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Inhibitory effects of *Acorus calamus* extracts on mast cell-dependent anaphylactic reactions using mast cell and mouse model

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ABSTRACT

Ethnopharmacological relevance: *Acorus calamus* Linn. (Araceae) is a traditional herbal plant used for centuries to treat various allergic symptoms including asthma and bronchitis.

Aim of the study: The present study was focused to provide a pharmacological basis for the traditional use of *Acorus calamus* in allergic symptoms using the mast cell-dependent anaphylactic reactions in *in vitro* and *in vivo* models.

Materials and methods: Cell viabilities were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Dinitrophenyl-human serum albumin (DNP-HSA) induced β -hexosaminidase and interleukin (IL)-4 productions in IgE-sensitized rat basophilic leukaemia (RBL-2H3) cells were measured by enzymatic assay and enzyme-linked immunosorbent assay (ELISA). Passive cutaneous anaphylaxis (PCA) reaction mouse model was implemented for *in vivo* studies.

Results: Hot water (HW), butylene glycol (BG), hexane (HE) and steam distilled (SD) extracts of *Acorus calamus* showed different cytotoxicity levels evaluated in RBL-2H3 cells. Sub-toxic doses of HW extract suppressed the β -hexosaminidase secretion and IL-4 production significantly and dose dependently in DNP-HSA induced IgE-sensitized RBL-2H3 cells compared to other extracts of *Acorus calamus*. Further, *in vivo* studies also revealed that the HW extract significantly inhibited the PCA reaction in mouse compared to the normal control group.

Conclusion: HW extract of *Acorus calamus* most effectively inhibited degranulation and IL-4 secretion in DNP-HSA-stimulated RBL-2H3 cells and also reduced the mast cell-mediated PCA reaction in mouse, providing a therapeutic evidence for its traditional use in ameliorating allergic reactions.

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1. Introduction

Acorus calamus Linn. (Araceae) has long been used as a folk remedy in treating various allergic symptoms including asthma, cough, bronchitis and throat irritation (Nadkarni, 1976). Pharmacologically *Acorus calamus* was reported to possess antimicrobial (Kim et al., 2011), antiinflammatory (Kim et al., 2009), neuroprotective (Shukla et al., 2006), antioxidant (Acuna et al., 2002) and antiasthmatic effects (Shah and Gilani, 2010). But so far the scientific basis supporting the traditional use of *Acorus calamus* in atopic symptoms and the mechanisms underlying were not explored.

Atopic dermatitis is a common inflammatory skin disease of variable severity characterized by eczematous skin lesions associated with itching. The disease has an intricate immunologic basis influenced by genetic/familial predisposition and certain environmental, lifestyle, and dietary factors (Sehgal et al., 2010) and often regarded as the first symptom in the manifestation of asthma, allergic rhinitis and eczema (Spergel and Paller, 2003). The hallmarks of atopic dermatitis are a chronic, relapsing form of skin inflammation, a disturbance of epidermal-barrier function that culminates in dry skin, and antigen (IgE)-mediated sensitization to food and environmental allergens (Bieber, 2008). Mast cell-derived mediators play a critical role in the initiation and progression of allergic inflammatory reactions associated with atopic and allergic disease (Brown et al., 2008; Shin et al., 2010). In immunoglobulin (Ig) E-dependent mast cell activation, activated mast cell can rapidly secrete the pre-formed and *de novo*

