



Gnetin H isolated from *Paeonia anomala* inhibits FcεRI-mediated mast cell signaling and degranulation

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ABSTRACT

Ethnopharmacological relevance: *Paeonia anomala* L. is used in Mongolian traditional medicine to treat various diseases including indigestion, abdominal pain, kidney disorders, inflammation, and female diseases. In this study we examined the effects of *Paeonia anomala* extract (PAE) and compounds derived from *Paeonia anomala* on immunoglobulin E (IgE)-mediated type I hypersensitivity responses *in vitro*.

Materials and methods: Degranulation assay, reverse transcription PCR, enzyme-linked immunosorbent assays, western blot analyses were performed to measure allergic and proinflammatory mediators in IgE-stimulated rat basophilic leukemia (RBL)-2H3 mast cells treated with or without PAE or gnetin H.

Results: Seventeen compounds were isolated, and β-hexosaminidase release from IgE-stimulated RBL-2H3 mast cells was measured. Of the seventeen isolated compounds, gnetin H, a resveratrol derivative, significantly inhibited β-hexosaminidase release from RBL-2H3 cells with an IC₅₀ value of 0.3 μM. Notably, Gnetin H reduced β-hexosaminidase release at lower concentrations than resveratrol. Furthermore, PAE and gnetin H inhibited histamine secretion, decreased the production and mRNA expression of tumor necrosis factor-α and interleukin-4 and suppressed translocation of nuclear factor κB. PAE and gnetin H also reduced the expression of cyclooxygenase-2 and production of prostaglandin E₂. PAE and gnetin H suppressed the phosphorylation of Syk, protein kinase C (PKC)μ, phospholipase Cγ, and the mitogen-activated protein kinases, c-Jun N-terminal kinase, p38, and extracellular signal-regulated kinase.

Conclusions: These results suggest that PAE and its active compound gnetin H could be promising therapeutic agents for allergic disorders.

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1. Introduction

The prevalence of antigen-related allergic disorders has increased worldwide over the last few decades (Kay, 2001). These allergic diseases are immunological disorders and are affected by genetic, environmental, dietary factors, and lifestyle (Ho, 2010). Typical symptoms include atopic dermatitis, allergic rhinitis, asthma, and eczema (Spergel, 2010).

Mast cells are inflammatory cells and are broadly distributed throughout various tissues and organs (Grivennikov et al., 2010). Mast cells play an important role in allergic inflammatory diseases, such as asthma, rhinitis, atopic dermatitis, and anaphylaxis (Leung et al., 2004). Mast cells express FcεRI, the high-affinity receptor for IgE, which stimulates downstream signaling pathways to activate mast cells (Grivennikov et al., 2010). Mast cell activation promotes degranulation and production of inflammatory mediators, such as

prostaglandins, leukotrienes, and several proinflammatory cytokines including interleukin (IL-4) and tumor necrosis factor (TNF)-α (Gilfillan and Tkaczyk, 2006). Mast cell degranulation is induced in part through nuclear translocation of nuclear factor κB (NF-κB) (Saito et al., 2009). NF-κB is a key transcription factor that modulates the expression of a variety of genes critical to immune responses (Marquardt and Walker, 2000).

During mast cell activation, histamine induces allergic inflammation either directly or indirectly through IgE sensitization. β-hexosaminidase is also concurrently released from activated cells (Theoharides et al., 2007). Thus, β-hexosaminidase release is a key marker for evaluating mast cell-mediated allergic reactions. RBL-2H3 cells can be used to assess the potential allergenicity of antigens to humans and other animals, such as dogs and horses, which are known to suffer from similar allergic manifestations (Rashid et al., 2012).

Paeonia anomala L. (Paeoniaceae) is used in Mongolian traditional medicine for treatment of exhaustion, hemorrhage, nocturnal enuresis, indigestion, blood clotting, respiratory tract disorders, abdominal pain, kidney disorders, bladder inflammation and gynecological diseases (Ligaa et al., 2006; Volodya et al., 2008). It is one of the

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most important crude drugs in traditional medicines of Euro-Asian countries including Russia, Mongolia, and China, used as anti-inflammatory, analgesic, and sedative agent and a remedy for female diseases (Zapesochay et al., 1992; Ligaa et al., 2006; Li et al., 2007). *Paeonia lactiflora*, the other species of *Paeonia* and a famous traditional Chinese medicine, has been used for more than 1200 years because of its anti-inflammatory and immune-regulatory properties (Ji et al., 2013). It was reported to possess antioxidant, anti-inflammation, anti-allergic, antibacterial properties (Kim et al., 2002; Lee et al., 2008; Jiang et al., 2011; Ngan et al., 2012).

Recently, our group demonstrated that *Paeonia anomala* has protective effects against tert-butylhydroperoxide-induced oxidative stress in HepG2 cells. Furthermore, several compounds isolated from *Paeonia anomala* mediate hepatoprotective effects (Oidovsambuu et al., 2013). Although anti-bacterial (Gonchig et al., 2008), anti-coagulant (Liapina et al., 2000), anti-thrombotic (Lyapina et al., 1997), and hepatoprotective effects of *Paeonia anomala* have been reported, its effects on allergic disease have not been investigated. In the present study, we investigated the effects of *Paeonia anomala* on IgE-stimulated allergic responses and FcεRI-mediated signaling.

2. Materials and methods

2.1. Reagents and materials

Fetal bovine serum (FBS), penicillin, and Dulbecco's-modified Eagle medium (DMEM) were purchased from Hyclone (South Logan, UT, USA). Anti-dinitrophenyl (DNP)-specific immunoglobulin E (IgE), DNP-bovine serum albumin (BSA), 1,4-piperazinediethanesulfonic

acid (PIPES), and PP2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies targeting cyclooxygenase 2 (COX-2), phospho-IκB (Ser32), IκB, NF-κB p65, phospho-Syk (Tyr525/526), phospho-Lyn (Tyr507), phospho-phospholipase C (PLC)γ (Tyr783), phospho-PKD/PKCμ (Ser916), phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK (Thr183/Tyr185), phospho-p44/42 MAPK (Erk1/2), p44/42 MAPK (Erk1/2), phospho-p38, p38, and β-actin were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA).

