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In-vitro and in-vivo anti-allergic actions of *Arecae semen*

Jun Ho Lee, Sung Ho Chang, Yun Sim Park, Erk Her, Hoi Young Lee, Jong Woo Park, Jeung Whan Han, Young Mi Kim and Wahn Soo Choi

Abstract

The effects of various extracts from oriental medicinal herbs on mast cell-mediated allergic reactions have been investigated. Among the extracts, *Arecae semen* was the most potent inhibitor of antigen-induced degranulation in RBL-2H3 mast cells. *A. semen* inhibited DNP-BSA- and compound 48/80-induced degranulation in RBL-2H3 mast cells with IC_{50} values of approximately 53 and 52 $\mu g\,mL^{-1}$, respectively, and inhibited compound 48/80-induced systemic anaphylaxis by 46% at 300 $mg\,kg^{-1}$ in mice. *A. semen* also inhibited the expression of TNF-α and the activation of mitogen activated protein kinase, ERK1/2, which is critical for the production of inflammatory cytokines in mast cells, as indicated by the suppression of the activating phosphorylation of ERK1/2. These results suggest that *A. semen* may be useful for the treatment of various immediate and delayed allergic diseases.

Bio-Food and Drug Research Center, Department of Immunology, College of Medicine, Konkuk University, Chungju, 380-701, South Korea

Jun Ho Lee, Sung Ho Chang, Yun Sim Park, Erk Her, Wahn Soo Choi

College of Medicine, Konyang University, Nonsan 320-711, South Korea

Hoi Young Lee

Department of Biochemistry and Molecular Biology, College of Pharmacy, Sungkyunkwan University, Suwon 440-746, South Korea

Jong Woo Park, Jeung Whan Han

Department of Pharmacy, Duksung Women's University, Seoul, 132-714, South Korea

Young Mi Kim

Correspondence:
Wahn Soo Choi, Bio-Food and Drug Research Center, Department of Immunology, College of Medicine, Konkuk University, Chungju, 380-701, South Korea.
E-mail: wahnchoi@kku.edu

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Introduction

In South Korea and other oriental countries, many herbs such as *Glycyrrhizae radix*, *Liriopis tuber*, *Castaneae semen*, *Rhemaniae radix*, *Citri tangerinae semen*, *Platycodi radix*, *Arecae semen*, *Phragmitis rhizoma*, *Aloe*, *Angelicae sinensis radix*, *Angelicae gigantis radix* and many others are used as traditional folk remedies for various diseases. However, the active components and mechanisms of action of most herbs are largely unknown and few have been screened for their pharmacologic activity or in in-vivo animal models. There is therefore a need for the systematic examination of many of these herbal remedies.

Arecae semen, known as 'Bin-rang' in Korea and China, is the dried ripe fruit of *Areca catechu* L. (Palmae). Traditionally, the extract of the dried fruit has been used for the treatment, usually by oral administration, of various diseases, such as taeniasis, ascariasis, fasciolopsiasis, abdominal pain due to intestinal parasitosis, diarrhoea, tenesmus due to accumulation of undigested food, oedema and weakness of the legs, and malaria, in Far Eastern countries including Korea (List & Horhammer 1969–1979; Emboden 1972). Some active constituents of *A. semen* have been identified (List & Horhammer 1969–1979; Emboden 1972; Robbers et al 1996) and examined for pharmacological activities in-vitro and in animal models. Interestingly, one constituent, tea catechin, has anti-allergic activity and suppresses the expression of the high-affinity IgE receptor, FcϵRI, in human basophils (Sano et al 1999; Fujimura et al 2002). However, the effects of catechins on mast cell degranulation have not been tested and it was not known if the tea catechins are similar to *A. semen*.

Mast cells and blood basophils are responsible for a variety of allergic disorders, such as allergic rhinitis, dermatitis, asthma and food allergies as well as catastrophic anaphylactic reactions to insect stings and some drugs. These cells respond to IgE-directed antigens via the high affinity receptor for IgE, namely FcϵRI, by releasing granules that contain preformed inflammatory mediators and by generating inflammatory lipids and cytokines (Kay 2001a, b).

