JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. XXXX, XXX, 000–000 A
DOI:10.1021/jf904356q

Mast Cell-Dependent Allergic Responses Are Inhibited by Ethanolic Extract of Adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) Testa

HONG-JHANG CHEN,[†] CHUN-KUANG SHIH,[‡] HSIN-YI HSU,[§] AND WENCHANG CHIANG*,[†]

[†]Graduate Institute of Food Science and Technology, College of Bioresources and Agriculture, National Taiwan University, Taipei, Taiwan, [‡]School of Nutrition and Health Sciences, Taipei Medical University, Taipei, Taiwan, and [§]Departmant of Sport Management, Aletheia University, Taipei, Taiwan

Allergy is an immune dysfunction caused by degranulation from mast cells in the early phase and cytokine secretion in the late phase of the cell. The purpose of this study was to investigate the effects of adlay (Job's tears, *Coix lachryma-jobi* L. var. *ma-yuen* Stapf) testa against β-hexosaminidase release as a marker of degranulation in rat basophilic leukemia (RBL)-2H3 cells. The ethyl acetate fraction from ethanolic extracts of adlay testa (ATE-EtOAc) exhibited potent inhibitory activity that suppressed degranulation from RBL-2H3 cells stimulated by 1 μM A23187. The 20%−80% EtOAc/Hex subfractions of ATE-EtOAc significantly inhibited histamine release with a IC_{50} of 75−100 μg/mL. In addition, the ATE-EtOAc subfractions suppressed interleukin (IL)-4, IL-6, and tumor necrosis factor-α secretion in RBL-2H3 cells, indicating that adlay testa were able to inhibit cytokine secretion. In order to explore the inhibitory mechanism of adlay testa in mast cell degranulation, we examined the activation of intracellular signaling molecules. Adlay testa inhibited the phosphorylation ERK expression. Furthermore, the two major active compounds, 4-hydroxyacetophenone and *p*-coumaric acid, were isolated from the ATE-EtOAc subfractions. These results suggest that ATE had an inhibitory effect on allergic response *via* the ERK signaling transduction in RBL-2H3 cells.

KEYWORDS: Allergy; adlay testa; degranulation; histamine; cytokines; ERK

INTRODUCTION

Mast cells are known to be key effector cells in allergic inflammation such as asthma and inflammatory arthritis (*1*). Chronic activation of mast cells contributes to the pathophysiology of many allergic diseases through the synthesis and release of numerous proinflammatory mediators and cytokines (*2*). Peritoneal mast cells can be obtained by peritoneal lavage of rat. However, they need to be purified, which can impede their ability to react to stimuli (*3*). In addition, it is impossible to maintain mast cells in primary culture over prolonged periods of time (*4*). In our study, we chose RBL-2H3 (rat basophilic leukemia) cells to investigate the antiallergic effects of adlay testa due to their high-affinity Fc receptor for IgE (FcεRI) and the release of proinflammatory mediators such as histamine, IL-4 and tumor necrosis factor (TNF)-α (*5*). Upon the degranulation of mast cells, β-hexosaminidase is also released along with histamine; therefore, here, this enzyme is used as a marker for mast cell degranulation (*6*, *7*).

Adlay (Job's tears, *Coix lachryma-jobi* L. var. *ma-yuen* Stapf), is an annual crop which has long been used in China to treat warts, chapped skin, rheumatism, and neuralgia. Adlay has been reported to have various immunomodulatory activities such as anticomplementary, increasing the activities of cytotoxic T cells and NK cells, and anti-inflammatory and antiallergic effects (*8*−*12*). Benzoxazinoid compounds isolated from adlay roots reduced histamine release in rat peritoneal mast cells (*12*). We showed that a 20% dehulled adlay diet can suppress the production of immunoglobulin E (IgE) against the ovalbumin (OVA) antigen, and modulate Th1/Th2 immune responses *via* reduced interleukin (IL)-5 secretion and increased IL-2 production (*11*). The methanolic extract of the adlay testa shows anti-inflammatory properties which involve inhibition of NO and $\cdot O_2^-$ production by activated macrophages (*10*). Therefore, adlay is expected to be effective in treating allergic symptoms. Previous studies have reported that adlay regulates the secretion of cytokines *in vivo*. However, allergic symptoms are caused not only by an imbalance of cytokines but also by an unusual degranulation of mast cells. The effects of adlay on mast cells remain unclear.

In this study, we prepared an ethanolic extract from adlay testa and evaluated its effects on allergic reactions caused by mast cells. The antiallergic mechanisms were explored.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), calcium ionophore (A23187), dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), and other chemicals were obtained from Sigma Chemical (St. Louis, MO). Fetal bovine serum (FBS) and trypsin-EDTA

*Corresponding author. Tel: 886-2-3366-4115. Fax: 886-2-2363-8673. E-mail: chiang@ntu.edu.tw. Postal address: 1, Roosevelt Road, Sec. 4, Taipei 10617 Taiwan.

© XXXX American Chemical Society

solution were obtained from Biological Industries (Haemek, Israel). Antibodies such as antiphospho-ERK (1/2), -phospho-Akt (Ser473), -phospho-p38, -phospho-JNK, and β-actin were from Cell Signaling (Danvers, MA).