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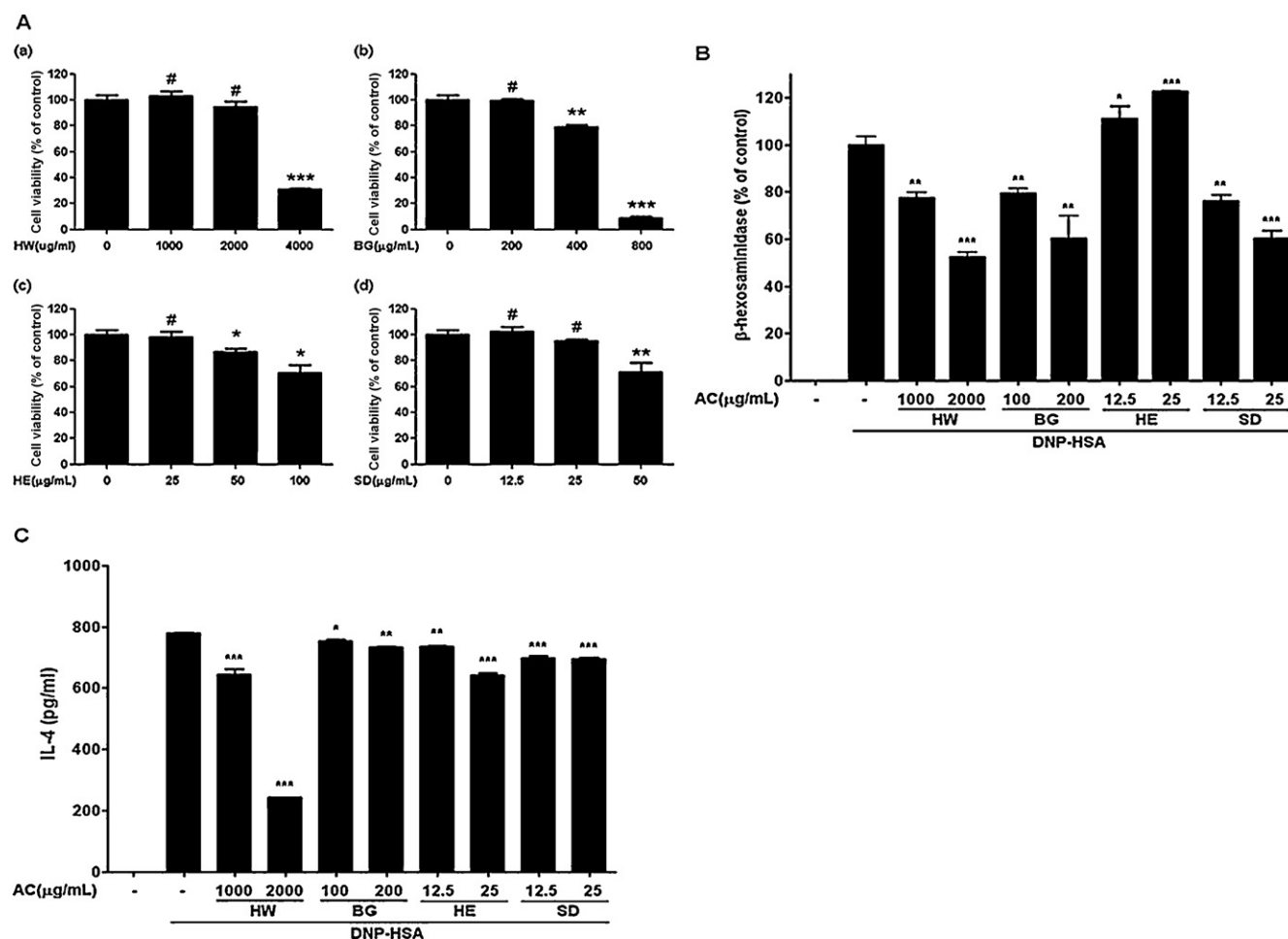


Fig. 1. Effect of selected extracts of *Acorus calamus* on cell viability, IgE-induced degranulation and IL-4 production in RBL-2H3 cells. (A) Cell viabilities of HW (a), BG (b), HE (c) and SD (d) extracts of *Acorus calamus* determined by MTT assay. (B) β-Hexosaminidase assay. (C) IL-4, levels of various extracts of *Acorus calamus* was assessed using IL-4 ELISA kits. Each bar shows the mean ± S.D. of three independent experiments. #No significant difference between the untreated group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, significantly different from DNP-HSA alone group. HW: hot water; BG: butylene glycol; HE: hexane; SD: steam distilled; IL: interleukin; AC: *Acorus calamus*.

synthesized allergic mediators such as histamine, proteases, arachidonic derivatives, proinflammatory cytokines and chemotactic cytokines (Galli et al., 2005).