In this study, we used RBL-2H3 mast cells and an in-vivo anaphylatic animal model to screen various oriental medicinal herbs for anti-allergic effects. The ethanol extract of *A. semen* was identified as a potential medicinal herb to cure allergic diseases.

Materials and Methods

Reagents

The dinitrophenol (DNP)-specific monoclonal IgE and DNP-BSA, arabic gum, Tween-80, compound 48/80, quercetin and cromolyn sodium were purchased from Sigma Chemical Co. (St Louis, MO). Minimal essential medium (MEM) and other cell culture reagents were obtained from GIBCO/Life Technologies, Inc. (Rockville, MD). Tris-glycine polyacrylamide gels were from Novex (San Diego, CA).

Animals

The male ICR mice (4 weeks old) were purchased from the Dae Han Experimental Animal Center (Daejon, South Korea), and the animals were housed in the animal facilities at the College of Medicine, Konkuk University. The mice were housed 10 per cage in a laminar air-flow cabinet maintained at a temperature of $22 \pm 1°C$ and relative humidity of $55 \pm 10\%$ throughout the study. The animal study was done in accordance with the guidelines of National Institutes of Health (NIH publication 85-23 1985) and the protocol was approved by the Institutional Ethics Committee at Konkuk University before study.

Preparation of crude extract of *A. semen*

The *A. semen* was imported from China and authenticated by Dr Hyung Kyu Lee at the Korea Research Institutes of Bioscience and Biotechnology. The extract was prepared by the Korea Research Institutes of Bioscience and Biotechnology as follows. Briefly, dried ripe seeds (100 g) of *Areca catechu* L. (Palmae) were extracted with 1000 mL of ethanol at 50°C by use of an ultrasonic cleaner (Branson Ultrasonics Corporation) and the extracted materials were concentrated with a speed bag (Biotron Corporation) at 40°C for 24 h. The concentrated extract was stored at −4°C. The yield of extraction was about 15% (w/w). The ethanol extract was purchased from the Korea Research Institutes of Bioscience and Biotechnology (extract number CA01-093) for study. A voucher specimen (number 2003-77517) was deposited at the College of Medicine, Konkuk University. Potency was assessed by the ability to inhibit degranulation of RBL-2H3 cells. The *A. semen* ethanol extract was dissolved in DMSO for the in-vitro assay and suspended in 5% arabic gum for the in-vivo animal studies.

Cell culture and measurement of degranulation in RBL-2H3 mast cells

RBL-2H3 cells were grown as monolayers in MEM with Earle's salts, supplemented with glutamine, antibiotics and 10% fetal bovine serum. For each experiment the cells were harvested by trypsinization, transferred to 24-well (2×10^5 cells $0.4\,mL^{-1}$ per well) cluster plates (Ali et al 1990), and incubated overnight in complete growth medium with $25\,ng\,mL^{-1}$ DNP-specific IgE to achieve 100% occupancy of FcεRI. Cultures were washed and the required buffered solution was added (0.2 mL per well). Experiments on intact cells were performed in a PIPES-buffered medium (25 mM PIPES, pH 7.2, 159 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose and 0.1% fatty acid-free fraction V from bovine serum) or in calcium-free PIPES-buffered medium (as above except 0.1 mM EGTA replaced 1 mM $CaCl_2$). Unless stated otherwise, cultures were incubated for 30 min with or without crude extracts before addition of stimulants for 10 min. Secretion of granules containing various allergic mediators was determined by measurement of the release of the granule marker, β-hexosaminidase, by use of a colorimetric assay in which release of p-nitrophenol from p-nitrophenyl-N-acetyl-β-D-glucosaminide is measured (Ozawa et al 1993). Values were expressed as the percentage of intracellular β-hexosaminidase that was released into the medium. The IC_{50} values, the concentration necessary to obtain 50% inhibition of the response, were calculated by non-linear regression analysis using GraphPad Software (San Diego, CA).

