

RESEARCH ARTICLE

Inhibitory effects of steroidal timosaponins isolated from the rhizomes of *Anemarrhena asphodeloides* against passive cutaneous anaphylaxis reaction and pruritus

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Abstract

To investigate the antiallergic effect of the rhizome of *Anemarrhena asphodeloides* (AA, family Liliaceae), which was found to inhibit the mouse passive cutaneous anaphylaxis (PCA) reaction induced by the antigen-immunoglobulin E (IgE) complex in preliminary experiments, main steroidal saponins, timosaponins AIII, BIII, and D, were isolated and their inhibitory effects against PCA reaction and scratching behaviors investigated in mice. Oral administration of three main steroidal sapogenins blocked the PCA reaction and scratching behaviors, timosaponin AIII was the most potent. However, intraperitoneal administration of timosaponin AIII showed weak inhibition. To understand its metabolism and antiallergic mechanism, timosaponin AIII was anaerobically incubated with human intestinal microflora to afford a main metabolite, sarsasapogenin. Intraperitoneal administration of sarsasapogenin inhibited allergic reaction more potently than timosaponin AIII. In addition, sarsasapogenin more potently inhibited degranulation and IL-4 protein expression of RBL-2H3 cells induced by IgE-antigen complex than timosaponin AIII. On the basis of these findings, antiallergic effect of AA may be due to those of its steroidal constituents, and that of timosaponin AIII may be activated by using intestinal microflora.

Keywords: *Anemarrhena asphodeloides*; passive cutaneous anaphylaxis reaction; sarsasapogenin; scratching behavior; timosaponin AIII

Abbreviations: AA, *Anemarrhena asphodeloides*; ANOVA, analysis of variance; DMEM, Dulbecco's modified Eagles medium; DMSO, dimethylsulfoxide; DNP-HSA, dinitrophenol-human serum albumin; ICR, institute of cancer research; PCA, passive cutaneous anaphylaxis; RBL, rat basophilic lymphoma.

Introduction

Allergies including anaphylaxis are an abnormal reaction to a substance, which can be represented by using food, pollen, dust mites, cosmetics, mold spores, and animal hair. Anaphylaxis causes acute systemic and severe type I hypersensitivity allergic diseases, such as asthma, allergic rhinitis, and atopic dermatitis afflict up to 10% of the population in most countries.⁽¹⁾ Allergy pathogenesis including anaphylaxis is based in part on immunoglobulin

E (IgE)-mediated stimulation of mast cell and basophils.⁽²⁾ Mast cells and basophils are well-known as critical participants in various biologic processes of allergic diseases.⁽³⁾ These cells express surface membrane receptors with high affinity and specificity for IgE.⁽⁴⁾ The interaction of antigen-bound IgE in surface membrane receptors releases histamine, prostaglandins, leukotrienes, and cytokines, which activate chemotaxis and phagocytosis of neutrophils and macrophages.^(5,6) Finally cytokine-induced reactions cause tissue inflammation.

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Antihistamines, steroids, and immunosuppressants can help treat allergic diseases,^(8,9) but the effectiveness of herbal medicines is receiving increasing attention.^(10,11)

The rhizome of *Anemarrhena asphodeloides* (AA, family Liliaceae) is an antipyretic, antiphlogistic, sedative, and diuretic agent in traditional Chinese medicine. It contains saponins, which improve learning and memory in rats,^(12,13) and inhibit platelet aggregation factor-induced platelet aggregation,⁽¹⁴⁾ as well as xanthenes, and phenols, which inhibit cyclic AMP phosphodiesterase.⁽¹⁵⁾ However, the antiallergic effect of these components remains to be thoroughly studied.

During a herbal medicine screening program for compounds that improved allergic reactions, such as anaphylaxis, AA was found to inhibit antigen-IgE complex-induced passive cutaneous anaphylaxis (PCA) reaction in mice. Therefore, we isolated main constituents, steroidal saponins, and examined their inhibitory effects against *in vitro* and *in vivo* allergic reactions, and investigated their structure-activity relationship.

