

Hot Water Extract of Adzuki (*Vigna angularis*) Suppresses Antigen-Stimulated Degranulation in Rat Basophilic Leukemia RBL-2H3 Cells and Passive Cutaneous Anaphylaxis Reaction in Mice

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The hot water extract of adzuki (HWEA), which is produced as a byproduct in the adzuki bean boiling process, has anti-tumor, antioxidative, and anti-diabetic activities. In this study, we fractionated HWEA to 4 fractions using stepwise gradient column chromatography with water and ethanol, and demonstrated the effects of each fraction on antigen (Ag)-stimulated degranulation in rat basophilic leukemia RBL-2H3 cells. The 40% ethanol eluate extract (EtEx.40) showed the strongest inhibition level of these fractions. To reveal the inhibitory mechanisms underlying degranulation by EtEx.40, we investigated intracellular reactive oxygen species (ROS) production, intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), and early intracellular signaling pathways. Treatment with EtEx.40 markedly inactivated Lyn following Ag stimulation, resulting in the suppressions of intracellular elevation of $[\text{Ca}^{2+}]_i$ and production of ROS. To identify the active compound in EtEx.40, we isolated 7 flavonoids from EtEx.40 and calculated their inhibition levels on Ag-stimulated degranulation. These flavonoids inhibited degranulation by about 25–60%. We further examined the *in vivo* effects of HWEA or EtEx.40 using a passive cutaneous anaphylaxis (PCA) reaction. Both extracts strongly suppressed the PCA reaction. These findings suggest that HWEA and/or EtEx.40 are beneficial for alleviating type I allergic symptoms. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: adzuki (*Vigna angularis*); hot water extract of adzuki; Lyn; degranulation; passive cutaneous anaphylaxis reaction.

INTRODUCTION

The number of patients suffering from allergic diseases such as bronchial asthma, rhinitis, and conjunctivitis and allergies against pollen and food is increasing worldwide. Allergies are classified into 5 types: type I, anaphylactic type (e.g. anaphylactic shock); type II, antibody-mediated cytotoxic type (e.g. autoimmune hemolytic anemia); type III, immune complex type (e.g. glomerulonephritis); type IV, cellular immunity type (e.g. tuberculin reaction); and type V, stimulative type (e.g. Graves' disease). Among these, the type I allergy patients form the largest group. Type I allergy is triggered by antigen (Ag) binding to the high-affinity immunoglobulin E receptor (FcεRI) on the surface of mast cells and basophils. This interaction causes the FcεRI receptors to aggregate, which induces a variety of cellular responses, including mobilization of Ca^{2+} , production of reactive oxygen species (ROS), and activation of signal transduction pathways and results in degranulation, cytokine production, and leukotriene secretion (Beaven and Metzger, 1993; Turner and Kinet, 1999; Mekori and Metcalfe, 2000). These chemical mediators cause asthma and inflammatory responses. Thus, inhibiting the early

intracellular events following Ag stimulation is important for alleviating type I allergic symptoms.

Adzuki beans (*Vigna angularis*) are mainly produced and consumed in East Asia. During the production of confectionaries, adzuki beans are boiled in a cooker; as a by-product of this process, a hot water extract (HWEA) is produced. The HWEA contains several chemical compounds such as catechin and saponins. These compounds are well known as bioactive ingredients (Hiro *et al.*, 2010; Tamura *et al.*, 2010; Takahashi *et al.*, 2010; Zhang and Popovich, 2010). Previously, we reported that the 40% ethanol-eluted fraction (EtEx.40) from HWEA treated with HP-20 resin is effective as an antitumor agent (Itoh *et al.*, 2002; Itoh *et al.*, 2004a; Itoh *et al.*, 2005a; Itoh *et al.*, 2005b); has antioxidant (Itoh *et al.*, 2005c), anti-metastasis (Itoh *et al.*, 2005c), and anti-diabetes (Itoh *et al.*, 2004b; Itoh *et al.*, 2009a) activities; lowers serum cholesterol levels (Itoh and Furuichi, 2009b); and enhances melanogenesis (Itoh and Furuichi, 2005d).

Many natural compounds suppress the Ag-stimulated degranulation in cellular systems (Han *et al.*, 2009; Jippo *et al.*, 2009; Chen *et al.*, 2010). To develop anti-allergic drugs or food, we investigated the effect of HWEA on Ag-stimulated degranulation in rat basophilic leukemia RBL-2H3 cells. We found that EtEx.40 significantly suppressed Ag-stimulated degranulation. To shed light on the suppression mechanisms, we examined the effects of

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HWEA and/or EtEx.40 on intracellular ROS production, elevation of intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), and early cell signal transduction following Ag stimulation. Furthermore, we identified the active compounds and investigated the inhibitory effects of oral administration of HWEA or EtEx.40 using a type I allergic mouse model.

MATERIALS AND METHODS

Reagents and materials. Mouse monoclonal anti-dinitrophenol (DNP) immunoglobulin E (IgE) was purchased from Yamasa Co. Ltd. (Tokyo, Japan); disodium cromoglycate, from ENZO Life science (Plymouth Meeting, PA, USA); $\times 25$ Complete[®] mixture of protease inhibitors, from Roche (Penzberg, Germany); and phosphatase Inhibitor Cocktail[®] 1 and 2, from Sigma (St Louis, MO, USA). The antibodies against p44/42 MAP kinase (ERK), phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204) (p-ERK), anti-SAPK/JNK (JNK), phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK), p38 MAP kinase (p38), phospho-p38 MAP kinase (Thr180/Tyr182) (p-p38), Lyn, phospho-Lyn, cytosolic phospholipase A_2 (cPLA $_2$), phospho-cPLA $_2$, phospho-PLC γ 1, phospho-PLC γ 2, and phospho-Syk were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibodies against -Syk, gp91^{phox}, p22^{phox}, p40^{phox}, p47^{phox}, p67^{phox}, and Rac were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against β -actin was purchased from Sigma. The Fc ϵ RI β antibody and *Rubus suavissimus* extract were kindly provided by Dr. J. Rivera (NIH, Molecular Immunology and Inflammation Branch, Bethesda, MD, USA,) and Suntory Holdings Limited (Osaka, Japan), respectively. Anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase (HRP) and the chemiluminescence (ECL) kit were obtained from GE Healthcare Science (GE Healthcare UK Ltd., Amersham Place, Buckinghamshire, UK).