Preparation of Ethanolic Extracts and Various Fractions from Testa of Adlay Seeds. The ethanolic extracts of adlay testa were obtained according to the procedure described by Huang et al. (*10*) as shown in **Figure 1**. Adlay testa powder (12 kg) was extracted three consecutive times with ethanol (95%, 120 L) at room temperature for 24 h, and filtered. The filtrate was concentrated under a vacuum to dryness affording 538 g (4.48%, based on the dry weight) of an ethanolic extract, which was then stored at −20 °C. The ethanolic extracts of adlay testa were referred to as ATE. ATE was then suspended in water with 10% methanol and partitioned with hexane until the hexane fraction was colorless. Thereafter, the hexane fraction (144 g, 1.2%, ATE-Hex) was obtained and dried under a vacuum. The defatted ATE was then partitioned with ethyl acetate (EtOAc) in order to prepare the EtOAc fraction (160 g, 1.33%, ATE-EtOAc). The residue was then partitioned with butanol and dried as described above to obtain the butanolic fraction (50 g, 0.42%, ATE-BuOH). The aliquot fraction was freeze-dried to form the H_2O fraction (122 g, 1.02%, ATE-H_2O). Silica gel is employed for further separation, and the mechanism of isolation depends on the polarity of compounds. Thus, the EtOAc faction was chromatographed successively on silica gel, and the eluting solution with increasing polarity was used to afford the different fractions. ATE-EtOAc was subjected to column chromatography on silica gel and eluted using a Hex/EtOAc/MeOH gradient. The combination was carried out according to the similarity of each collection in TLC to afford eight subfractions: A (∼0−10% EA/Hex), B (∼10−20% EA/Hex), C (∼20−30% EA/hex), D (∼30−50% EA/Hex), E (∼50−80% EA/Hex), F (∼80−100% EA/Hex), G (∼0−20% MeOH/EA), and H (∼20−100% MeOH/EA). Furthermore, the bioassay guided separation was conducted to screen the effective fractions.

Cell Culture. RBL-2H3 cells were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan) and were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 15% heat-inactivated FBS, 100 U/mL penicillin−streptomycin, and 2% glutamine in a 5% CO_2 incubator at 37 °C. Cells were detached with a trypsin-EDTA solution. After washing, cells were resuspended in fresh medium and used for subsequent experiments.

Assay of Cell Viability. Cell viability was evaluated by an MTT assay. Briefly, RBL-2H3 cells were seeded at 10^5 cells/well in a 96-well plate. After washing with Siraganian buffer (119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, and 40 mM NaOH; pH 7.4), cells were pretreated with various test samples and 1 μM A23187 for 12 h. Medium was removed, and then filtered MTT solution was added to each well (2.5 mg MTT/well) and incubated at 37 °C for 2 h. Unreacted dye was removed after 2 h. The insoluble MTT formazan crystals were dissolved in DMSO at room temperature for 15 min. Cell viability was determined by a microplate reader at 570 nm (Molecular Devices, Sunnyvale, CA). The cell viability from various test groups was determined by the equation: (absorbance of the test group/absorbance of the control) × 100. All test samples mentioned above were dissolved in DMSO, and the final concentration of DMSO was <0.1%; 0.1% DMSO was used as the control group.

Assay of β-Hexosaminidase Release. A β-hexosaminidase release assay was carried out according to the method described by Matsuda et al. (*7*) with some modifications. Briefly, RBL-2H3 cells were seeded into 24-well plates at 2×10^5 cells/well and incubated overnight. Cells were washed with Siraganian buffer supplemented with 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% BSA as the incubator buffer, and then incubated in 160 μL of incubator buffer for 10 min at 37 °C. After that, 20 μL of a test sample solution was added to each well and incubated for 10 min, followed by the addition of 20 μL of calcium ionophore (A23187, at a final concentration of 1 μM) at 37 °C for 10 min to stimulate cell degranulation. Luteolin served as the positive control. The reaction was stopped by cooling on ice for 10 min. The cell supernatant (50 μL) was transferred to a 96-well plate and incubated with 50 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 N Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured with a microplate reader at 405 nm (Molecular Devices). The percentage of β-hexosaminidase released was calculated at follows: (supernatant OD value of stimulated cells − supernatant OD value of unstimulated cells) × 100/(the total cell lysate optical density (OD) value − supernatant OD value of unstimulated cells). Total cell lysates were obtained by 1% Triton X-100. IC_{50} values were determined graphically.