A growing interest in allergy research using the traditional herbs with immense ethnopharmacological importance is advancing. Several medicinal plants have been studied for their antiallergic effects using mast cell activation and degranulation in cell lines and animal models (Moon et al., 2005).

In this study, we aimed to evaluate the antiallergic effect of different fractional extracts of *Acorus calamus* using the mast cell-dependent *in vitro* and *in vivo* PCA reaction mouse model.

2. Materials and methods

2.1. Plant material and extracts

Fresh rhizomes of *Acorus calamus* were collected from Jeon-Buk Province, Korea and were authenticated by a taxonomist. A voucher specimen (SMH-100402) has been deposited at Semyung University. The material was air-dried and subjected to extraction with hot water (HW), butylene glycol (BG), n-hexane (HE) and steam distilled (SD) with the final yields of 0.74% (v/w), 0.57% (v/w), 2.5% (v/w) and 0.11% (v/w), respectively.

2.2. Reagent

Modified Eagles Medium (MEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Hyclone (Logan, UT, USA). MTT, Evans blue, anti-dinitrophenyl (DNP)-IgE, DNP-HSA, 4-nitrophenyl N-acetyl-β-D-glucosaminide, and all other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Rat IL-4 ELISA kit (BD OptEIA™ Set) was purchased from BD Biosciences (Franklin Lakes, SD, USA).

2.3. Cell culture and animal treatment

Rat basophilic cell line RBL-2H3 cells were obtained from the American Type Culture Collection (ATCC, MD, USA) and grown in minimum essential medium (MEM) with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator with 5% CO₂/95% air atmosphere. For stimulation of RBL-2H3 cells via Fcε receptors, cells were incubated with anti-DNP-IgE (100 ng/mL) at 37 °C for 2 h, and then incubated with DNP-HSA (25 ng/mL) at 37 °C for 15 min in PIPES-buffer (25 mM PIPES, 119 mM NaCl, 40 mM NaOH, 5 mM KCl, 0.4 mM MgCl₂, 1 mM CaCl₂, 5.6 mM glucose, and 0.1% BSA, pH 7.2).

Male BALB/c mouse (6 weeks) were obtained from NARA biotech Co. Ltd (Seoul, Korea), and were kept in animal housing for 1 week,

maintained on a 12 h-light-dark cycle at $24 \pm 1^\circ\text{C}$ with free access to food and water *ad libitum*. All experiments were performed in accordance with the institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at Konkuk University, Korea.

2.4. MTT and β -hexosaminidase assay

Initially the effects of different fractional extracts of *Acorus calamus* were treated with indicated concentrations for 24 h to RBL-2H3 cells and their viabilities were determined by MTT assay as described previously (Carmichael et al., 1987). Degranulation of RBL-2H3 cells was evaluated by measuring the activity of the β -hexosaminidase release. RBL-2H3 cells were incubated with anti-DNP IgE for 2 h and then surface IgE was cross-linked with or without DNP-HSA. After 15 min, the percentage of specific β -hexosaminidase release was determined. The culture supernatant was incubated with 1.3 mg/mL p-nitrophenyl-N-acetyl β -D-glucosamine in 0.1 mol/L sodium citrate (pH 4.5) at 37°C for 1 h. The reaction was stopped by 0.1 mol/L sodium carbonate and 0.1 mol/L sodium bicarbonate. The enzyme activity was evaluated by measuring optical density at 405 nm.

2.5. IL-4 ELISA

RBL-2H3 cells were stimulated through Fc ϵ RI as described above. The supernatants of the cultured RBL-2H3 cells were collected after DNP-HSA stimulation for 4 h, and then the amount of IL-4 in the supernatant was measured using a rat IL-4 ELISA kit (BD Biosciences) according to the manufacturer's instruction.