Preparation of test materials. Adzuki beans (*Vigna angularis*; 2.5 kg) harvested in Tokachi, Hokkaido, Japan, were boiled, and HWEA (15 L) was obtained. This HWEA was concentrated 100 $\times$ by a plate heat exchanger (Izumi Food Machinery Co. Ltd., Hyogo, Japan). Concentrated HWEA (30 mL) was subjected to open column chromatography on Diaion HP-20 (Mitsubishi Chemical Co. Ltd., Tokyo, Japan) with a column size of $\phi 50 \times 300$ mm and a resin wet volume of 400 g. The column was eluted stepwise first with 5 L of distilled water followed by 40%, 60%, and 80% ethanol (WEx, EtEx.40, EtEx.60, and EtEx.80, respectively). Fractions were evaporated *in vacuo* to dryness (Figure 1). Each sample was dissolved with dimethyl sulphoxide (DMSO) to a final concentration of 50 or 100 $\mu\text{g}/\text{mL}$ directly prior to performing the experiment.

To determine the active compounds, EtEx.40 was separated by column chromatography over multiple columns (Sephadex LH20, Chromatorex DM-1020 T, and silica gel 60 [$<45 \mu\text{m}$]), preparative thin-layer chromatography (PTLC) on silica gel 60 F $_{254}$ or

RP-18 W F $_{254}$, and reverse-phase high-performance liquid chromatography (RP-HPLC) to give 6 compounds (Hori *et al.*, 2009). The chemical structures were identified using IR and mass spectra, and ^1H - and ^{13}C -NMR.

Cell culture. RBL-2H3 cells were obtained from Health Science Research Resource Bank (Tokyo, Japan). Cells were grown in Eagle's minimum essential medium (GIBCO, MD, USA) containing 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin, and 100 $\mu\text{g}/\text{mL}$ of streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

Degranulation assay

β -Hexosaminidase release assay. We used β -hexosaminidase as a marker of degranulation. RBL-2H3 cells (2×10^4 cells/well) were seeded onto 24-well plates and cultured for 12 h. Cells were then treated with anti-DNP IgE (0.45 $\mu\text{g}/\text{mL}$) and incubated for 24 h. After washing twice with Siraganian buffer A (119 mM NaCl, 5 mM KCl, 0.4 mM MgCl_2 , 25 mM PIPES, and 40 mM NaOH, pH 7.2), 160 μL of Siraganian buffer B (5.6 mM glucose, 1 mM CaCl_2 , and 0.1% bovine serum albumin (BSA)) was added to each well. After incubating at 37 °C for 10 min, cells were treated with the samples at 37 °C for 30 min. DNP-labeled bovine serum albumin (DNP-BSA; 20 μL) was then added to the culture medium to a final concentration of 10 $\mu\text{g}/\text{mL}$. After 10 min, cells were placed on ice for 10 min to terminate the reaction. The supernatants were then harvested by centrifuging at $300 \times g$ at 4 °C for 10 min. The supernatants (50 μL) were transferred into 96-well microplates and allowed to react with 50 μL of 0.1 M citrate buffer (pH 4.5) that contained 1 mM p-nitrophenyl-*N*-acetyl- β -D-glucosaminide (CB-PNAG) at 37 °C. The reaction was terminated after 1 h by adding stop buffer (0.1 M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 10.0). Absorbance was measured at 405 nm using a colorimetric microplate reader (Immuno-Mini NJ-2300; Nalge Nunc International K.K., Tokyo, Japan).

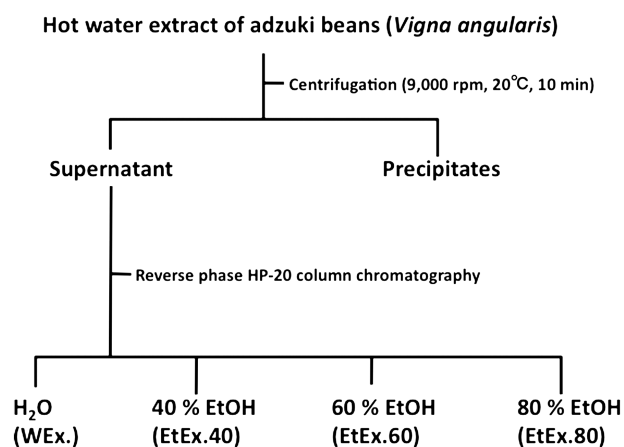


Figure 1. Concentrated HWEA was subjected to HP-20 column chromatography and eluted with a stepwise gradient of water and ethanol. Four fractions were obtained: water (WEx), 40% ethanol extract (EtEx.40), 60% ethanol extract (EtEx.60), and 80% ethanol extract (EtEx.80).