Compound 48/80-induced systemic anaphylaxis

The systemic anaphylaxis was induced by the mast cell degranulator, compound 48/80 (Alfonso et al 2000; Hong et al 2003). Briefly, each mouse was given an intraperitoneal injection of $8\,mg\,kg^{-1}$ of compound 48/80 in saline. *Arecae semen* extracts were suspended in 5% arabic gum and administered orally in doses of 20 to $300\,mg\,kg^{-1}$ 1 h before the injection of compound 48/80 to induce anaphylactic shock. Survival rate was monitored for 1 h after the injection of compound 48/80.

Immunoblotting analysis of mitogen activated protein (MAP) kinases in RBL-2H3 cells

The cells were harvested by trypsinization, transferred to 6-well (1×10^6 cells $3\,mL^{-1}$ per well) cluster plates, and incubated overnight in complete growth medium containing $25\,ng\,mL^{-1}$ DNP-specific IgE to achieve 100% occupancy of FcεRI. The cells were washed and the medium replaced with a PIPES-buffered medium (25 mM PIPES, pH 7.2, 159 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose and 0.1% fatty acid-free fraction V from bovine serum). The cells were stimulated by $10\,ng\,mL^{-1}$ DNP-BSA for 10 min without or with $100\,\mu g\,mL^{-1}$ *A. semen* and then washed twice with ice-cold phosphate-buffered saline (PBS). The cells were lysed in 0.25 mL ice-cold lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 60 mM octyl β-glucoside, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenylphosphate, $0.7\,\mu g\,mL^{-1}$ pepstatin and protease inhibitor cocktail tablet). Lysates were kept in ice for 30 min, centrifuged at $15\,000 \times g$ for 15 min at 4°C and the supernatant dissolved in 2 × Laemmli buffer (Laemmli 1970). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell Inc., NH).

The immunoreactive proteins were detected by use of horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences, Sweden).

Reverse transcription–polymerase chain reaction (RT–PCR) for TNF-α mRNA

RBL-2H3 cells were harvested by trypsinization, transferred to 6-well (1×10^6 cells 3 mL^{-1} per well) cluster plates, and incubated overnight in complete growth medium containing 25 ng mL^{-1} DNP-specific IgE to achieve 100% occupancy of FcϵRI. The cells were washed and the medium replaced with a PIPES-buffered medium (25 mM PIPES, pH 7.2, 159 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose and 0.1% fatty acid-free fraction V from bovine serum) and stimulated by 10 ng mL^{-1} DNP-BSA for 15 min without or with various concentrations of *Arecae semen* and then washed twice with ice-cold PBS. Total RNA was isolated using Trizol Reagent (Invitrogen) and reverse transcribed with the Superscript first strand synthesis system (Invitrogen) according to the manufacturer's protocol. PCR was performed at 94°C for 45 s, 55°C for 45 s and 72°C for 60 s for 30 cycles. The following primers were used: rat TNF-α forward 5′-CACCACGCTCTTCTGTCTACTGAAC-3′, reverse 5′-CCGGACTCCGTGATGTCTAAGTACT-3′; rat GAPDH forward 5′-GTGGAGTCTACTG GCGTCTTC-3′, reverse 5′-CCAAGGCTGTGGGCAAGGTCA-3′.

Statistical analysis

The data were presented as the mean ± s.e.m. from three or more separate experiments as indicated in the legend. Student's *t*-test was done to make a statistical comparison between each stimulated group and non-stimulated control by use of the SigmaPlot 2000 (Jandel Scientific, Corte Madera, CA). Asterisks indicate significant differences from the control group (*$P < 0.05$ and **$P < 0.001$).

Results

Activity of *A. semen* as assessed from degranulation of RBL-2H3 cells by antigen and compound 48/80

We used RBL-2H3 mast cells for the in-vitro assay to assess anti-allergic activity. RBL-2H3 cells were stimulated by DNP-BSA or compound 48/80. RBL-2H3 mast cells were pre-treated with 30 μM quercetin 24 h before stimulation with compound 48/80 and primed with 25 ng mL^{-1} DNP-specific IgE overnight before stimulation with DNP-BSA antigen. Consistent with previous reports, the degranulation of RBL-2H3 cells was dose- and time-dependently stimulated by DNP-BSA (Figure 1A, B) and compound 48/80 (Figure 1C, D). The maximal degranulation was induced by 10 ng mL^{-1} antigen and 15 min of stimulation. Also, the maximal degranulation was obtained with 10 μg mL^{-1} compound 48/80 and 15 min of stimulation. Next, we screened

