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Inhibitory effect of *Zizania latifolia* chloroform fraction on allergy-related mediator production in RBL-2H3 cells

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Abstract *Zizania latifolia* exhibits anti-inflammatory and anti-allergic effects; however, the mechanisms behind these effects are unknown. Here the ethanol extract of *Z. latifolia* was partitioned using hexane, chloroform, ethyl acetate, butanol, and water. Subsequently, the anti-allergic effects of these fractions were evaluated *in vitro*. The results showed that the chloroform fraction of *Z. latifolia* inhibited the release of β-hexosaminidase and tumor necrosis factor (TNF-α) from RBL-2H3 cells stimulated with dinitrophenyl-bovine serum albumin (DNP-BSA). In addition, this fraction suppressed the expression of cyclooxygenase-2 (COX-2) and inhibited the activation of mitogen-activated protein kinases (MAPKs). The results obtained suggest that the chloroform fraction of *Z. latifolia* inhibited mast cell-mediated allergic inflammatory responses.

Keywords: *Zizania latifolia*, anti-allergic, RBL-2H3 cells, COX-2, MAPK

Introduction

During past decades, the number of patients with allergies has increased substantially and around 10 to 20% of the world population suffers from allergies (1). Allergic responses are classified into four types, namely, anaphylactic hypersensitivity (Type I), cytotoxic hypersensitivity (Type II), immune complex hypersensitivity (Type III), and delayed hypersensitivity (Type IV), based on the mechanisms involved and time taken for the reaction. Type I hypersensitivity, commonly equated with allergy, is caused by the binding of antigen to the high-affinity immunoglobulin E (IgE) receptor (FcεRI) on the surface of mast cells or basophils (2). Both mast cells and basophils are histamine-containing granulated cells, and they release histamine upon activation (3), thus playing a crucial role in allergic reactions. The rat basophilic leukemia cells, RBL-2H3, are a tumor analogue of mast cells with a high expression of FcεRI on the cell surface and can be activated by the IgE-antigen complex. After stimulation with antigens, RBL-2H3 cells can also release histamine and various inflammatory cytokines (tumor necrosis factor (TNF)-α and interleukins) in the process of degranulation (4,5). Hence, these cells have been generally used for studying IgE–FcεRI interactions involving the intracellular signaling pathway of degranulation (6-8), which if inhibited, could be effective for preventing allergic disorders (9). Nonetheless, most medicines that antagonize the inflammatory mediators released from the activated mast cells or basophils for attenuating Type I allergies do not significantly inhibit the degranulation of basophils or mast cells (10).

Z. latifolia Turcz is an East Asian plant found in the margins of rivers and lakes. Both its grains and stems are edible and appreciated for their flavor, and the plant is commonly known as wild rice. Compositional analysis of the *Z. latiofolia* grain revealed that it has high content of protein, fiber, and lysine and low content of fat (11). Rats fed with *Z. latifolia* grains showed substantially improved blood lipid profiles and suppressed oxidative stress (12). Supplementation with *Z. latifolia* grains was also reported to suppress abnormal glucose metabolism and insulin resistance in rats fed with a diet comprising high contents of fat and cholesterol (13). Moreover, we previously described the anti-inflammatory and anti-allergic effects of the methanol extract and five tricin derivatives from the stems and leaves of *Z. latifolia* (14,15); however, the mechanism of action responsible for these anti-allergic effects is still unknown. Therefore, here we used RBL-2H3 cells stimulated by an IgE-antigen complex to elucidate the anti-allergic mechanism of the action of *Z. latifolia* leaf and stem extracts.