2.6. PCA reaction

Mast cell-dependent PCA reaction was generated by sensitizing the skin with an intra-dermal injection of anti-DNP IgE (0.5 $\mu\text{g}/30\ \mu\text{L}$) into the ear and 24 h later with an injection of DNP-HSA (200 μL of the 1:1 mixture of 100 μg) and 2% Evans blue *via* tail vein. One hour before this injection, each extract of *Acorus calamus* and azelastine for positive control were epicutaneously administered on ear. After sacrificing the animals, the Evans blue was extracted by incubating the ears pieces of each mouse with same size in 200 μL formamide for 12 h at 64°C . The absorbance of the Evans blue was measured at 650 nm.

2.7. Finger print analysis of *Acorus calamus* extract by high performance liquid chromatography (HPLC)

The hot water extract of *Acorus calamus* was dissolved in 100% methanol and filtrated through a 0.45 μm PTFE disposable syringe filter (Advantec, Toyo Roshi Kaisha Ltd., Japan) for fingerprint analysis. The components of *Acorus calamus* were determined through an HPLC system (SHIMADZU, Kyoto, Japan) equipped with CBM-20A communications bus module, LC-20AD liquid chromatograph, SPD-M20A diode array detector, SIL-20AC auto sampler, DGU-20A3 degasser and CTO-20AC column oven. The constituents of the extract were separated using Shim-pack VP-ODS (4.6 mm $\times$ 250 mm, 5 μm) at the flow rate of 1.0 mL/min. The mobile phase was methanol with the following gradient programs: linear gradient to 50% methanol for 10 min; linear gradient to 80% methanol for 25 min; linear gradient to 90% methanol for 5 min; and isocratic at 90% methanol for 5 min. The analytes were detected through UV absorption at 254 nm. The identification of major peaks was determined through an IT-TOP system (SHIMADZU, Kyoto, Japan), the mobile phase was 70% methanol,