***β*-Hexosaminidase inhibitory activity.** On 96-well microplates, each sample (5 µl) and conditioned medium prepared from Ag-stimulated RBL-2H3 cells (45 µl) were reacted with 50 µl of 0.1 M CB-PNAG at 37 °C for 1 h. After terminating the reaction, the absorbance at 405 nm was measured. The inhibition of degranulation was calculated as follows:

$$\text{Inhibition of degranulation}(\%) = \left(\frac{1 - (\text{A}_{405 \text{ nm of sample}} - \text{A}_{405 \text{ nm negative control}})}{(\text{A}_{405 \text{ nm positive control}} - \text{A}_{405 \text{ nm negative control}})} \times 100 \right) - \beta\text{-hexosaminidase inhibitory activity}(\%)$$

$$\beta\text{-hexosaminidase inhibitory activity}(\%) = \frac{1 - (\text{A}_{405 \text{ nm of sample}} - \text{A}_{405 \text{ nm negative control}})}{(\text{A}_{405 \text{ nm positive control}} - \text{A}_{405 \text{ nm negative control}})} \times 100$$

Measurement of intracellular Ca²⁺ concentrations. Intracellular Ca²⁺ levels were determined using Calcium Kit-Fluo 3™ (Dojindo Laboratories, Kumamoto, Japan). RBL-2H3 cells (5 × 10⁴ cells/well) were seeded onto 96-well microplates and incubated for 1 h. Then, cells were treated with anti-DNP IgE (0.45 µg/ml) and incubated for 24 h. After washing twice with PBS, 100 µl of loading buffer containing Fluo-3 AM (Calcium Kit-Fluo 3™) was added to culture medium. One hour later, cells were washed with PBS and incubated in 90 µl of loading buffer containing 50 µM of sample. After incubating for 30 min, cells were stimulated with DNP-BSA (10 µg/ml), and the fluorescence was measured using a fluorometric imaging plate reader (excitation, 490 nm; emission, 530 nm).

Measurement of intracellular ROS levels. Intracellular ROS levels were measured using CM-H₂DCF-DA. IgE-sensitized RBL-2H3 cells (5 × 10⁴ cells/well) were incubated with 10 µM 5-(and-6-)carboxy-2', 7'-dichlorodihydrofluorescein diacetate (CM-H₂DCF-DA) for 30 min at 37 °C. After washing twice, cells were incubated with 50 µM of sample for 30 min. Cells were then stimulated with DNP-BSA (10 µg/ml), and the fluorescence was measured using a fluorometric imaging plate reader (excitation, 490 nm; emission, 530 nm).

Immunoblot analysis. Ag-stimulated RBL-2H3 cells with or without EtEx.40 treatment were washed twice with PBS and harvested. The cytosolic and membrane fractions were separated using the ProteoExtract subcellular proteome extraction kit (Merck KGaA, Darmstadt, Germany). Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes. The membrane was blocked for 1 h in 5% nonfat milk, incubated with a primary antibody overnight at 4 °C, and then incubated with a HRP-conjugated secondary antibody at room temperature for 1 h. Immunoreactive proteins were detected with the enhanced ECL kit and chemiluminescence detector (LAS-4000; Fujifilm, Tokyo, Japan).

Real-time quantitative PCR. To measure the expression level of *TNFα* in EtEx. 40-treated RBL-2H3 cells after the Ag-stimulation, total RNA was extracted from the cells by TRIzol containing phenol/guanidium isothiocyanate (Invitrogen) with DNase I treatment. Subsequently, cDNA was extracted from total RNA samples using a PrimeScript™ reagent Kit (Takara

Bio). qRT-PCR was performed (Thermal Cycler Dice® TP800) using a SYBER® Premix Taq™ II Kit (Takara) and the following primer sets: *TNFα*-sense 5' - CACCACGCTCTTCTGTCTACTG-3' *TNFα*-antisense, 5' - CTCCTCTGCTTGGTGGTTT-3'; *β*-actin-sense, 5' - TGGCACCCAGCACAATGAA-3' and *β*-actin-antisense, 5' - CTAAGTCATAGTCCGCCTAGAAGCA-3'. The cDNA of *β*-actin was used as an internal control.

Animals and diets. Four-week-old ICR male mice (Japan SLC, Ltd., Hamamatsu, Japan) were housed in an air-conditioned room (22 ± 2 °C) with a 12-h light and dark cycle (lighting from 0700 to 1900). All mice were fed with commercial CE-2 pellets (Clea Japan Inc., Tokyo) and water *ad libitum* for 1 week to acclimatize them with the surroundings. This study was approved by the Gifu International Institute of Biotechnology Animal Use Committee, and animals were maintained according to the guidelines of the Gifu International Institute of Biotechnology for the care of laboratory animals.

Measurement of mouse passive cutaneous anaphylaxis (PCA) reaction. An IgE-induced passive cutaneous anaphylaxis reaction was measured according to a previously published method (Choo *et al.*, 2003) with the following slight modification. The back hair of the ICR mice were shaved, and 10 µg of anti-DNP-BSA was injected into 2 dorsal skin sites for each mouse. The sites were outlined with a water-insoluble marker. After 24 h, each mouse was treated with 100 µl of PBS (-) or EtEx.40 (100, 200, or 300 mg/Kg body weight) by oral administration. One hour after the corn oil or the extract administration, each mouse received an injection of 200 µl of DNP-BSA of 1% Evans blue (Sigma) in PBS, containing 200 µg of DNP-BSA. Treated mice were sacrificed 1 h later, and their dorsal skin was removed. Skin pieces × (1 cm) were dissolved with 1 N KOH at 37 °C for 24 h, and the extravasated Evans blue dye was extracted with a mixture of acetone and 0.2 M phosphoric acid (13:5) from the treated skin solution. The amount of dye was determined calorimetrically at 620 nm.

Statistical analysis. All data were analyzed first by one-way ANOVA and then by Fisher's multiple range test. The differences among the means were considered significant at *p* < 0.05.