Materials and Methods

Chemicals Chemicals and cell culture materials were obtained from the following sources: anti-dinitrophenyl (DNP)-bovine serum albumin (BSA), a crosslinking antigen from Alpha Diagnostic International (San Antonio, TX, USA); anti-DNP IgE, *p*-nitrophenyl-*N*-

acetyl-β-D-glucosaminide, 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), calcium ionophore A23187, and phorbol 13-myristate 13-acetate (PMA), a protein kinase C activator, from Sigma-Aldrich (St. Louis, MO, USA); fetal bovine serum (FBS), antibiotics, and cell culture medium from Gibco BRL (Rockville, MD, USA); rat TNF-α ELISA from Invitrogen Corporation (Carlsbad, CA, USA); anti-mouse cyclooxygenase-2 (COX-2), anti-mouse phospho-p44/42 mitogen-activated protein kinases (MAPK) (p-ERK), anti-mouse phospho-SAPK/JNK (p-JNK), anti-mouse phospho-p38 MAPK (p-p38), anti-mouse phospho-Akt (p-Akt), and HRP-linked anti-mouse IgG from Cell Signaling Technology (Danvers, MA, USA); ECL detection kit from Amersham Bioscience (Buckinghamshire, England); immobilon-P transfer membrane from Millipore (Billerica, MA, USA); and RBL-2H3 cells from the Korean Cell Line Bank (Seoul, Korea).

Plant extract Dried leaves and stems of *Z. latifolia* were bought from a market in Hayang-eup, Gyeongsangbuk-do, Republic of Korea. The plant material was extracted with 70% ethanol. The total extract was successively partitioned with n-hexane, $CHCl_3$, EtOAc, n-BuOH, and H_2O. Each fraction was dried and the yield was 0.94, 0.55, 0.23, 1.21, and 10.43%, respectively. Further, each fraction was placed in a plastic bottle and stored at −80°C until use.

Cell culture Rat basophilic leukemia RBL-2H3 cells were cultured in MEM medium (Gibco BRL) with 10% FBS and 1% penicillin/streptomycin. Cells were cultured at 37°C under humidified air with 5% CO_2.

Determination of cell viability Cell viability was determined by the MTT assay. RBL-2H3 cells were plated at a density of 3×10^4 cells/well in 96-well microplates and cultured with 450 ng/mL of DNP-specific IgE at 37°C for 24 h. The IgE-sensitized RBL-2H3 cells were then washed with fresh medium and treated with various sample concentrations. After 12 h of incubation, 100 μL of MTT (5 mg/mL) was added, and the cells were further incubated for 4 h. Finally, DMSO was added to solubilize the formazan salt formed, and the amount of formazan salt was determined by measuring the absorbance at 550 nm using a microplate reader (Spectra MAX 340 pc, Molecular Device, Sunnyvale, CA, USA). The density of formazan formed in the control cells indicated 100% viability.

Antigen-induced β-hexosaminidase release To investigate the inhibition of degranulation, β-hexosaminidase released from antigen-stimulated RBL-2H3 cells was assessed using the method of Matsuda *et al*. (16) with modifications. The cells (5×10^5 cells/well) were sensitized overnight with 450 ng/mL of DNP-specific IgE. The cells were then washed with Siraganian buffer (119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, 40 mM NaOH; pH 7.2) and cultured in 160 μL of the incubation buffer for 20 min at 37°C. Next, the cells were treated with *Z. latifolia* extracts (20 μL) for 10 min, followed by the addition of 20 μL of 10 μg/mL DNP-BSA and incubation for 10 min at 37°C to encourage cell granulation. The reaction was ended by cooling for 10 min in an ice bath. Subsequently, 25 mL of the supernatant was moved to a 96-well plate and cultured for 1 h with 25 μL substrate (1 mM ρ-nitrophenyl-N-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37°C. The reaction was ended by adding 200 μL of 0.1 M Na_2CO_3/$NaHCO_3$ (pH 10.0), and the absorbance was measured at a 405-nm wavelength on the microplate reader. The sample was dissolved in DMSO and was added to the incubation buffer to reach a final concentration of 0.1% DMSO.

