The Rhizomes of *Acorus gramineus* and the Constituents Inhibit Allergic Response *In vitro* and *In vivo*

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**Abstract**

The rhizomes of *Acorus gramineus* have frequently been used in traditional medicine mainly for sedation as well as enhancing brain function. In this study, the anti-allergic activity of *A. gramineus* was investigated. The 70% ethanol extract of the rhizomes of *A. gramineus* was found to inhibit the allergic response against 5-lipoxygenase (5-LOX)-catalyzed leukotriene (LT) production from rat basophilic leukemia (RBL)-1 cells and β-hexosaminidase release from RBL-2H3 cells with IC₅₀'s of 48.9 and >200 μg/ml, respectively. Among the 9 major constituents isolated, β-asarone, (2R,3R,4S,5S)-2,4-dimethyl-1,3-bis-(2',4',5'-trimethoxyphenyl) tetrahydrofuran (AF) and 2,3-dihydro-4,5,7-trimethoxy-1-ethyl-2-methyl-3-(2,4,5-trimethoxyphenyl)indene (AI) strongly inhibited 5-LOX-catalyzed LT production in A23187-treated RBL-1 cells. AI being the most potent (IC₅₀ = 6.7 μM). Against β-hexosaminidase release by antigen-stimulated RBL-2H3 cells, only AI exhibited strong inhibition (IC₅₀ = 7.3 μM) while β-asarone and AF showed 26.0% and 39.9% inhibition at 50 μM, respectively, In addition, the ethanol extract of *A. gramineus* showed significant inhibitory action against the hapten-induced delayed hypersensitivity reaction in mice by oral administration at 200 mg/kg. Therefore, it is suggested that *A. gramineus* possesses anti-allergic activity and the constituents including β-asarone and AI certainly contribute to the anti-allergic activity of the rhizomes of *A. gramineus*.

**Key Words:** *Acorus gramineus*, β-Asarone, 2,3-Dihydro-4,5,7-trimethoxy-1-ethyl-2-methyl-3-(2,4,5-trimethoxyphenyl)indene, 5-Lipoxygenase, β-Hexosaminidase, Anti-allergy

**INTRODUCTION**

Humans suffer from various allergic disorders in their life time including asthma, systemic allergic disorders and some skin disorders. It is difficult to completely cure these disorders. Many different kinds of small molecular weight-drugs and recently several biologics such as anti-TNF-α monoclonal antibody are used clinically (Walsh, 2011). However, there is a continual need for plant alternative medicine since they are relatively safe and cost-effective, and especially the cumulative record of their use over thousands of years exists. In this respect, the effect of many plant extracts were examined for their anti-allergic activity in our screening procedure, and the rhizomes of *Acorus gramineus* were found to possess some anti-allergic activity in vitro.

The rhizome of *Acorus gramineus* (Araceae) is a well-known Chinese traditional medicine. This plant material has been used widely as antipyretic, memory enhancement, analgiesia, sedative and digestive in China, Japan and Korea (Liao et al., 1998). The major constituents are β-asarone and phenylpropenes (Park et al., 2011). There have been many investigations concerning the effects of this plant material on the improvement of brain function (Liao et al., 1998; Chun et al., 2008), and an anti-allergic property has not been elucidated to date. In our preliminary experiment, the ethanol extract of the rhizomes of *A. gramineus* (AGE) were found to possess an anti-allergic action although the potency was not strong. Therefore, the anti-allergic activity of *A. gramineus* and the constituents was investigated using an *in vitro* and *in vivo* animal model of allergic responses in the present investigation.

**MATERIALS AND METHODS**

**Chemicals**

A23187 was obtained from Biomol (Plymouth Meeting, PA, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nordihydroguaiaretic acid (NDGA), prednisolone, quercetin, carboxymethylcellulose (CMC), dinitrophenyl (DNP)-BSA, anti-DNP mouse IgE and p-nitrophenyl-N-acetyl-
(β-D-glucosaminide were purchased from Sigma Chem (St. Louis, MO, USA). DMEM and other cell culture reagents including FBS were products of Gibco BRL (Grand Island, NY, USA). A protein assay kit was purchased from Bio-Rad ( Hercules, CA, USA).

Animals
Male ICR mice (4 weeks old, specific pathogen-free) were obtained from Orient-Bio Co. (Korea). The animals were maintained in the animal facility (KNU) at 20-22°C under 40-60% relative humidity and a 12 h/12 h (light/dark) cycle for at least 7 days prior to the experiment. The experimental design using the animals was approved by the local committee for animal experimentation, KNU (KIAUC-11-0007), and the animals were handled according to the guidelines described in the KFDA Guide for the Care and Use of Laboratory Animals.