Materials and methods

Reagents

Fetal bovine serum, dinitrophenol-human serum albumin (DNP-HSA), Dulbecco's modified Eagle's medium (DMEM), *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide, cremophor EL, compound 48/80 and histamine were purchased from Sigma Co. (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Pierce Biotechnology, Inc., Rockford, IL, USA.

Isolation of constituents from the rhizome of AA

The rhizomes of AA were purchased from KyungDong Market, Seoul, Korea, and were taxonomically identified by Prof. Nam-Jae Kim. A voucher specimen (KHUP061102) was deposited at the Herbarium of Kyung Hee University, Seoul, Korea. The pulverized rhizomes of AA (1 kg) were extracted three times with 70% EtOH under heat. Crude

70% EtOH extracts were combined and evaporated to dryness under reduced pressure, which yielded 84.69 g. This extract was partitioned with BuOH and H₂O. The BuOH fraction (50 g) was then separated over a silica gel and eluted with a gradient of MeOH in CH₂Cl₂. A total of six fractions (FB1–FB6) were collected on the basis of thin layer chromatography behavior. The fraction FB2 was then separated over another silica gel with a gradient of CH₂Cl₂/MeOH/H₂O (7:1:0.5, 7:2:0.5, and 7:3:1) to afford compound **1**⁽¹⁶⁾ (112 mg). The fraction FB3 was further purified with chromatography on a silica gel column eluted with CH₂Cl₂/MeOH/H₂O 7:3:1 to afford four subfractions (FB5-1–FB5-4). Fraction FB5-3 was further subjected to semipreparative high performance liquid chromatography (HPLC) (35% CH₃CN in 50% MeOH at a flow rate of 7 mL/min more than 60 min, GS-320 column, 30 × 500 mm, Japan Analytical Instrument) to afford compound **2**⁽¹⁶⁾ (222 mg). Compound **3**⁽¹⁷⁾ (13.5 mg) was purified by using HPLC (C30-UG-5, 28 × 250 mm, Develosil, Nomura Chemical Co.) by using 35% acetonitrile in water at a flow rate of 7 mL/min more than 40 min. The structures of all compounds (Figure 1) were elucidated by using chemical and spectroscopic means. The spectral data of each compound was compared with published data.

Timosaponin AIII (1) White amorphous powder: FABMS: *m/z* 763.5 [M+Na]⁺.

Timosaponin BIII (2) White amorphous powder: FABMS: *m/z* 926.2 [M][−].

Timosaponin D (3) White amorphous powder: FABMS: *m/z* 941.7 [M+Na]⁺.

Metabolism of timosaponin AIII

Timosaponin AIII (2, 200 mg) was added to an intestinal bacterial mixture (300 mL) and anaerobically incubated for 48 h at 37°C in an anaerobic box. The mixture was acidified with 3 M HCl and extracted with EtOAc (300 mL × 3). The organic layer was washed with H₂O and evaporated in vacuo. The residue was separated over silica gel and eluted successively with hexane, CH₂Cl₂, CH₂Cl₂/MeOH/H₂O (7:1:0.5) to afford compound **14**⁽¹⁸⁾ (47 mg).

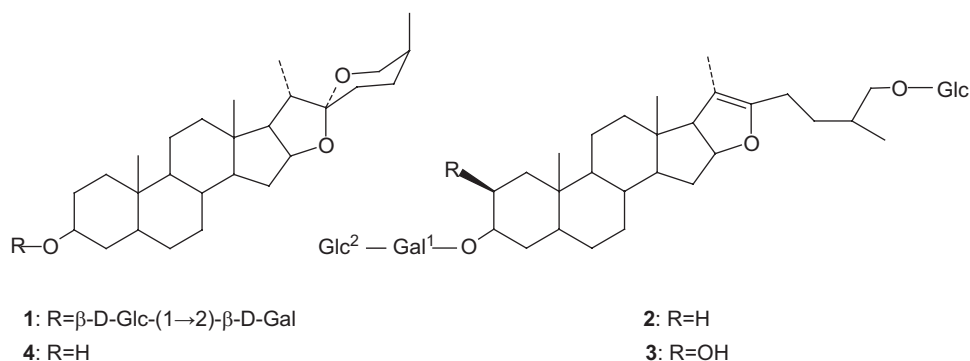


Figure 1. Chemical structures of timosaponins isolated from *A. asphodeloides*.