Production of TNF-α induced by A23187 and PMA The inhibition of A23187 and PMA-induced release of TNF-α from the RBL-2H3 cells by the extract was assessed by using a previously reported method (17) using an ELISA kit. The RBL-2H3 cells (2×10^5 cells/well) were cultured with 1 μM A23187 and 50 ng/mL PMA for 4 h at 37°C after exposure to 0, 5, 10, 25, or 50 μg/mL of the $CHCl_3$ fraction of *Z. latifolia*. Next, the supernatants were divided and stored at −80°C until use. The release of TNF-α was estimated from the absorbance measured at a 450-nm wavelength on the microplate reader.

Western blot analysis The RBL-2H3 cells (1×10^6 cells/well) were sensitized overnight with 450 ng/mL of DNP-specific IgE. The cells were pretreated with various concentrations of the $CHCl_3$ fraction (0, 5, 10, 25, and 50 μg/mL) and stimulated with 10 μg/mL of DNP-BSA. To prepare cell lysate, the cells were washed twice with ice-cold phosphate-buffered saline and harvested. The cell pellets were resuspended in RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 150 mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1% NP-40. The cells were disrupted and extracted for 30 min at 4°C. After centrifugation (9,447×*g*, 15 min), the cell lysate was obtained as a supernatant. The cell lysate was then boiled, separated on 10% SDS-PAGE, and moved to a nitrocellulose membrane, which was then incubated with 5% skim milk for 30 min at room temperature. Then, the membrane was incubated with primary antibodies and the appropriate secondary horseradish peroxidase-conjugated antibodies. The blots were probed with the ECL and Western blot detection system according to the manufacturer's instructions. Loading differences were normalized using polyclonal β-actin antibodies.

GC-MS analysis of the $CHCl_3$ fraction deivatization The most potent fraction of the *Z. latifolia* extract was subjected to GC-MS analysis using a GCMS-QP2010 Plus (Shimazu, Kyoto, Japan) equipped with an RTX-1 GC column (Restek, Bellefonte, PA, USA; 0.25 mm i.d.×30 m) and operated at 70 eV. Helium was used as the carrier gas. The initial column temperature was 120°C for 2 min, increased at a rate of 4°C/min to 220°C, 6°C/min to 250°C, 14°C/min to 320°C, and finally held at 320°C for 15 min. The injector, ion source, and interface temperatures were 220, 200, and 280°C,