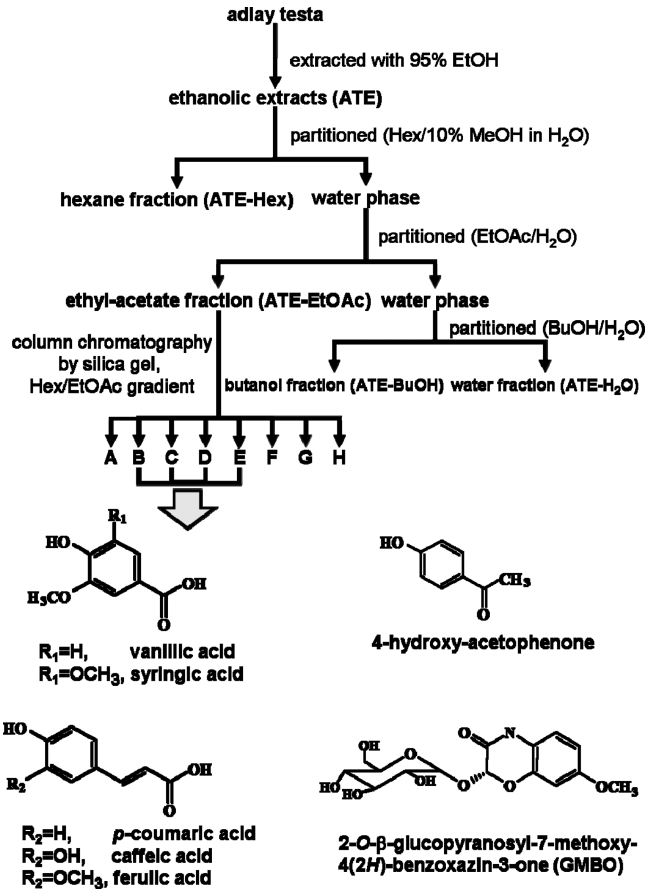

Figure 1. Procedure for extracting, partitioning, and fractionating anti-allergic fractions from adlay testa.

Assay of Histamine Release. The histamine release assay was carried out similarly to A23187-stimulated degranulation as described above. Briefly, RBL-2H3 cells were treated with various test samples and stimulated with 1 μM A23187. Supernatants (100 μL) were collected 20 min after stimulation and assayed for histamine by an enzyme-linked immunosorbent assay kit (IBL, Hamburg, Germany) according to the manufacturer's protocol.

Measurement of IL-4, IL-6 and TNF-α. The inhibitory effects on A23187/phorbol myristate acetate (PMA)-stimulated release of cytokines in RBL-2H3 cells were evaluated similarly to A23187-stimulated degranulation described above. Briefly, RBL-2H3 cells were treated with various test samples and stimulated with 1 μM A23187 and 50 nM PMA for 5 h. The cell supernatants were used to determinate the concentrations of IL-4, IL-6 and TNF-α using commercial ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's protocol.

Western Blotting. Cells were washed twice with ice-cold PBS and then lysed in RIPA lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 500 μM sodium ortho-vanadate, 10 μg/mL aprotinin, 10 mM NaF, 1% Triton X-100, and 0.1% sodium dodecylsulfate (SDS); pH 7.4) on ice for 20 min. The lysates were clarified by centrifugation at 10000*g* for 30 min at 4 °C. Equal amounts of samples were subjected to SDS−polyacrylamide gel electrophoresis (PAGE) using 8% running gels. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% nonfat milk for 1 h at room temperature, and then incubated overnight at 4 °C with antiphospho-p38, antiphospho-ERK, antiphospho-JNK, or anti-β-actin antibodies. After hybridization with primary antibodies, the membrane was incubated with a horseradish peroxidase (HRP)-labeled secondary antibody for 2 h. Final detection was performed with enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Pharmacia Biotech).

Table 1. Antiallergic Activity of Various Fractions and Subfractions of Adlay Testa on Cell Viability and β-Hexosaminidase Release in A23187-Stimulated RBL-2H3 Cells

	inhibition[a] (%)				
	50[b]	100[b]	200[b]	IC_{50} (μg/mL)	cell viability[c] (%)
ATE-Hex	24.6 ± 3.6*[d]	84.0 ± 7.6*	99.6 ± 2.7*	28.1	100.1 ± 6.9
ATE-EtOAc	14.5 ± 10.0	33.1 ± 3.4*	69.8 ± 1.5*	140.8	99.2 ± 7.1
ATE-BuOH	7.4 ± 5.0	15.9 ± 3.8	22.4 ± 5.1*	>200	102.2 ± 2.4
ATE-H_2O	−0.1 ± 6.3	6.7 ± 2.5	13.2 ± 0.6	>200	94.3 ± 5.2

	inhibition[a] (%)				
	25[b]	50[b]	100[b]	IC_{50} (μg/mL)	cell viability[c] (%)
ATE-EtOAc subfraction					
A	6.6 ± 10.9	12.5 ± 14.1	11.7 ± 8.3	>100	93.68 ± 0.4
B	22.3 ± 9.3*	93.5 ± 2.0*	95.5 ± 1.2*	53.5	103.7 ± 1.1
C	15.2 ± 3.1*	74.8 ± 3.3*	81.9 ± 14.3*	65.6	105.8 ± 1.3
D	30.0 ± 1.1*	87.1 ± 4.3*	88.6 ± 7.3*	54.3	102.9 ± 4.9
E	46.0 ± 0.1*	74.7 ± 2.2*	87.4 ± 1.1*	49.0	107.1 ± 0.3
F	19.3 ± 7.7	31.8 ± 5.0*	43.2 ± 2.5*	>100	98.5 ± 2.7
G	13.3 ± 0.5	15.1 ± 2.6*	18.4 ± 2.0*	>100	91.4 ± 2.8
H	0.7 ± 4.7	9.5 ± 0.4	15.0 ± 8.3	>100	92.9 ± 1.6

	inhibition[a] (%)				
	2.5[e]	5[e]	10[e]	IC_{50} (μM)	cell viability[c] (%)
luteolin	28.1 ± 2.7*	50.8 ± 5.2*	80.2 ± 3.7*	5.1	100.7 ± 7.6

[a] Each value represents the mean ± SD of three experiments. [b] Concentration (μg/mL). [c] Concentrations of adlay tests and luteolin used in the cell viability assay were 100 μg/mL and 20 μM, respectively. [d] * indicates significant differences from the control at $P < 0.05$. [e] Concentration (μM).

