The Rhizomes of *Alisma orientale* and Alisol Derivatives Inhibit Allergic Response and Experimental Atopic Dermatitis

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The 70% ethanol extract of the rhizome of *Alisma orientale* (Alismatis rhizome) (AOE) was prepared and found to significantly inhibit 5-lipoxygenase (5-LOX)-catalyzed leukotriene (LT) production from rat basophilic leukemia (RBL)-1 cells and β -hexosaminidase release by antigen-stimulated RBL-2H3 cells. It also attenuated delayed-type hypersensitivity (DTH) reaction in mice. Among the three major triterpene constituents isolated (*i.e.*, alisol B, alisol B 23-acetate, alisol C 23-acetate) as active principles, alisol B and its 23-acetate strongly and significantly inhibited LT production and β -hexosaminidase release between 1–10 μ M. On the other hand, all these alisol derivatives significantly and strongly inhibited DTH response after oral administration. In addition, AOE (200 mg/kg/d) was for the first time found to considerably alleviate hapten-induced dermatitis symptoms in NC/Nga mice, an animal model of atopic dermatitis. These results indicate that alisol derivatives possess inhibitory activities on immediate-type as well as delayed-type hypersensitivity reactions and may contribute to the anti-allergic action of AOE.

Key words *Alisma orientale*; alisol B; 5-lipoxygenase; β -hexosaminidase; anti-allergy; atopic dermatitis

The rhizome of *Alisma orientale* (Alismatis rhizome, Alismataceae) is a well-known Chinese traditional medicine. This plant material has been widely used as an anti-inflammatory drug in Asia¹⁾ and the major constituents are alisol derivatives.²⁾

Previously, the extract of *A. orientale* was reported to show anti-inflammatory and anti-allergic activities. Specifically, the methanol extract of *A. orientale* inhibited nitric oxide (NO) production.³⁾ The methanol extract also showed anti-complement and anti-inflammatory activities while several alisol derivatives (alisol A, alisol A acetate, alisol B and alisol B acetate) exhibited anti-complement activity.^{4,5)} The same plant material inhibited contact dermatitis and cotton pellet granuloma at a high dose of 20 g/kg.⁶⁾ In addition, the methanol extract showed anti-allergic actions against types I–IV hypersensitivity.⁷⁾ Several triterpenes and sesquiterpenes including alisol A, alisol B and alismol were found to be active against type III hypersensitivity reaction in rats with alisol B acetate being the most potent. Among the isolated compounds, alismol and several alisol derivatives such as alisol A and alisol B also inhibited NO production.^{3,8)} Recently, 13 β ,17 β -epoxy alisol A and alisol B acetate were found to have immunosuppressive properties on lymphocytes.⁹⁾ All these previous investigations have shown that *A. orientale* possesses anti-allergic activity and several constituents had inhibitory activity against type III hypersensitivity. However, these studies are far from complete. Anti-allergic activity of major constituents including alisol B, alisol B 23-acetate and alisol C 23-acetate against other types of hypersensitivity reaction have not been demonstrated and the effect of *A. orientale* on atopic dermatitis (AD) has not been investigated.

Thus, in the present study, the anti-allergic actions of these major compounds were examined. Since β -hexosaminidase is released along with histamine during an immediate-type allergic response (type I hypersensitivity),^{10,11)} inhibition of β -hexosaminidase release from the activated mast cells was

examined. Further, 5-lipoxygenase (5-LOX) inhibitory activity was measured because 5-LOX products, leukotrienes (LT), are deeply involved in several allergic disorders including asthma and AD.¹²⁾ In addition, the animal models of type IV hypersensitivity and experimental AD in mice were used to establish the anti-allergic effects of *A. orientale* and its major constituents as well as provide scientific rationale for its use in Chinese medicine.

MATERIALS AND METHODS

Chemicals A23187 was obtained from Biomol (Plymouth Meeting, PA, U.S.A.). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide, nordihydroguaiaretic acid (NDGA), carboxymethylcellulose (CMC), prednisolone, quercetin, dinitrophenol (DNP)-bovine serum albumin (BSA) and anti-DNP mouse immunoglobulin E (IgE) were purchased from Sigma Chem. (St. Louis, MO, U.S.A.). Picryl chloride (TNCB, 1-chloro-2,4,6-trinitrobenzene) was purchased from Nacalai Tesque (Japan). Dulbecco's modified Eagle's medium (DMEM) and other cell culture reagents including fetal bovine serum (FBS) were products of Gibco BRL (Grand Island, NY, U.S.A.). A protein assay kit was purchased from Bio-Rad (Hercules, CA, U.S.A.). Clobetasol-17-propionate ointment (0.05% w/w, Dermovate[®]) was obtained from GlaxoSmithKline Korea.