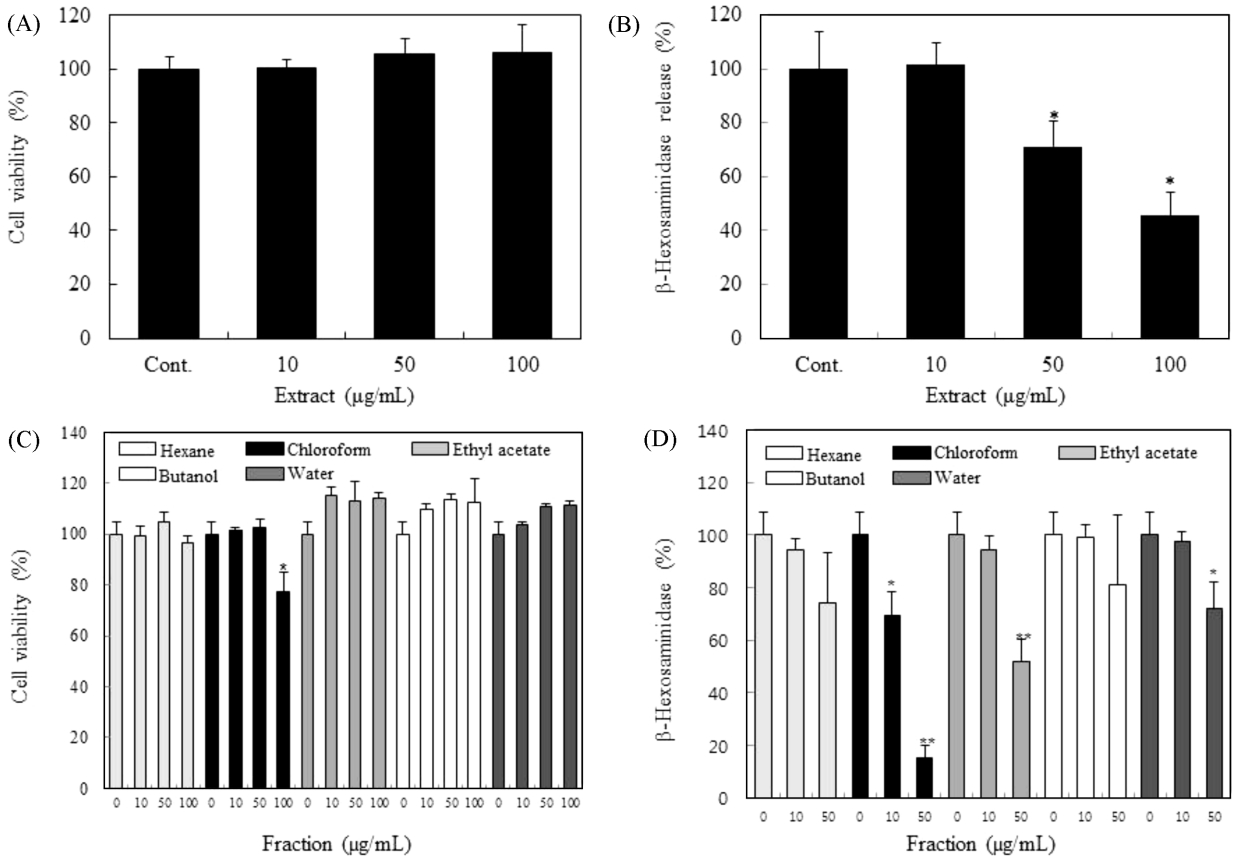

Fig. 1. Effects of *Z. latifolia* extract and its fractions on cell viability (A, C) and β-hexosaminidase release (B, D) from sensitized RBL-2H3 cells. RBL-2H3 cells (3×10^4 cells) were cultured overnight with 450 ng/mL of DNP-specific IgE. After treatment with various doses of *Z. latifolia* extract and its fractions, cells were cultured for 12 h. The cytotoxicities of *Z. latifolia* extract and its fractions on RBL-2H3 cells were determined by the MTT assay. The value represents mean±standard deviation (SD) of three independent experiments; **p*<0.05, ***p*<0.01 compared to the control.

respectively. Total-ion chromatograms were recorded in a mass range of 35–700 amu. The components were tentatively identified by comparing their mass spectra with those in the NIST and WILEY libraries of the GC-MS system and literature.

Statistical analysis All experiments were performed in triplicate. Data are shown as the mean±standard deviation. The Student's *t* test was used to detect significant differences; statistical significance was set at $p<0.05$.

Results and Discussion

Effects of the extract and its various fractions on the antigen-induced β-hexosaminidase release The anti-allergic effect was evaluated *in vitro* using RBL-2H3 cells. The cells were sensitized with 450 ng/mL of DNP-specific IgE overnight before treating them with the DNP-BSA antigen. An MTT assay was performed to assess the effects of the 70% ethanol extract from *Z. latifolia* and its various fractions on the viability of the RBL-2H3 cells. The viabilities of the IgE-sensitized RBL-2H3 cells after exposure to 0, 10, 50, and 100 μg/mL of the *Z. latifolia* extract and its fractions and 12 h of incubation are shown in Fig. 1A and 1C. No cytotoxicity was observed after treatment with the various samples except for the $CHCl_3$ fraction at 100 μg/mL, which decreased cell viability by approximately 23% (Fig. 1C).

β-Hexosaminidase release was used as a degranulation marker. The inhibitory effects of the 70% EtOH extract of *Z. latifolia* and its fractions on the DNP-BSA-induced β-hexosaminidase release from the IgE-sensitized RBL-2H3 cells are shown in Fig. 1B and 1D. β-Hexosaminidase release was inhibited by the extract in a dose-dependent fashion. At concentrations of 50 and 100 μg/mL, the EtOH extract inhibited β-hexosaminidase release by approximately 29 and 55%, respectively. Among the fractions, the $CHCl_3$ fraction exhibited the strongest inhibitory effect (85 and 31% inhibition at 50 and 10 μg/mL, respectively), followed by the EtOAc fraction (48% inhibition at 50 μg/mL).