Statistical Analysis. Results are expressed as the mean ± standard deviation (SD) for three experiments. Differences between specific means were analyzed by one-way analysis of variance (ANOVA) using SPSS, vers. 11.0 (SPSS, Chicago, IL). Group means were compared using one-way ANOVA followed by Dunnett's test. Probability values (P) of < 0.05 were considered statistically significant.

RESULTS

Effects of ATE Fractions on β-Hexosaminidase Release and Cell Viability. To screen for antiallergic fractions, we used the release of β-hexosaminidase by A23187 sensitized RBL-2H3 cells as a high throughput method. As shown in **Table 1**, β-hexosaminidase release was inhibited in the order ATE-Hex > ATE-EtOAc > ATE-BuOH > ATE-H_2O. The lower-polarity fraction of ATE had better inhibition, especially the ATE-Hex which had an IC_{50} value of 28.1 μg/mL. However, the ATE-Hex was difficult to isolate and identify. Therefore, we choose the other effective fraction of ATE, ATE-EtOAc, as the test sample. The ATE-EtOAc fraction effectively suppressed degranulation with an IC_{50} value of 140.8 μg/mL. Thus, ATE-EtOAc was separated into eight subfractions (A to H) according to chromatographic results. The lower-polarity subfractions (20−80% EtOAc/Hex) of ATE-EtOAc-B, -C, -D, and -E presented significant inhibition of cell degranulation at a concentration of 100 μg/mL. In addition, there was no cytotoxicity in any of the fractions in RBL-2H3 cells (**Table 1**). These data indicate that major antiallergic compounds possibly exist in the 20−80% EtOAc/Hex subfractions of ATE-EtOAc. Therefore, these four subfractions (ATE-EtOAc-B, -C, -D, and -E,) were subjected to further investigation.

Effects of ATE-EtOAc Subfractions on Histamine Release. We identified four effective subfractions of ATE-EtOAc which inhibited cell degranulation. Histamine is the major mediator of the allergic response. Therefore, we further examined whether these subfractions of ATE-EtOAc affected the secretion of histamine by cells. These ATE-EtOAc subfractions significantly decreased histamine release from A23187-stimulated RBL-2H3 cells at 50 μg/mL (**Table 2**). Inhibition of histamine release was strongest in the ATE-EtOAc-B subfraction where it reached 80% with 100 μg/mL. These results imply that these subfractions have the potential to mediate allergic activity in cells.

Table 2. Effects of ATE-EtOAc Subfractions on Histamine Release from A23187-Stimulated RBL-2H3 Cells

	inhibition[a] (%)			
	25[b]	50[b]	100[b]	IC_{50} (μg/mL)
ATE-EtOAc-B	27.7 ± 3.8*[c]	41.7 ± 1.2*	59.9 ± 6.4*	74.8
ATE-EtOAc-C	17.8 ± 0.1*	30.7 ± 8.1*	44.9 ± 11.8*	>100
ATE-EtOAc-D	18.2 ± 2.6*	42.9 ± 2.0*	58.4 ± 7.0*	78.5
ATE-EtOAc-E	4.0 ± 10.9	23.8 ± 3.5*	24.3 ± 11.3*	>100

	inhibition[a] (%)			
	5[d]	10[d]	20[d]	IC_{50} (μM)
luteolin	8.3 ± 5.1	46.4 ± 0.5	62.9 ± 0.6	7.1

[a] Each value represents the mean ± SD of three experiments. [b] Concentration (μg/mL). [c] * indicates significant differences from the control at $P < 0.05$. [d] Concentration (μM).

Effects of Subfractions of ATE-EtOAc on Secretion of Cytokines. IL-4, IL-6, and TNF-α are inflammatory mediators that are synthesized from cell membrane phospholipids by activated cells and are involved in allergic reactions. Mast cells secrete IL-4 as the major allergic cytokine. **Figure 2A** shows the inhibitory effects of subfractions of ATE-EtOAc on IL-4 secretion. There was modest inhibition of IL-4 secretion when cells were cultured with 25 and 50 μg/mL of the subfractions together. The high concentration (100 μg/mL) of each ATE-EtOAc subfractions resulted in significant decreases in IL-4 secretion, in particular, IL-4 secretion was inhibited by ∼98% and ∼94% by the ATE-EtOAc-D and -C subfractions, respectively. These obvious effects on IL-4 secretion by the ATE-EtOAc subfractions were comparable to those observed in luteolin-treated cells.

