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Inhibitory effect of the branches of *Hovenia dulcis* Thunb. and its constituent pinosylvin on the activities of IgE-mediated mast cells and passive cutaneous anaphylaxis in mice

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Hovenia dulcis Thunb. (Rhamnaceae) is a hardy tree native to Europe, the Middle East, and North Africa, and it is also grown in parts of Asia and has been used in traditional medicine to treat liver toxicity, stomach disorders, and inflammation. This study investigated the anti-allergy potential of an extract of the branches of *H. dulcis* (HDB) using the antigen-stimulated mast cell-like cell line rat basophilic leukemia (RBL)-2H3 and a passive cutaneous anaphylaxis (PCA) mouse model. Degranulation assay, reverse transcription PCR, enzyme-linked immunosorbent assays, western blot analyses, and PCA were performed to measure allergic responses and proinflammatory mediators in antigen-stimulated rat basophilic leukemia (RBL)-2H3 mast cells and the PCA mouse model. In antigen-stimulated RBL-2H3 cells, HDB inhibited the secretion of β -hexosaminidase (indicating the inhibition of degranulation) and histamine release; decreased expression and production of the inflammatory mediators, cyclooxygenase-2 and prostaglandin E_2 , and cytokines interleukin-4 and tumor necrosis factor- α ; and suppressed activation of nuclear factor κ B, a transcription factor involved in the response to cytokines. HDB attenuated phosphorylation of the mast cell downstream effectors Lyn, Syk, phospholipase C γ , protein kinase C μ , extracellular signal-regulated kinase and p38. In IgE-sensitized mice, HDB inhibited mast cell-dependent PCA. Furthermore, HDB contained pinosylvin and possessed significant anti-allergic activities. These results suggest that HDB would be of value in the prevention and treatment of allergic diseases.

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Introduction

The incidence of antigen-related allergic disorders has increased worldwide during the past few decades.¹ These allergic diseases, such as eczema, allergic rhinitis, asthma, and atopic dermatitis,² are the result of immunological disorders caused by genetic, environmental, dietary, and lifestyle factors.³

Mast cells are representative immune cells and are distributed throughout the entire body.⁴ They play an important role in allergic disorders such as asthma, rhinitis, atopic dermatitis, and anaphylaxis.⁵ Mast cells express the high-affinity immunoglobulin E (IgE) receptor Fc ϵ R1, which when cross-linked by IgE and antigens, causes activation.⁴ Activated mast cells undergo degranulation and release histamine. In

addition they produce inflammatory lipid mediators such as prostaglandin (PG), leukotriene, and cyclooxygenase-2 (COX-2) and several inflammatory cytokines and transcription factors, including interleukin 4 (IL-4), tumor necrosis factor alpha (TNF- α), and nuclear factor- κ B (NF- κ B).⁶ The rat basophilic leukemia (RBL)-2H3 cell line has similar characteristics to those of mast cells^{7,8} and is commonly used as a model in allergy research, for example, to screen potential mast cell stabilizers.⁹

Hovenia dulcis Thunb. (Rhamnaceae) has been used for centuries in East Asia as a source of both nutraceutical and pharmaceutical supplements, and its roots, barks, leaves, peduncles, and seeds have been used in traditional medicine to alleviate alcoholic intoxication, quench thirst, provide anti-febrile therapy, promote digestion, and strengthen the stomach.^{10–12} In ancient Chinese medicine, its fruit and peduncle were used as a febrifuge and administered to treat parasitic infections.¹³ Seeds were used as a diuretic and cure for alcohol overdosing, whereas the fruit was also used as anti-spasmodic, febrifuge, laxative and diuretic agent. Moreover, the stem bark was introduced as a natural drug to treat rectal diseases.¹⁴ Recent studies have demonstrated that the extracts of the fruits and seeds of *H. dulcis* can attenuate acute liver

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toxicity,^{15–17} ethanol-induced muscle relaxation^{14,18} and have anti-tumor and anti-lipid peroxidation activity.^{19,20}

The major constituents of *H. dulcis* are phenolic compounds such as ferulic acid (FA), 2,3,4-trihydroxybenzoic acid (TBA), vanillic acid (VA), methyl vanillate (MV), taxifolin, pinosylvin, dihydrokaempferol (DHK), and (–)-galocatechin (GC).²¹ The anti-inflammatory effects of pinosylvin in a rat model of adjuvant-induced arthritis have been recently reported.²² Although studies on the physiological functions of *H. dulcis*, including anti-inflammatory and anti-allergic effects,^{22,23} have been evaluated, the effects of *H. dulcis* and its constituents in improving allergic reactions have not been investigated. In this study, we investigated the effects of the branches of *H. dulcis* extract and its constituents on an antigen-induced allergic response and on the FcεRI-mediated signaling pathway.

Materials and methods

Reagents and materials

Fetal bovine serum (FBS), penicillin, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Hyclone (South Logan, UT, USA). Monoclonal anti-dinitrophenol (DNP)-specific IgE (DNP-IgE) antibody, DNP-bovine serum albumin (BSA), piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), and PP2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies reactive with phosphorylated (phospho)-Lyn (Tyr507), phospho-Syk (Tyr525/526), phospho-phospholipase C (PLC)γ (Tyr783) and phospho-protein kinase D/protein kinase Cμ (PKD/PKCμ; Ser916) and antibodies reactive with phospho-p38 mitogen-activated protein kinase (MAPK; Thr180/Tyr182), p38 MAPK, phospho-p44/42 MAPK (ERK1/2; Thr202/Tyr204), ERK1/2, phospho-stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) (Thr183/Tyr185), SAPK/JNK (Thr183/Tyr185), and β-actin were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA).