RESULTS AND DISCUSSION

We previously reported several biological functions of HWEA (Itoh *et al.*, 2002; Itoh *et al.*, 2004a; Itoh *et al.*, 2004b; Itoh *et al.*, 2005a; Itoh *et al.*, 2005b; Itoh *et al.*, 2005c; Itoh and Furuichi, 2005d; Itoh *et al.*, 2009a; Itoh and Furuichi, 2009b). In this study, we focused on determining the anti-allergic activity and active compound(s) of HWEA. We subjected HWEA to

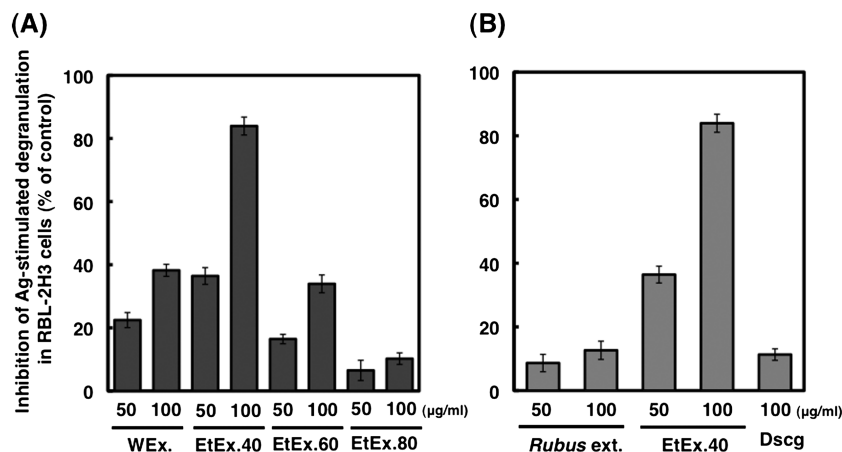


Figure 2. The inhibitory effects of each HWEA fraction obtained from HP-20 open column chromatography on Ag-stimulated β -hexosaminidase release (A) Effects of the 4 fractions of WEx, EtEx.40, EtEx.60, and EtEx.80 on Ag-stimulated degranulation in RBL-2H3 cells. (B) IgE-sensitized RBL-2H3 cells were treated with EtEx.40 (50 or 100 μ M), stimulated with antigen (DNP-BSA), and then subjected to β -hexosaminidase release assays. Values are given as mean $\pm$ SEM ($n = 10$). *Rubus suavis* extract (*Rubus ext.*) has been reported to be an anti-allergic food material. Disodium cromoglycate (DSCG) was used as anti-allergic positive control.

HP-20 column chromatography using stepwise ethanol elution methods and obtained 4 fractions: water extract (WEx), 40% ethanol extract (EtEx.40), 60% ethanol extract (EtEx.60), and 80% ethanol extract (EtEx.80). We examined the inhibitory effects of these fractions on Ag-stimulated degranulation *in vitro*. Among these, the suppression level of EtEx.40 was stronger than that of any other fractions (Figure 2A). Furthermore, the

suppression levels of EtEx.40 were higher than those of the *Rubus* (*Rubus suavis*) natural extract or the medical compound disodium cromoglycate (DSCG), both of which are well-known anti-allergenic agents (Figure 2B) (Golden *et al.*, 1986; Shin *et al.*, 2004; Sarfstein *et al.*, 2004).

To determine the mechanisms underlying the inhibitory effects of EtEx.40 on Ag-stimulated degranulation,

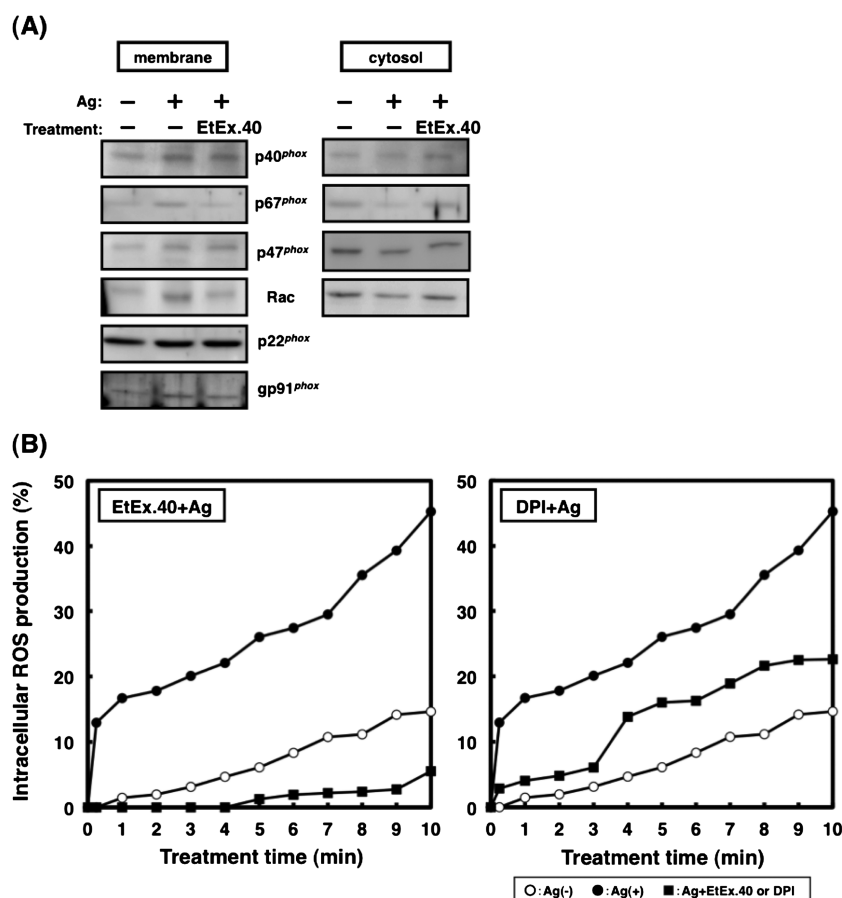


Figure 3. Effects of EtEx.40 on the intracellular ROS production in Ag-stimulated RBL-2H3 cells. (A) Inhibitory effects of EtEx.40 on translocation of cytosolic subunits of NADPH oxidase (p40^{phox}, p47^{phox}, p67^{phox}, and Rac) to the membrane. A representative blot from 3 independent experiments is shown. (B) IgE-sensitized RBL-2H3 cells were treated with CM-H₂DCF-DA and incubated with or without 50 μ g/ml EtEx.40 or 200 nM of diphenyleneiodonium chloride (DPI) for 30 min. Cells were then stimulated with or without DNP-BSA, and ROS-mediated DCF oxidation was measured. Values are given as mean $\pm$ SEM ($n = 10$).