IL-6 is the major inflammatory cytokine released from mast cells in the late phase of the allergic response. As shown in **Figure 2B**, effective subfractions of ATE-EtOAc significantly

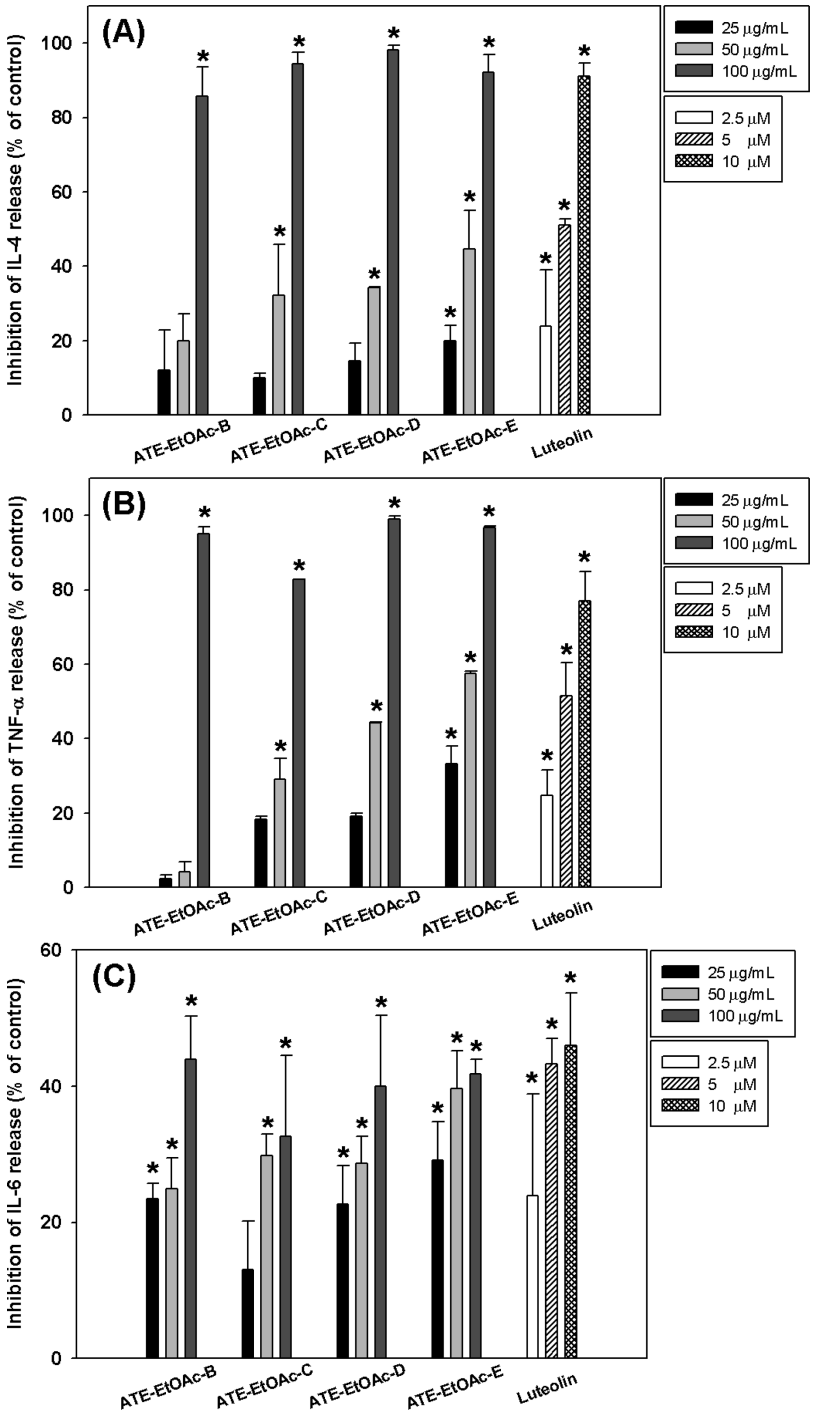

Figure 2. Inhibitory effects of various subfractions of ATE-EtOAc on (**A**) IL-4, (**B**) IL-6, and (**C**) TNF-α secretion in A23187/PMA-stimulated RBL-2H3 cells. Each column represents the mean ± SD of three experiments. * indicates statistically significant differences from the control at $P < 0.05$.

suppressed the secretion of IL-6. IL-6 secretion was inhibited in the order ATE-EtOAc-B > ATE-EtOAc-E > ATE-EtOAc-D > ATE-EtOAc-C; and the respective extents of inhibition were 44%, 42%, 40%, and 33%, at a concentration of 100 μg/mL. Interestingly, ATE-EtOAc-B and -E inhibited IL-6 secretion better the other subfractions. These results indicate that these subfractions regulated IL-4 and IL-6 secretion in different manners.

TNF-α is a potent inflammatory cytokine involved in many pathophysiological conditions including allergies. The ATE-EtOAc subfractions significantly suppressed TNF-α secretion by A23187/PMA-stimulated RBL-2H3 cells (**Figure 2C**). Although ATE-EtOAc-E showed less inhibition of TNF-α secretion, other subfractions suppressed TNF-α secretion in a concentration-dependent manner.