Animals Male ICR mice (6 weeks old, specific pathogen-free) and NC/Nga mice (6 weeks old, specific pathogen free) were obtained from Orient-Bio Co. (Korea) and from SLC (Japan), respectively. The animals were fed with standard laboratory chow and water freely available. They were maintained in the animal facility (KNU) at 20–22°C under 40–60% relative humidity and a 12h/12h (light/dark) cycle for at least 7 d prior to experimentation. The experimental design using the animals was approved by the local committee for animal experimentation, KNU (KIACUC-11-0007). The animals were

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handled according to the guidelines described in the KFDA Guide for the Care and Use of Laboratory Animals throughout the experiments.

Preparation of the Extract of *A. orientale* and Isolation of the Major Constituents The rhizomes of *Alisma orientale* were collected from Bosung, Chonnam, Korea, in 2007 and were authenticated by Prof. J. H. Lee at Dongkuk University. For the evaluation of pharmacological activity, the plant materials were extracted in water and 70% aqueous ethanol. By evaporation under vacuo, the water and ethanol extracts (AOE) were obtained, respectively. For the isolation of the active principles, the roots of *A. orientale* (15kg) were extracted with MeOH three times under reflux and 576 g of residue was produced. The MeOH extract was suspended in water and partitioned sequentially in *n*-hexane, CH_2Cl_2 , ethyl acetate (EtOAc), and *n*-BuOH. The CH_2Cl_2 fraction (10.5 g) was chromatographed over a silica gel column using a gradient solvent system of *n*-hexane–EtOAc (10:1→1:1) to give twelve subfractions, designated as D1–D12. Subfraction D6 (212.0 mg) was subjected to LiChroprep RP 18 column chromatography eluted with a gradient solvent system of MeOH– H_2O (1:1→1:0) to yield eighteen subfractions, D61–D618. Subfraction D617 (66.8 mg) was subjected to silica gel column chromatography eluted with a gradient solvent system of *n*-hexane–EtOAc (4:1→1:1) to give alisol B 23-acetate (49.9 mg) (Fig. 1). Subfraction D7 (138.2 mg) was subjected to LiChroprep RP 18 column chromatography eluted with a gradient solvent system of MeOH– H_2O (6:4→1:0) to yield twelve subfractions, D71–D712. Subfraction D79 (27.5 mg) was subjected to silica gel column chromatography eluted with a gradient solvent system of *n*-hexane–EtOAc (4:1→1:1) to give alisol C 23-acetate (10.8 mg). Subfraction D8 (565.5 mg) was subjected to LiChroprep RP 18 column chromatography eluted with a gradient solvent system of MeOH– H_2O (6:4→1:0) to yield seventeen subfractions, D81–D817. Subfraction D814 (226.1 mg) was subjected to silica gel column chromatography eluting with a gradient solvent system of *n*-hexane–EtOAc (3:1→1:1) to give alisol B (163.3 mg). The physico-chemical data including ^1H -NMR, ^{13}C -NMR, and heteronuclear single quantum coherence (HSQC) of these compounds were identical with those reported in the literature.^{2,13} In order to examine the content of alisol derivatives in 70% EtOH extract, we carried out additional HPLC chemical profile analysis of the 70% EtOH extract. The 70% EtOH extract of *A. orientale* dissolved in methanol was separated on a RP-C18 column (YMC-pak ODS-A column, 4.6×250 mm, 5 μm , YMC Co., Japan) by HPLC equipped with a Waters 600E solvent delivery system and a Waters 2487 UV detector (190–400 nm). The flow rate was 0.8 mL/min and the detection wavelength was set at 210 nm. The solvent gradient for HPLC was a mixture of 0.1% phosphoric acid (solvent A) in MeCN (solvent B): 0–50% B from 0 to 35 min, 50–100% B from 35 to 45 min. The contents of alisol C 23 acetate, alisol B, and alisol B 23 acetate were 1.62, 3.06, and 12.78% (w/w), respectively.

