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Inhibitory effect of the *Larix sibirica* and its various flavonoids on the IgE-stimulated mast cell activation and anaphylaxis

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ABSTRACT

Larix sibirica (Siberian larch) is a deciduous tree native to Russia, Mongolia, and China and has been reported to possess anti-viral, anti-oxidant, and neuroprotective properties. In this study, we assessed the anti-allergic effects of an *L. sibirica* extract (LSE) and its various flavonoids using an IgE-sensitized mast cell-like cell line, rat basophilic leukaemia (RBL)-2H3, and a passive anaphylaxis mouse model. In IgE-sensitized RBL-2H3 cells, LSE and several flavonoids reduced release and production of β -hexosaminidase, histamine, and inflammatory mediators (prostaglandin D₂, leukotriene C₄, interleukin-4, and tumour necrosis factor- α), reduced expression of cyclooxygenase-2 and 5-lipoxygenase, and suppressed activation of nuclear factor- κ B. LSE and several flavonoids reduced phosphorylation of downstream signalling molecules, such as Syk, protein kinase C μ , phospholipase C γ , and mitogen-activated protein kinases. Oral administration of LSE inhibited mast cell-dependent passive cutaneous anaphylactic reactions. These results suggest that LSE is a beneficial candidate for the prevention and treatment of allergic disorders.

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1. Introduction

The incidence of allergic disorders such as asthma, allergic rhinitis, and atopic dermatitis is a serious health problem. These diseases are classified as type I allergies caused by the release

of substances stored in mast cell granules. Notably, mast cells are key effectors in allergic and inflammatory responses. Cross-linking of FcεRI to antigens triggers production of pro-inflammatory mediators, including histamine, proteases, prostaglandin D₂ (PGD₂), and various chemokines and cytokines (Murakami, Austen, & Arm, 1995; Yamaguchi et al., 1999).

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Abbreviations: BSA, bovine serum albumin; COX, cyclooxygenase; DMSO, dimethyl sulfoxide; DNP, dinitrophenol; ECL, enhanced chemiluminescence; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; IgE, immunoglobulin E; IL-4, interleukin-4; JNK, c-Jun N-terminal kinase; LSE, *Larix sibirica* extract; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor- κ B; PCA, passive cutaneous anaphylaxis; PGD₂, prostaglandin D₂; PKC, protein kinase C; PLC γ , phospholipase C γ ; RBL, rat basophilic leukaemia; RT-PCR, reverse transcription-polymerase chain reaction; TNF- α , tumour necrosis factor- α .

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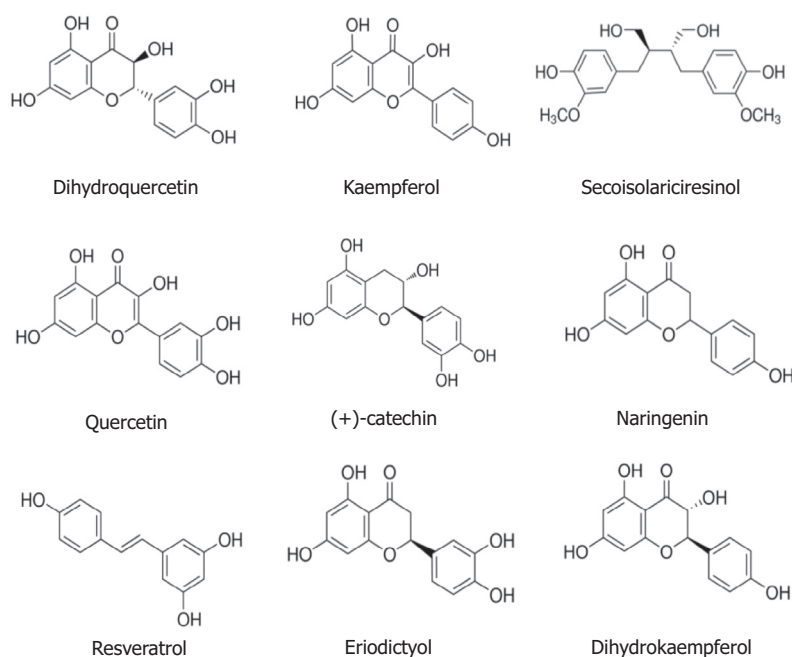


Fig. 1 – Chemical structure of flavonoids from *L. sibirica*.