2.2. Plant material

Paeonia anomala was collected in Bayanchandmani Sum, Tov province, near Ulaanbaatar, Mongolia and was identified by Dr. C. Sanchir, Institute of Botany, Mongolian Academy of Sciences. A voucher specimen was placed in the Flora and Plant Systematic Laboratory, Institute of Botany, Mongolian Academy of Sciences.

2.3. Extraction and isolation of compounds

Dried *Paeonia anomala* plants (3.8 kg) were extracted with 95% EtOH for 3 days at room temperature and filtered through Whatman no. 1 filter paper (GE Healthcare Life Sciences, PA, USA). The filtrates were evaporated in vacuo. The final weight of extracts was 57 g. Seventeen compounds (Fig. 1) were isolated from *Paeonia anomala* extract (PAE), as previously described (Oidovsambuu et al., 2013). Compound purity was determined by gas chromatography (GC) or high performance liquid chromatography (HPLC) analysis (Oidovsambuu et al., 2013).

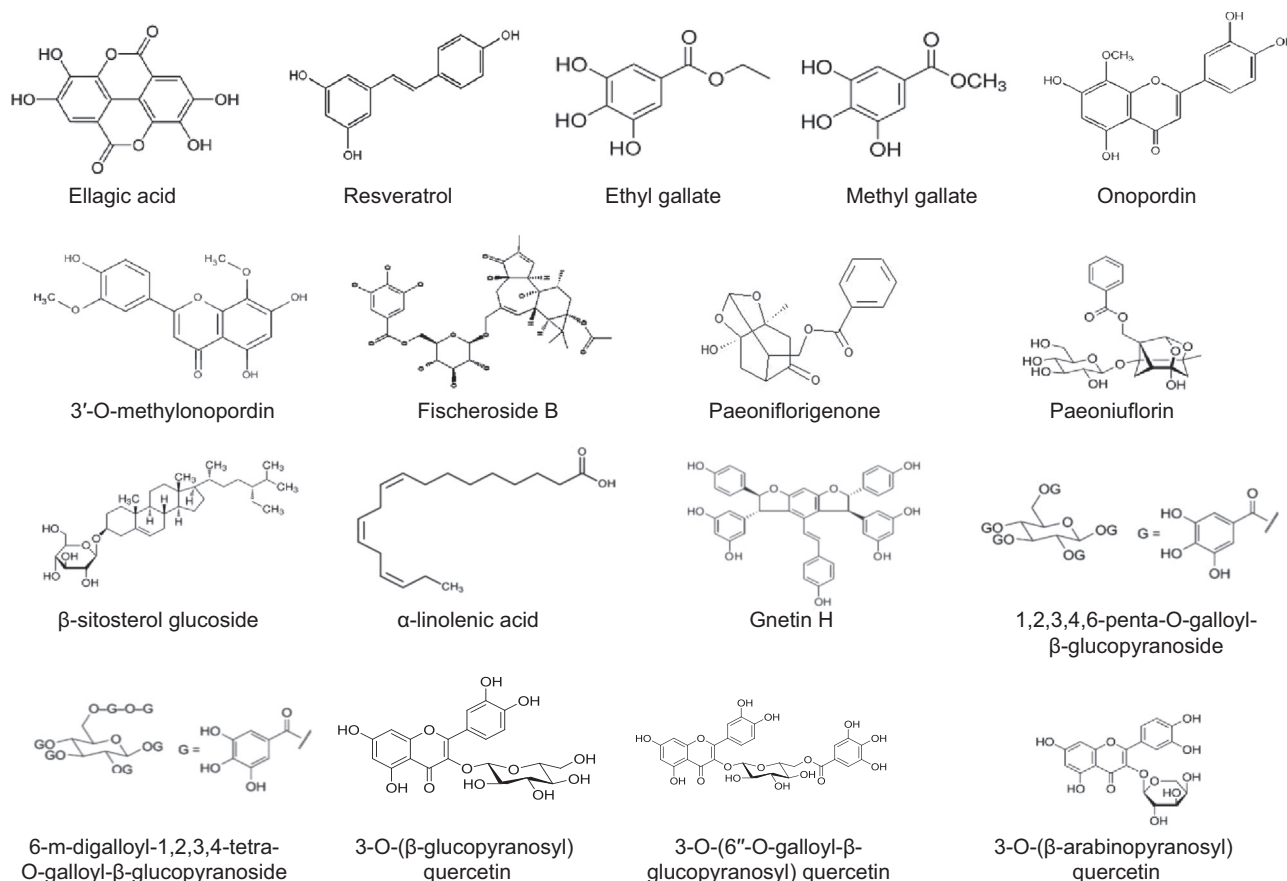


Fig. 1. Chemical structure of flavones and flavone glycosides from *Paeonia anomala*.

2.4. Cell culture

RBL-2H3 cells obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) via the Korean Cell Line Bank (KCLB no. 22256, Seoul, Korea) were cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin. Cells were grown in 100 π dishes at 37 °C with 5% CO₂ in a humidified atmosphere.

2.5. Cell viability assay

Cell viability was assayed using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich, St Louis, MO, USA). RBL-2H3 cells were grown in 24-well plates (2 $\times$ 10⁵ cells/mL) for 24 h. After treating cells with various concentrations of PAE and gnetin H for 6 h, cells were washed, treated with 200 μ L MTT (0.5 mg/mL) and then incubated for an additional 4 h. Cells were then washed and the insoluble formazan products were dissolved in 200 μ L DMSO. Absorbance was measured at 550 nm by spectrophotometry (Bio-Tek Instruments, Winooski, VT).

2.6. Measurement of histamine and β -hexosaminidase release

RBL-2H3 cells were sensitized with 0.2 μ g/mL monoclonal DNP-IgE. Cells were 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 PAE and compounds **1–17** at various concentrations for 1 h at 37 °C and then challenged with 0.2 μ g/mL DNP-BSA for 30 min at 37 °C. Histamine was then detected using an enzyme immunoassay kit (Oxford Biomedical Research, Rochester Hills, MI). To determine β -hexosaminidase release, supernatants and lysed pellets were 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, pH 10. Absorbance was measured by spectrophotometry at 405 nm.