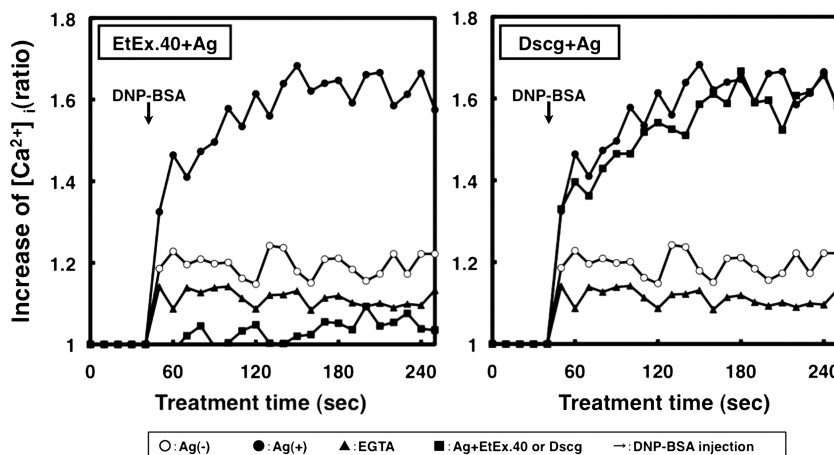


Figure 4. Effects of EtEx.40 on the Ag-stimulated elevation of intracellular $[Ca^{2+}]_i$ in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were treated with or without 50 μ g/ml EtEx.40 for 30 min. The cells were then stimulated with or without DNP-BSA, after which intracellular Ca^{2+} levels were measured. Values are given as the mean $\pm$ SEM ($n = 12$). Arrow, time at which DNP-BSA was added; filled circle, Ag-treated cells; open circle, non-Ag-treated cells; filled triangle, Ag- and EGTA-treated cells; filled square, Ag- and EtEx.40 or DSCG-treated cells.

we first examined intracellular ROS production and Ca^{2+} mobilization following Ag stimulation. Upon Ag-stimulated degranulation, intracellular ROS is produced by NADPH oxidase activation. NADPH oxidase is an enzyme complex composed of membrane-bound subunits (gp91^{phox} and p22^{phox}), cytosolic subunits (p40^{phox}, p47^{phox}, and p67^{phox}), and a monomeric GTP-binding protein of Rho family Rac2 (Bedard and Krause, 2007). To activate phagocytic NADPH oxidase, the cytosolic subunits are translocated to the membrane, thus initiating the production of superoxide (Yoshimaru *et al.*, 2002). As shown in Figure 3A, 50 μ g/mL of EtEx.40 suppressed the translocation of the cytosolic subunits of NADPH oxidase to the plasma membrane, consequently suppressing ROS production (Figure 3B). ROS produced following Ag stimulation regulates Ca^{2+} mobilization (Huang and Putney, 1998; Suzuki *et al.*, 2003; Swindle and Metcalfe, 2007). In allergic responses such as degranulation and

cytokine production, extracellular Ca^{2+} mobilization through the Ca^{2+} channel is a necessary event (Kinet, 1999; Vig and Kinet, 2009). In this study, 50 μ g/mL of EtEx.40 completely suppressed the elevation of $[Ca^{2+}]_i$ (Figure 4). Although we did not determine whether EtEx.40 directly affects Ca^{2+} channel function, the suppression of Ca^{2+} mobilization by EtEx.40 treatment was certainly due in part to suppression of ROS production by inactivation of NADPH oxidase.

Next, we examined early intracellular signaling transduction pathways. Aggregation of Fc ϵ RI on mast cells by multivalent antigen s activates multiple signal transduction pathways that play crucial roles in degranulation and cytokine production (Beaven and Metzger, 1993; Turner and Kinet, 1999; Mekori and Metcalfe, 2000). Data presented in Figure 5 show that 50 μ g/mL of EtEx.40 significantly suppressed the activation of Lyn and spleen tyrosine kinase (Syk) and their downstream targets, including PLC γ 1 and PLC γ 2 (PLC γ 1/2), ERK1/2, p38 MAP kinase, JNK, Akt, and cPLA₂. In addition, the

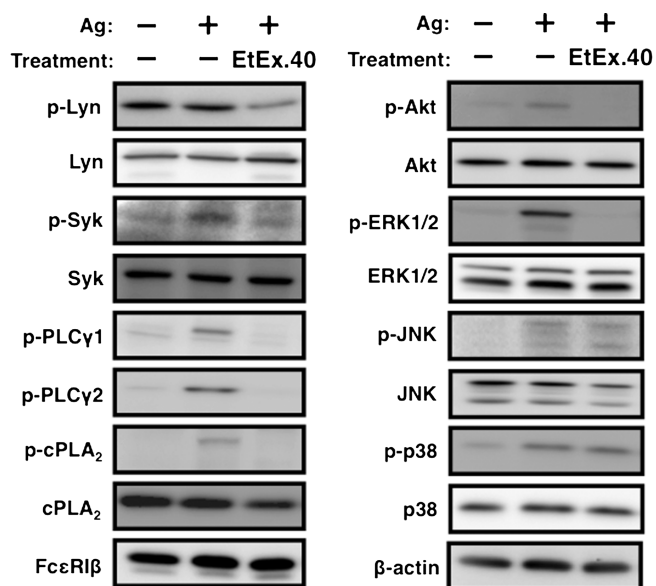


Figure 5. Effects of EtEx.40 on Ag-stimulated activation of intracellular signaling pathways in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were treated with or without 50 μ g/ml EtEx.40 for 30 min. The cells were then stimulated with or without DNP-BSA. Cell lysates were analyzed by western blot for the indicated proteins. A representative blot from 3 independent experiments is shown.