Sarsasapogenin (4) White amorphous powder: EIMS m/z 417 $[M+H]^+$.

Animals

The male institute of cancer research (ICR) mice (20–25 g) and male Sprague–Dawley rats were supplied by the Orient Experimental Animal Breeding Center (Seoul, Korea). All animals were housed in wire cages at 20–22°C and 50% ± 10% humidity, fed standard laboratory chow (Orient Experimental Animal Breeding Center, Seoul, Korea), and allowed water *ad libitum*. All procedures relating to the animals and their care conformed to the international guidelines in 'Principles of Laboratory Animals Care' (NIH publication no. 85–23, revised 1985).

Measurement of the PCA reaction

An IgE-dependent cutaneous reaction was measured according to the method of Choo et al.⁽¹⁹⁾ Male ICR mice were intradermally injected with 10 µg of anti-DNP IgE into two dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours later, each mouse received an injection of 200 µL of 3% Evans blue in phosphate-buffered saline, containing 200 µg of DNP-HSA, via the tail vein. The test agents were orally or intraperitoneally administered 1 h prior to the DNP-HSA injection. Thirty minutes after the DNP-HSA injection, the mice were killed, their dorsal skins removed and the pigmented area measured. After extraction with 1 mL 1.0 M KOH and 4 mL of a mixture of acetone and 0.2 M phosphoric acid (13:5), the amount of dye was determined colorimetrically at 620 nm.

Measurement of scratching frequency

Male BALB/c mice were placed in acrylic cages (22 × 22 × 24 cm) for about 10 min to become acclimatized. The behavioral experiments were performed according to the method of Sugimoto et al.⁽²⁰⁾ The rostral part of the skin on the back of the mice was clipped, and 50 µg/50 µL of compound 48/80 (dissolved in saline) intradermally injected into each mouse. Control mice received a saline injection instead of compound 48/80. Immediately after the intradermal injection, the mice (one animal/cage) were put back into the same cage and the scratching behaviors recorded by using an 8-mm video camera (SV-K80, Samsung, Seoul, Korea) under unmanned conditions. Scratching of the injected site with the hind paws was counted and compared with that of other sites, such as the ears. Each mouse was used for only one experiment. The mice generally showed several scratches per second, and a series of these behaviors was counted as one incident of scratching more than a 60 min period.

The test agents were orally administered 1 h before the scratching agent.

Assay of RBL-2H3 cell degranulation stimulated with IgE-antigen complex

We examined the ability of compounds to inhibit the release of β-hexosaminidase from rat basophilic lymphoma (RBL)-2H3 cells reported earlier.⁽¹⁹⁾ Briefly, RBL-2H3 cells were dispensed into 24-well plates at 2×10^5 cells/well by using DMEM containing 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 µg/mL), and 0.5 µg/mL of mouse monoclonal IgE, and these were incubated overnight at 37°C in 5% CO₂ for sensitization of the cells. Then the cells were washed twice with 500 µL of Siraganian buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂, 25 mM PIPES, and 40 mM NaOH), and incubated in 160 µL of Siraganian buffer (5.6 mM glucose, 1 mM CaCl₂, and 0.1% BSA were added) for an additional 10 min at 37°C. Aliquots (40 µL) of test sample solution were added to each well and incubated for 10 min, followed by the addition of 20 µL of antigen (DNP-HSA, final concentration 1 µg/mL) at 37°C for 10 min to stimulate the cells to evoke allergic reactions (degranulation). The reaction was stopped by cooling in an ice bath for 10 min. The reaction mixture was centrifuged at 3000 rpm for 2 min, and 25 µL aliquots of the supernatant were transferred to 96-well plates and incubated with 25 µL of substrate (1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37°C for 1 h. The reaction was stopped by adding 200 µL of stop solution (0.1 M Na₂CO₃/NaHCO₃, pH 10.0). The absorbance was measured by using a microplate reader at 405 nm. The test sample was dissolved in dimethylsulfoxide (DMSO), and the solution was added to Siraganian buffer (final DMSO concentration 0.1%). The percent inhibition of the release of β-hexosaminidase by the test material was calculated.