Effects of Subfractions of ATE-EtOAc on Mitogen-Activated Protein Kinase (MAPK) Cascades. Three major MAPKs, the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and p38, are activated in A23187-stimulated RBL-2H3 cells. As shown in **Figure 3**, RBL-2H3 cells treated with 1 μM A23187 alone showed dramatic inductions of the phosphorylation of ERK1/2, but not JNK or p38 (lane 2). The phosphorylation of ERK1/2 was suppressed by ATE-EtOAc subfractions when the concentration was 70 μg/mL (lanes 3−6). Our results showed no effects on the phosphorylation of JNK or p38 MAPKs where cells were treated with various subfractions of

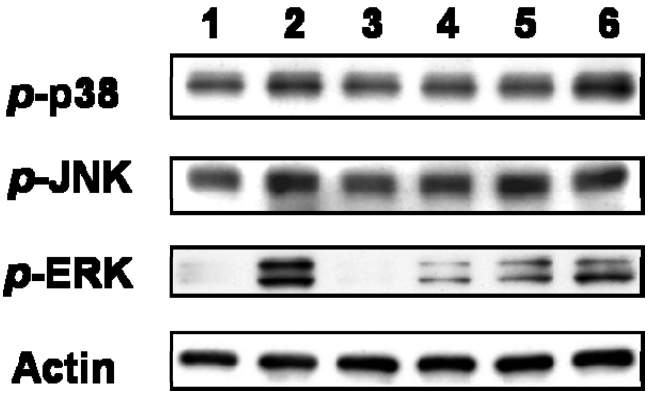

Figure 3. Effects of subfractions of ATE-EtOAc on the signaling pathway in A23187-stimulated RBL-2H3 cells. Cells were untreated (lane 1) or treated with 1 μM A23187 for 20 min (lane 2), and 70 μg/mL of either ATE-EtOAc-B (lane 3), ATE-EtOAc-C (lane 4), ATE-EtOAc-D (lane 5), or ATE-EtOAc-E (lane 6) and then coincubated with 1 μM A23187 for 20 min.

Table 3. Effects of Compounds Isolated from ATE-EtOAc Subfractions on β-Hexosaminidase Release from A23187-Stimulated RBL-2H3 Cells

	inhibition[a] (%)		
	50[b]	100[b]	200[b]
vanillic acid	4.4 ± 2.8	9.2 ± 5.2	10.0 ± 3.8
caffeic acid	2.8 ± 5.3	0.6 ± 3.4	2.7 ± 0.3
syringic acid	3.0 ± 9.9	4.2 ± 0.2	2.0 ± 2.7
GMBO	1.0 ± 8.8	5.3 ± 2.8	8.8 ± 6.7
4-hydroxyacetophenone	10.2 ± 2.8*[c]	18.2 ± 4.9*	30.7 ± 4.4*
p-coumaric acid	16.5 ± 10.6	17.9 ± 6.9*	22.9 ± 2.8*
ferulic acid	0.5 ± 5.9	3.9 ± 4.6	5.5 ± 6.5

[a] Each value represents the mean ± SD of three experiments. [b] Concentration (μg/mL). [c] * indicates significant differences from the control at $P < 0.05$.

ATE-EtOAc. It should be noted that ATE-EtOAc-B and -C suppressed the phosphorylation of ERK (lanes 3 and 4, respective) more strongly than the other fractions. These results again indicate that at least two antiallergic compounds exist in adlay testa.

Effects of Phenolic Compounds in ATE on β-Hexosaminidase Release from A23187-Induced RBL-2H3 Cells. The following components were found in ATE after purification and HPLC analysis: vanillic acid, caffeic acid, syringic acid, 4-hydroxyacetophenone, *p*-coumaric acid, ferulic acid, and 2-*O*-β-glucopyranosyl-7-methoxy-4(2*H*)-benzoxazin-3-one (GMBO) (*10*). The antiallergic activities of various phenolic compounds of ATE in the degranulation in RBL-2H3 cells are shown in **Table 3**. 4-Hydroxyacetophenone and *p*-coumaric acid had the greatest effects on the degranulation. The other compounds had no effects on the antiallergic activities.

DISCUSSION

Allergy (a type I hypersensitivity) is an immune dysfunction. It is a serious health problem worldwide. Taking advantage of dietary regimens to regulate the immune system, in order to reduce allergic symptoms and avoid costly drugs, has become a popular concept (*13*). Previously, antiallergic compounds have been isolated from adlay roots (*12*), and in our previous study, we also found compounds from ethyl acetate fractions of adlay testa, which had lower polarity than compounds exhibiting antiallergic activity. These results indicate that various antiallergic compounds exist in adlay.