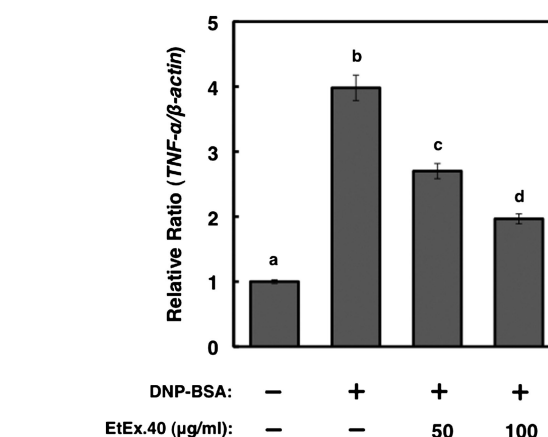


Figure 6. Effects of EtEx.40 on the expression level of *TNF α* mRNA in Ag-stimulated RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were treated with or without EtEx.40 for 30 min. The cells were then stimulated with or without DNP-BSA for 12 h. cDNA samples were prepared for analysis of *TNF α* gene expression using the PrimeScript[™] reagent Kit (Takara Bio), and the expression levels of *TNF α* mRNA were calculated using real-time PCR β . The cDNA of β -actin was used as an internal control. Values are given as the mean $\pm$ SEM ($n = 9$). Mean values with different letters are significantly different ($p < 0.05$; one-way analysis of variance followed by Fisher's multiple range test).

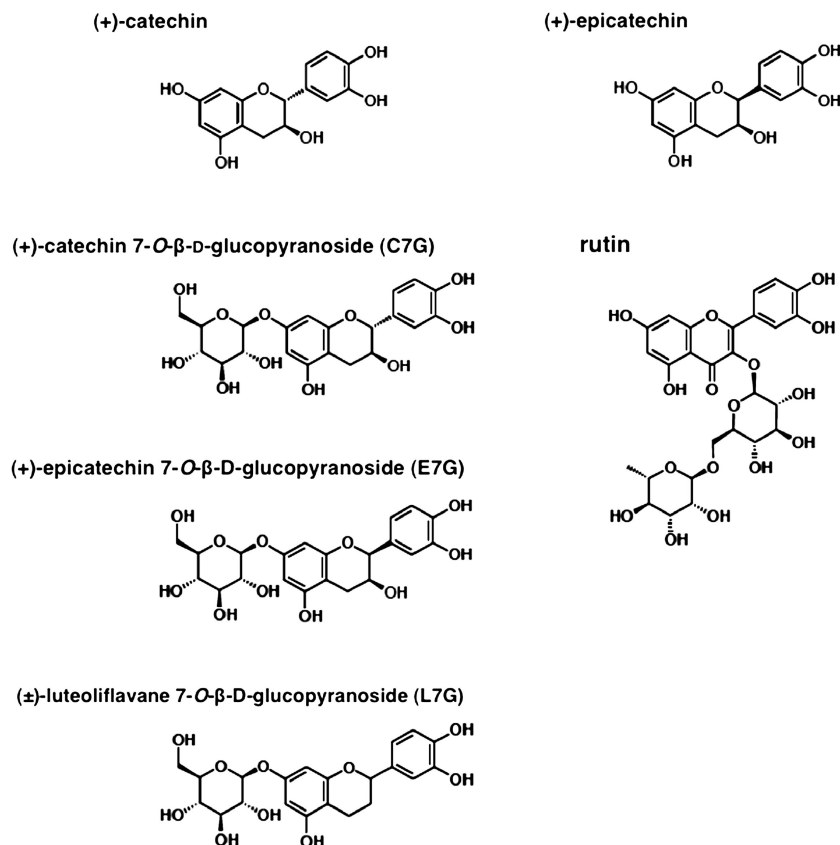


Figure 7. Structures of flavonoids isolated from EtEx.40.

mRNA expression level of *TNF α* , which is transactivated by Ag stimulation in RBL-2H3 cells, also significantly decreased in the EtEx.40-treated RBL-2H3 cells after the Ag-stimulation as compared to that of the antigen-only treated RBL-2H3 cells (Figure 6). Regulation of transcriptional activity by EtEx.40 may be also due to suppression of Lyn/Syk signaling. Fc ϵ RI consists of an α -chain (which binds an IgE), a β -chain, and 2 γ -chains

(Yamasaki and Saito, 2005). The β -chain and γ -chain have immunoreceptor tyrosine-based activation motifs (ITAMs) and are closely associated with intracellular signal transduction (Yamasaki and Saito, 2005). Aggregation of Fc ϵ RI following Ag stimulation leads to transphosphorylation of the β -chain and γ -chain ITAMs by the Src family protein tyrosine kinase Lyn (Xiao, *et al.*, 2005; Furumoto *et al.*, 2005; Alvarez-Erriico *et al.*, 2010). Therefore, Lyn plays a critical role as an “initiating kinase” in the degranulation process. Since Lyn is located upstream of Syk, inactivation of Lyn by EtEx.40 leads to the suppression of its downstream events, such as Syk/PLC γ pathways, intracellular ROS production, and Ca²⁺ mobilization. Furthermore, we have found in a previous study that the antioxidative activity of EtEx.40 is stronger than those of vitamins C and E (Itoh *et al.*, 2005c), suggesting that EtEx.40 acts as a radical scavenger in Ag-stimulated degranulation. Thus, we conclude that the inhibitory effects of EtEx.40 on Ag-stimulated degranulation are mainly due to the suppression of Lyn activation and its antioxidative activity.