Aggregation of FcεRI activates Src family kinases, including Fyn and Lyn, which phosphorylate the immunoreceptor tyrosine-based activating motif of FcεRI. Cross-linking of FcεRI also activates another key tyrosine kinase, Syk, which is crucial for the phosphorylation of many downstream effectors in mast cells (Gilfillan & Tkaczyk, 2006; Siraganian, 2003). Syk activation induces the phosphorylation of phospholipase Cγ (PLCγ) and protein kinase C (PKC), which are essential for the degranulation and mobilization of intracellular Ca²⁺ (Gilfillan & Rivera, 2009). Moreover, FcεRI signalling induces the activation of mitogen-activated protein kinases (MAPKs), such as c-Jun N-terminal kinases (JNKs), extracellular signal-regulated kinases (ERK1/2), and p38, and nuclear factor-κB (NF-κB) signalling pathways, which results in the production of pro-inflammatory mediators (Siraganian, 2003; Tak & Firestein, 2000). Furthermore, MAPKs contribute to the activation of cyclooxygenase (COX)-2, which plays important roles in the production of arachidonic acid, a common precursor of PGD₂ (Li et al., 2014; Lu et al., 2014).

Larix sibirica (Siberian larch) is a food plant that grows in Canada, Mongolia, and Russia. It is used in Mongolian traditional medicine for the treatment of chronic bronchitis, haemorrhage, cystitis, indigestion, wounds, chronic eczema, and psoriasis and it is one of the best wood materials for constructing buildings (Grieve, 1984). It has also been reported to possess anti-viral, antioxidant, skin whitening, and neuroprotective activities (Diwakar, Rana, & Scholten, 2012; Galochkina, Anikin, Babkin, Ostroukhova, & Zarubaev, 2016; Khairullina, Garifullina, Gerchikov, Ostroukhova, & Babkin, 2006; Loers, Yashunsky, Nifantiev, & Schachner, 2014). The major constituents of *L. sibirica* are flavonoids, such as dihydroquercetin, dihydrokaempferol, quercetin, kaempferol, secoisolariciresinol, (+)-catechin, naringenin, resveratrol, and eriodictyol (Ivanovaa, Gorshkovb, Kuzminb, Gordienkoa, & Babkina, 2012; Loers et al.,

2014; Ostroukhovaa, Ralduginb, Babkina, Onuchinaa, & Levchuka, 2012) (Fig. 1). Furthermore, dihydroquercetin is also a constituent of common food material such as vegetable oils, animal fat, milk powder, and dietary supplement ingredient (Schauss, Tselyico, Kuznetsova, & Yegorova, 2015; Tiukavkina, Rulenko, & Kolesnik, 1997).

Although the anti-allergic effects of flavonoids have been reported (Fujimura et al., 2002; Han et al., 2013; Kim, Lim, Kang, Um, & Nho, 2014; Koyama et al., 2006; Park, Park, & Kim, 2005; Yoo, Kim, Park, Kang, & Kim, 2012), the inhibitory effects of *L. sibirica* and its various flavonoids on allergic responses have not been investigated.

In our study, we demonstrated the molecular mechanism by which an *L. sibirica* extract (LSE) and its various flavonoids affect immunoglobulin E (IgE)-stimulated allergic reactions in rat basophilic leukaemia (RBL)-2H3 cells, which are similar to normal basophils and primary mast cells and have been used as an appropriate model for exploring anti-allergic activity (Kim, Lim, Lee, & Nho, 2015; Matsuda, Nakamura, & Yoshikawa, 2016; Passante & Frankish, 2009). In addition, the effect of LSE on passive cutaneous anaphylaxis (PCA) in mice was assessed. Herein, we describe the inhibitory effects of LSE on the degranulation of mast cells and on PCA in mice.