Extraction of plant material and isolation of compounds

Dried aerial parts of *H. dulcis* were purchased from Kyungdong oriental herbal market, Seoul, Korea, and their identity was confirmed by one of the authors (H.C. Lee). The *H. dulcis* voucher specimen was deposited at the Natural Products Research Center, KIST Gangneung Institute, Korea. Extraction was carried out by sonication using an RK158S ultrasonic bath (Bandelin, Germany). The dried fruits (1.7 kg), branches (11 kg), and leaves (100 g) of *H. dulcis* were ground and extracted three times with 95% ethanol for 3 hours, following which a fruit (HDF, 224.8 g), a branch (HDB, 357.2 g), and a leaf extract (HDL, 12.5 g) were obtained by filtration followed by solvent evaporation. Eight compounds were isolated from HDB, as previously described (Fig. 7A).²¹ The purity of the compound was determined by high performance liquid chromatography (HPLC) analysis. All samples were dissolved in dimethyl sulfoxide (DMSO) for *in vitro* assays, and HDB was suspended in Tween 20 for use *in vivo*.

Cell culture

RBL-2H3 cells were obtained from the ATCC (KCLB no. 22256; A Biological Resource Center, Manassas, Virginia) and cultured in DMEM supplemented with 10% FBS and 100 U mL^{−1} penicillin. The cells were grown in 75T-plasks at 37 °C with 5% CO₂ in a humidified atmosphere.

Cell viability assay

Cytotoxicity was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich). RBL-2H3 cells were treated with various concentrations of HDB or pinosylvin for 6 h. After washing, 0.5 mg mL^{−1} MTT was added to the cells in 24-well plates, and the plates were incubated for 4 h. The insoluble formazan products were dissolved in 200 μL DMSO, and the absorbance was measured with a microplate reader at 550 nm (Bio-Tek Instruments, Winooski, VT).

Measurement of degranulation and histamine release

RBL-2H3 cells were sensitized with 0.2 μg mL^{−1} DNP-IgE overnight and then washed with modified PIPES buffer (25 mM PIPES pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂·6H₂O, 1 mM CaCl₂, 5.6 mM glucose, 40 mM NaOH, and 0.1% BSA). Cells were treated with HDB and several compounds isolated from HDB at various concentrations for 1 h at 37 °C and then challenged with 0.2 μg mL^{−1} DNP-BSA for 30 min at 37 °C. Histamine was then detected using an enzyme immunoassay kit (Oxford Biomedical Research, Rochester Hills, MI). As a measure of mast cell degranulation, a β-hexosaminidase release assay was performed on supernatants and lysed pellets from the treated cells aliquoted into 96-well plates. Samples were mixed with substrate solution (1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine in 0.05 M citrate buffer, pH 4.5) and incubated for 1 h at 37 °C. Reactions were terminated by the addition of 0.05 M sodium carbonate buffer, having pH = 10. Absorbance was measured by spectrophotometry at 405 nm.

Measurement of prostaglandin E₂

RBL-2H3 cells were sensitized with 0.2 μg mL^{−1} DNP-IgE overnight. Cells were treated with various concentrations of HDB or pinosylvin for 1 h and then activated with DNP-BSA for 4 h. The concentration of prostaglandin E₂ (PGE₂) in the cell culture medium was determined using enzyme immunoassays according to the manufacturers' protocol (R&D Systems, MN, USA, and Cayman Chemical, MI, USA).

Measurement of IL-4 and TNF-α release

RBL-2H3 cells were sensitized with 0.2 μg mL^{−1} DNP-IgE overnight. Cells were treated with various concentrations of HDB or pinosylvin for 1 h and then activated with DNP-BSA for 4 h. The cell culture supernatants were collected and IL-4 and TNF-α concentrations were measured using enzyme-linked immunosorbent assay kits (Abcam, UK).

Western blot analysis

Immunoblotting analysis was performed as described previously.²⁴ Equal amounts of the protein samples (15 µg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes (Whatman GmbH, Dassel, Germany). The membranes were then incubated with primary antibodies for 16 h at 4 °C, followed by horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. The protein bands were detected with enhanced chemoluminescence (ECL; Amersham Biosciences, Little Chalfont, UK) and visualized using an ECL Advance system (GE Healthcare, Hatfield, UK).

Preparation of nuclear extracts

RBL-2H3 cells were sensitized with DNP-IgE (200 ng mL⁻¹) and pretreated with HDB (5–20 µg mL⁻¹) for 1 h, and then they were stimulated with DNP-BSA for 30 min. After washing twice, the cells were scraped into ice-cold phosphate-buffered saline (PBS) and pelleted at 14 000 g for 5 min. Cytoplasmic and nuclear extracts were prepared from cells using a NE-PER kit (Pierce Biotechnology, Rockford, IL, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from RBL-2H3 cells was extracted using RNAiso reagent (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol and quantified using a NanoDrop spectrophotometer at 260 nm. Reverse transcription (RT) was carried out in a 20 µL reaction volume containing 2 µg total RNA, oligo (dT), and reverse transcriptase premix (Elpis-Biotech, Inc., Taejeon, Korea). RT was initiated at 70 °C for 5 min, followed by incubation at 42 °C for 60 min, and was terminated at 94 °C for 5 min. The PCR amplification of the cDNA products (3 µL) was performed with a PCR premix (Elpis-Biotech) and the following primer pairs: TNF-α forward, 5'-CAA GGA GCA GAA GTT CCC AA-3' and TNF-α reverse, 5'-CGG ACT CCG TGA TGT CTA AG-3' (500 bp); IL-4 forward, 5'-ACC TTG CTG TCA CCC TGT TC-3' and IL-4 reverse, 5'-TTG TGA GCG TGG ACT CAT TC-3' (351 bp); COX-2 forward, 5'-TGA CTG TAC CCG GAC TGG AT-3' and COX-2 reverse, 5'-CAT GGG AGT TGG GCA GTC AT-3' (322 bp); NFκB1 forward, 5'-TGG CAG ACG ACG ATC CTT TC-3' and NFκB1 reverse, 5'-GGC CAT CTG TTG ACA GTG GTA T-3' (117 bp); NFκB2 forward, 5'-TCG ACC TCC ACC GGA TCT TT-3' and NFκB2 reverse, 5'-CCA CAA TGG AGG GGC TGA AT-3' (294 bp); β-actin forward, 5'-AGC CAT GTA CGT AGC CAT CC-3' and β-actin reverse, 5'-TCT CAG CTG TGG TGG TGA AG-3' (227 bp). Before PCR amplification, all the primers were denatured at 94 °C for 5 min. The amplification consisted of 28 cycles of denaturation at 94 °C for 30 s, annealing for 1 min (at 52 °C for IL-4; 55 °C for TNF-α and β-actin; 56 °C for NFκB1; and 57 °C for COX-2 and NFκB2), and extension at 72 °C for 1 min, followed by a final 5 min extension at 72 °C. PCR was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). PCR products were

