



Inhibitory effects of diterpene acids from root of *Aralia cordata* on IgE-mediated asthma in guinea pigs

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ABSTRACT

This study evaluated the anti-asthmatic activities of four diterpene acids isolated from *Aralia cordata* root that are proposed to be the active ingredients in its traditional use as a treatment for inflammation, overheating, pain and spasm in Korea. The diterpene acids were identified as kaurenoic acid, 7-oxo-sandaracopimaric acid, 17-hydroxy-ent-kaur-15-en-19-oic acid, and hederagenin, by comparing their phytochemical and spectroscopic data with previous reports. The effects of diterpene acids on asthma were evaluated by determining the specific airway resistance (sRaw) during the immediate asthmatic response (IAR) and the late-phase asthmatic response (LAR) in guinea pigs with IgE-mediated asthma. Recruitment of leukocytes and the presence of chemical mediators in bronchoalveolar lavage fluid (BALF) were determined, and histopathological surveys performed. The four diterpene acids dosed at 25–100 mg/kg had dose-dependently anti-asthmatic effects: 7-oxo-sandaracopimaric acid > 17-hydroxy-ent-kaur-15-en-19-oic acid > kaurenoic acid > hederagenin. 7-oxo-sandaracopimaric acid (25 mg/kg) significantly ($p < 0.05$) inhibited sRaw by 59.5% in IAR and LAR, and also dose-dependently inhibited recruitment of eosinophils and neutrophils into lung and release of chemical mediators, histamine, and the activity of phospholipase A₂ and eosinophil peroxidase in BALF. 7-Oxo-sandaracopimaric acid had the highest activity among the diterpene acids. But its effect was lower than cromolyn sodium, salbutamol, or dexamethasone in both the IAR and the LAR. These results suggested that C₇-oxo radical of 7-oxo-sandaracopimaric acid was more active than the C₇-hydroxy and hydrogen of the other compounds, and showed diterpene acids have anti-asthmatic effects, supporting the traditional application of this herb in treating IgE-mediated asthma.

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1. Introduction

Bronchial asthma is a chronic inflammatory disease of the airway that involves multiple components and is orchestrated by numerous cell types, particularly mast cells, eosinophils, and CD4⁺ lymphocytes. Activation of these cells lead to the release of proinflammatory mediators which in turn cause vascular leakage, bronchial smooth muscle contraction, inflammatory cell infiltration, mucous hypersecretion, airway hyperresponsiveness, and ultimately airway remodeling, which are expressed clinically as recurrent wheezing, dyspnea, chest tightness, cough, and airway obstruction [1]. Allergen

provocation induces an immediate asthmatic response (IAR) and a late-phase asthmatic response (LAR) [2–6]. The IAR and LAR can be observed specific airway resistance (sRaw) from 5 to 10 min and at 24 h after antigen challenge in a conscious guinea pig, which is similar to the segmental antigen challenge in human described by Pepys and Hutchcroft [7].

Inhaled corticosteroids are recommended for the first-line treatment of persistent disease. However, all patients not achieve asthma control even when these agents are used in high doses and in combination with other medications, including a long-acting β_2 -agonist or a leukotriene modifier [1]. In addition, prophylactic treatment with drugs such as cromolyn sodium and glucocorticoids inhibit both the IAR and the LAR, while treatment with a β_2 -selective adrenoceptor agonist such as salbutamol inhibits IAR, but not LAR [8,9].

We previously reported that the plant-based flavonoids, quercetin and rutin significantly inhibited asthmatic responses, based on observed inhibition of chemical mediator release in bronchoalveolar

Abbreviations: ACR, root of *Aralis cordata*; BALF, bronchoalveolar lavage fluids; EPO, eosinophil peroxidase; IAR, immediate asthmatic response; LAR, late-phase asthmatic response; OA, ovalbumin; PLA₂, phospholipase A₂; sRaw, specific airway resistance.

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lavage fluids (BALF) [10,11]. Furthermore, after screening 25 medicinal plants for anti-asthmatic effects, the ether- and ethanol-fractions of a methanol extract of ACR were found to have significant anti-asthmatic activity in guinea pigs with IgE-mediated asthma. The dried root of *Aralia cordata* Thunb (ACR, Araliaceae) is used in the traditional Korean folk medicine known as Dok Whal, for disorders such as inflammation, fever, pain, and spasms [12]. However, the active ingredients of ACR are unknown.

In this study, we investigated the ingredients of ACR that are active against inflammation by chromatographic isolation of four diterpene acids from the ether fractions of methanol extracts of ACR, and examining the activity of the fractions against asthma. The effects of the four diterpene acids on asthmatic responses were assayed by determining the specific airway resistance (sRaw) during the IAR and the LAR in guinea pigs with IgE-mediated asthma induced by sensitization and challenge with ovalbumin (OA). We also measured recruitment of leukocytes, levels of histamine, and phospholipase A₂ (PLA₂) and eosinophil peroxidase (EPO) activities in the BALF. Histopathological surveys were also conducted [9–11].

2. Materials and methods

2.1. Plant materials and chemicals

A. cordata Thunb (ACR) was collected at Sok Ri Mountain in Korea in August 2007, and identified by Professor W.K. Whang (Division of Natural Products, College of Pharmacy, Chung-Ang University). A voucher specimen no. ACR01/2007 has been submitted at the College of Pharmacy, Chung-Ang University. Chicken egg albumin (ovalbumin, OA, grade V), piprinhydrinate, bovine serum albumin (BSA), salbutamol, cromolyn sodium, dexamethasone, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1-palmitoyl-2-(10-pyrenyldecanoyl)-sn-glycero-3-phosphorylcholine (10-pyrene PC), O-phthalaldehyde (OPA), and ?-phenylen diamine dihydrochloride (OPD) were from Sigma Chemical Co. (St. Louis, Mo, USA). Thiopental sodium was from Samsung Pharmaceutical Co. (Seoul, Korea). The bicinchoninic acid (BCA, 4,4'-dicarboxy-2,2'-biquinoline) protein assay kit was from Pierce Co. (Rockford, IL, USA). All the other reagents were reagent grade. The composition of Hank's Balanced Salt Solution (HBSS)/BSA (pH 7.4) buffer was NaCl 132.0 mM, KCl 6.0 mM, HEPES 20.0 mM, KH₂PO₄ 1.2 mM, CaCl₂·H₂O 1.1 mM, MgSO₄ 1.0 mM, dextrose 5.5 mM, EDTA 10.0 mM and BSA 1.0%.

2.2. Isolation of diterpene acids from ACR

Dried and powdered ACR (20.0 kg) was extracted with 20 L hot MeOH three times to yield a solid dark extract (4000.0 g). This was suspended in water and partitioned three times with 10 L ethylether (EtOEt) to give successive 1500.0 g EtOEt fractions. EtOEt fractions were fractionated at 500.0 g per run by silica-gel column (10.0 × 85.0 cm) chromatography with elution with CHCl₃-MeOH (100:0 to 0:100), to give fractions **1** (99.7 g), **2** (115.3 g), **3** (63.8 g), **4** (37.2 g), **5** (43.1 g), **6** (39.7 g), and **7** (99.2 g). Fraction **4** (37.2 g) partitioned by silica-gel column chromatography (6.5 × 85 cm) eluted with hexane; EtOAc (1; 1), successively to give fraction **1** (4.5 g), **2** (5.5 g), **3** (6.3 g), **4** (12.0 g), **5** (4.5 g), and **6** (3.2 g). This fraction **4** was partitioned with an ODS column (6.5 × 85.0 cm) eluted with 95% MeOH, and then with a Polasil column (6.5 × 85.0 cm) eluted with hexane: EtOAc: CHCl₃ (4:1:0.5). Additional fractionation was by HPLC with a C₁₈ Waters µBondapak column (3.9 × 300 mm) eluted with 93% MeOH to give Compounds **1** (230 mg), **2** (6600 mg), **3** (120 mg) and **4** (150 mg). The four white compounds were recrystallized using the eluting solvent. Compounds **1**, **2**, **3**, and **4** were

identified by structure (Fig. 1) as ent-Kaur-16-en-18-oic acid (lauranoic acid) (**1**), 7-Oxo-ent-pimara-8(14),15-dien-18-oic acid (8-Oxo-sandaracopimaric acid) (**2**), 17-hydroxy-ent-kaur-15-en-18-oic acid (**3**), and hederagenin (**4**), by comparing their phytochemical and spectral data with previous reports [13–15]. All compounds used for this study were 98% pure, as assessed by HPLC with a Hypersil ODS C₁₈ column (150 × 4.6 mm, inner diameter 5 µm particle size). Voucher specimens no. ACR/EtOAc/diterpene/02~05/2007 have been submitted to the College of Pharmacy, Chung-Ang University.