2. Materials and methods

2.1. Reagents and materials

PIPES, PP2, mouse anti-dinitrophenol (DNP) IgE, and DNP-bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against the following proteins were used: phosphorylated Syk (Tyr525/526), PLCγ (Tyr783), protein kinase D/PKCμ (Ser916), p38

(Thr180/Tyr182), p44/42 (ERK1/2, Thr202/Tyr204), JNK (Thr183/Tyr185), I κ B α (Ser32), and IKK α / β (Ser176/180) as well as total Syk, PLC γ , PKC μ , p38, ERK1/2, JNK, I κ B α , IKK α , and β -actin (Cell Signaling Technology Inc., Danvers, MA, USA). COX-2 was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Dihydroquercetin, kaempferol, quercetin, secoisolariciresinol, (+)-catechin, naringenin, resveratrol, eriodictyol, and dihydrokaempferol were purchased from Sigma-Aldrich and were >95% pure, as determined by high-performance liquid chromatography-mass spectroscopy. All compounds were dissolved in dimethyl sulfoxide (DMSO) and final concentrations of DMSO were adjusted to 0.1% (v/v) in culture media.

2.2. Extraction of plant material

Dried bark of *L. sibirica* was collected in Bayanchandmani Sum, Tov province, near Ulaanbaatar, Mongolia and was identified by Dr. C. Sanchir (Institute of Botany, Mongolian Academy of Sciences, Ulaanbaatar, Mongolia). A voucher specimen was placed in the Flora and Plant Systematic Laboratory, Institute of Botany, Mongolian Academy of Sciences. Dried *L. sibirica* plants (1 kg) were extracted with 95% ethanol for 3 days at room temperature and filtered through Whatman No. 1 filter paper (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The filtrates were evaporated in a vacuum. The final weight of extracts was 123 g.

2.3. Cell culture and cell viability assay

RBL-2H3 mast cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Cell culture and the cell viability assay were performed as described (Kim et al., 2015). When the effects of the indicated concentrations of LSE and various flavonoids were examined, they were added 1 h prior to the addition of DNP-BSA.

2.4. Degranulation assays

Release of β -hexosaminidase and histamine into the medium was determined as previously described (Kim et al., 2015). Briefly, RBL-2H3 cells were treated with various concentrations of LSE and nine compounds for 1 h before being sensitized with 0.2 μ g/mL DNP-IgE overnight and then challenged with 0.2 μ g/mL DNP-BSA for 30 min. The amount of β -hexosaminidase released from RBL-2H3 cells was measured by incubating cell supernatants with the substrate solution. Sodium carbonate buffer was added to stop the reaction and absorbance was measured by spectrophotometry at 405 nm. After the supernatants were collected, histamine release was measured with a histamine enzyme immunoassay (EIA) kit (Oxford Biomedical Research, Rochester Hills, MI, USA).

2.5. PGD₂ ELISAs

RBL-2H3 cells were treated with various concentrations of LSE and nine compounds for 1 h before being sensitized with DNP-IgE (200 ng/mL) overnight and then stimulated with DNP-BSA (200 ng/mL) for 4 h. The concentrations of PGD₂ (for 4 h) in the supernatants were determined using EIA kits (Cayman Chemical, Ann Arbor, MI, USA).

2.6. Interleukin-4 (IL-4) and tumour necrosis factor- α (TNF- α) enzyme-linked immunosorbent assays (ELISAs)

To examine IL-4 and TNF- α release, RBL-2H3 cells were treated with various concentrations of LSE and nine compounds for 1 h before being sensitized with DNP-IgE (200 ng/mL) overnight and then stimulated with DNP-BSA (200 ng/mL) for 4 h. The concentrations of IL-4 and TNF- α in the supernatants were determined using ELISA kits (Abcam, UK).

2.7. Immunoblot analysis

Immunoblot analysis was performed as described previously (Kim et al., 2015). Equal volumes of cell culture supernatants were electrophoresed by 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany), and subjected to immunoblotting. The membranes were incubated with primary antibodies for 16 h at 4 °C, followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. The signals were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Little Chalfont, UK) and an ECL Advance system (GE Healthcare, Hatfield, UK).