Alisol B: Amorphous powder; electron ionization (EI)-MS, m/z : 472 (M^+ , $\text{C}_{30}\text{H}_{48}\text{O}_4$: 472); IR (KBr) ν_{max} 3430, 1700 cm^{-1} ; ^1H -NMR (300 MHz, CDCl_3) δ : 3.87 (1H, ddd, $J=5.9$, 10.6, 10.6 Hz, H-11), 3.21 (1H, ddd, $J=2.0$, 8.1, 10.6 Hz, H-23), 2.90 (2H, m, Hb-22, 20), 2.79 (1H, dd, $J=5.9$, 13.3 Hz, H-12), 2.69 (1H, d, $J=8.1$ Hz, H-24), 1.73 (1H, d, $J=10.6$ Hz, H-9), 1.55 (1H, ddd, $J=3.7$, 10.6, 14.2 Hz, Ha-22), 1.05 (3H, d, $J=8.8$ Hz,

Alisol B, R=H

Alisol B 23-acetate, R=Ac

Alisol C 23-acetate

Fig. 1. Chemical Structures of Alisol B, Alisol B 23-Acetate and Alisol C 23-Acetate

1.32, 1.24, 1.12, 1.07, 1.06, 1.02, 0.99 (3H each, s); ^{13}C -NMR (75 MHz, CDCl_3) δ : 220.2 (C-3), 137.9 (C-13), 135.0 (C-17), 70.1 (C-11), 69.1 (C-23), 67.7 (C-24), 59.2 (C-25), 57.0 (C-14), 49.7 (C-9), 48.5 (C-5), 46.9 (C-4), 40.6 (C-8), 38.7 (C-22), 36.9 (C-10), 34.5 (C-7), 34.3 (C-12), 33.7 (C-2), 30.9 (C-1), 30.6 (C-15), 29.6 (C-28), 29.1 (C-16), 27.7 (C-20), 25.5 (C-19), 24.9 (C-27), 24.0 (C-30), 23.4 (C-18), 20.2 (C-21), 20.1 (C-6), 20.0 (C-29), 19.2 (C-26).

Alisol B 23-Acetate: Amorphous powder; EI-MS, m/z : 514 (M^+ , $\text{C}_{32}\text{H}_{50}\text{O}_5$: 514); IR (nujol) ν_{max} 3490, 1740, 1700 cm^{-1} ; ^1H -NMR (500 MHz, CDCl_3) δ : 4.61 (1H, ddd, $J=2.6$, 8.4, 10.6 Hz, H-23), 3.81 (1H, ddd, $J=5.5$, 10.6, 11.0 Hz, H-11), 2.73 (1H, d, $J=8.4$ Hz, H-24), 2.56 (1H, dd, $J=6.0$, 13.3 Hz, H_a-12), 2.07 (3H, s, OAc), 1.72 (1H, d, $J=11.0$ Hz, H-9), 1.06 (3H, d, $J=8.0$ Hz), 1.33, 1.31, 1.14, 1.04, 1.03, 1.01, 0.97 (3H each, s); ^{13}C -NMR (125 MHz, CDCl_3) δ : 220.1 (C-3), 170.0 (–OAc), 138.1 (C-13), 134.2 (C-17), 71.5 (C-23), 70.2 (C-11), 65.1 (C-24), 58.4 (C-25), 57.0 (C-14), 50.0 (C-9), 48.5 (C-5), 46.9 (C-4), 40.7 (C-8), 36.9 (C-10), 36.7 (C-2), 34.5 (C-12), 34.2 (C-7), 33.7 (C-22), 30.9 (C-1), 30.7 (C-15), 29.5 (C-28), 29.2 (C-16), 27.8 (C-20), 25.7 (C-19), 24.7 (C-27), 23.8 (C-30), 23.2 (C-18), 21.2 (–OAc), 20.1 (C-6), 20.1 (C-21), 20.0 (C-29), 19.4 (C-26).

Alisol C 23-Acetate: Amorphous powder; EI-MS, m/z : 528 (M^+ , $\text{C}_{32}\text{H}_{48}\text{O}_6$: 528); IR (KBr) ν_{max} 3470, 1742, 1700, 1680, 1640 cm^{-1} ; ^1H -NMR (500 MHz, CDCl_3) δ : 4.52 (1H, ddd, $J=2.2$, 8.4, 11.4 Hz, H-23), 4.00 (1H, ddd, $J=5.9$, 12.1, 12.1 Hz, H-11), 2.72 (1H, d, $J=8.4$ Hz, H-24), 2.69 (1H, ddd, $J=6.2$, 11.4, 14.5 Hz, H-2), 2.60 (1H, ddd, $J=4.0$, 7.0, 12.4 Hz, H-20), 2.42 (1H, d, $J=18.1$ Hz, Hb-15), 1.88 (1H, d, $J=12.1$ Hz, H-9), 1.84 (1H, d, $J=18.1$ Hz, Ha-15), 1.76 (1H, ddd, $J=4.0$, 11.4, 15.3 Hz, Ha-22), 2.07 (3H, s), 1.20 (3H, d, $J=7.0$ Hz), 1.33, 1.29, 1.23, 1.10, 1.08, 1.07, 0.89 (3H each, s); ^{13}C -NMR