When mast cells are activated by an antigen or degranulation inducer, histamine is released together with β-hexosaminidase. Therefore, β-hexosaminidase is a marker for histamine release from mast cells. Several inhibitors against β-hexosaminidase release have been reported, including coumarins, flavonoids, alkaloids, terpenoids, and peptides (18).

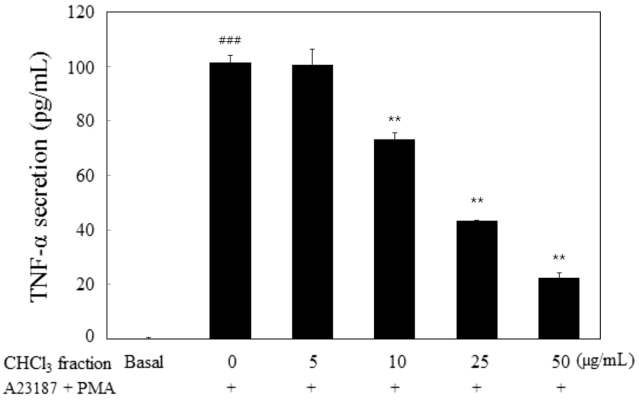

Fig. 2. Effect of the $CHCl_3$ fraction on A23187 and PMA-induced TNF-α secretion from RBL-2H3 cells. The RBL-2H3 cells (2×10^5 cells) were pretreated with various concentrations of the $CHCl_3$ fraction and then stimulated with PMA (50 ng/mL) and A23187 (1 mM) for 4 h before overnight incubation in a 24-well plate. The TNF-α concentration in the supernatant was measured using ELISA. The value represents the mean±SD of three independent experiments; $^{###}p<0.001$ compared to the basal, $^{**}p<0.01$ compared to the control (stimulated with A23187 and PMA).

Inhibitory effect of the $CHCl_3$ fraction on TNF-α production The effect of the $CHCl_3$ fraction on the production of TNF-α by the RBL-2H3 cells stimulated with calcium ionophore A23187 and PMA, which acted as agents capable of bypass the IgE receptor and stimulating mast cells, was examined. Calcium ionophore A23187 increases intracellular Ca^{2+} and induces the release of inflammatory mediators alongside PMA, a PKC activator, to a level of secretion equivalent to that produced by an antigen (19,20). As shown in Fig. 2, exposure to calcium ionophore A23187 and PMA augmented the production of TNF-α by the RBL-2H3 cells, whereas treatment with the $CHCl_3$ fraction significantly suppressed the secretion of TNF-α in a dose-dependent manner. At concentrations of 10, 25, and 50 μg/mL, the inhibition rates were approximately 27, 56, and 78%, respectively.

Mast cells activated with antigens can generate proinflammatory mediators (cytokines and chemokines) that induce allergic reactions. One of these mediators is TNF-α, a well-known proinflammatory cytokine. Thus, to prevent allergic reactions, inhibiting mediator release from mast cells is considered a promising strategy (18). Here the $CHCl_3$ fraction from *Z. latifolia* appears to exert anti-allergic effects by inhibiting the release of proinflammatory TNF-α from mast cells.