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

Total RNA from treated cells was prepared using RNAiso reagent (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. A reverse transcriptase premix (Elpis-Biotech., Inc., Taejeon, Korea) was used for reverse transcription of each sample. RT-PCR was run using a PCR premix (Elpis-Biotech) and the sequences of the primers (5'→3') were as follows: IL-4 forward, CGT GAT GTA CCT CCG TGC TT and reverse, ATT CAC GGT GCA GCT TCT CA (108 bp); TNF- α forward, GGC TTT CGG AAC TCA CTG GA and reverse, CCC GTA GGG CGA TTA CAG TC (544 bp); NF κ B1 forward, TTC AAC ATG GCA GAC GAC GA and reverse, CCA TCT GTT GAC AGT GGT ATA TCT G (122 bp); NF κ B2 forward, CTC CAC CGG ATC TTT CCC GC and reverse, AGC GTA GCC GTA CAA TGC TC (793 bp); and β -actin forward, AGG GAA ATC GTG CGT GAC AT and reverse, GCA GCT CAG TAA CAG TCC GC (533 bp). PCR products were visualized by ethidium bromide staining and ultraviolet illumination. mRNA expression levels were normalized against that of β -actin, the internal control.

2.9. Induction of PCA in mice

PCA was induced as described previously (Kim et al., 2015). LSE (50 or 200 mg/kg) or cetirizine (20 mg/kg) was orally administered to examine their anti-allergic activity. After 1 h, Evans Blue dye together with DNP-BSA (250 μ g) was intravenously injected into mice. The dye was extracted from each ear in 700 μ L of formamide at 63 °C overnight. Absorbance was measured with a microplate reader at 630 nm. The experiments were approved by the Animal Care and Use Committee of KIST.

2.10. Statistical analysis

Results are expressed as the means $\pm$ standard deviation. A one-way analysis of variance followed by Scheffé's test was used

to assess the significance of the difference between the vehicle (DMSO) and treatment groups (SPSS 17.0, Chicago, IL, USA). * $p < 0.05$, # $p < 0.01$, * $p < 0.05$, and ** $p < 0.01$ were considered statistically significant.

3. Results

3.1. LSE and its active compounds inhibit antigen-stimulated degranulation

To examine the effects of LSE and various compounds on the activation of mast cells, we tested the cytotoxicity of LSE and nine compounds using the MTT assay. When cells were treated with various concentrations of LSE (5–40 $\mu\text{g/mL}$) and nine compounds (5–40 μM) for 8 h, no cytotoxicity was observed (data not shown). For this reason, we decided to use LSE at concentrations of 1–10 $\mu\text{g/mL}$ and the nine compounds at a concentration of 20 μM for all *in vitro* studies. First, we assessed the effects of LSE and various compounds on degranulation in IgE-sensitized RBL-2H3 cells. Granulated mast cells release β -hexosaminidase and histamine when stimulated with IgE (Razin et al., 1983). LSE reduced the release of β -hexosaminidase in a dose-dependent manner in antigen-stimulated RBL-2H3 cells (Fig. 2A). Furthermore, quercetin and kaempferol suppressed β -hexosaminidase release to a greater degree than dihydroquercetin, secoisolariciresinol, (+)-catechin, naringenin, resveratrol, eriodictyol, and dihydrokaempferol (Fig. 2B). To further examine the inhibitory effects of LSE and the compounds on mast cell degranulation, we measured histamine release. LSE, quercetin, and kaempferol also decreased the production of histamine in antigen-stimulated mast cells (Fig. 2C and D).

3.2. LSE and its active compounds attenuate PGD_2 generation by reducing COX-2 expression

PGD_2 is produced in IgE-sensitized mast cells by initiation of Fc ϵ RI-activated signalling (Roth, Chen, & Lin, 2008). To investigate the effects of LSE and various compounds on the production of PGD_2 , which is a metabolite of arachidonic acid, IgE-sensitized RBL-2H3 cells were pretreated with LSE or the compounds and then stimulated with DNP-BSA. LSE and the compounds markedly inhibited PGD_2 generation in IgE-sensitized mast cells (Fig. 3A and B). Arachidonic acid is used as a substrate by COX-2 to generate PGD_2 . We assessed the effects of LSE and the compounds on COX-2 protein expression by immunoblot analysis. LSE and the compounds strongly inhibited both COX-2 expression (Fig. 3C and D).