2.3. Instrumental analysis, and phytochemical and spectral data

All melting points were determined on a BUCHI melting point B-540 (Swiss). High resolution mass spectrometer were recorded Micromass Autospec (England) with gas chromatograph (HP6890), sample inlet system high temperature direct insertion probe, gas-chromatograph HP6890, LSIMS system (FAB), ion source (EI, CI, FAB), analyzer (tri-sector double-focusing type (EBE)), detector (photo multiplier dual detector) and mass range (~3000 daltons). The UV spectra were recorded with a Hitachi 624 digital spectrophotometer and the IR spectra with a Bruker 48 (Germany). The ¹H-NMR spectra were recorded with a Varian gemini 2000, 200 MHz (USA) and Bruker AM-500, 500 MHz (Germany) with tetramethylsilane as an internal standard. The mass spectra were measured with Hitachi double-focusing and JEOL JMS-01-SG-2 mass spectrometers. The specific rotations were measured with a JASCO DIP-SL unit. GLC was run on a Hitachi 073 unit with a hydrogen flame ionization detector. Silica-gel (Kieselgel 60, Merck) was used for column chromatography. Thin-layer chromatography (TLC) was carried out on Merck plates precoated with Kieselgel 60F₂₅₄ and preparative layer chromatography (PLC) on plates (20 × 20 cm, 0.75 mm thick) coated with Kieselgel GF₂₅₄ (Merck).

Compound 1: Colorless crystals: mp: 218–220 °C, EI-MS(*m/z*): 302[M⁺], ¹H-NMR (500 MHz, pyridine-*d*₅): δ 4.70, 4.81 (each 1H, s, H-17), 2.52 (1H, brs, H-13), 1.88–2.05 (4H, m, H-3a, H-6a, H-13, H-13a), 1.71–1.79 (4H, m, H-1a, H-2a, H-6b, H-7a), 1.42–1.50 (4H, H-2b, H-11a, H-12a, H-12b), 1.15 (2H, brd, *J* = 6.4 Hz, H-15), 1.13 (3H, s, H-18), 1.01 (1H, dd, *J* = 4.6, 7.2 Hz, H-14b), 0.92 (1H, t, *J* = 7.0 Hz, H-5), 0.91 (1H, m, H-3b), 0.89 (1H, m, H-9), 0.81 (3H, s, H-20), 0.69 (1H, m, H-1b), ¹³C-NMR (125 MHz, pyridine-*d*₅): δ 186.0 (C-19), 155.9 (C-16), 103.2 (C-17), 57.1 (C-5), 55.2 (C-9), 49.0 (C-15), 44.3 (C-8), 43.9 (C-4), 43.7 (C-13), 41.3 (C-7), 40.7 (C-1), 39.8 (C-10), 39.9 (C-14), 37.8 (C-3), 33.2 (C-12), 29.0 (C-18), 21.9 (C-6), 19.2 (C-2), 18.6 (C-2), 18.5 (C-11), 15.7 (C-20).

Compound 2: Colorless needles: mp: 247–249 °C, EI-MS(*m/z*): 316.4[M⁺], ¹H-NMR (500 MHz, pyridine-*d*₅): δ 6.81 (1H, s, H-14), 5.67 (1H, dd, *J* = 10.2, 16.2 Hz, H-15), 5.02 (1H, dd, *J* = 1.4, 10.2 Hz, H-16b), 4.80 (1H, dd, *J* = 1.4, 16.2 Hz, H-16a), 3.72 (1H, dd, *J* = 13.6, 19.2 Hz, H-6b), 3.13 (1H, dd, *J* = 5.1, 19.2 Hz, H-6a), 2.45 (1H, br, *J* = 13.2 Hz, H-3b), 2.20 (1H, dt, *J* = 3.3, 13.6 Hz, H-2b), 2.00 (1H, m, H-9), 1.75 (1H, 1H, brd, *J* = 12.5 Hz, H-1b), 1.71 (1H, brd, *J* = 13.5 Hz, H-5), 1.56–1.61 (1H, m, H-12b), 1.51–1.61 (2H, m, H-11b, H-2a), 1.31 (3H, s, H-18), 1.25–1.31 (1H, m, H-12a), 1.06–1.13 (2H, m, H-1a, H-3a), 1.07 (3H, s, H-17), 0.95 (3H, s, H-20), ¹³C-NMR (125 MHz, pyridine-*d*₅): δ 199.8 (C-7), 179.4 (C-19), 145.1 (C-15), 141.0 (C-14), 137.2 (C-8), 114.3 (C-16), 51.0 (C-5), 50.5 (C-9), 43.8 (C-10), 39.4 (C-6), 39.2 (C-13), 39.1 (C-1), 38.5 (C-3), 37.3 (C-4), 35.1 (C-12), 28.7 (C-18), 28.0 (C-2), 19.2 (C-11), 13.1 (C-20).

Compound 3: Colorless needles: mp: 218–220 °C, EI-MS(*m/z*): 318[M⁺], 300, 285, 193, ¹H-NMR (500 MHz, pyridine-*d*₅): δ 5.61 (1H, s, H-15), 4.49 (1H, d, *J* = 1.5 Hz, H-17), 2.68 (1H, brs, H-13), 2.43 (1H, brd, *J* = 13.2 Hz, H-7b), 2.27 (1H, dt, *J* = 3.5, 10.3 Hz, H-2b), 2.15 (1H, dd, *J* = 3.3, 12.5 Hz, H-6b), 2.13 (1H, d, *J* = 10.9 Hz, H-14b), 2.20 (1H, dd, *J* = 2.5, 12.6 Hz, H-6a), 1.85 (1H, brd, *J* = 12.9 Hz, H-1b), 2.20 (1H, dd, *J* = 2.5, 12.5 Hz, H-6a), 1.84 (1H, brd, *J* = 12.9 Hz, H-1b),

1.63–1.68(1H,m, H-3b), 1.32, 1.18(each 3H,s, H-18, H-20), 1.00–1.08(3H, m, H-7, H-5, H-9). ^{13}C -NMR(125 MHz, pyridine- d_5): δ 180.2(C-19), 148.2(C-16), 134.8(C-15), 60.5(C-17), 56.7(C-5), 49.2(C-8), 48.2(C-9), 44.2(C-14), 43.8(C-4), 41.7(C-13), 41.2(C-1), 40.3(C-10), 39.8(C-3), 38.8(C-7), 29.4(C-18), 25.8(C-12), 21.7(C-6), 19.8(C-2), 19.3(C-11), 15.8(C-20).