Determination of IL-4 and TNF-α by ELISA

RBL-2H3 cells (5×10^5 cells), previously cultured in DMEM, were treated with 0.5 µg/mL of mouse monoclonal IgE to sensitize the cells. The cells (1.8 mL) were exposed to 0.2 mL of the test agents (dissolved in 0.5% dimethyl sulfoxide) for 4 h, followed by treatment with 0.2 mL DNP-HSA (1 µg/mL) for 40 min at 37°C. The supernatant (50 µL) was transferred to a 96-well plate, and the IL-4 and TNF-α concentrations then determined by using commercial ELISA kits.⁽²¹⁾

Statistical analysis

All data are expressed as the mean ± SEM, and analyzed by using analysis of variance, with Turkey's post-hoc test. Significance was accepted at the 0.05 level of probability.

Results

In the preliminary experiment, AA extract (70% ethanol) at 50 and 100 mg/kg inhibited the PCA reaction by using 31% and 51%, respectively. Therefore, we isolated main steroidal constituents from AA and measured their inhibitory effect against the PCA reaction (Table 1). The PCA reaction was induced by the injection of IgE and antigen, with isolated compounds administered orally or intraperitoneally 1 h prior to the challenge. Timosaponin AIII was the most effective (39.1% and 55.3% at 20 and 50 mg/kg, respectively), followed by timosaponin BIII (33.8% and 50.3%, 20 and 50 mg/kg, respectively), with timosaponin D showing weak inhibition. Interestingly, administration of compounds at 5 and 20 mg/kg, intraperitoneally, only weakly inhibited the PCA reaction.

We also measured scratching behavior induced by compound 48/80 (Table 2). Compound 48/80 treatment significantly increased scratching behavior like the previous report.⁽¹⁹⁾ The oral administration of compounds at 20 and 50 mg/kg potently and dose-dependently decreased scratching frequency. Timosaponin AIII was the most effective, inhibiting scratching by 33.0% and 47.9% at 20 and 50 mg/kg, respectively, followed by timosaponin BIII and timosaponin D. Intraperitoneal administration at 5 and 20 mg/kg only weakly decreased scratching frequency.

To understand the differences in activity based on the route of administration, we anaerobically incubated timosaponin AIII with human fecal microflora and isolated a main metabolite, sarsasapogenin, and measured their inhibitory effects against PCA reaction and scratching behavior. Oral and intraperitoneal administration

of sarsasapogenin, the aglycone of timosaponin AIII, inhibited the PCA reaction (Figure 2) and scratching behavior (Figure 3), with intraperitoneally administered sarsasapogenin more than orally administered

Table 2. Inhibitory effect of steroidal components isolated from *A. asphodeloides* on scratching behavior induced by compound 48/80 in mice.

Compound	Dose (mg/kg)	Inhibition ^a (%)	
		p.o.	i.p.
Timosaponin AIII (1)	5	— ^b	16.8 ± 7.4 ^c
	20	33.0 ± 7.4 ^d	18.7 ± 7.5 ^c
	50	47.9 ± 7.2 ^e	—
Timosaponin BIII (2)	5	—	11.5 ± 5.2 ^c
	20	27.9 ± 11.2 ^{c,d}	12.7 ± 5.4 ^c
	50	45.1 ± 10.8 ^{d,e}	—
Timosaponin D (3)	5	—	13.5 ± 3.9 ^c
	20	19.3 ± 4.2 ^c	17.5 ± 9.6 ^c
	50	26.9 ± 6.2 ^{c,d}	—
Azelastine	10	62.5 ± 8.0 ^f	72.5 ± 5.5 ^d

Each value represents means ± SEM ($n=5$).