2.7. Measurement of prostaglandin E₂

Cell culture medium was harvested, and secretion of prostaglandin E₂ (PGE₂) was determined using competitive radioimmunoassay kits (R&D Systems, MN, USA, and Cayman Chemical, MI, USA) according to the manufacturers' protocol.

2.8. Measurement of cytokine release

IgE-sensitized RBL-2H3 cells were incubated with PAE and gnetin H (various concentrations) for 1 h and stimulated with DNP-BSA for 4 h. Interleukin (IL)-4 and tumor necrosis factor (TNF)- α concentrations in cell culture supernatants were measured using enzyme-linked immunosorbent assay kits (Abcam, UK).

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's protocol and quantified by spectrophotometry at 260 nm. The cDNA was synthesized in a 20 μ L reaction containing 1 μ g total RNA, oligo (dT), and reverse transcriptase premix (Elpis-Biotech., Inc., Taejeon, Korea). Reverse transcription (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. PCR amplification of the cDNA products (5 μ L) 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); NFKB1 forward, 5'-TGG CAG ACG ACG ATC CTT TC-3', and NFKB1 reverse, 5'-GGC CAT CTG TTG ACA GTG GTA T-3' (117 bp); NFKB2 forward, 5'-TCG ACC TCC ACC GGA TCT TT-3', and NFKB2 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, primers for cytokines and β -actin were denatured at 94 °C for 5 min. Amplification consisted of 28 cycles of denaturation at 94 °C for 30 s, annealing for 1 min (52 °C for IL-4; 55 °C for TNF- α and β -actin; 56 °C for NFKB1; 57 °C for COX-2 and NFKB2), 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 visualized by ethidium bromide staining and UV illumination.

2.10. Preparation of nuclear extracts

Cells were pretreated with PAE (5–20 μ g/mL) and gnetin H (0.1–5 μ M) for 1 h, treated with DNP-BSA for 30 min, then immediately washed twice, scraped into 1.5 ml ice-cold PBS (pH 7.8) and pelleted at 14,000 g for 30 s. Cytoplasmic and nuclear extracts were prepared from cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA).

2.11. Western blot analysis

RBL-2H3 cells were lysed in RIPA buffer containing protease inhibitors and then held on ice for 10 min. Lysate protein concentrations were determined by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (30 μ g) in each sample were loaded, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Blotted membranes were then incubated with primary antibodies for 16 h at 4 °C, followed by horseradish peroxidase-linked secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. Proteins were detected with enhanced chemoluminescence (Amersham Biosciences, Little Chalfont, UK) and visualized using an ECL advanced system (GE Healthcare, Hatfield, UK).

2.12. Statistical analysis

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

3. Results

3.1. Identification of seventeen compounds from *Paeonia anomala*

Using LC-NMR/MS, we identified seventeen compounds including ellagic acid (> 98%), resveratrol (> 99%), ethyl gallate (> 98%), methyl gallate (> 98%), onopordin (> 98%), 3'-O-methylnopordin (> 97%), fischeroside B (> 93%), paeoniflorigenone (> 98%), paeoniflorin (> 98%), β -sitosterol glucoside (> 98%), α -linolenic acid (> 98%), gnetin H (> 92%), 1,2,3,4,6-penta-O-galloyl- β -glucopyranoside (PGG) (> 90%), 6-m-digalloyl-1,2,3,4-tetra-O-galloyl- β -glucopyranoside (HGG) (> 92%), 3-O-(β -glucopyranosyl) quercetin (> 85%),