Compound **4**: Colorless needles: mp>307 °C, EI-MS(m/z): 472[M^+], 454, 426, 409, 248, ^1H -NMR(500 MHz, pyridine- d_5): δ 5.48(1H, brs, H-12), 4.21(1H, dd, J = 6.0, 10.8 Hz, H-3), 3.73, 3.31(1H, m, H-18), 0.91, 0.95, 0.99, 1.03, 1.04, 1.24(each 3H, s, 6 $\times$ CH $_3$)

^{13}C -NMR(125 MHz, pyridine- d_5): δ 180.2(C-28), 144.7(C-13), 122.5(C-12), 73.4(C-3), 68.0(C-23), 48.6(C-5), 48.3(C-9), 46.6(C-17), 46.4(C-19), 43.0(C-4), 42.3(C-14), 42.1(C-18), 39.9(C-8), 38.7(C-1), 37.3(C-10), 34.2(C-21), 33.3(C-22), 33.2(C-29), 33.2(C-7), 30.8(C-20), 28.4(C-15), 27.5(C-2), 26.3(C-27), 23.8(C-11), 23.7(C-30), 23.6(C-16), 18.5(C-6), 17.4(C-26), 16.1(C-25), 13.2(C-24).

2.4. Animals

Male Dunkin-Hartley guinea pigs (350–400 g) were from the HanLim Laboratory Animal Co. (Hwa Sung-Gun, Korea) and were housed in an air-conditioned room (temperature: 24 ± 2 °C; humidity: $50 \pm 5\%$; light: 300–500 Lux) and fed a standard laboratory diet (Samyang Co.) with free access to water. Animals were cared for in accordance with *The Guidance Principles in the Care and Use of Animals*. Experiments were approved by the Institutional Animal Care and Use Committee of the Institute for Molecular-Based New Drug Development at Chung-Ang University.

2.5. Active sensitization of guinea pigs to ovalbumin

Guinea pigs were actively sensitized by *i.p.* and *s.c.* injection of 0.5 ml of 10% w/v ovalbumin (OA) in saline on the same day. Twenty-one days after sensitization, 1% OA (0.1 ml per site) was injected intradermally and skin thickness was measured 1 h later. A doubling of skin thickness indicated successful sensitization [4,10,11]. Animals that did not respond to intradermal OA were not used.

2.6. Drug treatment and antigen challenge

At 21 d after sensitization, animals were placed in a double-chambered plethysmograph (HSE type 855, Hugo Sachs Elektronik, Germany) and baseline airway function was measured. At 30 min before antigen challenge, the animals were pretreated with the histamine H_1 -receptor antagonist, piprinhydrinate (5 mg/kg, *p.o.*), to prevent lethality from endogenously released histamine during the IAR. Test drugs and reference drugs were suspended in 0.9% saline containing 0.5% sodium carboxy methyl cellulose, respectively, and administered orally 1 h prior to allergen challenge, and 12 and 20 h afterward. Animals were challenged with 5 min of inhalation of aerosolized-OA (10 mg/ml in 0.9% saline) from a jet nebulizer in the head chamber of the plethysmograph at an operating pressure of approximately 1.5 bar. This gave a particle size of below 10 μm , 60% below 2.5 μm .

2.7. Measurement of airway function

Airway function in conscious guinea pigs was measured with the double-chambered plethysmograph [2,4,10,11]. Animals were positioned with the neck extending through the partition of the head and body chamber. IAR and LAR, tidal volume, tidal airflow, respiratory rate and specific airway resistance (sRaw) were measured by a volumetric differential pressure transducer (PT5, Grass Co. USA), a noninvasive respiratory analyzer and an ink-

writing oscilloscope with a polygraph (7E, Grass Co. USA). 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 by the Pennock program [16]. Increases in sRaw were expressed as percentage of the baseline value before drug treatment and compared to control (OA) using the following equation: $\text{sRaw increase}(\%) = \{(\text{sRaw after challenge} / \text{sRaw before challenge}) - 1\} \times 100$. Furthermore, inhibition in sRaw were expressed as percentage of value of drug-treated group against that of OA-challenged control group using the following equation: $\text{Inhibition of sRaw}(\%) = [1 - (\text{sRaw of drug-treated group} / \text{sRaw of OA-challenged control})] \times 100$.

2.8. Bronchoalveolar lavage and cytologic examination

Lung function parameters were measured 24 h after allergen challenge on animals anesthetized with thiopental sodium (30 mg/kg, *i.p.*). Tracheotomy was performed, the trachea was cannulated and the lungs were lavaged four times with 5 ml of Ca^{2+} , Mg^{2+} -free HBSS containing EDTA (10 mM) and HEPES (10 mM), pre-warmed to 37 °C. The recovered fluid were combined and was centrifuged ($400 \times g$ for 5 min at 4 °C) to remove the supernatant and the supernatant stored in microtubes at -70 °C until being assayed for histamine contents, and eosinophil peroxidase (EPO) and phospholipase A_2 (PLA $_2$) specific activity. The cell pellets were immediately resuspended in 5 ml of HBSS containing 1% BSA. Total leukocyte counts in the resuspended cell pellets were done with a hemacytometer (Superior, Germany). Differential leukocyte counts were performed on cyto-centrifuged preparations (Cytospin II; Shandon Southern Instruments, Pittsburgh, PA) stained with Wright's or Turk's stain. More than 300 cells were counted per sample, using standard morphologic criteria for the classification of macrophages (mononuclear cells), eosinophils, neutrophils and lymphocytes.

2.9. Histamine assay

Histamine in BALF was measured using a fluorometric method [17]. Briefly, 1 ml BALF was transferred to a test tube with 1 ml H_2O , and 0.4 ml of 1 N NaOH was added, followed 4 min later by 0.1 ml of OPA reagent and 0.2 ml of 3 N HCl. The sample was thoroughly mixed after each addition. The solution was transferred to a 96-well plate and fluorescence measured at excitation 350 nm, emission 650 nm with a fluorospectrophotometer (FL600 Microplate Fluorescence Reader, Bio-Tek, USA).

2.10. Phospholipase A_2 assay

PLA $_2$ activity was measured using 10-pyrene PC in the presence of serum albumin using the fluorospectrophotometer as above at excitation 345 nm, emission 398 nm. Spectrofluorometric analysis of pyrene phospholipids and fatty acids was carried out in reaction solution [18]. The 10-pyrene PC was dried under nitrogen and suspended in ethanol at 0.2 mM. Stock solutions were kept at -20 °C for up to one month. Reaction solution was prepared by sequential addition of 1 ml 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 10% BSA (to 0.1% final concentration) and 6 μl of 1 M CaCl_2 (to 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 and per milligram protein (nmol/min/mg) was obtained by dividing the activity by the amount of protein in mg. To calculate the specific activity, protein content was analyzed with the BCA protein assay kit.

2.11. 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 Nagase and co-workers [19]. 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 μL 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 μL 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.

2.12. Histological staining

Animals anesthetized with thiopental sodium (30 mg/kg, *i.p.*), and the trachea was cannulated by the tracheotomy. The lung tissues were fixed by inflating the lungs with phosphate-buffered formalin saline (PBSS-FS) via the tracheal cannula to a pressure of 15 cmH₂O, and removed by chest surgery after death, and then stored in PBSS-FS at 4°C for histological examination. A length of trachea and two lung slices from similar sites were also examined from each animal. Tissues were dehydrated with a graded aqueous ethanol series and embedded in paraffin for 3 μm sectioning and staining with Mayer's H & E before mounting in canada balsam. Eosinophil/neutrophil infiltration into bronchiolar and bronchial tissue was assessed according to the method of Richard [20]. 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 to the peribronchial and peribronchiolar tissues). Leukocytes were negligible in the bronchi and bronchiolar tissues after saline inhalation in antigen-sensitized animals.

2.13. Data analysis

All values were represented as mean $\pm$ standard error (SE). Results of airway function measurement were expressed as percent change from baseline. Significant differences were calculated by one-way analysis of variance for fully randomized or repeated measures design, followed by Bonferroni method and *p* values < 0.05 were considered significant. All statistics were performed with a Sigma plot.