^aNumber of scratches in normal controls treated with saline alone, and the control group, which was treated with compound 48/80 and saline, for 1 h were 3 ± 2 and 235 ± 22 , respectively.

^bNot determined.

^{c,d,e,f}Means followed by the same letter are not significantly different at $P < 0.05$.

Table 1. Inhibitory effect of steroidal components isolated from *A. asphodeloides* on passive cutaneous anaphylaxis reaction induced by IgE-antigen complex in mice.

Compound	Dose (mg/kg)	Inhibition ^a (%)	
		p.o.	i.p.
Timosaponin AIII (1)	5	— ^b	26.7 ± 7.9 ^c
	20	39.1 ± 6.0 ^d	25.8 ± 6.8 ^c
	50	55.3 ± 6.3 ^e	—
Timosaponin BIII (2)	5	—	19.4 ± 14.8 ^c
	20	33.8 ± 13.1 ^{c,d}	24.2 ± 13.5 ^c
	50	50.3 ± 6.9 ^e	—
Timosaponin D (3)	5	—	18.2 ± 13.7 ^c
	20	20.6 ± 9.3 ^c	28.6 ± 7.6 ^c
	50	31.5 ± 9.1 ^{c,d}	—
Azelastine	10	76.5 ± 5.8 ^f	65.7 ± 9.0 ^d

Each value represents means ± SEM ($n=5$).

^aAmounts of extravasated Evans blue in the dorsal skin (1×1 cm) of control stimulated with IgE-antigen complex and vehicle-treated groups were 27 ± 3 µg and 12 ± 2 µg, respectively.

^bNot determined.

^{c,d,e,f}Means followed by the same letter are not significantly different at $P < 0.05$.

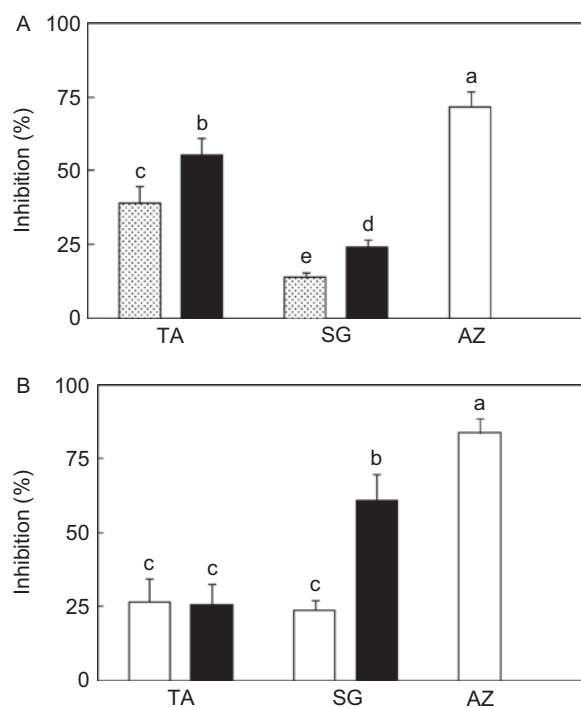


Figure 2. Inhibitory effect of timosaponin AIII (TAIII), sarsasapogenin (SG), and azelastine (AZ) against IgE-induced passive cutaneous anaphylaxis reaction in mice. Compounds were administered orally (A) at 10 mg/kg (white bar), 20 mg/kg (dotted bar), and 50 mg/kg (black bar) or intraperitoneally (B) at doses of 5 mg/kg (white bars) and 20 mg/kg (black bars). Bars are means ± SD ($n=5$). Values with the same letter above the error bars were not significantly different at $P < 0.05$.