Effects of the $CHCl_3$ fraction on antigen-induced expression of COX-2 It has been reported that induction of allergic reactions and mast cell-mediated inflammation is regulated by COX-2 (21,22). COX is present in two different enzyme isoforms: COX-1 and COX-2. COX-1 produces low levels of prostaglandins, which are required for normal

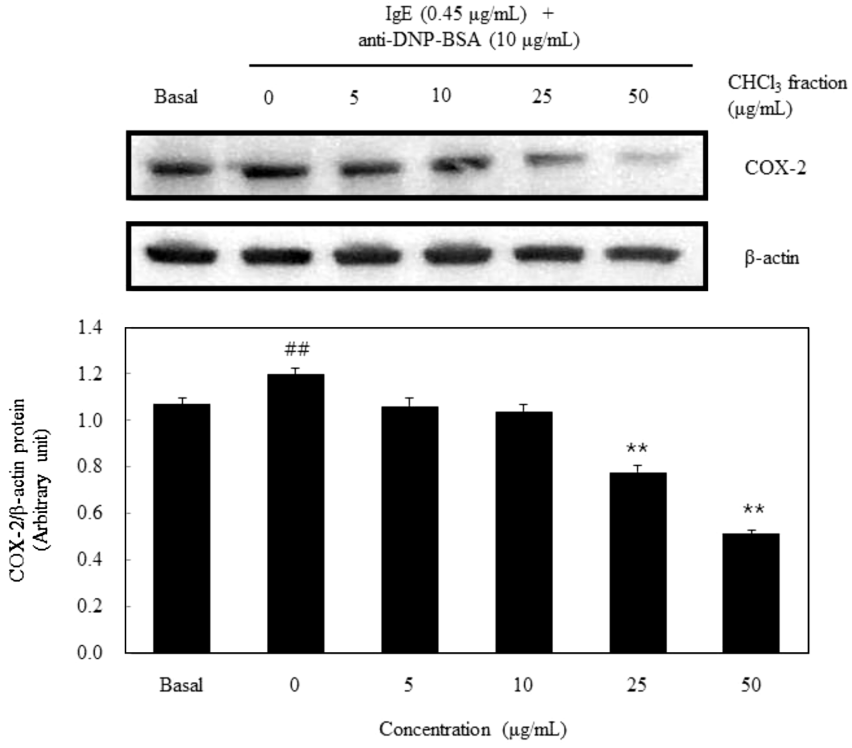

Fig. 3. Effect of the $CHCl_3$ fraction on the expression of COX-2 in RBL-2H3 cells. RBL-2H3 cells (1×10^6 cells/mL) were sensitized overnight with 450 ng/mL of DNP-specific IgE. After washing, cells were pretreated with various doses of the $CHCl_3$ fraction (5, 10, 25, and 50 μg/mL) in PIPES buffer for 30 min and then stimulated with 10 μg/mL of DNP-BSA for 4 h. The proteins derived from the RBL-2H3 cell lysates were subjected to Western blot analysis to detect COX-2 expression (β-actin was used as a protein loading control). Representative results of three independent experiments are shown; $^{##}p<0.01$ compared to the basal, $^{**}p<0.01$ compared to the control (sensitized with anti-DNP IgE and stimulated with DNP-BSA).