(125 MHz, CDCl_3) δ : 219.4 (C-3), 208.0 (C-16), 176.8 (C-13), 170.1 (–OAc), 138.4 (C-17), 71.9 (C-23), 69.9 (C-11), 64.9 (C-24), 58.6 (C-25), 49.7 (C-14), 48.8 (C-9), 48.4 (C-5), 46.9 (C-4), 45.7 (C-15), 40.1 (C-8), 36.9 (C-10), 35.6 (C-12), 35.0 (C-22), 34.8 (C-7), 33.5 (C-2), 30.8 (C-1), 29.5 (C-28), 26.7 (C-20), 25.5 (C-19), 24.6 (C-27), 23.1 (C-18), 23.0 (C-30), 21.2 (–OAc), 20.0 (C-21), 19.9 (C-6), 19.7 (C-26), 19.3 (C-29).

Rat Basophilic Leukemia-1 (RBL-1) Cell Culture and Measurement of Leukotrienes (LT) To evaluate the 5-LOX inhibitory activity, RBL-1 cells purchased from the American Type Culture Collection (ATCC, Rockville, VA, U.S.A.) were cultured in RPMI 1640 with 10% fetal calf serum under 5% CO_2 at 37°C. The test compounds dissolved in dimethyl sulfoxide (DMSO) (0.1%, w/v) were added to the cells. The control group also received the same amount of DMSO. The cells were then pre-incubated with the test compounds for 10 min. A23187 (3 μM) was added to activate 5-LOX and the cells were incubated for 15 min as previously described, with slight modification.¹⁴ The media was then collected and the concentration of the 5-LOX products, cysteinyl-leukotrienes ($\text{LTC}_4/\text{D}_4/\text{E}_4$), were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chem.) as recommended by the manufacturer. Cell viability was assessed using MTT assay as previously described.¹⁵

RBL-2H3 Cell Culture and Antigen-Induced Degranulation of β -Hexosaminidase RBL-2H3 cells (ATCC) were cultured in DMEM with 10% fetal calf serum. According to the previously described procedure,¹⁶ anti-DNP mouse IgE was added for sensitization. Twenty-four hours later, the cells were washed, test compounds dissolved in DMSO were added and the cells were incubated for 30 min. DNP-BSA (1 $\mu\text{g}/\text{mL}$) was then added for degranulation and after a 10 min incubation, the reaction was stopped by cooling in an ice bath for 10 min. After centrifugation, the supernatant was transferred into 96-well plates. Substrate (1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide) was added and incubated for 1 h at 37°C. The reaction was stopped by adding 0.1 M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ (200 $\mu\text{L}/\text{well}$) and the absorbance was measured at 405 nm.

Delayed-Type Hypersensitivity (DTH) To assess inhibitory activity against delayed-type hypersensitivity (type IV hypersensitivity), 7% picryl chloride in acetone (100 $\mu\text{L}/\text{mouse}$) was smeared onto the shaved abdomen of ICR mice for the elicitation phase groups. For the animals in the control group and the induction phase group, acetone (100 μL) was applied instead of picryl chloride. One week later, the elicitation phase of delayed hypersensitivity (P-P) was induced by application of 1% picryl chloride in acetone (20 $\mu\text{L}/\text{ear}$) to the right ears of the sensitized mice. To elicit the induction phase reaction (A-P), 1% picryl chloride (20 $\mu\text{L}/\text{ear}$) was applied to the right ears of the acetone-treated mice. For the control group (A-A, control), only acetone (20 μL) was applied to the right ears of the acetone-treated mice. After 24 h, ear thickness was measured using a dial thickness gauge (Mitutoyo, Japan). Test compounds dissolved in 0.4% carboxymethylcellulose (CMC) were orally administered 1 h after initial treatment of sensitizer or acetone and 1 h after final picryl chloride or acetone treatment.

Atopic Dermatitis (AD) in NC/Nga Mice According to the previously described procedures,^{17,18} 200 μL of 1% picryl chloride (TNCB) solution in acetone–olive oil (3:1) were topically applied to the hair-removed backs of NC/Nga mice.

Three days later, a picryl chloride solution was applied again. One week after the initial treatment, a 0.4% picryl chloride solution (150 μL) was applied three times per week for 4 weeks to induce dermatitis. According to the slightly modified criteria¹⁹ of a previously described procedure,^{20,21} skin severity was scored based on clinical symptoms on the last day which included dry skin and lichenification, edema and excoriation, and erythema. Scratching behavior was measured by observing each mouse for 20 min. Mice were sacrificed and blood was withdrawn; the IgE concentration in serum was measured using an ELISA kit (Shibaya Co., Japan). For histological examination, skin was removed and stored in formalin solution. AOE dissolved in 0.4% CMC was administered orally at 200 mg/kg/d, 5 d/week for 5 weeks during the experiment. Clobetasol-17-propionate ointment (30 mg/mouse/d, 5 d/week) was applied topically as a reference drug.