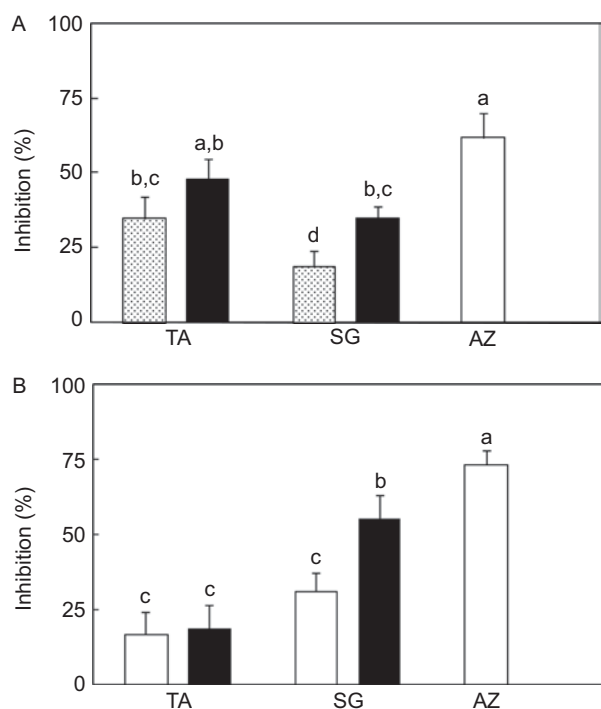


Figure 3. Inhibitory effect of timosaponin AIII (TAIII), sarsapogenin (SG), and azelastine (AZ) on compound 48/80-induced scratching behavior in mice. Compounds were administrated orally (A) at 10 mg/kg (white bars), 20 mg/kg (dotted bars), and 50 mg/kg (black bars) or intraperitoneally (B) at 5 mg/kg (white bars) and 20 mg/kg (black bars). Bars are means $\pm$ SD ($n=5$). Values with the same letter above the error bars were not significantly different at $P<0.05$.

one. Intraperitoneally administered sarsapogenin more potently inhibited the PCA reaction and scratching behavior. However, orally and intraperitoneally administered azelastine, a commercial drug, showed a potent inhibition against PCA reaction and scratching behavior.

To investigate their antiallergic mechanism, we examined their inhibitory effect against the RBL-2H3 cell degranulation induced by IgE-antigen complex (Figure 4). Sarsapogenin was at least twofold more potent than its parent glycoside, timosaponin AIII. Sarsapogenin also inhibited the expression of IL-4 and TNF- α in RBL-2H3 cells induced by IgE-antigen complex more potently than timosaponin AIII.

Discussion

Antihistamines such as azelastine, steroids such as betametasone, and some flavonoids and immunosuppressants such as FK-506 and cyclosporin A have been used to treat allergic diseases caused by anaphylactic reactions.⁽²²⁻²⁴⁾ However, these agents have significant side effects, such as severe nephrotoxicity and neurotoxicity.^(7,25-27) Herbal medicines have also been used for allergic diseases, and their efficacy is attracting increasing attention.^(10,11) Therefore, in order to investigate if AA is an effective treatment for allergic diseases and the active components in AA against allergic diseases, the main steroidal components from AA were isolated