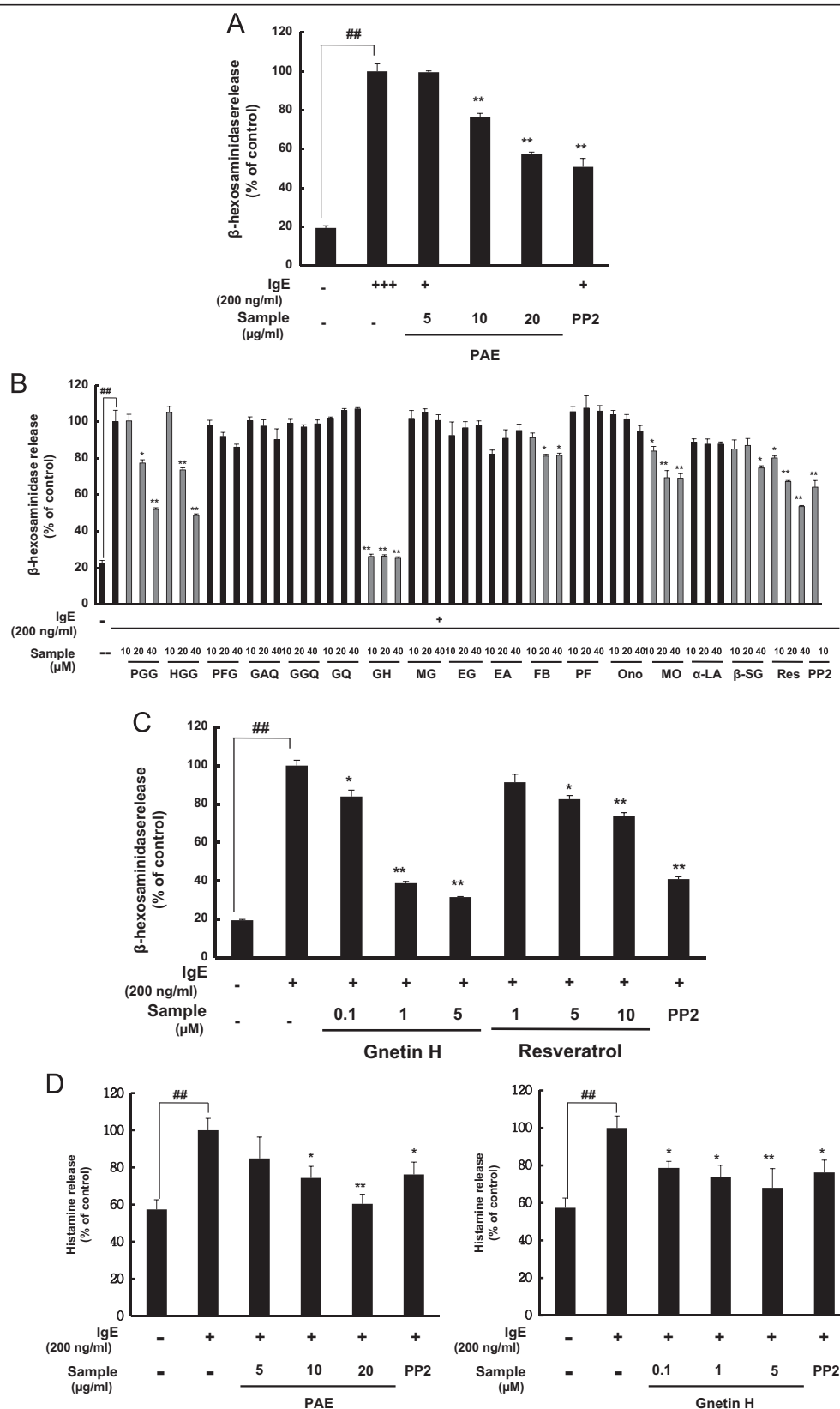


Fig. 2. Effects of PAE and gnetin H on β -hexosaminidase and histamine release. (A) The degranulation was determined by measuring β -hexosaminidase release in antigen-stimulated RBL-2H3 cells. The RBL-2H3 cells were treated in the presence or absence of PAE (5, 10 or 20 μ g/mL) for 1 h and then stimulated for 1 h with antigen. (B) The RBL-2H3 cells were treated in the presence or absence of 1,2,3,4,6-penta-O-galloyl- β -glucopyranoside (PGG), 6-m-digalloyl-1,2,3,4-tetra-O-galloyl- β -glucopyranoside (HGG), paeoniflorigenone (PFG), 3-O-(2''-O-galloyl- β -arabinopyranosyl) quercetin (GAQ), 3-O-(6''-O-galloyl- β -glucopyranosyl) quercetin (GGQ), 3-O-(β -glucopyranosyl) quercetin (GQ), gnetin H (GH), methyl gallate (MG), ethyl gallate (EG), ellagic acid (EA), fischeroside B (FB), paeoniflorin (PF), onopordin (Ono), 3'-O-methylonopordin (MO), α -linolenic acid (α -LA), β -sitosterol glucoside (β -SG), resveratrol (Res) (10, 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 gnetin H (0.1, 1 or 5 μ M) or resveratrol (1, 5 or 10 μ M) for 1 h and then stimulated for 1 h with antigen. (D) Histamine release was determined with enzyme-linked immunosorbent assay kit. The RBL-2H3 cells were treated in the presence or absence of PAE (5, 10 or 20 μ g/mL) or gnetin H (0.1, 1 or 5 μ M) 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).

3-O-(6''-O-galloyl- β -glucopyranosyl) quercetin (> 98%), and 3-O-(2''-O-galloyl- β -arabinopyranosyl) quercetin (> 87%). Compound purity was determined by HPLC or GC (Fig. 1).

3.2. Cytotoxicity of PAE and gnetin H on IgE-stimulated RBL-2H3 cells

RBL-2H3 cells were treated with PAE and gnetin H, and then cell viability was evaluated by the colorimetric MTT assay. PAE (5–20 μ g/mL) and gnetin H (0.5–5 μ M) had no effect on RBL-2H3 cell viability (data not shown).

3.3. Effects of PAE and *Paeonia anomala*-derived compounds on β -hexosaminidase and histamine release from IgE-stimulated RBL-2H3 cells

Previously we identified and isolated several compounds from *Paeonia anomala* (Oidovsambuu et al., 2013). To determine the effect of PAE and these compounds on mast cell degranulation, β -hexosaminidase release was examined. PAE inhibited the release of β -hexosaminidase from RBL-2H3 cells treated with IgE at

concentrations of 5, 10, and 20 μ g/mL in a dose-dependent manner (Fig. 2A). In addition, we found that PGG, HGG, gnetin H, resveratrol, fischeriside B, 3'-O-methylnopordin, and β -sitosterol glucoside inhibited IgE-stimulated degranulation of RBL-2H3 cells. Notably, gnetin H, a resveratrol trimer, had the most potent inhibitory effect on β -hexosaminidase release among all of the compounds tested (Fig. 2B and C). Furthermore, the effects of PAE and gnetin H on histamine release were evaluated to determine anti-allergic activity (Fig. 2D). PAE and gnetin H also inhibited histamine release in a dose-dependent manner in RBL-2H3 cells treated with IgE (Fig. 2E).

3.4. Effects of PAE and gnetin H on PGE₂ production and COX-2 expression in IgE-stimulated RBL-2H3 cells

The Fc ϵ RI-activated signaling initiates the arachidonate cascade in IgE-stimulated mast cells (Roth et al., 2008). Therefore, we investigated the effect of PAE and gnetin H on the production of PGE₂. PAE and gnetin H markedly suppressed PGE₂ production in IgE-sensitized RBL-2H3 cells (Fig. 3A). The effect of PAE and gnetin H on the COX-2 expression, an enzyme that produces PGs from