3.3. LSE and its active compounds decrease production and mRNA expression of IL-4 and TNF- α

IL-4 and TNF- α , which are pro-inflammatory cytokines, are reported to play a significant role in inflammatory and allergic disorders (Theoharides & Kalogeromitros, 2006). For this reason, we examined the effects of LSE and the compounds on the release and gene expression of IL-4 and TNF- α in IgE-sensitized RBL-2H3 cells. LSE and the compounds (especially quercetin,

kaempferol, and eriodictyol) markedly inhibited IL-4 and TNF- α release (Fig. 4A–D) and mRNA expression (Fig. 4E and F) in antigen-stimulated RBL-2H3 cells.

3.4. LSE and its active compounds attenuate NF- κB activation

To further demonstrate the mechanism by which LSE and the compounds suppress pro-inflammatory mediators, the level of IgE/Ag-stimulated NF- κB activation was assessed by immunoblot analysis. LSE and the compounds suppressed IgE/Ag-induced phosphorylation of IKK α/β (Fig. 5A and B). Furthermore, degradation of I κB , which suppresses nuclear translocation of NF- κB , was inhibited in RBL-2H3 cells exposed to LSE or the compounds (Fig. 5A and B). LSE and the compounds inhibit NF- κB activation via inhibiting the degradation of I κB and blocking the phosphorylation of IKK α/β .

3.5. LSE and its active compounds inhibit the Fc ϵ RI signalling pathway

Binding of antigens to IgE triggers Fc ϵ RI cross-linking and phosphorylates Syk protein, inducing MAPK activation. Furthermore, activation of the Syk pathway promotes the activation of PLC γ and PKC (Li et al., 2014). Therefore, we verified the effect of LSE and the compounds on the Fc ϵ RI pathway in IgE-sensitized mast cells. LSE and the compounds (especially eriodictyol) inhibited antigen-induced phosphorylation of Syk, PLC γ , and PKC ζ (Fig. 6A and B). Activation of MAPKs induces the production of pro-inflammatory mediators (Zhang, Baumgartner, Yamada, & Beaven, 1997). To determine the downstream signalling molecules that affect degranulation and production of pro-inflammatory mediators, we examined the phosphorylation of JNK, ERK, and p38. LSE and the compounds suppressed phosphorylation of MAPKs, including JNK, ERK, and p38, in IgE-sensitized mast cells (Fig. 6C and D).

3.6. LSE suppresses the anaphylactic reaction in mice

To investigate the anti-allergic effects of LSE *in vivo*, IgE-mediated PCA was induced. Mice were subcutaneously sensitized with DNP-IgE in their ears for 24 h and orally administered LSE or cetirizine, a typical anti-histamine drug. After 1 h, DNP-BSA containing 4% Evans blue dye was intravenously injected into mice. Treatment with 50 and 200 mg/kg LSE markedly decreased the amount of dye by 43% ($p < 0.05$) (Fig. 7A and B).

4. Discussion

Mast cells are crucial effector cells in allergic diseases (Galli & Tsai, 2012). In our study, we showed that LSE and its active compounds had anti-allergic activities by inhibiting mast cell activation and PCA. Furthermore, we demonstrated that LSE and its compounds suppressed the release of pro-inflammatory mediators, such as histamine, prostaglandins, and pro-inflammatory cytokines, in mast cells.