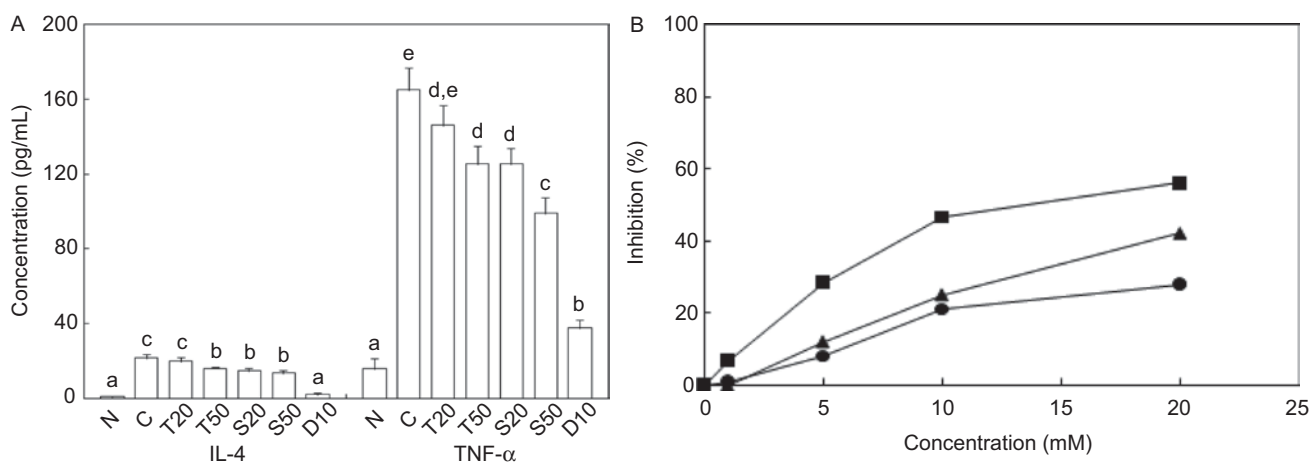


Figure 4. Inhibitory effect of timosaponin AIII (TAIII) and sarsapogenin (SG) on (A) IL-4 and TNF- α expression and (B) degranulation in RBL-2H3 cells stimulated with the IgE-antigen complex. RBL-2H3 cells (5×10^5 cells) were treated with 0.5 μ g/mL of mouse monoclonal IgE, exposed to 0.2 mL of agents [N, normal; C, vehicle alone; T20, 20 μ M TAI; T50, 50 μ M TAI; S20, 20 μ M SG; S50, 50 μ M SG; D10, 10 μ M dexamethasone for 4 h, followed by treatment with 0.2 mL of dinitrophenol-human serum albumin (1 μ g/mL) for 4 h at 37°C, and then IL-4 and TNF- α levels were determined with ELISA. The normal group was treated with vehicle alone instead of agents and IgE-antigen. Values represent the mean $\pm$ SD for duplicate experiments. Values with the same letter above the error bars were not significantly different at $P<0.05$. Closed circle, TAI; closed square, SG; closed triangle, azelastine.

and the antiallergic effect of AA and its components was evaluated in the mouse model induced PCA reaction by IgE-antigen complex, although allergic models have been developed for antianaphylactic agents.^(20,28,29)

AA and its components inhibited IgE-induced PCA reaction and compound 48/80-induced scratching behavior. Of them, timosaponin AIII was the most effective, followed by timosaponin BIII and timosaponin D. Oral and intraperitoneal administration of sarsasapogenin, the aglycone of timosaponin AIII, more potently inhibited the PCA reaction and scratching behavior than intraperitoneally administered one. These results suggest that orally administered timosaponin AIII may be metabolized to sarsasapogenin by intestinal microflora and timosaponin AIII as well as sarsasapogenin inhibit PCA and scratching behavioral reactions *in vivo*. The sugar side chain (glycone) of timosaponins may reduce intestinal absorption due to increased hydrophilicity. Thus, orally administered steroidal glycosides may be metabolized to their aglycones by intestinal microflora, and then their metabolites may be absorbed into the blood. Sarsasapogenin and timosaponin AIII are spirostane saponins, whereas timosaponin BIII and timosaponin D are furostanes. Timosaponin AIII showed better antiallergic effects than the furostane-type saponins. Although furostane-type saponins have an additional glycoside substitution at the 26-position, orally administered saponins may be metabolized to their aglycones, which would mediate the antiallergic activity. Orally administered timosaponin BIII potently inhibited the PCA reaction and scratching behavior, but timosaponin D, which has a hydroxyl group at the 2-position of the aglycone, did not reduce these reactions.

Sarsasapogenin, a metabolite of timosaponin AIII, inhibited the degranulation of RBL-2H3 cells and the expression of IL-4 and TNF- α in RBL-2H3 cells more potently than timosaponin AIII. These results suggest that sarsasapogenin and timosaponin AIII may inhibit the allergic reaction by inhibiting cytokine expression in mast cells and basophils and their degranulation.

On the basis of these findings, antiallergic effect of AA may be due to those of its steroidal components, and that of timosaponin AIII may be activated by intestinal microflora.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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