separated by 1.5% agarose gel electrophoresis and were visualized by ethidium bromide staining and UV illumination.

Passive cutaneous anaphylaxis (PCA) in mice

DNP-IgE (0.5 mg) was injected intradermally into the left ears of BALB/c mice. After IgE sensitization, HDB (100 or 300 mg kg⁻¹) or cetirizine (20 mg kg⁻¹) was orally administered. One hour later, the mice were challenged with an intravenous tail vein injection of 250 µg DNP-BSA in 250 µL PBS containing 5% Evans Blue. After 1 h, mice were killed and the treated ears were removed. The dye absorbed by the ear tissue was extracted overnight in 800 µL formamide at 63 °C. The intensity of the dye dissolved in formamide was measured at 620 nm. Procedures used in this study were approved by the Animal Care and Use Committee of Korea Institute of Science and Technology.

Statistical analysis

Each experiment was performed at least three times. Results are expressed as the mean ± standard deviation (SD). The vehicle (DMSO) and treated groups were compared by the one-way analysis of variance followed by Scheffé's test (SPSS 17.0, Chicago, IL, USA). $p < 0.01$ (##), $p < 0.05$ (*), and $p < 0.01$ (**) were considered statistically significant.

Results

HDF, HDB, and HDL is not cytotoxic to antigen-stimulated RBL-2H3 cells

DNP-BSA-stimulated RBL-2H3 cells were treated with HDF, HDB, and HDL, and then cytotoxicity was measured using the colorimetric MTT assay. HDF, HDB, and HDL (5–20 µg mL⁻¹) did not affect the RBL-2H3 cell viability (data not shown).

HDB suppresses β-hexosaminidase and histamine release

To determine the effect of HDF, HDB, and HDL on mast cell degranulation, β-hexosaminidase release was examined. Of these, HDB demonstrated the greatest inhibition of mast cell degranulation from IgE-sensitized RBL-2H3 cells (Fig. 1A) and was therefore chosen for further study. To further investigate the anti-allergic activity of HDB, we assessed its effect on histamine release. HDB also inhibited histamine release in RBL-2H3 cells treated with IgE (Fig. 1B).

HDB suppresses pro-inflammatory cytokine production and gene expression

The pro-inflammatory cytokines, IL-4 and TNF-α are known to have a crucial role in allergic inflammatory disease.²⁵ We, therefore, investigated the effect of HDB on the production and mRNA expression of IL-4 and TNF-α in antigen-stimulated mast cells. HDB significantly suppressed both IL-4 and TNF-α production at 20 µg mL⁻¹ concentration (Fig. 2A and B) and IL-4 and TNF-α mRNA expression (Fig. 2C) in IgE-sensitized RBL-2H3 cells at all the concentrations treated.

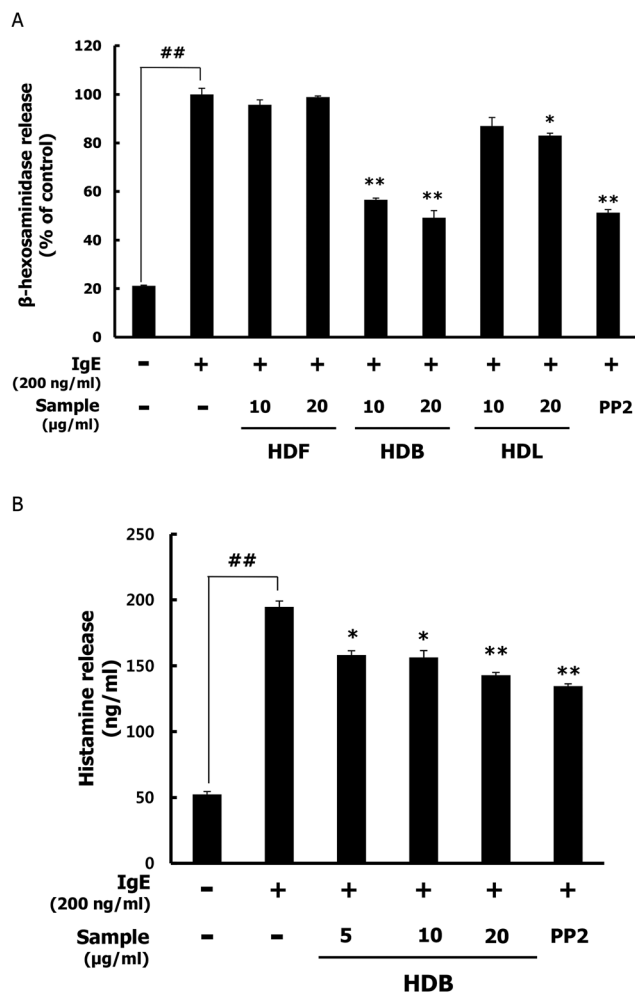


Fig. 1 Effects of the branches of *Hovenia dulcis* extract (HDB) on β -hexosaminidase and histamine release. (A) The degranulation was determined by measuring β -hexosaminidase release in antigen-stimulated RBL-2H3 cells. The RBL-2H3 cells were treated in the presence or absence of the fruits (HDF), HDB, and leaves (HDL) of *Hovenia dulcis* extract (10 or 20 μ g mL⁻¹) for 1 h and then stimulated for 1 h with antigen. (B) Histamine release was determined with an enzyme-linked immunosorbent assay kit. The RBL-2H3 cells were treated in the presence or absence of HDB (5, 10 or 20 μ g mL⁻¹) for 1 h and then stimulated for 1 h with antigen. PP2 (10 μ M) is a general Src-family kinase inhibitor. Results are expressed as the mean $\pm$ SD of three independent experiments (** p < 0.01, * p < 0.05 and ** p < 0.01).