We further examined the effect of flavonoids isolated from EtEx.40 on Ag-stimulated degranulation to determine the active compounds. The active compounds were isolated following previously reported procedures (Figure 7) (Hori *et al.*, 2009). Although each flavonoid showed an inhibitory effect higher than that of DSCG, the inhibitory effects of its glycoside groups (rutin, C7G, E7G, and L7G) were weaker than the effects of the aglycone types (Figure 8). Moreover, the inhibitory effect of (+)-epicatechin was significantly stronger than that of (–)-epicatechin. Tea catechins are epimerized by heat treatment (Seto *et al.*, 1997; Chen *et al.*, 2001; Kobayashi *et al.*, 2005). We have also observed epimerization from

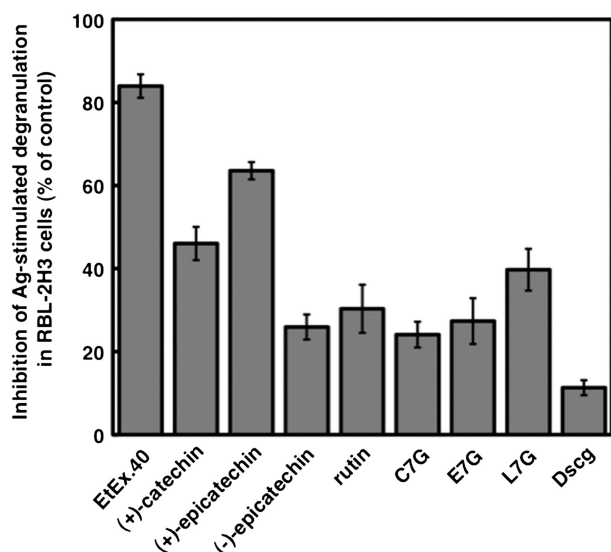


Figure 8. Effects of each compound isolated from EtEx.40 on Ag-stimulated degranulation in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were treated with or without 50 μ M of each compound, stimulated with antigen (DNP-BSA), and then subjected to β -hexosaminidase release assays. Values are mean $\pm$ SEM ($n = 10$). DSCG-treated cells were used as anti-allergic positive controls.

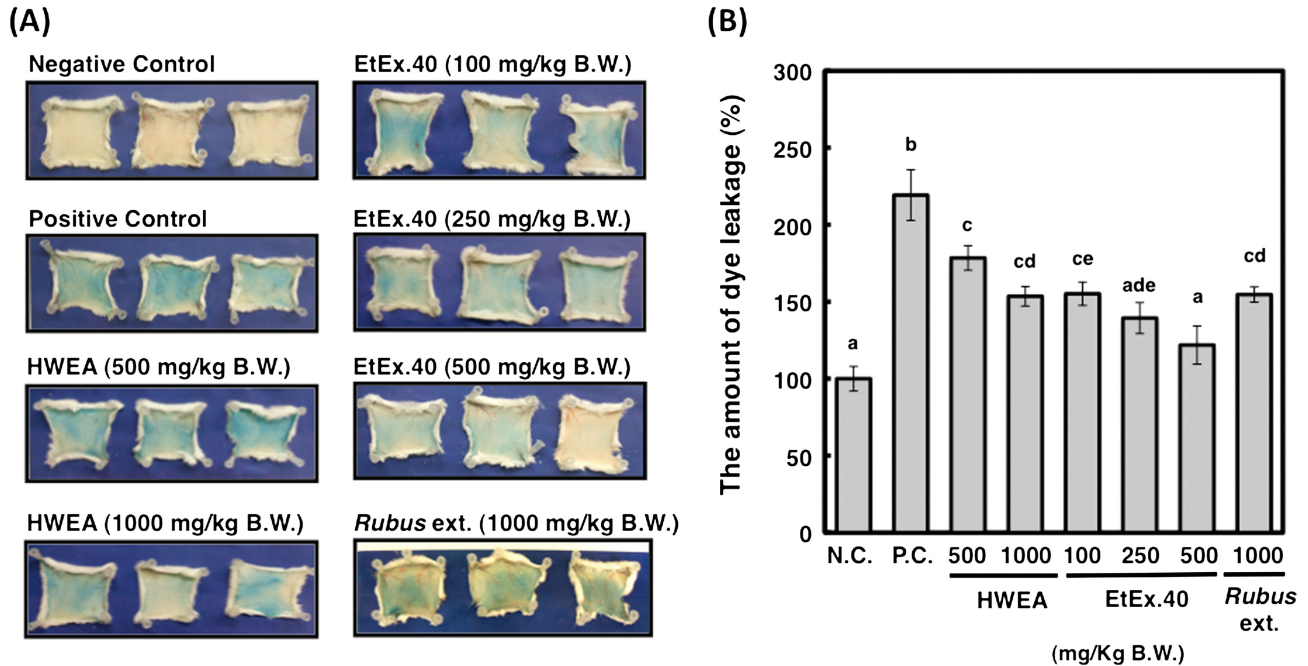


Figure 9. Effects of HWEA and EtEx.40 on mouse passive cutaneous anaphylaxis (PCA) reactions induced by IgE-antigen complex. (A) Imaging of extravasated Evans blue on dorsal skin. *Rubus* extract was used as an anti-allergic positive control. Images shown represent 3 independent experiments. (B) The amount of extravasated Evans blue dye from dorsal skin of mice treated with HWEA and EtEx.40. Values are given as the mean $\pm$ SEM ($n = 9$). Mean values with different letters depict those that are significantly different ($p < 0.05$; one-way analysis of variance followed by Fisher's multiple range test). This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