Statistical Analysis All data were represented as arithmetic mean $\pm$ S.D. One-way analysis of variance (ANOVA), followed by Dunnett's test was used to determine the statistical significance.

RESULTS

When A23187 (ionophore) was treated, RBL-1 cells produced large amounts of cysteinyl-LTs (1,574.6 $\pm$ 138.4 pg/mL) by activated 5-LOX for 15 min. The basal level of the cysteinyl-LTs was 39.3 $\pm$ 1.5 pg/mL ($n=3$). Under these conditions, the 70% ethanol extract of *A. orientale* (AOE) reduced LT production by 2.4–89.7% at 50–200 $\mu\text{g}/\text{mL}$ while the water extract did not have an effect (Fig. 2A). These results led to the successful isolation of the major three triterpenoids (alisol B, alisol B 23-acetate, alisol C 23-acetate) from *A. orientale*. Among the isolated compounds, alisol B showed cytotoxicity on RBL-1 cells at $\geq 50 \mu\text{M}$ measured by the MTT assay in the initial experiment (data not shown). When three major terpene derivatives were treated at non-cytotoxic concentrations, alisol B and its 23-acetate (1–10 μM) showed strong inhibitory effects on 5-LOX-catalyzed LT production, while alisol C 23-acetate only showed weak inhibition (16.5% inhibition at 50 μM ; Fig. 2B). The IC_{50} values of alisol B and its 23-acetate were 3.5 and 3.7 μM , respectively. NDGA (1 μM) used as a reference compound strongly inhibited LT production (99.2%) as expected.

When antigen was treated, β -hexosaminidase was degranulated in the media from RBL-2H3 cells after a 10 min incubation period (from 0.0 $\pm$ 4.4 to 100.0 $\pm$ 29.1% release, $n=3$). Under these conditions, AOE strongly inhibited the degranulation reaction between 50–200 $\mu\text{g}/\text{mL}$ while the water extract only showed a 9.1% inhibition at 200 $\mu\text{g}/\text{mL}$ (Fig. 2C). These results are similar to previous findings⁷ showing that the methanol extract of *A. orientale* was active in an *in vivo* type I allergy model (immediate-type), but the water extract was not. Since MTT assay revealed that alisol B and its acetate showed cytotoxicity on RBL-2H3 cells at $\geq 50 \mu\text{M}$ (data not shown), the concentrations of these two compounds tested were limited to 25 μM . Alisol B and alisol B 23-acetate significantly and strongly inhibited the degranulation reaction, whereas alisol C 23-acetate did not show significant inhibition between 2–50 μM (Fig. 2D). The IC_{50} values of alisol B and alisol B 23-acetate were 3.9 and 3.3 μM , respectively. The reference compound, quercetin (degranulation inhibitor, 20 μM),

Fig. 2. Inhibition of LT Production and β -Hexosaminidase Release by *A. orientale* and the Constituents

(A) Effects of the water and ethanol extracts on 5-LOX-catalyzed LT production in A-23187-treated RBL-1 cells. (B) Effects of the constituents on 5-LOX-catalyzed LT production in A-23187-treated RBL-1 cells. (C) Effects of the water and ethanol extracts on β -hexosaminidase release in antigen-treated RBL-2H3 cells. (D) Effects of the constituents on β -hexosaminidase release in antigen-treated RBL-2H3 cells. Water extract (○), ethanol extract (●), alisol B (Δ), alisol B 23-acetate (▲), alisol C 23-acetate (◇). Data and bars represent the arithmetic mean $\pm$ S.D. ($n=3$). * $p<0.05$, ** $p<0.01$, significantly different from the control group.

Fig. 3. Inhibition of DTH Response by the Constituents from *A. orientale*

All data and bars represent the arithmetic mean $\pm$ S.D. ($n=6$). The ear thickness increased in picryl chloride (PiCl)-treated group was 0.160 ± 0.026 mm. Prednisolone (Pred), * $p<0.05$, ** $p<0.01$, significantly different from the picryl chloride-treated control group (P-P).

inhibited β -hexosaminidase release (88.9%) as expected. These results indicate that alisol B and alisol B 23-acetate may contribute to the anti-allergic activity of *A. orientale* against immediate-type hypersensitivity.