HDB inhibits PGE₂ production and COX-2 expression

Fc ϵ RI-activated signaling initiates the arachidonate cascade in antigen-stimulated mast cells.²⁶ We therefore investigated the effect of HDB on the production of PGE₂, a COX metabolite of arachidonic acid. HDB markedly suppressed PGE₂ production from 20 μ g mL⁻¹ concentration in IgE-sensitized RBL-2H3 cells (Fig. 3A). The enzyme COX-2 is a mediator of the production of PGs from arachidonic acid. We therefore examined the effect of HDB on the expression of COX-2 in IgE-sensitized RBL-2H3 cells using RT-PCR and western blot analysis. The level of both COX-2 mRNA and protein increased in the IgE-sensitized

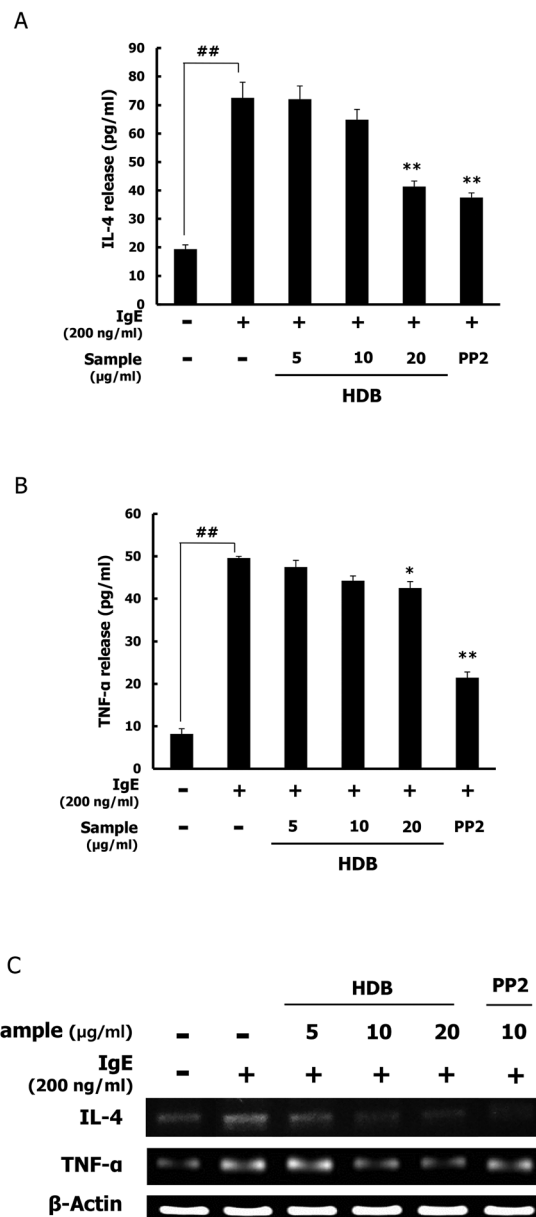


Fig. 2 Effects of HDB on IL-4 and TNF- α production and expression. RBL-2H3 cells were treated with HDB for 1 h and then stimulated with DNP-BSA for 4 h. (A and B) IL-4 and TNF- α production was determined by an enzyme-linked immunosorbent assay kit. (C) IL-4 and TNF- α mRNA levels were determined with RT-PCR. PP2 (10 μ M) is a general Src-family kinase inhibitor. Results are expressed as the mean $\pm$ SD of three independent experiments (** p < 0.01, * p < 0.05 and ** p < 0.01).

RBL-2H3 cells; however, treatment with HDB inhibited this upregulation (Fig. 3B and C).

HDB attenuates the activation and mRNA expression of NF- κ B

To examine the mechanism by which HDB inhibits NF- κ B transcriptional activity, we assessed its effects on nuclear translocation and phosphorylation of NF- κ B. HDB inhibited antigen-induced nuclear translocation of NF- κ B p65 protein (Fig. 4A).