3. Results

3.1. Decrease of sRaw by diterpene acids

The mean baseline value for specific airway resistance (sRaw) was 5.52 ± 0.95 mmHg per sec for negative control saline-challenged guinea pigs, and 5.52 ± 0.84 mmHg per sec for positive control OA-challenged guinea pigs. Compared to the saline-challenged control, the sRaw of the OA-challenged positive control guinea pigs was increased by $432.43 \pm 45.29\%$ during the IAR and $240.68 \pm 29.12\%$ during the LAR (Fig. 2).

Diterpene acids showed dose-dependent anti-asthmatic activity. 7-Oxo-sandaracopimaric acid decreased the sRaw during the LAR in a dose-dependent manner ($F = 6.309$, $p < 0.01$), and was the most active of the tested diterpene acids. Treatment with

7-Oxo-sandaracopimaric acid (25 mg/kg) significantly ($p < 0.05$) decreased the sRaw by about 59.5% during both of IAR and LAR, respectively. Kaurenoic acid (50 mg/kg) significantly ($p < 0.05$) decreased the sRaw by $40.52 \pm 3.75\%$ during LAR. Hedragenin and 17-hydroxy-ent-kaur-15-en-18-oic acid (100 mg/kg) showed significant anti-asthmatic action by inhibition of sRaw during IAR and LAR, compared to the positive control, respectively (Fig. 3). In addition, dexamethasone has a dose-dependent inhibitory effect during IAR and LAR, and also dexamethasone and salbutamol (5 mg/kg) and cromolyn sodium (10 mg/kg) decreased the sRaw by 86.00 ± 7.25 , 76.30 ± 6.55 and $68.67 \pm 5.46\%$ during IAR, and by $90.58 \pm 7.72\%$, $62.55 \pm 5.25\%$ and $60.97 \pm 5.95\%$ during LAR, compared to the OA-challenged group, respectively (Fig. 3). These results showed that inhibitory effect of 7-oxo-sandaracopimaric acid on sRaw in the asthmatic response at 50 mg/kg has nearly same as that of cromolyn sodium (10 mg/kg) and dexamethasone (5 mg/kg), whereas less to that of salbutamol (5 mg/kg).

3.2. Decrease of recruitment of leukocytes in BALF by diterpene acids

OA-challenge of OA-sensitized guinea pigs significantly ($p < 0.01$) increased the total leukocytes in BALF by approximately four-fold, compared to the negative control ($7.75 \pm 0.45 \times 10^5$ cells vs. $30.78 \pm 1.15 \times 10^5$ cells) (Fig. 4). Eosinophils and neutrophils increased significantly ($p < 0.01$) compared to the negative control ($0.70 \pm 0.19 \times 10^5$ to $6.66 \pm 0.95 \times 10^5$ cells vs. $2.45 \pm 0.30 \times 10^5$ to $8.85 \pm 0.95 \times 10^5$ cells). Lymphocytes and monocytes also increased significantly ($p < 0.01$) compared to the negative control ($2.61 \pm 0.25 \times 10^5$ cells to $8.21 \pm 0.94 \times 10^5$ and $1.99 \pm 0.44 \times 10^5$ cells to $7.06 \pm 0.55 \times 10^5$ cells, respectively) (Fig. 4). These data showed that OA-challenge increased eosinophils in BALF by ten-fold, and the other leukocytes by three-fold over the negative control.

7-Oxo-sandaracopimaric acid inhibited the recruitment of eosinophils ($p < 0.05$) and neutrophils ($F = 6.939$, $p < 0.01$) into the lung in a dose-dependent manner (Fig. 5). 7-Oxo-sandaracopimaric acid (50 mg/kg) significantly inhibited leukocyte recruitment, and recruitment of eosinophils ($6.66 \pm 0.95 \times 10^5$ cells to $4.40 \pm 0.60 \times 10^5$ cells, $p < 0.05$) and neutrophils ($8.85 \pm 0.95 \times 10^5$ cells to $3.61 \pm 0.91 \times 10^5$ cells, $p < 0.05$). Kaurenoic acid (50 mg/kg) significantly inhibited recruitment of leukocytes into lung, and recruitment of neutrophils ($8.85 \pm 0.95 \times 10^5$ cells to $3.51 \pm 0.70 \times 10^5$ cells, $p < 0.05$) (Fig. 5). In addition, 17-hydroxy-ent-kaur-15-en-18-oic acid significantly inhibited recruitment of leukocytes into lung at 25 mg/kg, and recruitment of neutrophils ($8.85 \pm 0.95 \times 10^5$ cells to $4.20 \pm 0.54 \times 10^5$ cells, $p < 0.01$) at 50 mg/kg. In addition, kaurenoic acid (100 mg/kg) significantly ($p < 0.05$) decreased lymphocytes and monocytes, but their inhibitory effect of other tripenic acids, as well as dexamethasone and cromolyn sodium were not significantly. Furthermore, the reference drugs cromolyn sodium (10 mg/kg) and dexamethasone (5 mg/kg) significantly ($p < 0.01$) inhibited recruitment of leukocytes into the lung ($80.85 \pm 5.76 \times 10^5$ cells to $19.00 \pm 1.55 \times 10^5$ and $20.95 \pm 1.82 \times 10^5$ cells, respectively), and also significantly ($p < 0.01$) inhibited recruitment of eosinophils and neutrophils into the lung, respectively, whereas salbutamol (5 mg/kg) did not inhibit recruitment of leukocyte in BALF. They at 5–10 mg/kg inhibited recruitment of eosinophils and neutrophils in same manner as the diterpene acids at 50–100 mg/kg, but more effectively.

3.3. Decrease of histamine in BALF by diterpene acids

OA-challenge of OA-sensitized guinea pigs significantly ($p < 0.01$) increased the histamine content of BALF compared to the negative control (0.065 ± 0.002 to $0.271 \pm 0.07 \mu\text{g/ml}$). Thus,

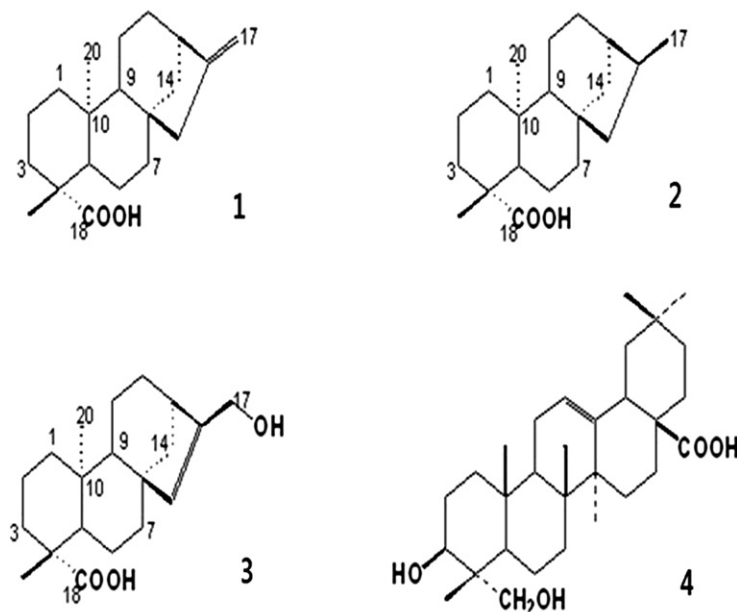


Fig. 1. Structures of diterpene acids isolated from roots of *Aralis cordata*. ent-Kaur-16-en-18-oic acid (kaurenoic acid, KRA) (1), 7-Oxo-ent-pimara-8(14),15-dien-18-oic acid (8-Oxo-sandaracopimaric acid, OSA) (2), 17-hydroxy-ent-kaur-15-en-18-oic acid (HKA) (3), and hederagenin (HDG) (4).

histamine released in the BALF of OA-challenged animals increased by 417% during LAR compared to the negative control group. These results showed that histamine release in BALF increased during the asthmatic response induced by aerosolized-OA.