RBL-2H3 cells were used due to the high affinity Fc receptor for IgE (FcεRI) and the release of proinflammatory mediators such as histamine, interleukin-4 (IL-4) and tumor necrosis factor-alpha (TNF-α) (*5*). Mast cell secretory granules contain β-hexosaminidase, which is released in a quantitative relation to histamine after the immunologic stimulation of cells (*14*). In order to characterize the effects of various fractions of adlay testa, we used a β-hexosaminidase release assay as a high-throughput method to screen adlay samples. Ca^{2+} ionophores such as A23187 have been shown to induce histamine release by directly increasing intracellular Ca^{2+} level (*15*). Therefore, we chose 1 μM A23187 as the stimulation agent to induce ~30−60% ratios of β-hexosaminidase release. Luteolin, a common flavonol abundant in plants, served as an antiallergic agent (*16*). **Table 1** shows that 5 μM of luteolin markedly decreased β-hexosaminidase release (51%, $P < 0.05$), indicating that this throughput model can be used to assess antiallergic effects. Our results showed that adlay testa significantly inhibited the allergic response in RBL-2H3 cells. Furthermore, the ATE-EtOAc-B and -D subfractions suppressed histamine release by A23187-stmulated RBL-2H3 cells at IC_{50} values of 49−53 μg/mL (**Table 1**). The rates of inhibition of histamine release were ~90% for ATE-EtOAc-B and -D at concentrations of 100 μg/mL. This finding suggests that adlay testa has potent antiallergic activity *via* suppression of the degranulation of mast cells.

Mast cells are a potential source of inflammatory cytokines such as IL-4, IL-6, and TNF-α and play key roles in allergic diseases (*5*). The ATE-EtOAc subfractions significantly suppressed inflammatory cytokines, especially the secretion of IL-4 (**Figure 2A**). Furthermore, in A23187/PMA-stimulated RBL-2H3 cells, the ATE-EtOAc subfractions decreased the secretion of TNF-α (**Figure 2C**). The ATE-EtOAc subfractions decreased the phosphorylation of ERK, suggesting the release of anti-TNF-α. Although inhibition of IL-6 secretion by the ATE-EtOAc subfractions was not as strong as the inhibition of TNF-α (**Figure 2B**), the ATE-EtOAc subfractions significantly decreased IL-6 secretion, indicating that adlay testa suppressed Th2 cytokines as well as inflammatory cytokines. Various compounds may be involved in different mechanisms to suppress the allergic response. It was previously reported that two major antiallergic compounds isolated from the rhizomes of *Dioscorea membranacea* showed different effects. Dioscorealide B isolated from *D. membranacea* inhibited the degranulation of mast cells in the early phase of the allergic response, but dioscorealide A suppressed cytokine secretion in the late phase (*6*). Similarly, we also found that major subfractions in ATE-EtOAc-B showed better inhibition of the degranulation of mast cells, while ATE-EtOAc-D and -E suppressed the secretion of cytokines.

MAPK pathways are major target mechanisms for asthma therapy (*17*). The phosphorylation of ERK is the main signal for the degranulation of mast cells in the early phase of the allergic response. The ATE-EtOAc subfractions showed antidegranulation effects through suppression of ERK phosphorylation (**Figure 3**). Several studies have shown similar effects from various antiallergic compounds. For example, Ginsenodise Rb1 from *Panax ginseng* has antiallergic effects which are evidenced by decreased IL-4 secretion through the MAPK pathway (*18*). Therefore, these MAPK-activated mediators, including antihistamines, IL-4 antagonists, and anti-inflammatory compounds, are useful targets in current allergic therapy (*19*). Together, the combined effects of modulating the signaling transduction in RBL-2H3 cells show that adlay testa has antiallergic effects.

Cereals are known to have antioxidative stress effects due to their phenolic compounds (*20*). Our study shows that adlay testa contains *p*-coumaric acid and 4-hydroxyacetophenone as antiallergic compounds. It should be noted that the GMBO had no effect in our experiment. Otsuka et al. reported that the aglycon of

GMBO displayed strong antiallergic activity. *In vivo*, the glycoside is hydrolyzed; thus, results may be different (*12*). Furthermore, ferulic acid has strong antioxidant and anti-inflammation activities. It has been reported that ferulic acid has antiallergic activity in animal model (*21*). However, in our study, the ferulic acid cannot suppress the degranulation of mast cell. Therefore, the antiallergic activity of ferulic acid may not occur through the inhibition of degranulation of mast cells. Besides, food has complex constituents which have different functions. Therefore, further studies to analyze the profile of compounds are being undertaken using LC/MS.

In conclusion, the ATE suppresses the histamine and inflammatory cytokine releases in sensitive-RBL-2H3 cells resulting in antiallergic activity. Furthermore, the active compounds were identified from the subfractions of ATE. These results show that ATE is a candidate for effective therapeutic material for allergic diseases.

ABBREVIATIONS USED

ATE, ethanolic extract of adlay testa; ATE-BuOH, 1-butanol-soluble fraction from the ethanolic extract of adlay testa; ATE-EtOAc, ethyl acetate-soluble fraction from the ethanolic extract of adlay testa; ATE-Hex, *n*-hexane-soluble fraction from the ethanolic extract of adlay testa; ATE-H_2O, water-soluble fraction from the ethanolic extract of adlay testa; TLC, thin layer chromatography; DMSO, dimethyl sulfoxide; FcεRI, Fc receptor for IgE; IL, interleukin; MAPK, mitogen-activated protein kinases; NF, nuclear factor; OVA, ovalbumin; RBL, rat basophilic leukemia; TNF, tumor necrosis factor.

LITERATURE CITED

(1) Puxeddu, I.; Piliponsky, A. M.; Bachelet, I.; Levi-Schaffer, F. Mast cells in allergy and beyond. *Int. J. Biochem. Cell Biol.* **2003**, *35* (12), 1601–7.