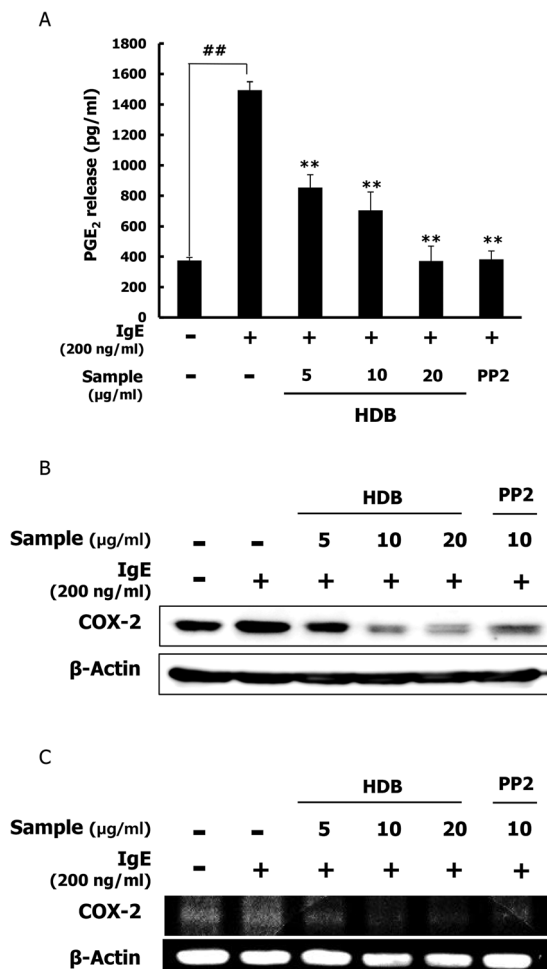


Fig. 3 Effects of HDB on PGE₂ secretion and COX-2 mRNA expression. RBL-2H3 cells were treated with HDB (5, 10 or 20 μg mL⁻¹) for 1 h and then stimulated for 4 h with antigen. (A) PGE₂ secretion was determined by an enzyme-linked immunosorbent assay kit. (B and C) COX-2 protein and mRNA level were determined with Western blot assay and RT-PCR, respectively. PP2 (10 μM) is a general Src-family kinase inhibitor. Results are expressed as the mean ± SD of three independent experiments (***p* < 0.01 and ****p* < 0.001).

Moreover, in antigen-stimulated cells, HDB inhibited the phosphorylation and degradation of IκB, which regulates the nuclear translocation of NF-κB (Fig. 4B). Furthermore, HDB reduced mRNA expression of NFKB1 and NFKB2 (Fig. 4C). Thus, it is likely that HDB inhibits the releases of allergic mediators *via* the inhibition of NF-κB transcriptional activation and post-translational modification.

HDB suppresses the FcεRI signaling pathway

The crosslinking of FcεRI by IgE and antigen initiates downstream signaling and consequent mast cell activation (leading to degranulation) *via* the phosphorylation of Lyn, Syk, PKC, and PLCγ.²⁷ To determine the effect of HDB on this pathway, FcεRI downstream signals were assessed in antigen-stimulated RBL-2H3 cells after treatment with the extract. Antigen stimu-

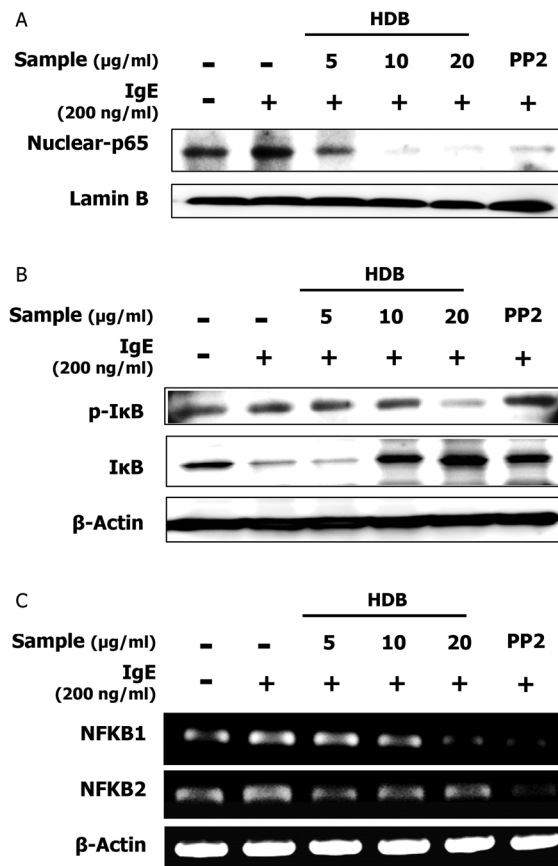


Fig. 4 Effect of HDB on NF-κB pathway and mRNA expression. (A) RBL-2H3 cells were treated with HDB for 1 h and then stimulated with DNP-BSA for 30 min. After nuclear proteins were extracted, NF-κB p65 was determined by western blot analysis. (A) RBL-2H3 cells were treated with HDB for 1 h and then stimulated with DNP-BSA for 30 min. Phosphorylation of IκB and IκB was determined by western blot analysis. (C) NFKB1 and NFKB2 mRNA levels were determined with RT-PCR. PP2 (10 μM) is a general Src-family kinase inhibitor.

lation increased the phosphorylation of Lyn, Syk, PKD/PKCμ, and PLCγ, and treatment with HDB reduced this phosphorylation (Fig. 5A). Phosphorylation of MAPKs leads to the production of cytokines;²⁸ therefore, to investigate the downstream effectors that directly regulate degranulation and cytokine production, we assessed the phosphorylation status of JNK, ERK, and p38. HDB inhibited phosphorylation of ERK and p38 in IgE-sensitized RBL-2H3 cells at higher concentrations, but the phosphorylation of JNK was not affected at any concentration (Fig. 5B).

HDB inhibits mast cell-mediated PCA in mice

The anti-allergic activity of HDB was then tested *in vivo* using a mast cell-dependent PCA model. To induce PCA, DNP-IgE was injected intradermally into mouse ear tissue, followed by the intravenous injection of DNP-BSA containing 5% Evans Blue dye. PCA was successfully induced through the intradermal injection of IgE in the mouse ear. Orally-administered HDB (100 or 300 mg kg⁻¹) or the widely used anti-histamine drug