Table 1. Inhibition of DTH Response in Mice by the 70% Ethanol Extract of *A. orientale* (AOE)

Compounds	Dose (mg/kg/treatment $\times 2$)	Ear thickness (mm)	% inhibition
Control (A-A)	—	0.270 ± 0.015	
Control (A-P)	—	0.295 ± 0.0	
AOE (A-P)	400	0.295 ± 0.0	
Picryl chloride-treated (P-P)	—	0.368 ± 0.040	
Prednisolone (P-P)	20	$0.307 \pm 0.019^*$	84.1
	25	0.372 ± 0.021	— ^{a)}
AOE (P-P)	100	0.360 ± 0.015	11.4
	400	$0.325 \pm 0.021^*$	59.1

All data are the arithmetic means $\pm$ S.D. ($n=6$). ^{a)} Not active, * $p<0.05$, significantly different from the picryl chloride-treated group.

The *in vivo* anti-allergic activity against delayed-type hypersensitivity (DTH) was examined in ICR mice. Picryl chloride treatment to the sensitized mice strongly increased the ear thickness after 24 h in a classical delayed-type response (Table 1). Under these conditions, AOE (25–400 mg/kg) inhibited the DTH response after oral administration. In particular, at 400 mg/kg, AOE significantly inhibited the DTH response (59.1%). Prednisolone as a reference drug also showed potent inhibition. When the effects of major constituents were examined, all three triterpene derivatives showed significant inhibitory action after oral administration at doses of 10 and 50 mg/

Fig. 4. Inhibition of Experimental AD in NC/Nga Mice by AOE

(A) Skin severity score: three categories were checked on the skin of NC/Nga mice (dry skin and lichenification, edema and excoriation, erythema), and each category was scored based on the severity as follows: no symptom (0), weak (1), medium (2), severe (3). The scores were combined in each animal. (B) Scratching behavior: Numbers of scratching were counted for 20 min period. (C) Serum IgE concentration. Data and bars are represented as the arithmetic mean $\pm$ S.D. ($n=5$). (D) Histology of the skin lesions of NC/Nga mice. Note: many inflammatory cells were infiltrated in the dermal area, but epidermal and dermal hyperplasia were not seen (control), while massive epidermal and dermal hyperplasia along with many inflammatory cells infiltrated in the dermal area was observed (TNCB-treated control). Oral administration of AOE (200 mg/kg/d) moderately reduced dermal hyperplasia and inflammatory cell infiltration. Strong reduction of these two parameters was seen in the clobetasol 17-propionate-treated group. Magnification: $\times 40$ [†] $p < 0.1$, ^{**} $p < 0.01$, significantly different from the TNCB-treated control group.

kg (Fig. 3). At 50 mg/kg, alisol B, alisol B 23-acetate and alisol C 23-acetate showed 44.3%, 62.0% and 69.6% inhibition of the DTH reaction, respectively.

Finally, the effect of AOE on an animal model of atopic dermatitis was examined. NC/Nga mice developed human AD-like symptoms in response to application of the hapten such as picryl chloride or dinitrochlorobenzene (DNCB). A repetitive application of picryl chloride for 5 weeks dramatically increased severity score on the affected skin area (Fig. 4A) and also augmented scratching behavior (Fig. 4B). Picryl chloride treatment increased serum IgE concentration to $19.9 \pm 4.2 \mu\text{g/mL}$ from a basal level of $0.2 \pm 0.3 \mu\text{g/mL}$ (Fig. 4C). Histology of the affected skin area showed epidermal and dermal hyperplasia as well as recruitment of inflammatory cells in the skin (Fig. 4D). Under these conditions, it was for the first time found that AOE (200 mg/kg/d, oral) alleviated severity scores and scratching behavior. Dermatitis symptoms assessed by severity score decreased by 37.5% and scratching frequency was reduced by 32.1%. However, AOE treatment

did not reduce IgE concentration in the serum. Histological examination indicated that AOE treatment moderately reduced dermal hyperplasia and inflammatory cell infiltration. Topical steroid treatment (clobetasol-17-propionate) as a reference drug strongly reduced severity score (62.5%) as well as scratching behavior (83.5%) as expected. Steroid treatment also reduced IgE concentration (92.8% reduction).

DISCUSSION

The present investigation has clearly demonstrated that AOE inhibits LT production and β -hexosaminidase release from mast cell lines as well as DTH response *in vivo* and AD-like symptoms. The major constituents, alisols, also showed the inhibitory action on immediate-type and delayed-type allergic responses.

Among the isolated compounds, alisol B and its 23-acetate were active in *in vitro* assays (LT production and β -hexosaminidase release), while alisol C 23-acetate was not,

suggesting that the C-16 keto group in alisol C 23-acetate may provide unfavorable effect on these parameters. On the other hand, all the alisol derivatives were active on the DTH response in mice after oral administration. These differences may be due to the differences of the intrinsic activity of the compounds because immediate-type hypersensitivity is mainly mediated by mast cells and basophils, while the delayed-type response is mainly mediated by macrophages, lymphocytes and cytokines released from them. Or it could be speculated that alisol C 23-acetate may be quickly metabolized to active compound(s) *in vivo* by oral administration. But this cannot be confirmed because bioavailability/metabolism data of the alisols is not available to date.