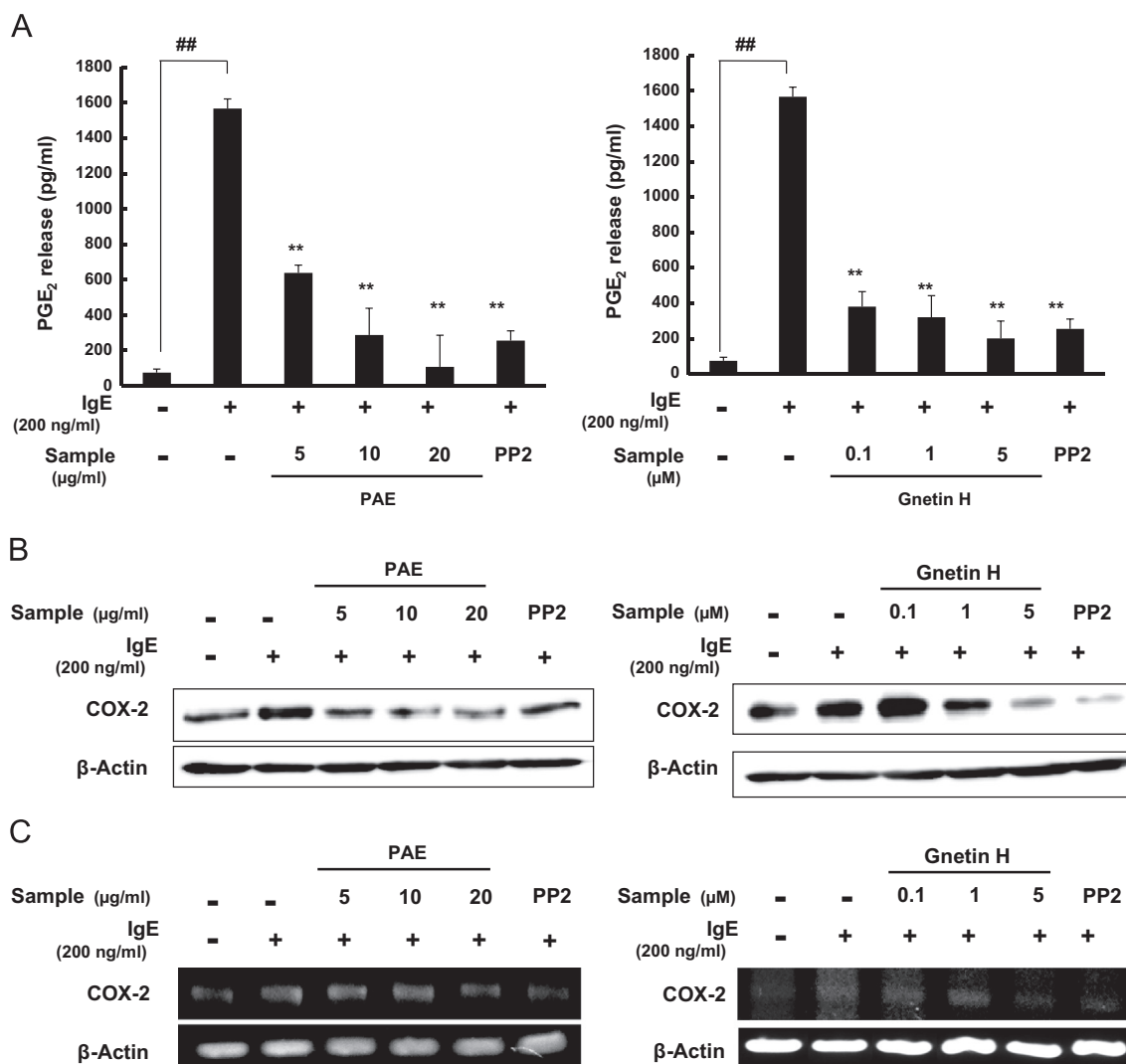


Fig. 3. Effects of PAE and gnetin H on PGE₂ secretion and COX-2 mRNA expression. RBL-2H3 cells were treated with PAE (5, 10 or 20 μ g/mL) or gnetin H (0.1, 1 or 5 μ M) for 1 h and then stimulated for 4 h with antigen. (A) PGE₂ secretion was determined by enzyme-linked immunosorbent assay kit. (B,C) COX-2 protein and mRNA level was determined with Western blot assay and RT-PCR, respectively. 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 and ^{**} p < 0.01).

arachidonic acid, was also confirmed by western blot analysis and RT-PCR. COX-2 protein and mRNA expression are increased in IgE-sensitized RBL-2H3 cells. However, PAE and gnetin H inhibited the upregulation of COX-2 in IgE-stimulated mast cells (Fig. 3B and C).

3.5. Effects of PAE and gnetin H on proinflammatory cytokine production and gene expression in IgE-stimulated RBL-2H3 cells

Proinflammatory cytokines, IL-4 and TNF- α , are crucial for allergic inflammatory disease (Theoharides and Kalogeromitros, 2006). We investigated whether effect of PAE and gnetin H on the production and mRNA expression of IL-4 and TNF- α in IgE-stimulated RBL-2H3 cells. PAE and gnetin H suppressed both IL-4

and TNF- α production (Fig. 4A and B) and mRNA expression (Fig. 4C).

3.6. Effect of PAE and gnetin H on activation and mRNA expression of NF- κ B in IgE-stimulated RBL-2H3 cells

To examine the mechanism by which PAE and gnetin H inhibit NF- κ B transcriptional activity, we assessed their effects on nuclear translocation and phosphorylation of NF- κ B. PAE and gnetin H inhibited antigen-induced nuclear translocation of NF- κ B p65 protein (Fig. 5A). PAE and gnetin H also reduced the antigen-induced activation of serine 536-phosphorylated NF- κ B p65 (Fig. 5B). Moreover, in antigen-stimulated cells, PAE and gnetin H inhibited the phosphorylation and degradation of I κ B, which