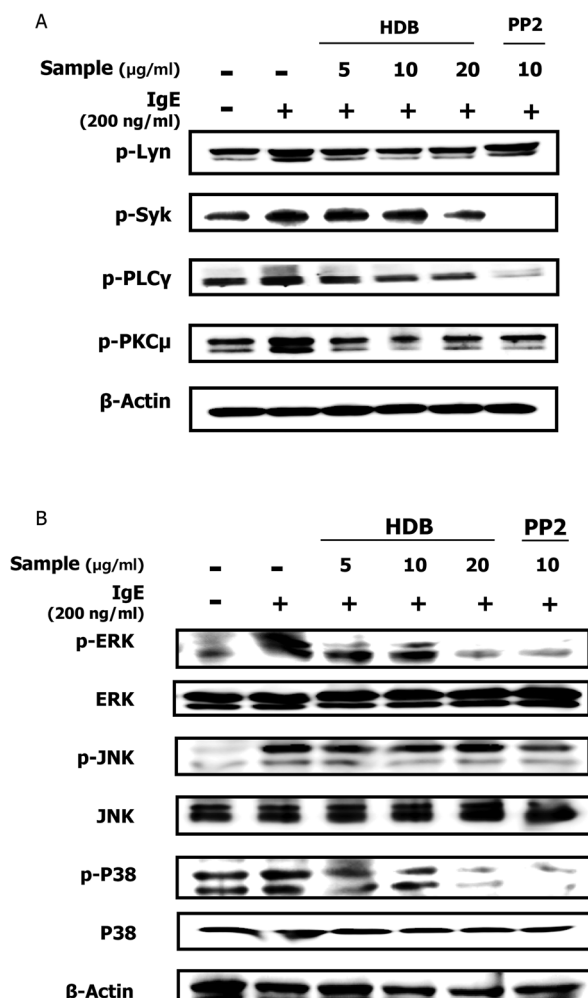


Fig. 5 Effect of HDB on FcεRI signaling. (A) RBL-2H3 cells were treated with HDB for 1 h and then stimulated with DNP-BSA for 10 min. Phosphorylation of Lyn, Syk, PLCγ, and PKD/PKCμ was determined by western blot analysis. (B) RBL-2H3 cells were treated with HDB for 1 h and then stimulated with DNP-BSA for 30 min. Phosphorylation of JNK, ERK, and p38 was determined by western blot analysis. PP2 (10 μM) is a general Src-family kinase inhibitor.

cetirizine (20 mg kg⁻¹) suppressed the antigen-stimulated PCA response (Fig. 6A and B).

Effects of pinosylvin on degranulation and proinflammatory mediators in antigen-stimulated RBL-2H3 cells

To determine the effect of several compounds isolated from HDB on mast cell degranulation, β-hexosaminidase release was examined. We found that taxifolin, pinosylvin, and dihydrokaempferol inhibited the antigen-stimulated degranulation of RBL-2H3 cells. Notably, pinosylvin had the most potent inhibitory effect on β-hexosaminidase release among all of the compounds tested (Fig. 7B). To further investigate the mechanism study of pinosylvin on IgE-sensitized RBL-2H3 cells, cytotoxicity was measured using the colorimetric MTT assay. Pinosylvin (5–20 μg mL⁻¹) did not affect the RBL-2H3 cell

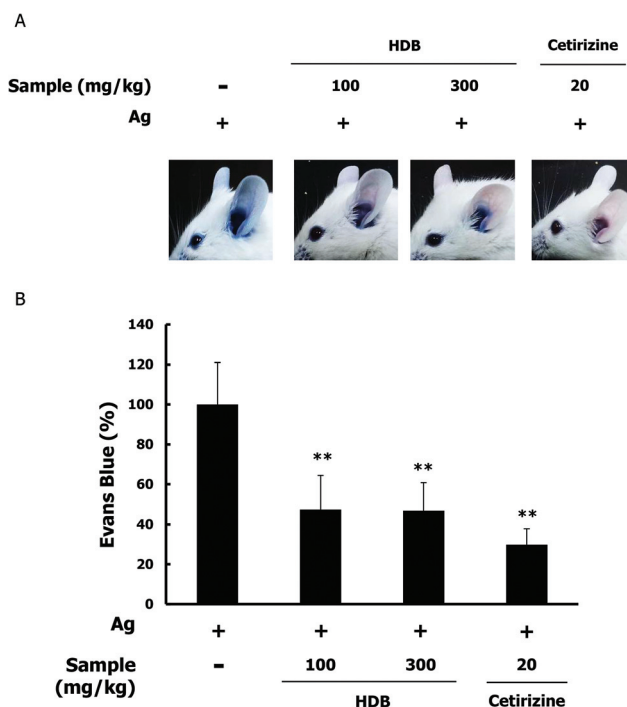


Fig. 6 Effect of HDB on IgE-mediated passive cutaneous anaphylaxis (PCA). (A) HDB (100 or 300 mg kg⁻¹) or cetirizine (20 mg kg⁻¹) was orally administered to mice for 1 h before IgE sensitization. Evans blue stained ears were photographed. (B) The ear absorbed dye was extracted with formamide. The quantification data is presented as % of IgE-stimulated control. Cetirizine (20 mg kg⁻¹) is an anti-histamine reference drug. Results are expressed as the mean ± SD of three independent experiments. (**p < 0.01).

viability (Fig. 7C). Furthermore, the effects of pinosylvin on proinflammatory mediators were evaluated to determine anti-allergic activity. Pinosylvin inhibited the release of IL-4, TNF-α and PGE₂ (Fig. 7D–F) and the expression of IL-4, TNF-α, COX-2, NFKB1, and NFKB2 in a dose-dependent manner in RBL-2H3 cells treated with IgE (Fig. 7G).

Discussion

Mast cells are key effector cells in allergic disorders. They produce allergic mediators, including histamines and PGE₂, and regulate allergic inflammatory responses.²⁹ In addition, the studies of mast cell-deficient mice show that mast cell-derived mediators (histamines, proteases, and cytokines) play a crucial role during allergic inflammation.³⁰ Many studies have investigated various mediators of mast cell activation, and they have revealed active components that target allergic mediators.^{31–33}

Previous reports have demonstrated that the extracts of *H. dulcis* have antioxidative, anti-diabetic, anti-fatigue, neuroprotective, hepatoprotective, and immunostimulatory activities.^{21,34–38} However, the effects of the branches of *H. dulcis* on allergic disease have not been revealed previously.