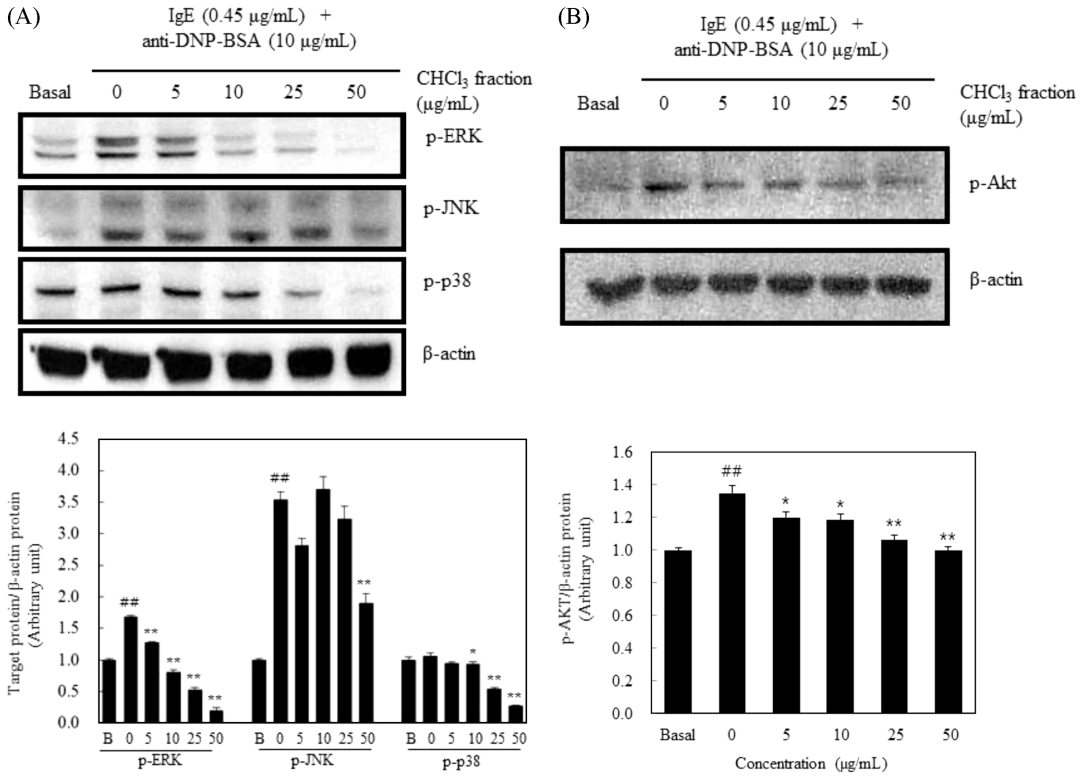

Fig. 4. Effects of the $CHCl_3$ fraction on MAPKs (A) and Akt (B) phosphorylation in RBL-2H3 cells. RBL-2H3 cells (1×10^6 cells/mL) were sensitized overnight with 450 ng/mL of DNP-specific IgE. After washing, the cells were pretreated with various doses of the $CHCl_3$ fraction (5, 10, 25, and 50 μg/mL) in PIPES buffer and then stimulated with 10 μg/mL of DNP-BSA for 15 min. The proteins derived from the RBL-2H3 cell lysates were subjected to Western blot analysis to detect phosphorylated forms of Akt and three typical MAPKs (β-actin was used as a protein loading control). Representative results of three independent experiments are shown; $^{\#\#}p<0.01$ compared to the basal, $^{*}p<0.05$, $^{**}p<0.01$ compared to the control (sensitized with anti-DNP IgE and stimulated with DNP-BSA).

cell functioning, whereas COX-2 is an inducible enzyme that can be upregulated by substances such as cytokines and endotoxins and produces prostaglandins involved in inflammation (22,23). Therefore, allergy-related signaling molecules have attracted increased attention as potential therapeutic targets for mitigating many allergic inflammatory disorders (18).

To determine the effect of the $CHCl_3$ fraction of *Z. latifolia* on COX-2 expression in IgE-sensitized RBL-2H3 cells, Western blot analysis was performed using a COX-2 specific antibody. The cells were pretreated with the $CHCl_3$ extract (5, 10, 25, and 50 μg/mL) for 30 min and then stimulated with DNP-BSA for 4 h; unstimulated cells were used as a control. As a result, COX-2 was present in the unstimulated RBL-2H3 cells, but its expression was significantly and dose-dependently decreased after treatment with the $CHCl_3$ fraction (Fig. 3), demonstrating the inhibition of antigen-induced COX-2 expression in antigen-exposed mast cells. This result suggests that the compounds in the $CHCl_3$ fraction of *Z. latifolia* can effectively block the production of proinflammatory substances by inhibiting the enzyme responsible for their catalysis, and thus could be used to deter mast cell-dependent inflammatory events.