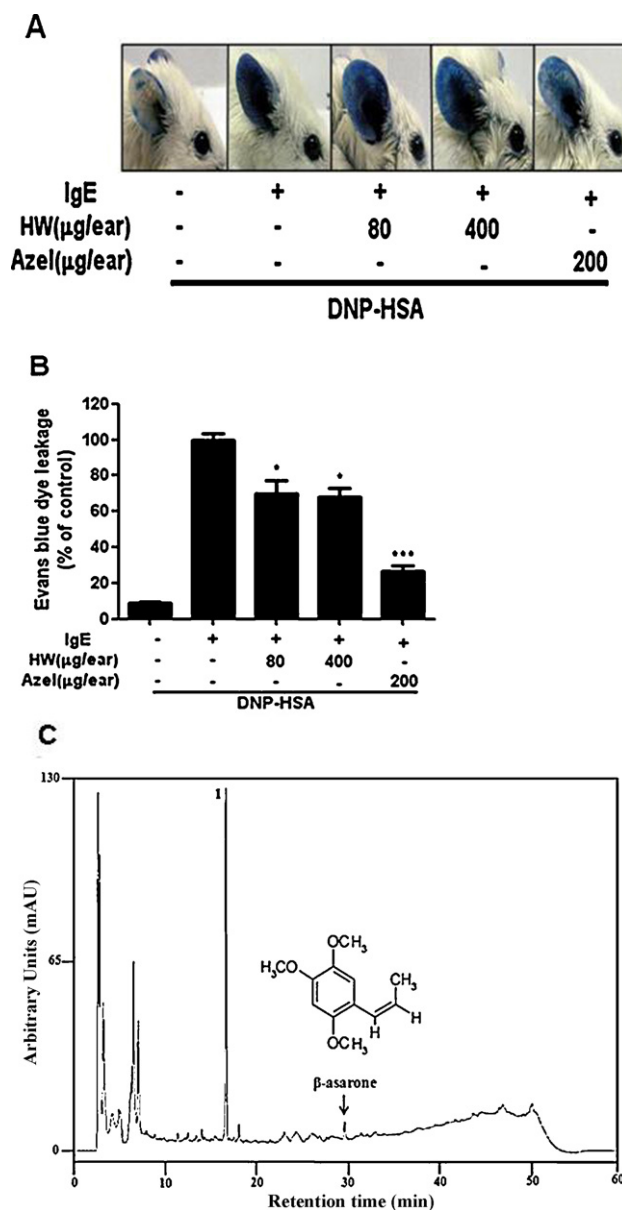


Fig. 2. Effects of HW of *Acorus calamus* on mast cell-dependent PCA reaction in mouse and HPLC fingerprint. After PCA reaction, the ear was excised in order to measure the Evans blue extravasation. (A) Representative pictures of the ear were shown. (B) Vascular leakage assayed by Evans blue extravasation into ear tissues. Azelastine (Azel) was used as a representative anti-allergic drug. (C) HPLC fingerprint of HW extract of *Acorus calamus* was performed as described in Section 2.7. Reference standard of β -asarone was used to compare the obtained peak. Each bar shows the mean $\pm$ S.D. of three independent experiments, $n = 3$. *Significantly different from control group. * $P < 0.05$ and *** $P < 0.001$, significantly different from IgE and DNP-HSA treated group. HW: hot water.

flow rate was 0.2 mL/min, injection volume was 20 μL , and mass range (m/z) was 100–1500 amu.

2.8. Statistical analyses

All data was expressed as means $\pm$ S.D. Statistical significances were determined by Student's *t*-test. The regression equation and the correlation coefficient were determined by the least square method.

3. Results and discussion

This study examined the effects of different fractional extracts from *Acorus calamus* on IgE-induced mast cell activation in RBL-2H3 cells. HW, BG, HE and SD extracts up to 2000, 200, 25 and 25 µg/mL respectively, did not significantly affect the cytotoxicity in RBL-2H3 cells for 24 h (Fig. 1A). Further, at non-toxic concentrations, HW, BG and SD extracts suppressed the DNP-HSA induced β-hexosaminidase secretion in IgE-sensitized RBL-2H3 cells significantly (Fig. 1B), whereas HE extract did not exhibit such effect. The results showed that each extract differently inhibited antigen-induced mast cell degranulation.

IL-4 is an important factor in facilitating mast cell activation, as well as in inducing the secretion of other Th2 cytokines (Ricci et al., 1997). In our study, IgE-sensitized RBL-2H3 cells were treated with HW, BG, HE and SD extracts of *Acorus calamus*, and challenged with DNP-HSA. It was found that only HW extract significantly decreased the levels of IL-4 production (Fig. 1C). The IL-4 expression at mRNA level was also decreased (data not shown).

PCA is one of the most important *in vivo* models of anaphylaxis in local allergic reactions (Yang et al., 1997). In our study, compared to the normal mouse group, intra-dermal administration of anti-DNP-IgE induced a prominent enhancement of Evans blue dye leakage into the ears in the control mouse (Fig. 2A). The leakage of injected Evans blue dye was significantly suppressed by HW pretreatment (Fig. 2B). Azelastine (200 µg/ear), an anti-allergic drug also significantly inhibited the leakage of Evans blue dye.

To confirm the extract used in the study possess the major active constituent, HW extract was subjected to HPLC fingerprint and the peak of major active constituent β-asarone was identified with UV absorption at 254 nm. It was confirmed comparing with the reference standard (Fig. 2C). The results are in agreement with previous reported works that the rhizome of *Acorus calamus* contains α and β forms of asarone (Rodríguez-Páez et al., 2003; Ka et al., 2005). The unknown peak (peak 1) shown in Fig. 2C was analyzed at 254 nm absorbance through LC/MS and identified a molecular ion *m/z* 224. However, we could not identify other reported compounds as they were below the levels of quantification.

4. Conclusion

The present scientific report supported the traditional usage of *Acorus calamus* in treating various allergy related symptoms. This study is the first to reveal the antiallergic potential of *Acorus calamus* extract as a mast cell stabilizer and inhibitor to atopic dermatitis. The HW extract of *Acorus calamus* significantly inhibited degranulation and IL-4 cytokine secretion in DNP-HSA-stimulated RBL-2H3 cells and reduced the mast cell-mediated PCA reaction in mouse. Further work is required to isolate the active principles from HW extract of *Acorus calamus* and explore the mechanisms involved in preventing various allergic diseases.

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