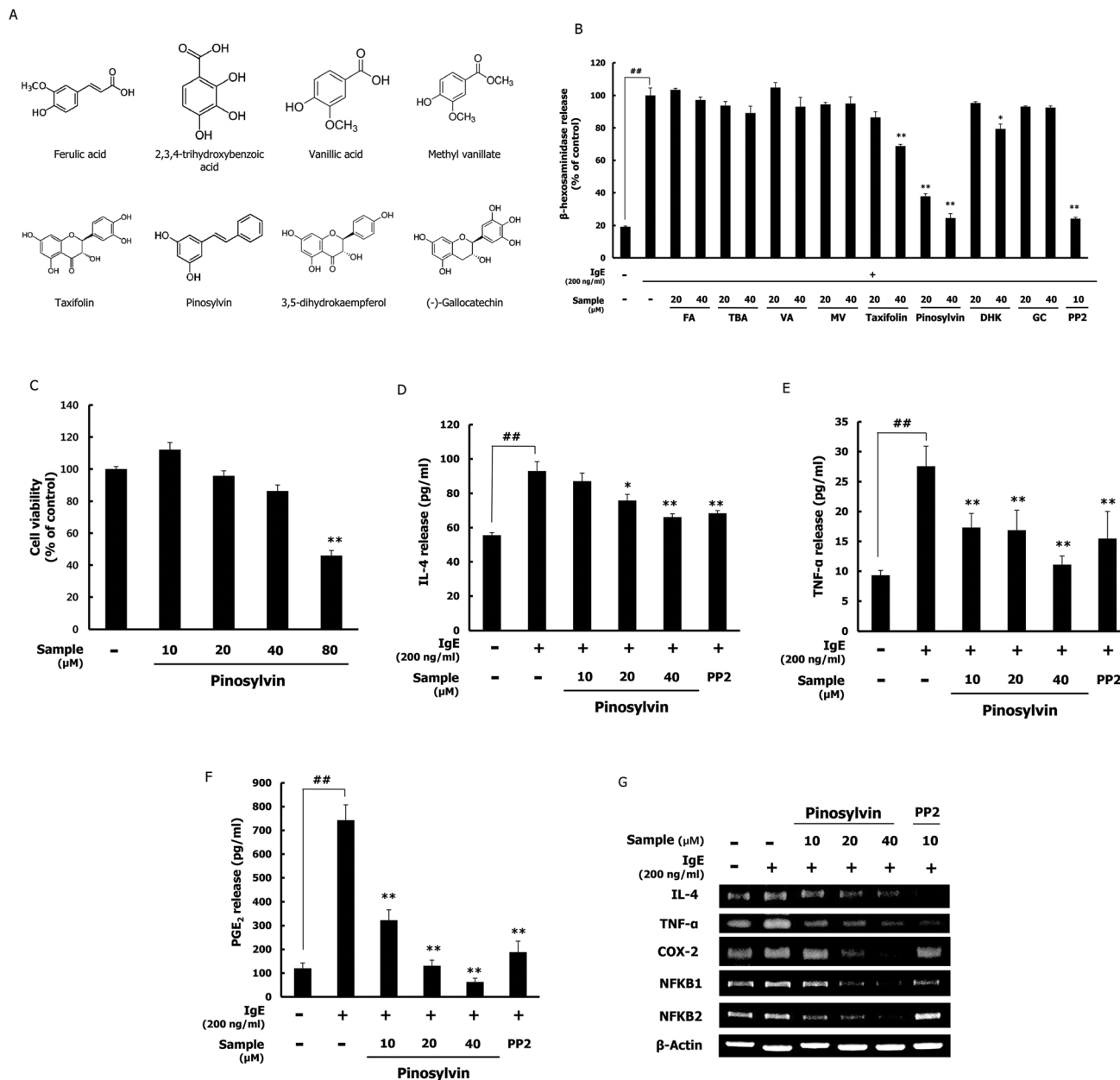


Fig. 7 Effects of pinosylvin on degranulation and proinflammatory mediators. (A) Chemical structure of phenolic compounds from HDB. (B) The degranulation was determined by measuring β -hexosaminidase release in antigen-stimulated RBL-2H3 cells. The RBL-2H3 cells were treated in the presence or absence of ferulic acid (FA), 2,3,4-trihydroxybenzoic acid (TBA), vanillic acid (VA), methyl vanillate (MV), taxifolin, pinosylvin, dihydrokaempferol (DHK), and (-)-gallocatechin (GC) (20 or 40 μ M) for 1 h and then stimulated for 1 h with antigen. (C) The RBL-2H3 cells were treated in the presence or absence of pinosylvin (5–80 μ M) for 6 h. Cell viability was determined by an MTT assay. (D–F) The RBL-2H3 cells were treated in the presence or absence of pinosylvin (10, 20 or 40 μ M) for 1 h and then stimulated for 4 h with antigen. IL-4, TNF- α , and PGE₂ production was determined by an enzyme-linked immunosorbent assay kit. (G) IL-4, TNF- α , COX-2, NFKB1, and NFKB2 mRNA levels were determined with RT-PCR. PP2 (10 μ M) is a general Src-family kinase inhibitor. Results are expressed as the mean $\pm$ SD of three independent experiments. ($^{##}p < 0.01$, $^{*}p < 0.05$ and $^{**}p < 0.01$).

In this study, we investigated the inhibitory effects of the HDB and its constituents on allergic mediators in mast cells *in vitro* and in a PCA mouse model *in vivo*.