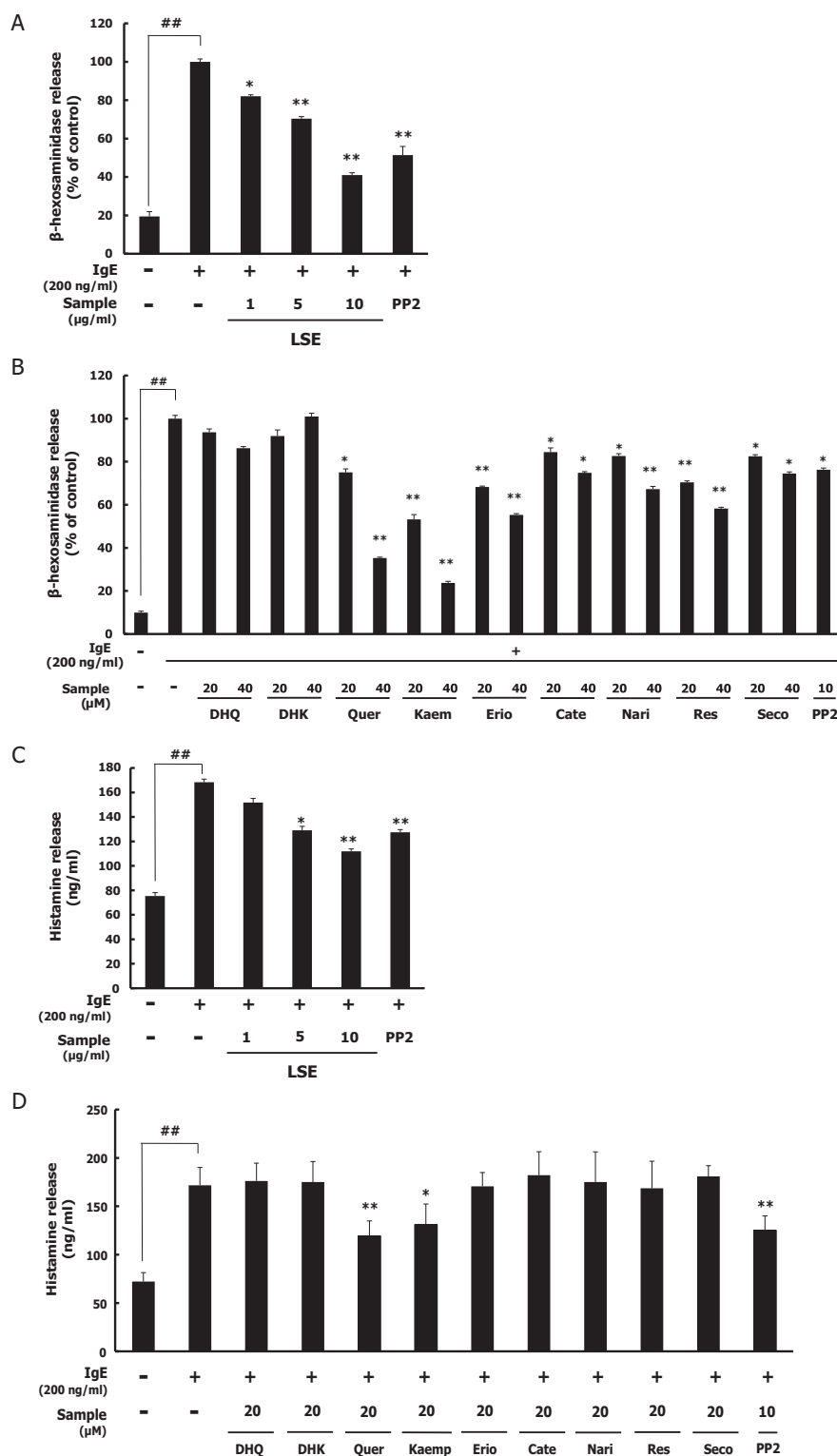


Fig. 2 – Effects of LSE and various flavonoids on β -hexosaminidase and histamine release. (A) The degranulation was determined by measuring β -hexosaminidase release in antigen-stimulated RBL-2H3 cells. The RBL-2H3 cells were treated in the presence or absence of LSE (1, 5 or 10 μ g/mL) for 1 h and then stimulated for 1 h with antigen. (B) The RBL-2H3 cells were treated in the presence or absence of dihydroquercetin (DHQ), dihydrokaempferol (DHK), quercetin (Quer), kaempferol (Kaem), eriodyctiol (Erio), catechin (Cate), naringenin (Nari), resveratrol (Res), secoisolariciresinol (Seco) (20 or 40 μ M) for 1 h and then stimulated for 1 h with antigen. (C,D) Histamine release was determined with enzyme-linked immunosorbent assay kit. The RBL-2H3 cells were treated in the presence or absence of LSE (1, 5 or 10 μ g/mL) or various flavonoids (20 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 ** $p < 0.01$).