Diterpene acids inhibited histamine content in BALF in a dose-dependent manner (Fig. 6, kaurenoic acid; $p < 0.05$, 17-hydroxy-ent-kaur-15-en-18-oic acid; $p < 0.01$, respectively). 7-Oxo-sandaracopimaric acid and hederagenin dosed at 25 mg/kg significantly ($p < 0.05$) inhibited histamine content by $31.98 \pm 4.68\%$ and $29.96 \pm 8.33\%$ in BALF, compared to the positive control, respectively. Kaurenoic acid (50 mg/kg) and 17-hydroxy-ent-kaur-15-en-18-oic acid (100 mg/kg) significantly ($p < 0.05$) inhibited histamine content by $38.66 \pm 8.35\%$ and $37.86 \pm 9.23\%$ in BALF, compared to the positive control, respectively. In addition, cromolyn sodium

(10 mg/kg) and dexamethasone (5 mg/kg) significantly ($p < 0.05$) inhibited histamine content by $69.12 \pm 5.78\%$ and $65.16 \pm 5.45\%$ in BALF, compared to the positive control, respectively, whereas salbutamol did not inhibit histamine content. These results indicated that inhibitory effect of diterpene acids at 25–100 mg/kg on histamine release in BALF were less than that of cromolyn sodium (10 mg/kg)- and dexamethasone (5 mg/kg)-treated groups.

3.4. Decrease phospholipase A_2 activity in BALF by diterpene acids

PLA₂ activity in the BALF of the negative control was 2.18 ± 0.14 nmol/min/mg, increasing by 161% in the OA-challenged groups to 5.11 ± 0.23 nmol/min/mg during the LAR (Fig. 6). Therefore, PLA₂ activity related to eicosanoid inflammatory mediators increased during an asthmatic response induced by aerosolized-OA, in OA-sensitized guinea pigs.

17-Hydroxy-ent-kaur-15-en-18-oic acid and 7-oxo-sandaracopimaric acid inhibited PLA₂ activity in BALF in a dose-dependent manner (Fig. 6, 17-Hydroxy-ent-kaur-15-en-18-oic acid; $P < 0.01$). 7-Oxo-sandaracopimaric acid (25 mg/kg) significantly ($p < 0.05$) inhibited PLA₂ activity by $39.26 \pm 5.24\%$ in BALF, compared to the positive control. 17-Hydroxy-ent-kaur-15-en-18-oic acid (50 mg/kg), and kaurenoic acid and hederagenin (100 mg/kg) significantly ($p < 0.05$) inhibited PLA₂ activity by 48.55 ± 9.22 , 31.45 ± 7.23 and $30.05 \pm 9.42\%$ in BALF, compared to the positive control, respectively. 7-Oxo-sandaracopimaric acid was the most active among the tested diterpene acids. In addition, dexamethasone (5 mg/kg) and cromolyn sodium (10 mg/kg) significantly ($p < 0.05$) inhibited PLA₂ activity by $65.55 \pm 6.12\%$ and $60.01 \pm 5.66\%$ in BALF, compared to the positive control, respectively, whereas salbutamol did not inhibit PLA₂ activity in BALF. These results indicated that inhibitory effect of diterpene acids at 25–100 mg/kg on PLA₂ activity in BALF were less than that of cromolyn sodium (10 mg/kg)- and dexamethasone (5 mg/kg)-treated groups (Fig. 6).

3.5. Decrease EPO activity in BALF by diterpene acids

EPO activity in the BALF of the negative control was 12.09 ± 3.55 mU/mL, increasing by 335.3% in the OA-challenged

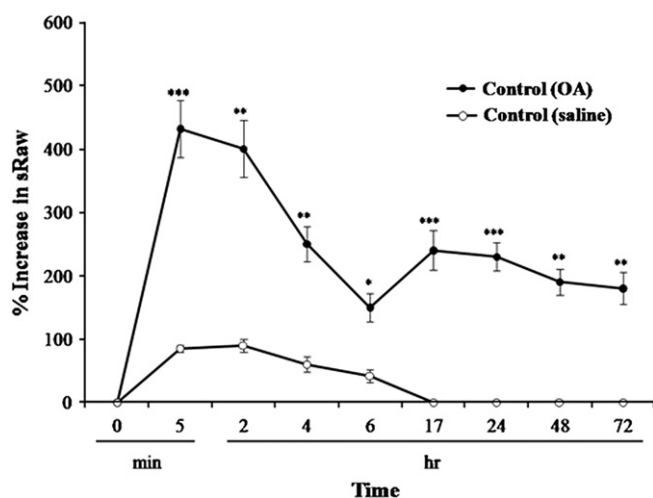


Fig. 2. Time-related changes of specific airway resistance (sRaw) after aerosolized-OA challenge. Animals were sensitized by i.p. and s.c. injection of 0.5 ml of 10% w/v ovalbumin (OA) in saline on the same day. Time-related changes of sRaw were determined for 48 h by challenge with aerosolized-OA inhalation at twenty-one days after sensitization. % Increase in sRaw = (sRaw after challenge/sRaw before challenge-1) $\times$ 100. Each value represents the mean $\pm$ S.E.M ($n = 6$). Significantly different from control (saline), * $P < 0.05$ and ** $P < 0.01$. Control: saline- or AOA-inhaled control.

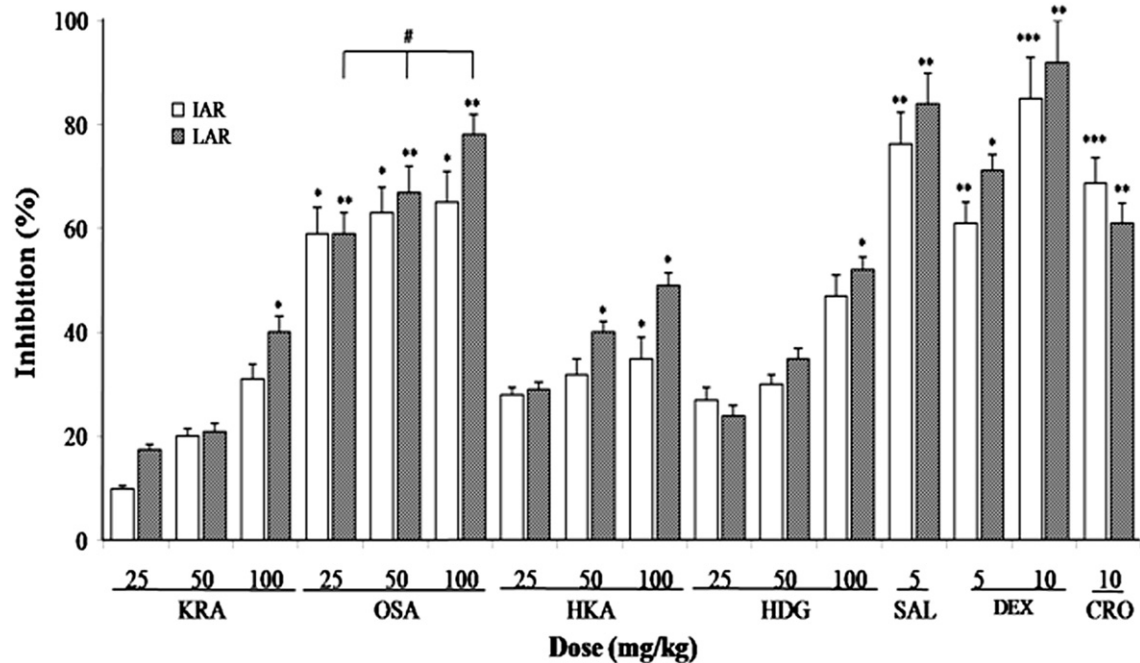


Fig. 3. Inhibitory activity of diterpene acids from *Aralis cordata* root on specific airway resistance (sRaw) of the IAR at 5 min and LAR at 24 h after ovalbumin (OA)-aerosolized challenge in OA-sensitized guinea pigs. Drugs were orally administered 1 h prior to OA-challenge, additionally 12 and 20 h later, respectively. Increase of sRaw (%) in IAR and LAR shows at 3–10 min and 24 h after allergen challenge, respectively. Decrease of sRaw (%) = $[1 - (\text{sRaw of drug-treated group} / \text{sRaw of OA-challenged control})] \times 100$. Each value represents the mean $\pm$ S.E.M ($n = 6$). Significantly different from OA-inhaled control, * $P < 0.05$ and ** $P < 0.01$. Significantly dose-dependent, # $P < 0.05$ and ## $P < 0.01$. Con (saline); saline-inhaled control, Con (OA); OA-inhaled control, KRA: kaurenoic acid, OSA: 7 α -hydroxy-sandaracopimaric acid, HKA: 17-hydroxy-ent-kaur-15-en-19-oic acid, HDG: hederagenin, DEX: dexamethasone, CRO: cromolyn sodium, SAL: salbutamol.