Granule-associated β -hexosaminidase is released along with other allergic mediators (e.g., serotonin and histamine) upon IgE crosslinking of Fc ϵ RI,³⁹ leading to smooth muscle contrac-

tion, vascular permeability, and inflammation. We found that HDB inhibits degranulation and histamine release from activated mast cells *via* suppressing the release of both β -hexosaminidase (Fig. 1A) and histamine (Fig. 1B) in a dose-dependent manner. In the previous reports, the triterpene glycosides from the fruit of *Hovenia dulcis* exhibited inhibitory

activity on the histamine release from rat mast cells induced by compound 48/80 or calcium ionophore A23157.⁴⁰ In our study, however, the fruit of *Hovenia dulcis* did not inhibit mast cell degranulation, and HDB exhibited the strongest inhibitory effect of allergic response among HDF, HDB, and HDL. It is reported that HDB contained the phenolic compounds such as vanillic acid, ferulic acid, 3,5-dihydroxystilbene, (–)-catechin, (+)-afzelechin, and (+)-aromadendrin,²¹ and these compounds exhibited the anti-allergic activity.^{41–43} Therefore, we suggest that these compounds influenced the effect of HDB on allergic response.

HDB markedly decreased the production and expression of the proinflammatory cytokines TNF- α and IL-4 (Fig. 2A–C). TNF- α and IL-4 induce immune reactions and play a critical role in initiating allergic responses in mast cells. IL-4 is crucial for the Th2 cellular response and triggers B-cell class switching to IgE.⁴⁴ Although inflammation is a necessary process in the defense against pathogens, excessive cytokine overexpression can induce serious inflammatory disorders such as allergic rhinitis, asthma, and atopic dermatitis. Thus, the finding in the present study that HDB suppressed the expression of TNF- α and IL-4 indicates that this extract could be beneficial for the treatment of allergic disorders.

COX-2 expression is primarily regulated by the NF- κ B signaling pathway in various cells, and NF- κ B is activated by ubiquitination, phosphorylation, and subsequent proteolytic degradation.⁴⁵ NF- κ B is a dimeric transcription factor formed by the hetero- or homo-dimerization of proteins in the Rel family, including p50 and p60.⁴⁶ It plays an important role in the immune response and in cell differentiation induced by cytokines, growth factors, and other cell activators.⁴⁷ The NF- κ B transcription factor translocates to the nucleus and binds to κ B motifs in the promoters of several target genes, including TNF- α , IL-4, and COX-2, to promote their transcription.⁴⁵ HDB attenuated the release of the inflammatory mediator PGE₂ (Fig. 3A), an arachidonic acid metabolite⁴⁸ and the expression of COX-2, which mediates the production of PGs from arachidonic acid (Fig. 3B and C). HDB also inhibits the translocation of NF- κ B in antigen-stimulated mast cells (Fig. 4). These results suggest that HDB suppresses the production of inflammatory mediators in IgE-induced mast cells by inhibiting the COX pathway and NF- κ B activation.

Crosslinking of the tetrameric complex Fc ϵ RI by IgE triggers the Fc ϵ RI signaling cascade, which includes Syk, Lyn, PKC, and PLC γ , bringing about an allergic response.⁴⁹ The Fc ϵ RI signaling pathway is initiated by the phosphorylation of Syk and Lyn, which can then act as docking sites for adaptor molecules, such as Gads, Grb2, SLP76, PLC γ and PKC, which in turn activate downstream signaling.⁵⁰ It has been reported that the regulation of Syk may be the main target for the treatment of allergic diseases. For example, aerosolized Syk antisense oligonucleotides are used as a therapeutic agent for allergic rhinitis and asthma.⁵¹ To determine the mechanisms of HDB action, we examined the phosphorylation of signaling molecules. In our study, HDB suppressed the phosphorylation of multiple downstream signaling such as Syk, Lyn, PKD/PKC μ ,

and PLC γ (Fig. 5A). These results indicate that HDB may have anti-allergic effects in antigen-stimulated mast cells by the inactivation of Fc ϵ RI signaling cascade.

The extensive MAPK signaling pathway is an important therapeutic target in allergic disorders.⁵² To assess the mechanism by which HDB attenuated the activation of mast cells, we analyzed the phosphorylation status of the MAPK pathway components JNK, ERK, and p38. HDB suppressed the antigen-stimulated phosphorylation of ERK and p38, but not JNK. Studies with Syk kinase activity showed an important role for ERK and p38 but not JNK in cytokine secretion.⁵³ This report supports our results showing the regulatory role of ERK and p38 in activated mast cells. Our data suggest that HDB inhibited the activation of mast cells by suppressing ERK and p38 signaling (Fig. 5B).

To identify the ingredients responsible for inhibiting degranulation in antigen-stimulated mast cells, we tested the eight constituents in HDB to determine whether they suppress mast cell activation (Fig. 7A). Of those, we determined that pinosylvin is the most effective active ingredient that decreases mast cell degranulation (Fig. 7B). Pinosylvin is present in many plants, including the stem bark of *Pinus* species and HDB, and it has been reported to have anti-oxidant, anti-inflammation, anti-bacterial, and anti-cancer activity.^{54–57} Here, we showed that pinosylvin suppressed the release and expression of allergic and inflammatory mediators in IgE-induced mast cells (Fig. 7D–G). We show that treatment with HDB or pinosylvin is not critically cytotoxic to RBL-2H3 cells and does not significantly affect the weights of mice used *in vivo* experiments.

Conclusion

In summary, this study investigated the beneficial effects of the HDB and its constituents on allergic responses and PCA for the first time. HDB and pinosylvin suppressed degranulation, release of histamine, and the production of PGE₂ and inflammatory cytokines in antigen-induced mast cells *in vitro*. HDB suppressed the Fc ϵ RI signaling pathway by inhibiting the phosphorylation of Syk, Lyn, PKC, PLC γ , and MAPKs. Furthermore, HDB attenuated PCA *in vivo*. Taken together, our findings suggest that HDB would be a useful agent for treating allergic diseases.

Conflict of interest

The authors declare that there are no conflicts of interest.

Author contributions

M.K and S.J.L. contributed equally to this work.

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