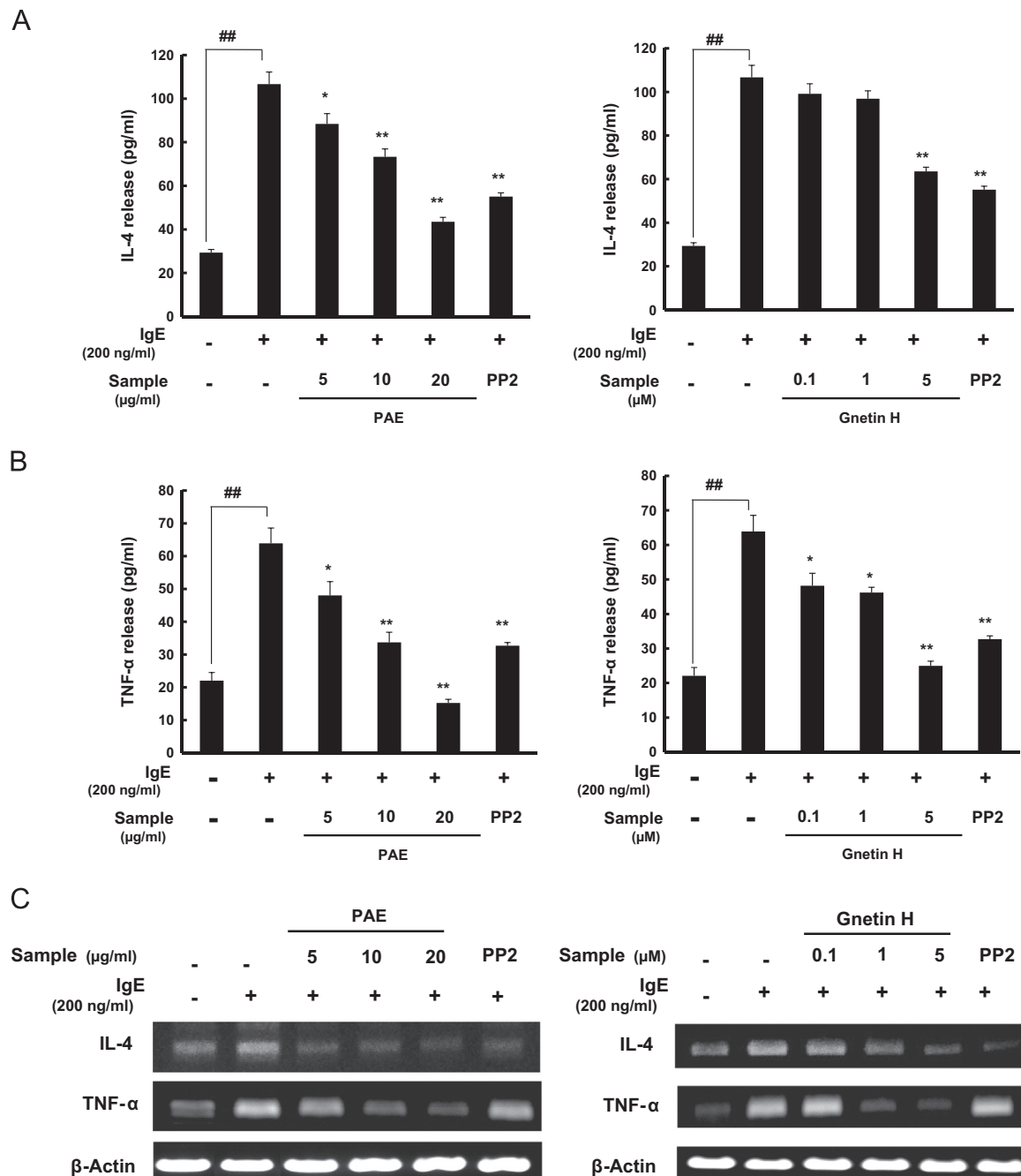


Fig. 4. Effects of PAE and gnetin H on IL-4 and TNF- α production and expression. RBL-2H3 cells were treated with PAE and gnetin H for 1 h and then stimulated with DNP-BSA for 4 h. (A and B) IL-4 and TNF- α production was determined by enzyme-linked immunosorbent assay kit. (C) IL-4 and TNF- α mRNA levels were determined by 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 $^{**}p < 0.01$).

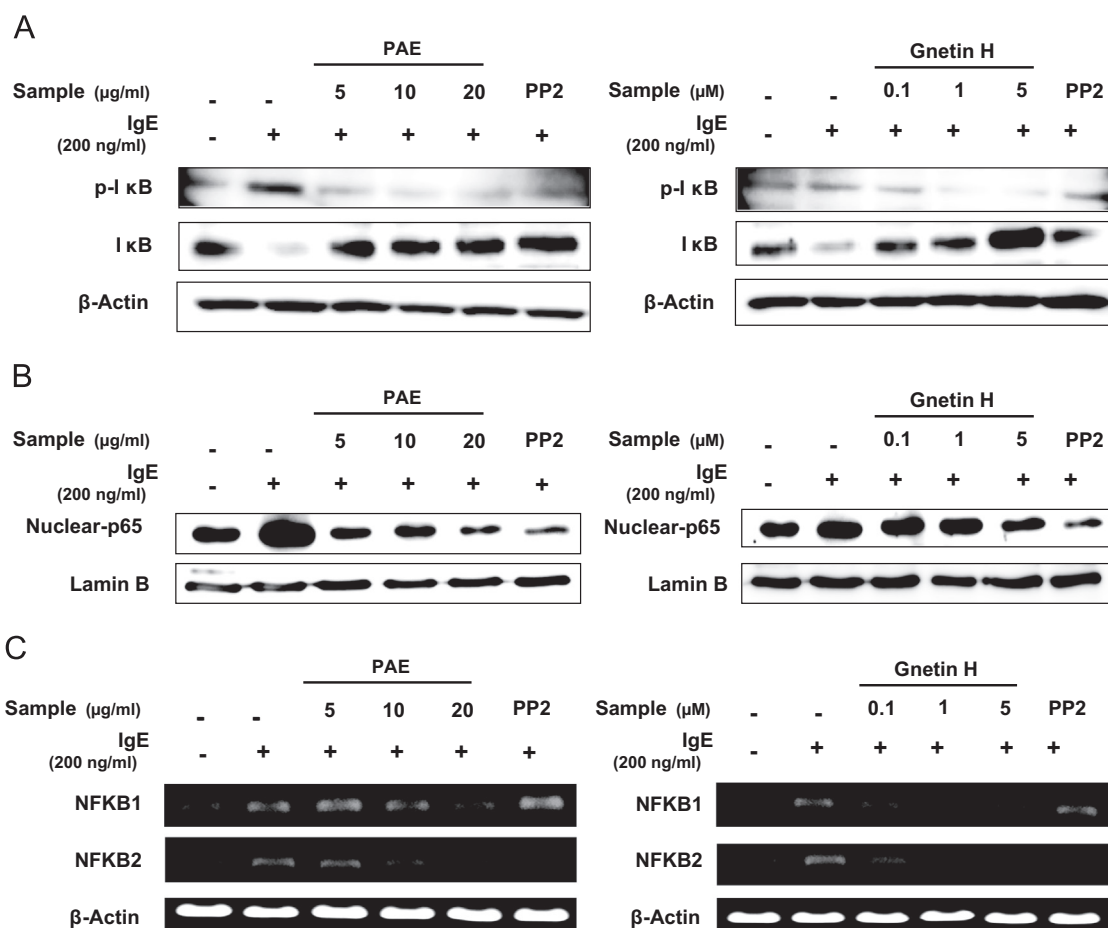


Fig. 5. Effect of PAE and gnetin H on NF-κB pathway and mRNA expression. (A) RBL-2H3 cells were treated with PAE and gnetin H for 1 h and then stimulated with DNP-BSA for 30 min. Phosphorylation of IκB and IκB was determined with Western blot analysis. (B) RBL-2H3 cells were treated with PAE and gnetin H for 1 h and then stimulated with DNP-BSA for 30 min. After nuclear proteins were extracted, NF-κB p65 was determined with Western blot analysis. (C) NFKB1 and NFKB2 mRNA levels were determined with RT-PCR. PP2 (10 μM) is a general Src-family kinase inhibitor.