It is well known that histamine release (and β -hexosaminidase release) is related with scratching frequency and LTs produced by 5-LOX are deeply involved in AD etiology.¹²⁾ Alisol B and its 23-acetate were shown to inhibit these parameters. These inhibitory actions of alisols might reduce AD-like symptoms. Thus, it is suggested that the some effectiveness of AOE on AD may be caused by its constituents, alisol B and alisol B 23-acetate, at least in part.

Alisol derivatives were previously found to inhibit type III hypersensitivity reaction *in vivo*.⁷⁾ The present investigation has clearly shown that alisol B and its 23-acetate inhibited immediate-type allergic response as well as delayed-type hypersensitivity, and alisol C 23-acetate inhibited the DTH reaction. We also found that alisol B and its acetate were cytotoxic on mast cell lines at high concentrations in the present study. These results are well correlated with previous reports demonstrating that alisol derivatives, including alisol B and alisol B acetate, were cytotoxic on cancer cell lines.^{22,23)} The cytotoxic properties of these compounds may be mediated *via* the apoptotic process as previously described.^{24–26)} It is significant to mention that alisol B was recently found to inhibit osteoclast formation, suggesting its preventive effect on bone loss.²⁷⁾ All these studies suggest that certain alisol derivatives may be potentially useful agents for treating allergic disorders, cancer and protection of bone loss.

In conclusion, the results of the present study clearly demonstrate, for the first time, that AOE moderately inhibits AD-like symptoms and the constituents, including alisol B and alisol B 23-acetate inhibit immediate-type as well as delayed-type hypersensitivity reactions. In addition, alisol C 23-acetate inhibits delayed-type hypersensitivity. These anti-allergic activities of the constituents may contribute to the action of *A. orientale*, at least in part. Furthermore, our results provide a scientific basis for the anti-allergic use of *Alismatis* rhizomes in traditional medicine.