groups to 40.54 ± 4.24 mU/mL during the LAR (Fig. 6). Therefore, EPO activity related to eosinophil recruitment increased during an asthmatic response induced by aerosolized-OA-challenge in OA-sensitized guinea pigs (Table 1).

7-Oxo-sandaracopimaric acid (25 mg/kg) inhibited EPO activity in BALF in a dose-dependent manner (Fig. 6. 7-Oxo-sandaracopimaric acid; $p < 0.001$). 7-Oxo-sandaracopimaric acid and 17-hydroxy-ent-kaur-15-en-18-oic acid (25 mg/kg)

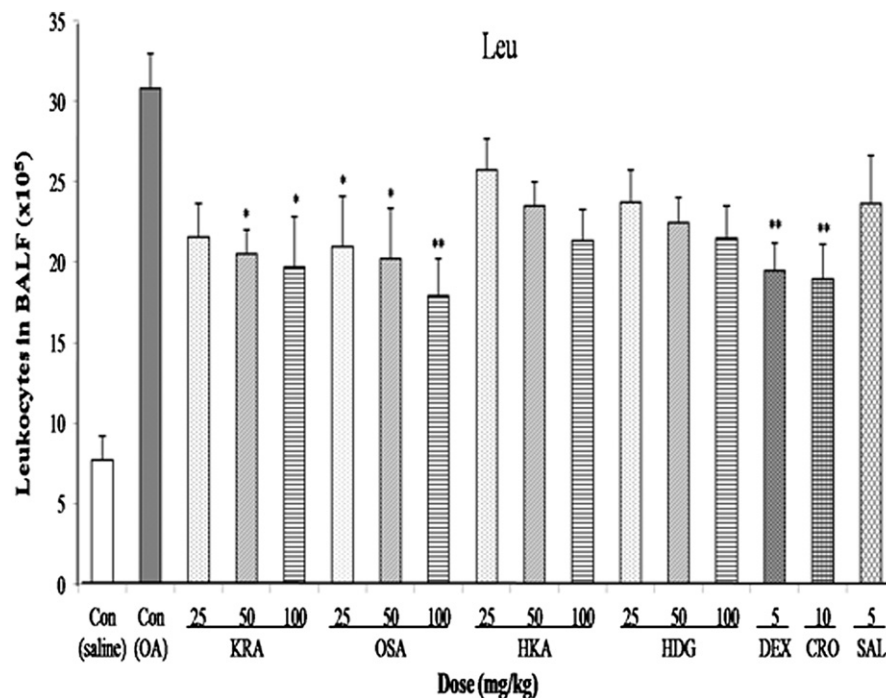


Fig. 4. Inhibitory activity of diterpene acids roots of *Aralis cordata* on the recruitment of leukocytes into the lung 24 h after aerosolized-OA-challenge in the OA-sensitized guinea pigs. Drugs were orally administered 1 h prior to aerosolized-OA-challenge, additionally 12 and 20 h later, respectively. Each value represents the mean $\pm$ S.E.M ($n = 6$). Significantly different from OA-inhaled control, * $P < 0.05$ and ** $P < 0.01$. Significantly dose-dependent, # $P < 0.05$ and ## $P < 0.01$. All abbreviations are same as Fig. 3.

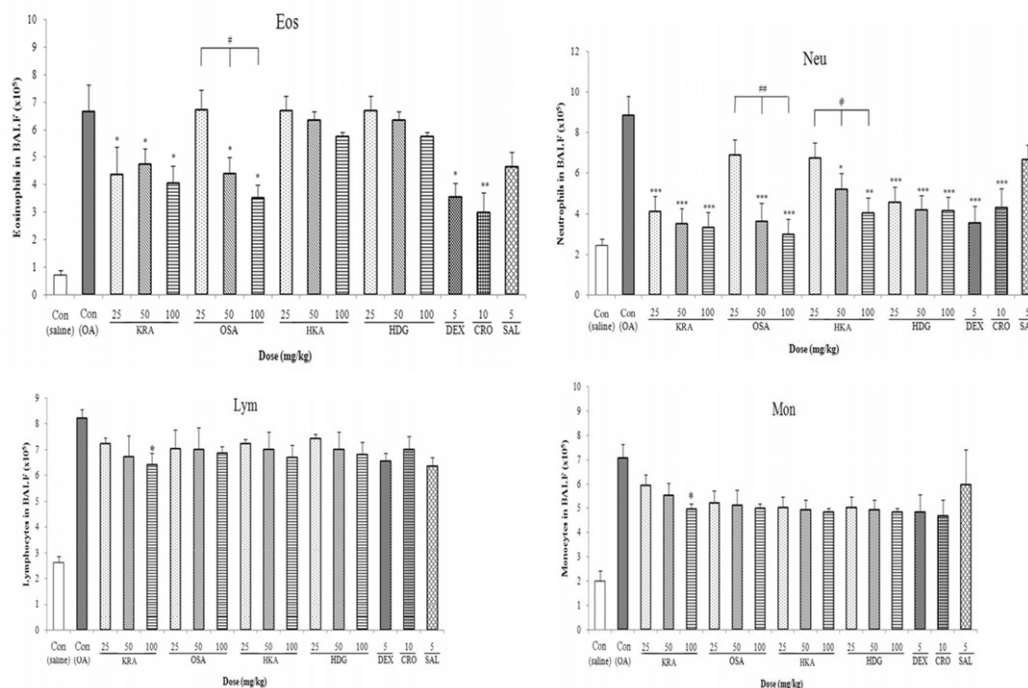


Fig. 5. Inhibitory activity of diterpene acids from roots of *Aralis cordata* on the recruitment of differential leukocytes into the lung 24 h after aerosolized-OA-challenge in the OA-sensitized guinea pigs. Drugs were orally administered 1 h prior to OA-challenge, additionally 12 and 20 h later, respectively. Each value represents the mean $\pm$ S.E.M ($n = 6$). Significantly different from OA-inhaled control, * $P < 0.05$ and ** $P < 0.01$. Significantly dose-dependent, # $P < 0.05$ and ## $P < 0.01$. All abbreviations are same as Fig. 3.

significantly ($p < 0.05$) inhibited EPO activity by 40.27 ± 8.46 and $30.25 \pm 2.98\%$ in BALF, compared to the positive control. 17-Hydroxy-ent-kaur-15-en-18-oic acid (50 mg/kg), and hederagenin and kaurenoic acid (100 mg/kg) significantly ($p < 0.05$) inhibited EPO activity by 69.55 ± 3.69 , 36.77 ± 11.32 and $36.66 \pm 9.45\%$ in BALF, compared to the positive control, respectively. In addition, dexamethasone (5 mg/kg), cromolyn sodium (10 mg/kg) and salbutamol (5 mg/kg) significantly ($p < 0.05$) inhibited EPO activity by 80.25 ± 8.15 , 68.51 ± 6.65 and $37.25 \pm 2.78\%$ in BALF, compared to the positive control, respectively. These results indicated 7-oxo-sandaracopimaric acid was the most active of the tested diterpene acids which was less than cromolyn sodium- and dexamethasone-treated groups (Fig. 6).