regulates nuclear translocation of NF-κB (Fig. 5B). Furthermore, PAE and gnetin H reduced mRNA expression of NFKB1 and NFKB2 (Fig. 5C). Thus, PAE and gnetin H likely inhibit the activities of allergic mediators via inhibition of NF-κB transcriptional activation and mRNA expression.

3.7. Effect of PAE and gnetin H on FcεRI signaling in IgE-stimulated RBL-2H3 cells

FcεRI downstream signaling induces phosphorylation of Lyn, Syk, PKC, and PLCγ (Leung et al., 2004). To determine the effect of PAE and gnetin H on the inhibiting mechanism of degranulation, FcεRI downstream signals were assessed. IgE treatment induced phosphorylation of Syk, PKD/PKCμ, and PLCγ, and treatment with PAE and gnetin H reduced this phosphorylation (Fig. 6A). To further identify the relevant downstream effectors that directly modulate cytokine production following PAE and gnetin H treatment, phosphorylation of ERK, JNK and p38 was examined since phosphorylation of MAPKs leads to the production of cytokines (Zhang et al., 1997). PAE and gnetin H inhibited phosphorylation of ERK, JNK and p38 in IgE-sensitized RBL-2H3 cells (Fig. 6B).

4. Discussion

Immune cells such as mast cells, eosinophils, and basophils release inflammatory mediators (histamines and PGs) and

cytokines (TNF-α and IL-4). In particular, mast cells produce proinflammatory mediators including histamines and PGE₂ and regulate allergic inflammatory responses (Galli et al., 2005). In addition, mast cell-derived mediators (histamines, proteases, and cytokines) play a crucial role during allergic inflammation in mast cell-deficient mice (Wershil, 2000). Thus, many studies have investigated various mediators of mast cell activation, and revealed active compounds targeting these inflammatory mediators (Westcott et al., 1996; Itoh et al., 2008; Tanifuji et al., 2010).

Previous reports demonstrated that extracts of *Paeonia* have antioxidative, antitumor, antiviral, cardioprotective, and immunomodulatory activities (Shon and Nam, 2004; Wang et al., 2005; He et al., 2010; Su et al., 2010). Among the *Paeonia* species, *Paeonia lactiflora* has been reported to have an anti-allergic effect against passive cutaneous anaphylaxis (Lee et al., 2008). Moreover, PGG, isolated from *Paeonia anomala*, also suppresses FcεRI cell surface expression in IgE-mediated mast cell activation (Kageyama-Yahara et al., 2010). However, the effects of *Paeonia anomala* and its constituents on allergic disease have not previously been revealed. In the present study, we investigated the inhibitory effects of PAE and its constituents on allergic mediators in RBL-2H3 cells. First, we isolated seventeen compounds from *Paeonia anomala*, and found that gnetin H, a resveratrol trimer, significantly inhibited IgE-mediated degranulation of RBL-2H3 mast cells (Fig. 2B). Therefore, we evaluated the anti-allergic effects of PAE and gnetin H in IgE-activated mast cells.

FcεRI receptors present on mast cells are activated by high-affinity binding and aggregation of IgE and activation of these

