nauta A. J., Fredi Engels F., Knipples L. M., Garssen J., Nijkamp F. P., Redegeld F. A., *Eur. J. Pharmacol.*, **585**, 354–360 (2008).
- 5) Crompton G., *Prim. Care Respir. J.*, **15**, 326–331 (2006).
- 6) Nishibe S., Chiba M., Hisada S., *Yakugaku Zasshi*, **97**, 1134–1137 (1977).
- 7) Chiba M., Hisada S., Nishibe S., *Shoyakugaku Zasshi*, **32**, 194–197 (1978).
- 8) Lee J. S., Min B. S., Bae K. W., *Yakhak Hoeji*, **40**, 462–467 (1996).
- 9) Kang H. S., Lee J. Y., Kim C. J., *J. Ethnopharmacol.*, **116**, 305–312 (2008).
- 10) Jung C. H., Lee J. Y., Cho C. H., and Kim C. J., *Arch. Pharm. Res.*, **30**, 1599–1607 (2007).
- 11) Pennock B. E., Pennock C. P., Rogers R. M., Cain W. A., and Wells J. H., *J. Appl. Physiol.*, **46**, 399–406 (1979).
- 12) Sanjar S., Aoki S., Kristersson S. D., Moley J., *Br. J. Pharmacol.*, **99**, 679–686 (1990).
- 13) Aguirre M. C., Delporte C., Backhouse N., Erazo S., Letelier M. E., Cassels B. K., Silva X., Alegria S., Negrete R., *Bioorg. Med. Chem.*, **14**, 5673–5677 (2006).
- 14) Manoharan K. P., Song F. J., Benny T. K., Yang D., *Magn. Reson. Chem.*, **45**, 279–281 (2007).

- 15) Turkmen Z., Klymchenko A. S., Oncul S., Duportail G., Topcu G., Demchenko A. P., *J. Biochem. Biophys. Methods*, **64**, 1—18 (2005).
- 16) Gua J. Q., Franzblau S. G., Montenegro G., Timmermann B. N., *Z. Naturforsch. C*, **59**, 797—802 (2004).
- 17) Galgon T. H., Höke D., Dräger B., *Phytochem. Anal.*, **10**, 187—190 (1999).
- 18) Shore P. A., Burkhalter A., Cohn V. H. Jr., *J. Pharmacol. Exp. Ther.*, **127**, 182—186 (1959).
- 19) Radvanyi F., Jordan L., Russo-Marie F., Bon C., *Anal. Biochem.*, **177**, 103—109 (1989).
- 20) Strath M., Warren D. J., Sanderson C. J., *J. Immunol. Methods*, **83**, 209—215 (1985).
- 21) Richard V. N., Griffin R. L., Oosteen J. A., Morris J., Wiska D. G., Dunn C. J., *Am. Rev. Respir. Dis.*, **140**, 1712—1718 (1989).
- 22) Matsumoto T., Ashida Y., Tsukuda R., *J. Pharmacol. Exp. Ther.*, **269**, 1236—1244 (1994).
- 23) Kane G. C., Tollino M., Pollice M., Kim C. J., Cohn J., Murray J. J., Dworski R., Sheller J., Fish J. E., Peters S. P., *Prostaglandins*, **50**, 1—18 (1995).
- 24) Kim M. S., Na H. J., Han S. W., Jin J. S., Song U. Y., Lee E. J., Song B. K., Kim H. M., *Inflammation*, **27**, 129—135 (2003).
- 25) Dzubak P., Hajduch M., Vydra D., Hustova A., Kvasnica M., Biedermann D., Markova L., Urban M., Sarek J., *Nat. Prod. Rep.*, **23**, 394—411 (2006).
- 26) Svendsen U. G., Frølund L., Madsen F., Nielsen N. H., Holstein-Rathlou N. H., Weeke B. J., *Allergy Clin. Immunol.*, **80**, 68—74 (1987).