(2) Bradding, P.; Walls, A. F.; Holgate, S. T. The role of the mast cell in the pathophysiology of asthma. *J. Allergy Clin. Immunol.* **2006**, *117* (6), 1277–84.

(3) Coutts, S. M.; Nehring, R. E., Jr.; Jariwala, N. U. Purification of rat peritoneal mast cells: occupation of IgE-receptors by IgE prevents loss of the receptors. *J. Immunol.* **1980**, *124* (5), 2309–15.

(4) Horigome, K.; Bullock, E. D.; Johnson, E. M., Jr. Effects of nerve growth factor on rat peritoneal mast cells. Survival promotion and immediate-early gene induction. *J. Biol. Chem.* **1994**, *269* (4), 2695–702.

(5) Bischoff, S. C. Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. *Nat. Rev. Immunol.* **2007**, *7* (2), 93–104.

(6) Tewtrakul, S.; Itharat, A. Anti-allergic substances from the rhizomes of *Dioscorea membranacea*. *Bioorg. Med. Chem.* **2006**, *14* (24), 8707–11.

(7) Matsuda, H.; Tewtrakul, S.; Morikawa, T.; Nakamura, A.; Yoshikawa, M. Anti-allergic principles from Thai zedoary: structural requirements of curcuminoids for inhibition of degranulation and effect on the release of TNF-alpha and IL-4 in RBL-2H3 cells. *Bioorg. Med. Chem.* **2004**, *12* (22), 5891–8.

(8) Takahashi, M.; Konno, C.; Hikino, H. Isolation and hypoglycemic activity of coixans A, B and C, glycans of *Coix lachryma-jobi* var. *ma-yuen* seeds. *Planta Med.* **1986**, No. 1, 64–5.

(9) Hidaka, Y.; Kaneda, T.; Amino, N.; Miyai, K. Chinese medicine, Coix seeds increase peripheral cytotoxic T and NK cells. *Biotherapy* **1992**, *5* (3), 201–3.

(10) Huang, D. W.; Kuo, Y. H.; Lin, F. Y.; Lin, Y. L.; Chiang, W. Effect of Adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) Testa and its phenolic components on Cu^{2+}-treated low-density lipoprotein (LDL) oxidation and lipopolysaccharide (LPS)-induced inflammation in RAW 264.7 macrophages. *J. Agric. Food Chem.* **2009**, *57* (6), 2259–66.

(11) Hsu, H. Y.; Lin, B. F.; Lin, J. Y.; Kuo, C. C.; Chiang, W. Suppression of allergic reactions by dehulled adlay in association with the balance of TH1/TH2 cell responses. *J. Agric. Food Chem.* **2003**, *51* (13), 3763–9.

(12) Otsuka, H.; Hirai, Y.; Nagao, T.; Yamasaki, K. Anti-inflammatory activity of benzoxazinoids from roots of *Coix lachryma-jobi* var. *ma-yuen*. *J. Nat. Prod.* **1988**, *51* (1), 74–9.

(13) Paul, A. T.; Gohil, V. M.; Bhutani, K. K. Modulating TNF-alpha signaling with natural products. *Drug Discovery Today* **2006**, *11* (15−16), 725–32.

(14) Schwartz, L. B.; Austen, K. F.; Wasserman, S. I. Immunologic release of beta-hexosaminidase and beta-glucuronidase from purified rat serosal mast cells. *J. Immunol.* **1979**, *123* (4), 1445–50.

(15) Baumgartner, R. A.; Yamada, K.; Deramo, V. A.; Beaven, M. A. Secretion of TNF from a rat mast cell line is a brefeldin A-sensitive and a calcium/protein kinase C-regulated process. *J. Immunol.* **1994**, *153* (6), 2609–17.

(16) Kimata, M.; Shichijo, M.; Miura, T.; Serizawa, I.; Inagaki, N.; Nagai, H. Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. *Clin. Exp. Allergy* **2000**, *30* (4), 501–8.

(17) Gilfillan, A. M.; Tkaczyk, C. Integrated signalling pathways for mast-cell activation. *Nat. Rev. Immunol.* **2006**, *6* (3), 218–30.

(18) Liao, B. C.; Hou, R. C.; Wang, J. S.; Jeng, K. C. Enhancement of the release of inflammatory mediators by substance P in rat basophilic leukemia RBL-2H3 cells. *J. Biomed. Sci.* **2006**, *13* (5), 613–9.

(19) Walsh, G. M. Novel therapies for asthma—advances and problems. *Curr. Pharm. Des.* **2005**, *11* (23), 3027–38.

(20) Baublis, A. J.; Lu, C.; Clydesdale, F. M.; Decker, E. A. Potential of wheat-based breakfast cereals as a source of dietary antioxidants. *J. Am. Coll. Nutr.* **2000**, *19* (3 Suppl.), 308S–311S.

(21) Hu, H. J.; Hang, B. Q. [Effects of ferulic acid on allergic reaction]. *Zhongguo Yaoli Xuebao* **1991**, *12* (5), 426–30.

Received for review December 9, 2009. Revised manuscript received January 10, 2010. Accepted January 10, 2010.