Preparation of the extracts of the rhizomes of A. gramineus and isolation of the constituents
The rhizomes of A. gramineus were collected in Jeju Island, Korea in March 2009, and identified by one of authors (Dr. K. R. Lee). A voucher specimen (SKKU-NPL-0910) was deposited at the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea. The dried and chopped rhizomes of A. gramineus (150.0 g) were extracted with water and 70% EtOH under reflux and then filtered. The filtrates were evaporated under reduced pressure to give water extract and EtOH extract. These are used for the pharmacological assays. For an isolation of active constituents, the rhizome parts of A. gramineus (15 kg) were extracted at room temperature with 80% MeOH to give a MeOH extract (1.2 kg). The isolation and identification of β-asarone, asaraldehyde, propioveratrone, acoraminol A, acoraminol B, 7S,8R-dihydodiconiferyl alcohol (AA), 2,4,5-trimethoxyallylbenzene (AB), (2R,3R,4S,5S)-2,4-dimethyl-1,3-bis(2',4',5'-trimethoxyphenyl)tetrahydrofuran (AF), 2,3-dihydro-4,5,7-trimethoxy-1-ethyl-2-methyl-3-(2,4,5-trimethoxyphenyl)indene (AI) from the MeOH extract of A. gramineus were reported previously (Fig. 1) (Park et al., 2011).

Rat basophilic leukemia-1 (RBL-1) cell culture and measurement of leukotriene (LT) concentration
To evaluate the 5-lipoxygenase (5-LOX) inhibitory activity, RBL-1 cells purchased from the American Type Culture Collection (ATCC, Rockville, VA, USA) were cultured in RPMI 1640 with 10% FBS under 5% CO₂ at 37°C. The test compounds were dissolved in DMSO and diluted to appropriate concentrations with serum-free DMEM. The final concentration of DMSO was adjusted to 0.1% (v/v). The cells were pre-incubated with the test compounds for 10 min. Then, A23187 (3 μM) was added to activate the 5-LOX and the cells were further incubated for 15 min as previously described, with slight modification (Tries et al., 2002). The media was then collected and the concentration of the 5-LOX product, cysteinyl leukotrienes (LTC₄/D₄/E₄), was measured using an ELISA kit (Enzo Life Sciences) as recommended by the manufacturer. The cell viability was assessed using an MTT assay as previously described (Mossman, 1983).

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RBL-2H3 cell culture and antigen-induced degranulation of β-hexosaminidase

Sensitization and degranulation procedures were followed by the previously described procedure (Choi et al., 1996). In brief, anti-DNP mouse IgE was added to RBL-2H3 cells (ATCC) for sensitization and incubated overnight. Twenty four hours later, the cells were washed with siraganian buffer (pH 7.2). The test compounds dissolved in DMSO were added and the cells were incubated for 30 min. Then, DNP-BSA (1 μg/ml) was added, and after 10 min incubation, the reaction was stopped by cooling in an ice bath. The supernatant was obtained by centrifugation. The 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 NaHCO₃ (200 μl/well). The absorbance was measured at 405 nm.

Picryl chloride-induced delayed hypersensitivity in mice

For measuring inhibitory activity against delayed type hypersensitivity (DTH, type IV hypersensitivity), 7% picryl chloride (Nacalai Tesque Inc., Japan) in acetone (100 μl/mouse) was smeared to the abdomen of mice to sensitize the animals for the elicitation phase group. For the animals of the control group, acetone (100 μl) was applied instead of picryl chloride. Seven days later, the elicitation phase of delayed hypersensitivity was induced by application of 1% picryl chloride in acetone (20 μl/ear) to right ears of the sensitized mice. For obtaining the induction phase reaction, 1% picryl chloride (20 μl/ear) was applied to right ears of acetone-treated mice. For the control group, only acetone (20 μl) was applied to right ears of acetone-treated mice. After 24 h, ear thickness was measured. Test compounds dissolved in 0.4% CMC were orally administered 1 h after initial treatment of sensitizer or acetone. The same amounts of test compounds in vehicle were treated again 1 h after final picryl chloride or acetone treatment.

Statistical analysis

All data were represented as arithmetic mean ± SD. One-way analysis of variance (ANOVA), followed by Dunnett's test was used to determine the statistical significance.

RESULTS

In A23187-treated RBL-1 cells, cysteinyl-LTs (1,811.7 ± 134.5 pg/ml) were synthesized by 5-LOX for 15 min. The basal level of the cysteinyl-LTs without A23187 treatment was 50.3 ± 18.9 pg/ml (n=3). Under these conditions, the water and ethanol extracts considerably inhibited LT production at 12.5-200 μg/ml (Fig. 2A). The IC₅₀ values for the water and ethanol extracts were 120.0 and 48.9 μM, respectively. When the constituents were tested, β-asarone, AF and AI showed a strong inhibitory action (Fig. 2B), while AB showed weak inhibition. Their IC₅₀ values were represented in Table 1. NDGA (LOX inhibitor, 1 μM) used as a reference strongly inhibited LT production (99.3%).

In antigen-treated RBL-2H3 cells, β-hexosaminidase was degranulated and released into the media for a 10 min incubation period (from 0.0 ± 3.2% to 100.0 ± 1.2%, n=3). Under these conditions, the ethanol extract of A. gramineus significantly inhibited the degranulation reaction at 50-200 μg/ml (Fig. 3A). The ethanol extract showed 31.4% inhibition at 200 μg/ml.