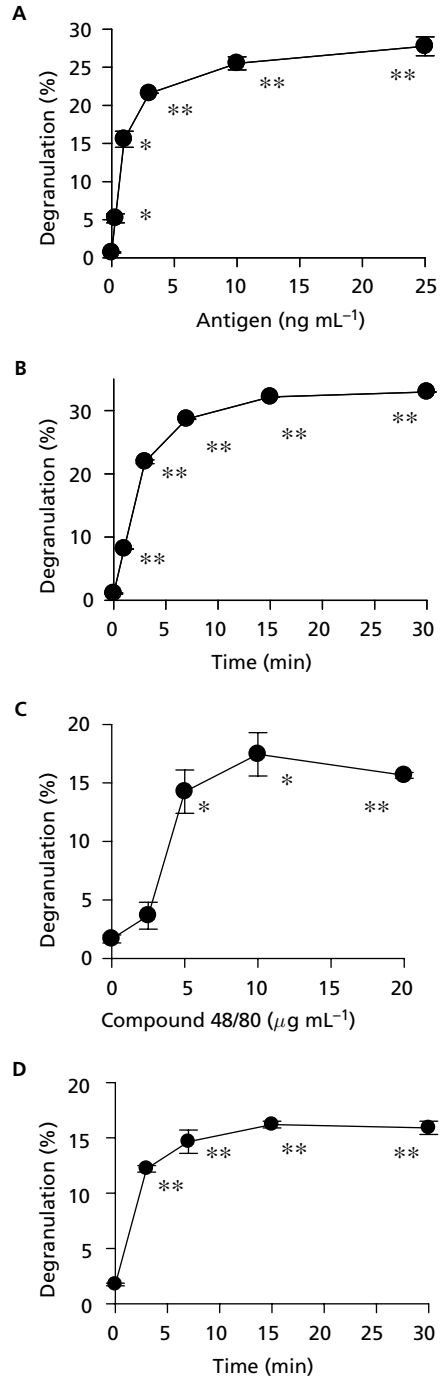

Figure 1 Antigen and compound 48/80 stimulate degranulation in a dose- and time-dependent manner. RBL-2H3 cells were incubated overnight in 24-well cluster plates (2×10^5 cells 0.4 mL^{-1} per well) with DNP-specific IgE for stimulation by antigen or 30 μM quercetin for stimulation by compound 48/80 in complete growth medium. Next day, the medium was replaced with a PIPES-buffered medium as described in the Methods section. Cells were stimulated with 10 ng mL^{-1} DNP-BSA (A and B) or 10 μg mL^{-1} compound 48/80 (C and D) at the concentrations and times indicated for the determination of the release of β-hexosaminidase. The values are means ± s.e.m. from three independent experiments and the asterisks indicate significant differences (*$P < 0.05$; **$P < 0.001$) compared with non-stimulated cells of 0 ng mL^{-1} (A, C) or 0 min (B, D).