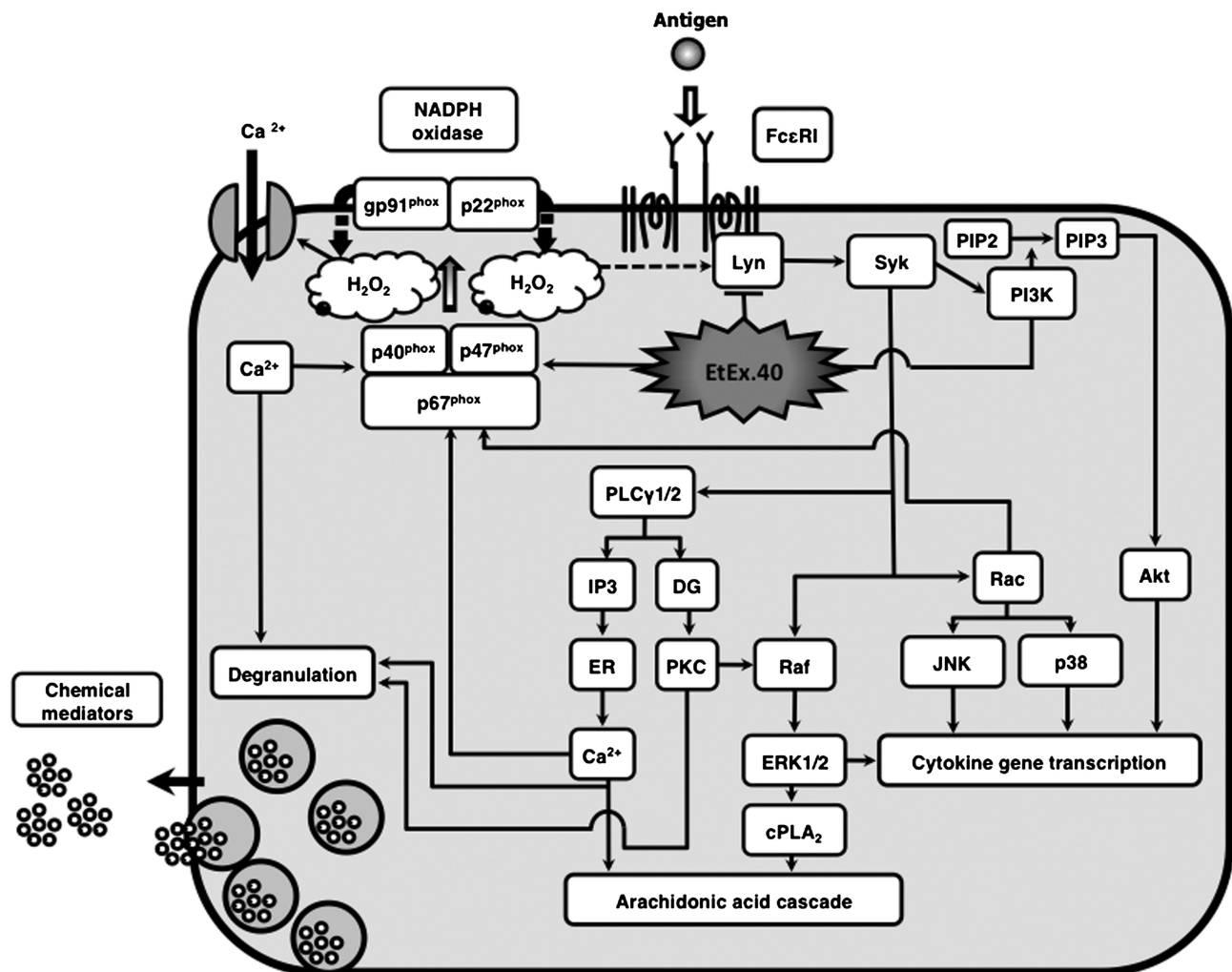


Figure 10. A scheme showing the inhibitory effects of EtEx.40 on Ag-stimulated degranulation in RBL-2H3 cells. EtEx.40 treatments inhibit Ag-stimulated degranulation through direct inhibition of Lyn activation.

(+)-catechin to (+)-epicatechin by using FAB-MS, optical rotation, and ^1H - and ^{13}C -NMR analyses (data not shown). Thus, EtEx.40, which was fractionated from HWEA using HP-20 column chromatography, contains many active compounds that inhibit Ag-stimulated degranulation. It can be presumed that these compounds cooperatively act as anti-degranulation agents.

In order to gain further insight into the effect of EtEx.40 on IgE-mediated allergic responses, we examined the effect of EtEx.40 *in vivo* using a PCA reaction that is widely used as a type I allergy animal model. Oral administration of HWEA (500–1000 mg/Kg mouse body weight) or EtEx.40 (100–500 mg/Kg mouse body weight) significantly suppressed mouse PCA reaction (Figures 9A and 9B). These inhibitory effects are similar to the effect of *Rubus* extract. These results suggest that HWEA or EtEx.40 intake is effective for alleviating allergic reaction.

In summary, the inhibitory effect of EtEx.40 on Ag-stimulated degranulation is mainly due to the

inactivation of the Lyn/Syk/PLC γ s pathways and their antioxidative activities (Figure 10). Our *in vitro* and *in vivo* data suggest that HWEA and/or EtEx.40 are effective foods for alleviating type I allergy symptoms.

Other Abbreviations Used

ERK, extracellular signal-regulated kinase; PLC, phospholipase C; SAPK/JNK, stress-activated protein kinase/c-jun-N-terminal kinase.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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