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REFERENCES

- 1) Bae K. *The medicinal plants of Korea*. (Bae K ed.) Kyo-Hak Pub., Seoul, p. 519 (2000).
- 2) Nakajima Y, Satoh Y, Katsumata M, Tsujiyama K, Ida Y, Shoji J. Terpenoids of *Alisma orientale* rhizome and the crude drug *alismatis rhizoma*. *Phytochemistry*, **36**, 119–127 (1994).
- 3) Matsuda H, Kageura T, Toguchida I, Murakami T, Kishi A, Yoshikawa M. Effects of sesquiterpenes and triterpenes from the rhizome of *Alisma orientale* on nitric oxide production in lipopolysaccharide-activated macrophages: absolute stereostructures of alismaketones-B 23-acetate and -C 23-acetate. *Bioorg. Med. Chem. Lett.*, **9**, 3081–3086 (1999).
- 4) Matsuda H, Tomohiro N, Yoshikawa M, Kubo M. Studies on *Alismatis* Rhizoma. II. Anti-complementary activities of methanol extract and terpene components from *Alismatis* Rhizoma (dried rhizome of *Alisma orientale*). *Biol. Pharm. Bull.*, **21**, 1317–1321 (1998).
- 5) Lee SM, Kim JH, Zhang Y, An RB, Min BS, Joung H, Lee HK. Anti-complementary activity of protostane-type triterpenes from *Alismatis* rhizoma. *Arch. Pharm. Res.*, **26**, 463–465 (2003).
- 6) Dai Y, Hang B, Huang Z, Li P. Anti-inflammatory activities and effect of rhizoma *Alismatis* on immune system. *Zhongguo Zhong Yao Za Zhi*, **16**, 622–625 (1991), abstract.
- 7) Kubo M, Matsuda H, Tomohiro N, Yoshikawa M. Studies on *Alismatis* rhizoma. I. Anti-allergic effects of methanol extract and six terpene components from *Alismatis* rhizoma (dried rhizome of *Alisma orientale*). *Biol. Pharm. Bull.*, **20**, 511–516 (1997).
- 8) Kim NY, Kang TH, Pae HO, Choi BM, Chung HT, Myung SW, Song YS, Sohn DH, Kim YC. *In vitro* inducible nitric oxide synthesis inhibitors from *Alismatis* Rhizoma. *Biol. Pharm. Bull.*, **22**, 1147–1149 (1999).
- 9) Zhang C, Zhou A, Zhang M. Chemical constituents of *Alisma orientale* and their immunosuppressive function. *Zhongguo Zhong Yao Za Zhi*, **34**, 994–998 (2009), abstract.
- 10) Schwartz LB, Lewis RA, Seldin D, Austen KF. Acid hydrolases and tryptase from secretory granules of dispersed human lung mast cells. *J. Immunol.*, **126**, 1290–1294 (1981).
- 11) Marquardt DL, Wasserman SI. Modulation of rat serosal mast cell biochemistry by *in vivo* dexamethasone administration. *J. Immunol.*, **131**, 934–939 (1983).
- 12) Rubin P, Mollison KW. Pharmacotherapy of diseases mediated by 5-lipoxygenase pathway eicosanoids. *Prostaglandins Other Lipid Mediat.*, **83**, 188–197 (2007).
- 13) Zhao M, Xu LJ, Che CT. Alisolid, alisols O and P from the rhizome of *Alisma orientale*. *Phytochemistry*, **69**, 527–532 (2008).
- 14) Tries S, Neupert W, Laufer S. The mechanism of action of the new antiinflammatory compound ML3000: inhibition of 5-LOX and COX-1/2. *Inflamm. Res.*, **51**, 135–143 (2002).
- 15) Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65**, 55–63 (1983).
- 16) Choi OH, Kim JH, Kinet JP. Calcium mobilization *via* sphingosine kinase in signalling by the Fc epsilon RI antigen receptor. *Nature*, **380**, 634–636 (1996).
- 17) Matsuda H, Watanabe N, Geba GP, Sperl J, Tsudzuki M, Hiroi J, Matsumoto M, Ushio H, Saito S, Askenase PW, Ra C. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int. Immunol.*, **9**, 461–466 (1997).
- 18) Suto H, Matsuda H, Mitsuishi K, Hira K, Uchida T, Unno T, Ogawa H, Ra C. NC/Nga mice: a mouse model for atopic dermatitis. *Int. Arch. Allergy Immunol.*, **120** (Suppl. 1), 70–75 (1999).
- 19) Jin JH, Ngoc TM, Bae K, Kim YS, Kim HP. Inhibition of experimental atopic dermatitis by rhubarb (rhizomes of *Rheum tanguticum*) and 5-lipoxygenase inhibition of its major constituent, emodin. *Phytother. Res.*, **25**, 755–759 (2011).
- 20) Leung DY, Hirsch RL, Schneider L, Moody C, Takaoka R, Li SH, Meyerson LA, Mariam SG, Goldstein G, Hanifin JM. Thymopentin therapy reduces the clinical severity of atopic dermatitis. *J. Allergy Clin. Immunol.*, **85**, 927–933 (1990).
- 21) Suzuki R, Shimizu T, Kudo T, Ohtsuka Y, Yamashiro Y, Oshida K. Effects of n-3 polyunsaturated fatty acids on dermatitis in NC/

- Nga mice. *Prostaglandins Leukot. Essent. Fatty Acids*, **66**, 435–440 (2002).
- 22) Lee S, Kho Y, Min B, Kim J, Na M, Kang S, Maeng H, Bae K. Cytotoxic triterpenoids from *Alismatis Rhizoma*. *Arch. Pharm. Res.*, **24**, 524–526 (2001).
- 23) Lee S, Min B, Bae K. Chemical modification of alisol B 23-acetate and their cytotoxic activity. *Arch. Pharm. Res.*, **25**, 608–612 (2002).
- 24) Huang YT, Huang DM, Chueh SC, Teng CM, Guh JH. Alisol B acetate, a triterpene from *Alismatis rhizoma*, induces Bax nuclear translocation and apoptosis in human hormone-resistant prostate cancer PC-3 cells. *Cancer Lett.*, **231**, 270–278 (2006).
- 25) Law BY, Wang M, Ma DL, Al-Mousa F, Michelangeli F, Cheng SH, Ng MH, To KF, Mok AY, Ko RY, Lam SK, Chen F, Che CM, Chiu P, Ko BC. Alisol B, a novel inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase pump, induces autophagy, endoplasmic reticulum stress, and apoptosis. *Mol. Cancer Ther.*, **9**, 718–730 (2010).
- 26) Xu YH, Zhao LJ, Li Y. Alisol B acetate induces apoptosis of SGC7901 cells via mitochondrial and phosphatidylinositol 3-kinases/Akt signaling pathways. *World J. Gastroenterol.*, **15**, 2870–2877 (2009).
- 27) Lee JW, Kobayashi Y, Nakamichi Y, Udagawa N, Takahashi N, Im NK, Seo HJ, Jeon WB, Yonezawa T, Cha BY, Woo JT. Alisol-B, a novel phyto-steroid, suppresses the RANKL-induced osteoclast formation and prevents bone loss in mice. *Biochem. Pharmacol.*, **80**, 352–361 (2010).