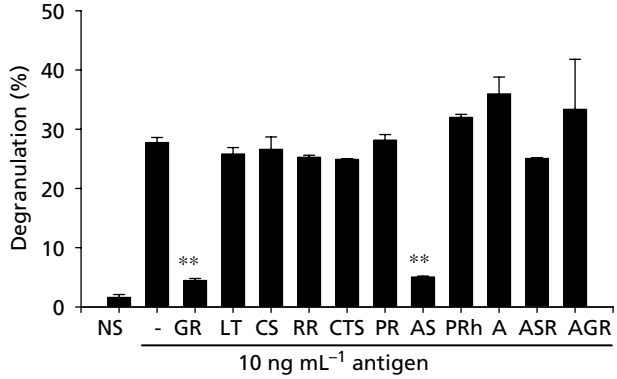

Figure 2 Inhibitory activities of various plant extracts on degranulation. IgE-primed RBL-2H3 cells were stimulated with 10 ng mL^{-1} DNP-BSA for 10 min with or without the pretreatment of 100 μg mL^{-1} ethanol extracts of various herbs for the determination of release of β-hexosaminidase as described in the Methods section. Data are means ± s.e.m. of values from three independent experiments and asterisks indicate significant differences (**$P < 0.001$) compared with antigen-stimulated cells without a plant extract (–). NS, non-stimulated; GR, *Glycyrrhizae radix*; LT, *Liriopis tuber*; CS, *Castaneae semen*; RR, *Rhemaniae radix*; CTS, *Citri tangerinae semen*; PR, *Platycodi radix*; AS, *Arecae semen*; PRh, *Phragmitis rhizoma*; A, *Aloe*; ASR, *Angelicae sinensis radix*; AGR, *Angelicae gigantis radix*.

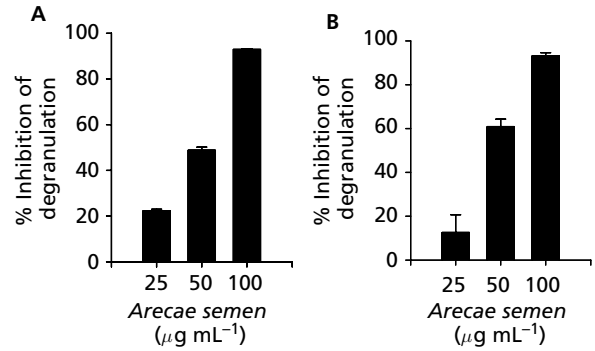

Figure 3 *Arecae semen* inhibits degranulation in a dose-dependent manner. RBL-2H3 cells were incubated overnight in 24-well cluster plates with DNP-specific IgE or 30 μM quercetin in complete growth medium as described for Figure 1. The medium was replaced with a PIPES-buffered medium that contained the indicated concentration of *A. semen* extract before stimulation with DNP-BSA (A) or compound 48/80 (B) for measurement of the release of β-hexosaminidase. The values are the mean ± s.e.m. from three independent experiments.

various oriental medicinal herbs such as *Glycyrrhizae radix*, *Liriopis tuber*, *Castaneae semen*, *Rhemaniae radix*, *Citri tangerinae semen*, *Platycodi radix*, *Arecae semen*, *Phragmitis rhizoma*, *Aloe*, *Angelicae sinensis radix* and *Angelicae gigantis radix* to determine anti-allergic activity (Figure 2). As observed previously, *G. radix* significantly inhibited degranulation in the cells (Kim 2001). *Arecae semen* dramatically suppressed degranulation (Figure 2) in a dose-dependent manner induced by compound 48/80 as well as antigen (Figure 3). The IC_{50} values for antigen and compound 48/80 were approximately 53 and 52 μg mL^{-1}, respectively. Other herbal extracts were inactive in this test. These results indicate that *A. semen* may have a potent anti-allergic activity in the in-vivo animal model.

Table 1 Protective activity of *A. semen* on compound 48/80-induced anaphylaxis

Dose (mg kg^{-1})[a]	Compound 48/80 (8 mg kg^{-1})[b]	Survival rate (%)[c]
Vehicle	+	0.0
20	+	6.3
100	+	43.3
300	+	46.2
300	–	100.0

[a]Administered p.o. in 200 μg 20 g^{-1} mouse 1 h before the i.p. injection of compound 48/80. *Arecae semen* extract was suspended in 5% arabic gum. Eight mice were tested for each of the indicated doses. [b]Administered i.p. in 200 μL 20 g^{-1} mouse. [c]Measured 1 h after injection of compound 48/80. Values are the means of three independent experiments.

Arecae semen extracts inhibit compound 48/80-mediated anaphylaxis in mice

To measure the in-vivo anti-allergic activity of *A. semen* we used the compound 48/80-mediated anaphylactic animal model. Compound 48/80 successfully induced anaphylactic death within 1 h after intraperitoneal injection of 8 mg kg^{-1} to ICR mice. As shown in Table 1, compound 48/80-induced anaphylactic death was dose-dependently inhibited by *A. semen* extract. The anaphylatic death was inhibited by 46% in mice given 300 mg kg^{-1} of *A. semen* extract.