3.6. Improvement of morphological changes by diterpene acids

The lungs of negative control guinea pigs at 24 h after exposure to aerosolized-saline showed normal structural architecture (Fig. 7A), but asthmatic lungs at 24 h after challenge with aerosolized-OA showed marked recruitment of leukocytes, in particular, eosinophils were found in alveolar sacs, and a pavement of leukocytes was seen in venules (Fig. 7B). The lungs of 7-Oxo-sandaracopimaric acid and 17-hydroxy-ent-kaur-15-en-18-oic acid-treated animals (50 mg/kg) at 24 h after exposure to aerosolized-OA showed dose-dependent improvements in the infiltration of inflammatory cells, particularly eosinophils in the alveolar sacs and around the venule, respectively (Fig. 7C and D).

4. Discussion

In this study, as the our previous work, the model for asthmatic responses in the IAR and the LAR which produced by pretreatment of an antihistamine, piprinhydrinate (5 mg/kg), without using an adjuvant such as $Al(OH)_3$ to prevent sudden death due to systemic

hypersensitivity, was a conscious, OA-sensitized guinea pigs [2,3]. In this model, the antigen delivery route is similar to human asthma. After challenge with aerosolized-OA of OA-sensitized guinea pigs, the sRaw increased by over 432.43% during the IAR, and over 240.68% during the LAR, compared to the saline-challenged control (Fig. 2). In this study, the IAR and LAR were observed 5 and 24 h min after antigen challenge, respectively (Fig. 2), similar to the segmental antigen challenge in human described by Kane et al. [21]. Analysis of BALF, and histopathological examinations, showed that leukocytes, particularly eosinophils and neutrophils infiltrated the airway in the guinea pig asthma model (Figs. 4, 5 and 6, and Table 1), which was similar to the segmental antigen challenge in asthmatic humans with IgE-mediated asthma described by Kane et al. [21]. The infiltrated eosinophils may also release cytotoxic products, such as major basic protein, eosinophilic cationic protein, and other lipid mediators, including platelet activated factor and cysteinyl-leukotrienes, which lead to tissue damage [22]. However, recently the inbred strain, Brown-Norway rats is increasingly being used for the development of in vivo asthma models [23].

In our preliminary experiments, water and ethylether fractions of methanolic extract of ACR showed inhibitory effect of inflammation and asthma in the murine models, and also 7-Oxo-sandaracopimaric acid showed not only anti-inflammatory action in rat peritoneal mast cell models of histamine release, but also activity against phenylquinone-induced writhing syndrome and capillary permeability, as well as anti-COX-1 and anti-COX-2 activity in a concentration-dependent manner (data did not show until now): The inhibitory activity of 7-Oxo-sandaracopimaric acid against COX-1 and 2 was similar to that of indomethacin at 10 μ M, but 7-Oxo-sandaracopimaric acid was less active than celebrex against COX-1 and 2. Other reports showed that diterpene acids such as kaurenoic acid and hederagenin had anti-inflammatory effect on acetic acid-induced colitis [24] and antinociceptive action [25], but not anti-asthmatic effect.

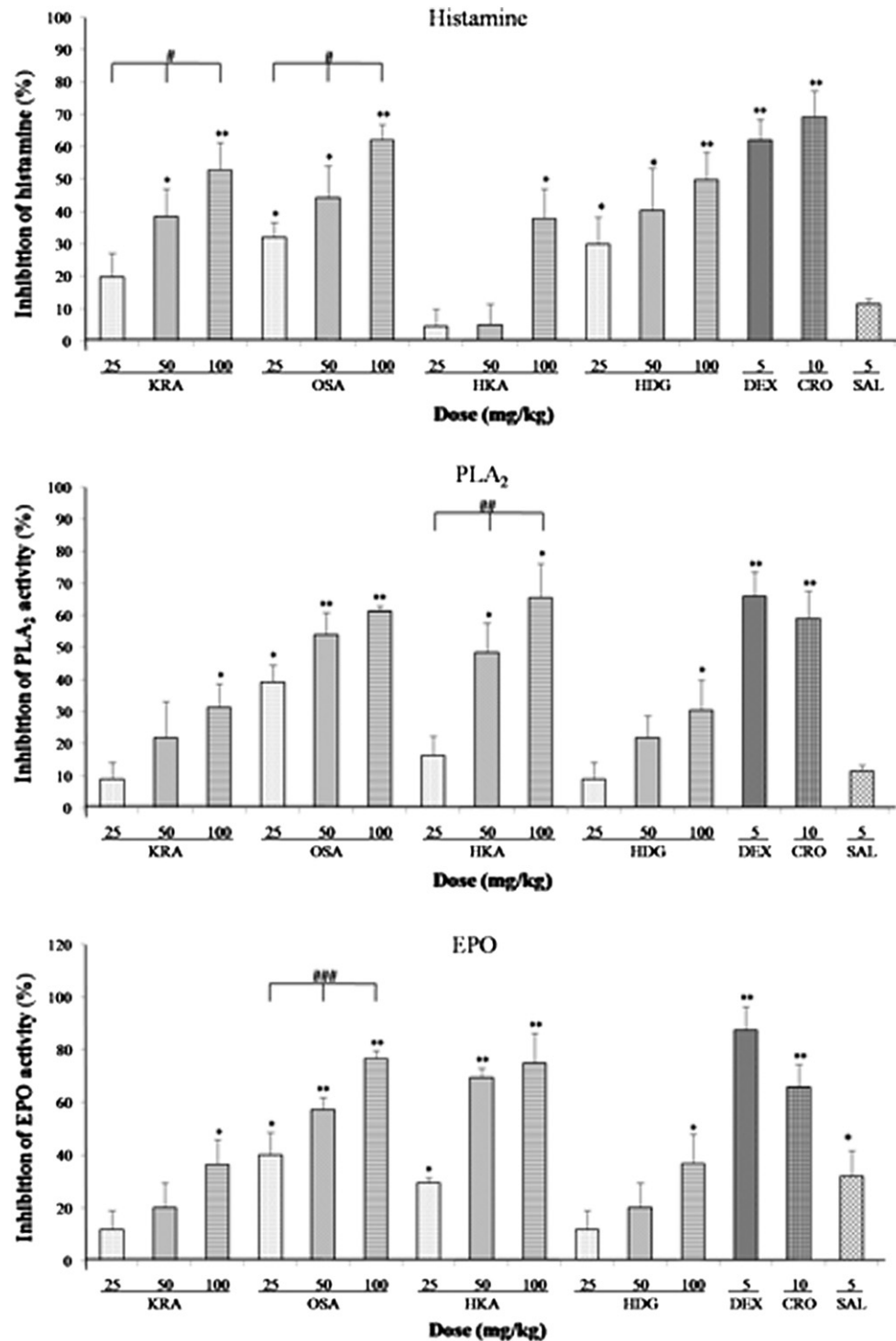


Fig. 6. Inhibitory activity of diterpene acids from roots of *Aralis cordata* on the contents of histamine, PLA₂ and EPO activities in BALF 24 h after aerosolized-OA-challenge in the OA-sensitized guinea pigs. OA-challenge of OA-sensitized guinea pigs significantly ($p < 0.01$) increased the histamine content (0.065 ± 0.002 to 0.271 ± 0.07 $\mu\text{g/ml}$), and activities of PLA₂ (2.18 ± 0.14 to 5.11 ± 0.23 nmol/min/mg) and EPO (12.09 ± 3.55 to 40.54 ± 4.24 mU/mL) of BALF compared to the negative control. Data showed Mean $\pm$ S.E. ($n = 6$) of the inhibition of test drugs on the OA-challenged increase of histamine, PLA₂ and EPO activities. Drugs were orally administered 1 h prior to OA-challenge, additionally 12 and 20 h later, respectively. Significantly different from OA-inhaled control, * $P < 0.05$ and ** $P < 0.01$. Significantly dose-dependent, # $P < 0.05$ and ### $P < 0.01$. All abbreviations are same as Fig. 3.