Table 1. Inhibition of the constituents of the rhizomes of A. gramineus against 5-LOX catalyzed LT production and β-hexosaminidase degranulation

<table>
<thead>
<tr>
<th>Compounds</th>
<th>% inhibition at 50 μM²</th>
<th>5-LOX</th>
<th>β-hexosaminidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Asarone</td>
<td>94.9 (31.1)³</td>
<td>39.9</td>
<td>12.9</td>
</tr>
<tr>
<td>Asaraldehyde</td>
<td>(-)</td>
<td>12.9</td>
<td>12.9</td>
</tr>
<tr>
<td>Propioveratrone</td>
<td>(-)</td>
<td>8.4</td>
<td>8.4</td>
</tr>
<tr>
<td>Acoraminol A</td>
<td>(-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acoraminol B</td>
<td>(-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AA</td>
<td>5.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AB</td>
<td>29.9</td>
<td>26.0</td>
<td>26.0</td>
</tr>
<tr>
<td>AF</td>
<td>90.8 (32.0)</td>
<td>81.5</td>
<td>81.5</td>
</tr>
</tbody>
</table>

*All values are arithmetic mean of % inhibition at 50 μM, n=3, ²The values of the parenthesis are IC₅₀ values in μM. ³not active, ⁴cytoxic at 2-50 μM by MTT assay.


μg/ml, while the water extract did not show significant inhibitory action at 50-200 μg/ml. When the inhibitory action of the constituents was examined, only AI showed strong inhibitory activity (Fig. 3B, Table 1). On the other hand, β-asarone, asaraldehyde and AF showed weak inhibitory action at 50 μM (less than 50% inhibition). The reference agent, quercetin (degranulation inhibitor, 20 μM), strongly inhibited β-hexosaminidase release (92.2%).

In addition, the inhibitory action of A. gramineus on a delayed-type hypersensitivity in mice was evaluated. A treatment of hapten, picryl chloride, to the sensitized mice produces strong ear edema of delayed-type hypersensitivity. In this condition, when orally administered at 22.2-200 mg/kg, the ethanol extract of A. gramineus significantly inhibited the DTH response (29.9%) only at 200 mg/kg as shown in Fig. 4. The reference drug, prednisolone, potently inhibited DTH (80.5%) at 10 mg/kg. Therefore, it is suggested that A. gramineus possesses anti-allergic activity against the immediate-type as well as against the delayed-type hypersensitivity.

**DISCUSSION**

In the present investigation, inhibitions of 5-LOX inhibition and β-hexosaminidase release were studied for investigating anti-allergy activity in vitro. Leukotrienes (LT) are produced from arachidonic acid by 5-LOX. LTs are deeply involved in several allergic disorders such as asthma and atopic dermatitis (Rubin and Mollison, 2007). Thus 5-LOX inhibitory activity was examined using a mast cell line (RBL-1). And it is well known that the immediate hypersensitivity reaction is provoked at least in part by histamine released from antigen-stimulated mast cells and basophils. Along with a histamine release, β-hexosaminidase is also degranulated from the antigen-stimulated cells, which is frequently used as a biomarker of an immediate-type allergic response (Schwartz et al., 1981; Marquardt and Wasserman, 1983). Thus, RBL-2H3 cells were used and the amounts of β-hexosaminidase release were checked. On these parameters, A. gramineus and its constituents showed anti-allergic activity.

Many pharmacological studies have revealed that A. gramineus and its major constituent, β-asarone, could protect brain damage, alleviate memory function and inhibit Alzheimer’s symptoms (Liao et al., 1998; Chun et al., 2008; Geng et al., 2010; Pages et al., 2010; Zou et al., 2011). However, the anti-allergic and anti-inflammatory activities of this plant material and its constituents including β-asarone were rarely demonstrated. There have been reports of cyclooxygenase-1 inhibitory action of trans-asarone (Momin et al., 2003) and passive cutaneous anaphylaxis inhibition of γ-asarone (Hashimoto et al., 1994). To our best knowledge, this is the first report of the anti-allergic property of A. gramineus and the constituents.

In conclusion, the rhizomes of A. gramineus and its several constituents possess anti-allergic activity in vitro against 5-LOX-catalyzed LT production and antigen-induced β-hexosaminidase release from mast cell lines. In particular, 2,3-dihydro-4,5,7-trimethoxy-1-ethyl-2-methyl-3-(2,4,5-trimethoxyphenyl)indene (AI) showed strong inhibitory activity against these two parameters. β-Asarone, a major constituent of A. gramineus, also showed anti-allergic action. Moreover, A. gramineus showed inhibitory activity on DTH reaction although the potency was not strong. It is suggested that β-asarone and 2,3-dihydro-4,5,7-trimethoxy-1-ethyl-2-methyl-3-(2,4,5-trimethoxyphenyl)indene (AI) certainly contribute to the anti-allergic property of A. gramineus.

**ACKNOWLEDGMENTS**

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