Effects of *Arecae semen* on the phosphorylation of MAP kinase and the expression of TNF-α in RBL-2H3 cells

MAP kinases are stimulated through antigen-induced aggregation of receptors with high affinity for IgE (FcϵRI), which leads to activation of Syk and, ultimately, to the activation of phosphatidylinositol 3-kinase (Turner & Kinet 1999) and the MAP kinases, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAP kinase (Hirasawa et al 1995a; Jabril-Cuenod et al 1996; Zhang et al 1997; Hirasawa et al 1998; Ishizuka et al 1999). Activation of the MAP kinases is associated with the production of various late-phase inflammatory cytokines. Recently, ERK1/2 has been reported as the signal for the production of IL-5, TNF-α, IL-3 and IL-13 in mast cells (Lorentz et al 2003). Also, ERK2 activates phospholipase A2 (cPLA2), which results in the production of arachidonic acid, the precursor of various inflammatory mediators such as leukotriene C_4/B_4 and PGD_2 (Hirasawa et al 1995a, b). We therefore measured the effect of *A. semen* extract on the phosphorylation of ERK1/2 at sites known to promote activation of ERK1/2 in RBL-2H3 cells. As

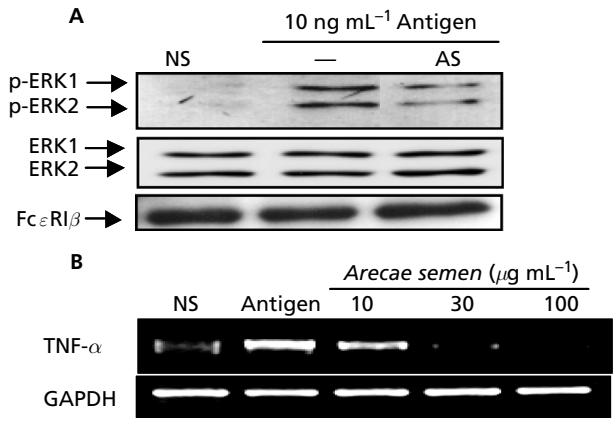

Figure 4 Effect of *A. semen* on the activating phosphorylation of ERK1/2 and the expression of TNF-α. RBL-2H3 cells were incubated overnight in 6-well plates with DNP-specific IgE in complete growth medium. Cells were stimulated with 10 ng mL^{-1} DNP-BSA without or with *A. semen* extract for 15 min and chilled on ice to terminate stimulation. Cells were washed twice with ice-cold PBS. For immunoblotting, cells were lysed in 0.25 mL ice-cold lysis buffer. After centrifugation, the supernatant was dissolved in 2× Laemmli buffer (Laemmli 1970) and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell Inc., NH). The immunoreactive proteins to phospho-ERK1/2, ERK1/2 and FcϵRIβ were detected by use of horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech) (A). After antigen stimulation, total RNA was extracted and the levels of mRNA for TNF-α and GAPDH were determined by RT–PCR as described in the Methods section (B). NS, non-stimulated.

shown in Figure 4A, ERK1/2 phosphorylation was significantly inhibited by *A. semen* extract. This result raised the possibility that *A. semen* may inhibit the generation of inflammatory cytokines at later stages of the allergic response in addition to inhibiting degranulation. We therefore measured the effect of *A. semen* on the expression of TNF-α mRNA. *Arecae semen* significantly suppressed the expression of TNF-α stimulated by antigen in a dose-dependent manner (Figure 4B). Interestingly, TNF-α expression was suppressed under the basal level at 30 and 100 μg mL^{-1} *A. semen*. The inhibition of activation of ERK1/2 and the expression of TNF-α was not associated with the down-regulation of IgE high affinity receptor, FcϵRI (Figure 4A).