The diterpene acids tested in this study have a dose-dependent anti-asthmatic activity. 7-Oxo-sandaracopimaric acid and kaurenoic acid have more active than other diterpene acids, probably due to the inhibition of sRAW in IAR and LAR, and recruitment of leukocytes, especially eosinophils and neutrophils, and histamine, and activity of

PLA₂ and EPO in BALF. Kaurenoic acid (100 mg/kg) significantly ($p < 0.05$) decreased recruitment of lymphocytes which may play a key role of Th-lymphocytes in the regulation of immune and inflammatory reactions through the release of cytokines [26–28]. And also 17-hydroxy-ent-kaur-15-en-18-oic acid and hederagenin

Table 1

Histological assessment of eosinophil infiltration in the lungs of vehicle-e-treated and diterpene acids-treated guinea pigs 24 h after OA-aerosolized inhalation.

Drug	Dose (mg/kg)	Infiltration of eosinophil (% inhibition) ^a
Control (OA)	–	3.05 ± 0.25 (–)
KRA	25	2.82 ± 0.16 (7.54)
	50	2.76 ± 0.18 (9.95)
	100	2.55 ± 0.17 (18.03) ^b
OSA	25	2.78 ± 0.16 (8.85)
	50	2.48 ± 0.15 (18.99)
	100	2.20 ± 0.12 (27.87) ^b
HKA	25	2.58 ± 0.19 (15.41)
	50	2.45 ± 0.15 (19.67)
	100	2.32 ± 0.12 (23.93) ^b
HDG	25	2.85 ± 0.18 (6.56)
	50	2.81 ± 0.19 (9.54)
	100	2.73 ± 0.22 (10.49)
DEX	5	1.88 ± 0.08 (40.60) ^b
CRO	10	1.92 ± 0.09 (36.63) ^b
SAL	5	2.93 ± 0.22 (3.30)

KRA, ent-Kaur-16-en-18-oic acid (Kaurenoic acid); OSA, 7-oxo-ent-pimara-8(14),15-dien-18-oic acid (8-oxo-sandaracopimaric acid); HKA, 17-hydroxy-ent-kaur-15-en-19-oic acid; HDG, hederagenin; DEX, dexamethasone; CRO, cromolyn sodium; SAL, salbutamol.

^a Graded 0 to 4 (See method).

^b Significantly different from aerosolized-OA control ($p < 0.05$).

have the significant anti-asthmatic effects. However, 17-hydroxy-ent-kaur-15-en-18-oic acid significantly inhibited recruitment of neutrophils, PLA₂ and EPO in BALF, although hardly (100 mg/kg) inhibit histamine content in BALF.

Furthermore, the results of the present study indicate that the effect of glucocorticoid, mast cell stabilizer and β_2 -agonist on IAR and LAR in this guinea pig model are similar to the clinical effects of the drugs in human asthma [8]. Dexamethasone, cromolyn

sodium and salbutamol significantly inhibited not only sRaw during IAR and LAR, but also recruitment of eosinophils and neutrophils, histamine, and activity of PLA₂ and EPO in BALF during LAR, however, salbutamol did not inhibit recruitment of eosinophils and neutrophils, histamine, and activity of PLA₂ and EPO in BALF during LAR. These results indicate that the effects of diterpene acids on IAR and LAR in this guinea pig model are similar to the clinical effects of the anti-asthmatic drugs, dexamethasone and cromolyn sodium. However, the observation in this study that administration of salbutamol did not affect recruitment of eosinophils and neutrophils, histamine, and activities of PLA₂ and EPO in BALF during LAR (Figs. 5 and 6) suggests that factors other than the contraction of airway smooth muscle may be involved in LAR development.

In addition, 7-oxo-sandaracopimaric acid has a C₇-Oxo radical and a C₁₈-COOH group, and was the most active among the four tested diterpene acids. 17-Hydroxy-ent-kaur-15-en-18-oic acid, which has a C₇-hydroxy and a C₁₈-COOH, and kaurenoic acid and hederagenin, which have a C₇-hydrogen and a C₁₈-COOH, showed less anti-asthmatic activity than 7-Oxo-sandaracopimaric acid. The inhibitory action of diterpene acids was the same as flavonoids, which was the same as the anti-microbial activity of methyl *cis*- and *trans*-7-oxo-13-deiso-propyldehydroabietate, in which a mixture of both isomers exhibits activity against fungi and bacteria [29]. These results suggested that the C₇-oxo radical and the C₁₈-COOH in the structure of primadiene are the functional groups for anti-asthmatic activity.

However, Tomatsu et al. reported that aralin from the shoots of *Aralia elata* has the cytotoxicity which is brought about primarily through the induction of apoptosis [30]. In addition, diterpene acids may act as larval growth inhibitors [31]. Therefore the toxicity of diterpene acids have to further study for the clinical use.

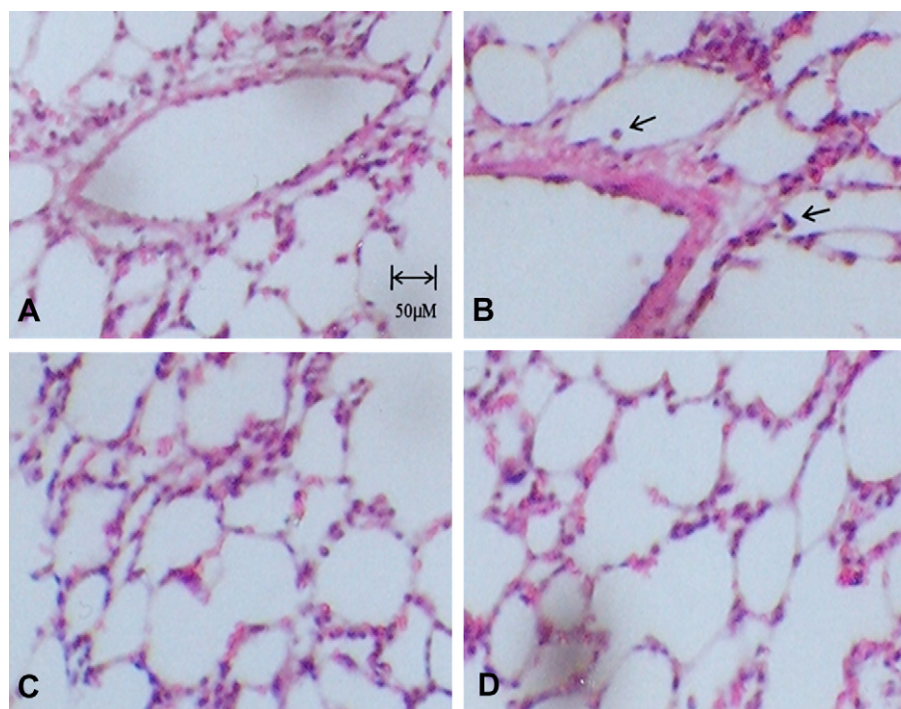


Fig. 7. The morphological changes in the lung 24 h after aerosolized-OA-challenge in the OA-sensitized guinea pigs in the lung tissue. Drugs were orally administered 1 h prior to OA-challenge, additionally 12 and 20 h later, respectively. A: Negative control 24 h after exposure of aerosolized-saline (H & E, $\times 100$). B: Lung of positive control 24 h after exposure of aerosolized-OA (H & E, $\times 100$). C: Lung of OSA (50 mg/kg) treated groups 24 h after exposure of aerosolized-OA, respectively (H & E, original magnitude $\times 400$).

Acknowledgements

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