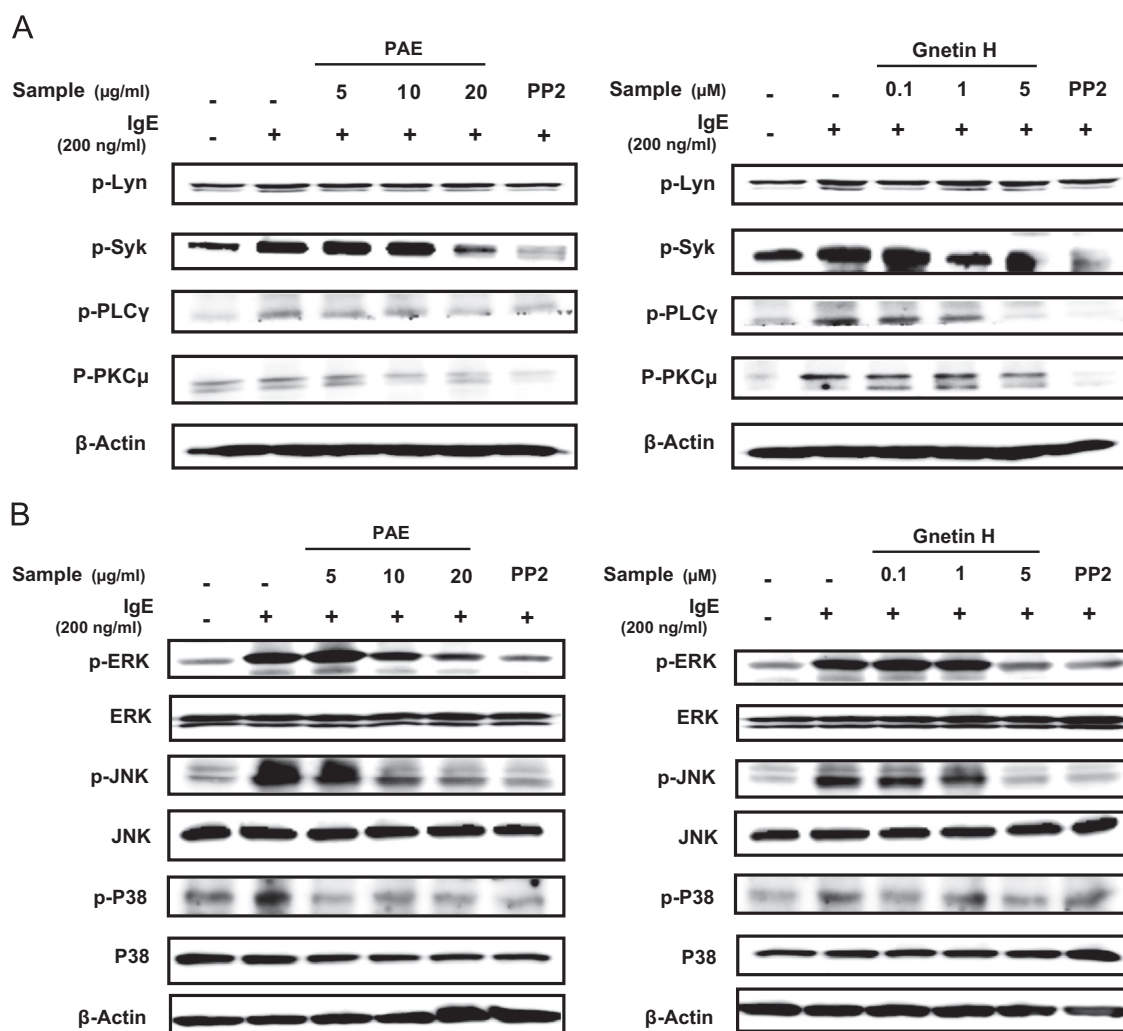


Fig. 6. Effect of PAE and gnetin H on FcεRI Signaling. (A) RBL-2H3 cells were treated with PAE and gnetin H for 1 h and then stimulated with DNP-BSA for 10 min. Phosphorylation of Lyn, Syk, PLCγ, and PKD/PKCμ was determined with Western blot analysis. (B) RBL-2H3 cells were treated with PAE and gnetin H for 1 h and then stimulated with DNP-BSA for 30 min. Phosphorylation of JNK, ERK, and p38 was determined with Western blot analysis. PP2 (10 μM) is a general Src-family kinase inhibitor.

receptors initiates a signaling cascade that results in secretion of inflammatory mediators and degranulation (Beaven and Metzger, 1993). We identified that PAE and gnetin H suppressed the release of both β-hexosaminidase (Fig. 2A and C) and histamine (Fig. 2D) in a dose-dependent manner. These results suggest that PAE and gnetin H inhibit the histamine release, known to cause smooth muscle contraction, vascular permeability, and inflammation.

PAE and gnetin H also attenuated release of the inflammatory mediator PGE₂ (Fig. 3A), which is an arachidonic acid metabolite (Gonzalez-Periz and Claria, 2007), likely via inhibition of the COX pathway, as both compounds inhibited COX-2 expression, a key enzyme in this pathway (Fig. 3B and C). These results suggest that PAE and gnetin H suppress production of inflammatory lipid mediators by inhibiting the COX pathway.

Notably, PAE and gnetin H markedly decreased the expression of the proinflammatory cytokines TNF-α and IL-4 (Fig. 4A–C). TNF-α and IL-4 play a critical role in initiating allergic responses in mast cells. IL-4 switches B-cells to the IgE isotype and activates T-cell development. TNF-α also induces physiological immune reactions by triggering leukocyte infiltration and tissue fibrosis, and promotes proinflammatory states through secretion of cytokine (Gilfillan and Tkaczyk, 2006). Although inflammation is a necessary process because of physiological defense mechanism against pathogens, Excessive cytokines overexpression can cause serious inflammatory disorders such as allergic rhinitis, asthma and atopic

dermatitis. In terms of the above, PAE and gnetin H suppressed the expression of TNF-α and IL-4, indicate that PAE and gnetin H could be beneficial for the treatment of allergic disorders.

NF-κB is a dimeric transcription factor formed by the hetero- or homodimerization of proteins in the Rel family, including p50 and p60 (Hanada and Yoshimura, 2002). It plays an important role in the immune response and in cell differentiation induced by cytokines, growth factors, and other cell activators (Marquardt and Walker, 2000). Our data demonstrate that PAE and gnetin H inhibit the translocation of NF-κB in antigen-stimulated mast cells. These results suggest that PAE and gnetin H may block the induction of IL-4 and TNF-α in antigen-stimulated mast cells by inhibiting NF-κB activation.

The FcεRI receptor signals regulate degranulation through Lyn, Syk and PLCγ (Theoharides and Kalogeromitros, 2006). The FcεRI receptor is a tetrameric complex, which binds IgE and initiates intracellular signaling to induce allergic responses. Activated FcεRI induces phosphorylation of Syk, which binds immunoreceptor tyrosine-based activation motifs (ITAMs) present on FcεRI β and γ chains. In turn, Syk induces phosphorylation of PLCγ and PKC (Siraganian et al., 2010). In our study, PAE and gnetin H also decreased the phosphorylation of Syk, PLCγ, and PKD/PKCμ (Fig. 6A). These results indicate that PAE and gnetin H may have anti-allergic effects in antigen-induced mast cells, most likely by suppressing the activation of Syk, PLCγ, and PKC, and the consequent inhibition of mast cell degranulation.

MAPK signaling pathways are an important therapeutic target in allergic disorders (Abdel-Raheem et al., 2005). To assess the mechanism by which PAE and gnetin H inhibit the activation of RBL-2H3 cells, ERK, p38, and JNK phosphorylation was analyzed. PAE and gnetin H suppressed IgE-induced phosphorylation of ERK, p38, and JNK, suggesting that PAE and gnetin H inhibit mast cell activation by regulating MAPK signaling (Fig. 6B).

In summary, PAE and its active compound, gnetin H, suppressed degranulation and the release of histamine, lipid mediators, and proinflammatory cytokines in IgE-sensitized RBL-2H3 cells. PAE and gnetin H also suppressed signaling pathway from the FcεRI receptor by inhibiting phosphorylation of Syk, PLCγ, PKC, and MAPK. Taken together, our findings suggest that PAE and gnetin H could be valuable therapeutic agents for preventing or treating IgE-mediated allergic diseases.

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