Discussion

In many Asian countries, folk herb medicines have been used to alleviate various diseases without the identification of a clear mechanism or the active components involved. As part of the systematic investigation of some of their herbal medicines we have found that *A. semen* may be a valuable anti-allergic medication. In the present study, we measured the anti-allergic effects of various plant extracts using in-vitro and in-vivo animal models. Among the extracts, *A. semen* was selected as a potent plant extract to treat allergic diseases. We showed that *A. semen* pretreatment significantly suppresses compound 48/80-induced systemic anaphylactic reaction in mice and inhibits degranulation induced by DNP-BSA and compound 48/80 in RBL-2H3 cells. *Arecae semen* also inhibits the activation of ERK1/2 and the expression of TNF-α by DNP-BSA.

Stimulation of mast cells with antigen and compound 48/80 initiates the activation of signal transduction pathways that lead to degranulation and generation of various inflammatory cytokines. Several studies have demonstrated that compound 48/80 and other polybasic compounds can activate G proteins (Mousli et al 1990). Recently, Chahdi et al (2000) reported that compound 48/80 activated RBL-2H3 mast cell phospholipase D (PLD) via heterotrimeric GTP-binding proteins. Although compound 48/80 is still used as an experimental model in vivo (Alfonso et al 2000), the compound acts only on certain subtypes of mast cells, such as rat peritoneal mast cells, to induce the rapid release of inflammatory mediators. Rat mucosal mast cells and RBL-2H3 mast cells are not activated by the compound (Swieter et al 1993; Ogasawara et al 1997). However, as shown in this (Figure 1) and a previous study (Senyshyn et al 1998), RBL-2H3 cells become sensitive to compound 48/80 after treatment with 30 μM quercetin for 24 h. The treatment with quercetin increased expression of Gi proteins by more than seven-fold in RBL-2H3 cells and transformed the cell from an unresponsive to a compound 48/80-responsive phenotype, thus providing a useful cell line for our experiments (Senyshyn et al 1998). As shown in Figure 1, the degranulation in RBL-2H3 cells was induced by compound 48/80 with the maximal response of approximately 17% release of granules. *Arecae semen* strongly suppressed degranulation induced by compound 48/80 and DNP-BSA antigen (Figure 3) at concentrations considerably lower than those reported for other anti-allergic medicinal herbs (Yi et al 2002; Hong et al 2003). However, the mechanism of inhibition of degranulation by *A. semen* is unknown and we are currently investigating the effects of *A. semen* on signalling pathways that lead to degranulation.

Stimulated mast cells also produce a variety of cytokines, including interleukins 1, 3, 4, 5 and 6 as well as TNF-α and granulocyte-macrophage colony-stimulating factor (Galli 1993; Baumgartner & Beaven 1996). Typically, increased expression of cytokine mRNA and protein is detectable 30 min and several hours after the addition of stimulant (Gordon et al 1990). Such cytokines, particularly TNF-α, are thought to mediate pathogenic inflammatory reactions at later stages of the allergic reaction (Galli 1993). The activation of ERK1/2 was reported as being an essential signal for the production of IL-5, TNF-α, IL-3 and IL-13 in mast cells (Lorentz et al 2003), and the production of TNF-α in the RBL-2H3 mast cells is dependent on the activation of the ERK2 cascade (Zhang et al 1997). In investigating the mechanism of actions of *A. semen*, we have focused on the ERK activation cascade because of these reports. Based on our findings that both expression of TNF-α and ERK1/2 activation were suppressed by *A. semen*, we suggest that

A. semen inhibits the expression of TNF-α through inhibition of the ERK1/2 cascade. We also suggest that *A. semen* may be useful in the treatment of late-phase allergic symptoms as well as reactions to antigen challenge in allergic diseases.

Conclusions

In this study, we report for the first time that *A. semen* dose-dependently inhibits degranulation of RBL-2H3 cells by DNP-BSA antigen and compound 48/80 with IC_{50} values of approximately 50 $\mu g\,mL^{-1}$ and effectively suppresses in-vivo anaphylatic reaction. In addition, *A. semen* inhibits the expression of TNF-α in a dose-dependent manner as well as activation of ERK1/2, which is a critical signalling event for production of TNF-α. The results suggest that *A. semen* may be a potential candidate for treatment of various allergic diseases. However, further study is necessary to identify the mechanism of action and the active components of *A. semen*.

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