Effects of the $CHCl_3$ fraction on MAPKs and Akt phosphorylation

To evaluate the mechanisms underlying the anti-allergic effects of *Z. latifolia*, the potential activation of MAPKs and PI3K signaling by the $CHCl_3$ fraction was examined. MAPKs are activated by growth factors, cytokines, and environmental stress and are implicated in the proliferation, differentiation, and death of cells (24). The phosphorylation of Akt, which is a marker of PI3K activation, and that of signaling molecules linked to allergic reactions play a critical role in the activation of mast cells as well as the initiation of the inflammatory cytokines and MAPK signaling cascades that are critical for mast cell degranulation (25,26). Activation of MAPKs and Akt encourages the production of proinflammatory cytokines such as TNF-α, IL-3, and IL-4 in RBL-2H3 cells stimulated by an antigen (26). Thus, here we evaluated whether the $CHCl_3$ fraction hindered the activation of these pathways.

Stimulation of the RBL-2H3 cells with DNP-BSA resulted in an increased phosphorylation of all three types of MAPKs (ERK, JNK, and p38) after 15 min. Nevertheless, treatment with the $CHCl_3$ fraction significantly and dose-dependently inhibited the phosphorylation of these MAPKs (Fig. 4A), which play a vital function in regulating the

Table 1. Compounds detected in the $CHCl_3$ fraction of *Z. latifolia* by GC-MS (tentative identification by the mass spectral library)

Compounds	RT (min)	Peak area (%)	Mol. mass	Mol. formula
Unknown 1	8.910	7.05	164	$C_5H_{10}O_5$
Unknown 2	9.100	2.73	178	$C_6H_{12}O_6$
Unknown 3	9.322	43.51	164	$C_6H_{14}O_6$
Phytol	20.589	42.77	296	$C_{20}H_{40}O$
11,14,17-Eicosatrienoic acid	21.899	3.93	320	$C_{21}H_{36}O_2$

transcription activity of proinflammatory cytokines in mast cells (26). Furthermore, antigen-induced phosphorylation of Akt was inhibited by the $CHCl_3$ fraction in IgE-sensitized RBL-2H3 cells (Fig. 4B). Thus, the inhibition of Akt and MAPKs phosphorylation by the *Z. latifolia* $CHCl_3$ fraction demonstrates that the release of these proinflammatory cytokines and the antigen-stimulated degranulation pathway in RBL-2H3 cells were hindered.

Tentative identification of compounds in the $CHCl_3$ fraction The $CHCl_3$ fraction was analyzed by GC-MS. The constituents of the $CHCl_3$ fraction of *Z. latifolia* were tentatively identified by their mass spectra and quantified, and the results are summarized in Table 1. Carbohydrates predominated in the $CHCl_3$ extract. By peak area, the major compounds were a possible D-mannitol derivative (RT=9.322) and phytol (RT=20.589). Phytol is a diterpene alcohol widely found in plants, and it was identified as one of the compounds in the active fraction of *Anchieta salutaris* that inhibited histamine release (27). Moreover, phytol was reported to decrease TNF-α and IL-1β levels, thus exhibiting anti-inflammatory properties (28). For this reason, phytol is probably a key compound for the anti-allergic effects observed here, although further research is necessary to elucidate the specific anti-allergic mechanism of this compound at the molecular level. Conversely, mannitol is a non-toxic and inert sugar alcohol widely present in plant products and is commonly used as an excipient in food products. Mannitol is known to be insoluble in $CHCl_3$; however, a compound with a mass spectrum closely resembling that of mannitol was detected by the mass spectral library used here. This compound could probably be a mannitol derivative, but further analysis such as NMR would be needed to confirm its identity. Thus, the data reported may still serve as the basis for further identification studies of the active compounds in the $CHCl_3$ fraction.

Therefore, it can be concluded that *Z. latifolia* extract, in particular its $CHCl_3$ fraction, dose-dependently inhibited β-hexosanimidase and TNF-α release, COX-2 activity, and Akt and MAPKs phosphorylation, thus hindering the degranulation of sensitized RBL-2H3 cells. This outcome demonstrates the anti-allergic effects of *Z. latifolia* and supports its use as a functional food.

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Disclosure The authors declare no conflict of interest.

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