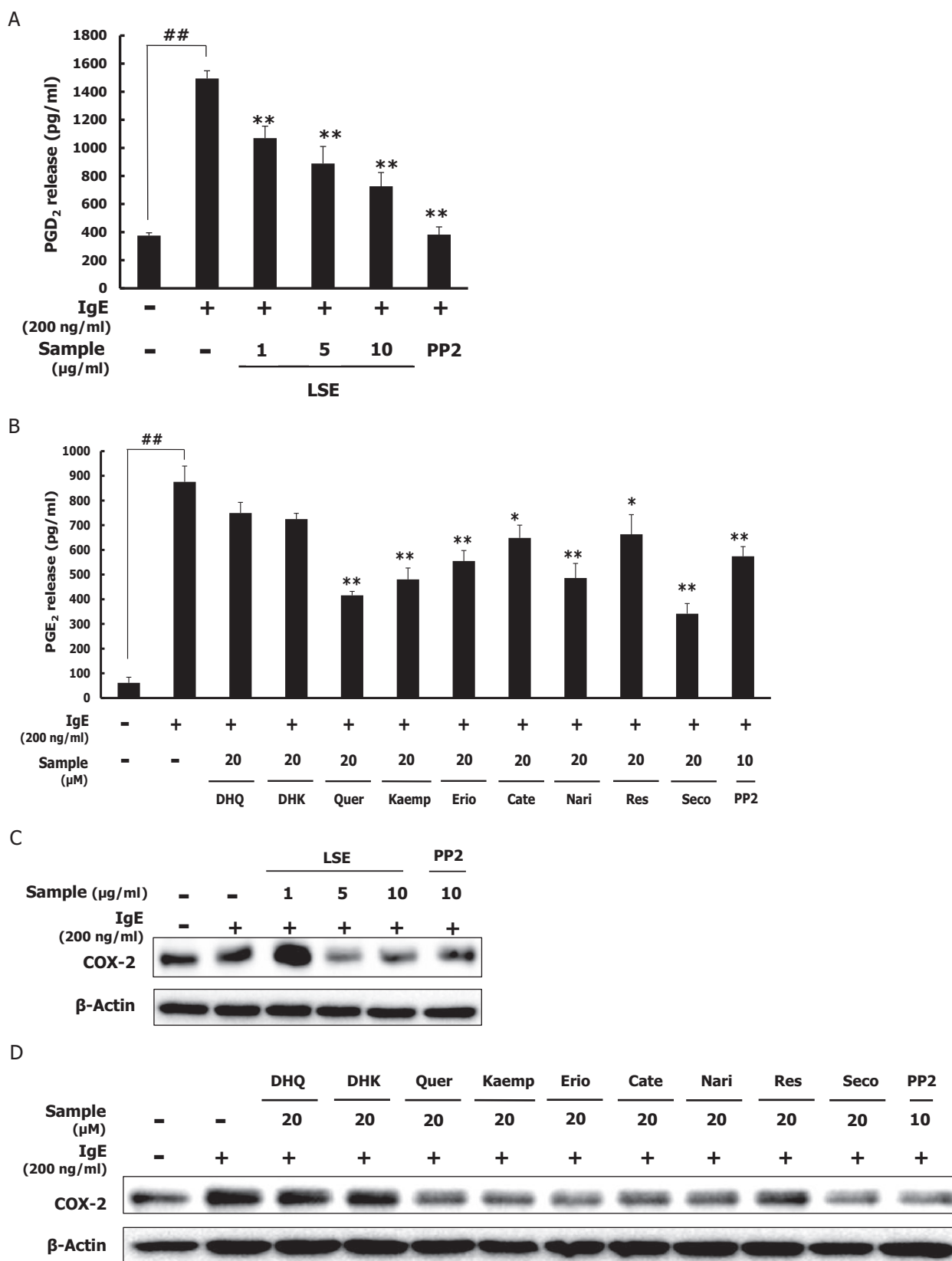


Fig. 3 – Effects of LSE and various flavonoids on PGD₂ secretion and COX-2 protein expression. RBL-2H3 cells were treated with LSE (1, 5 or 10 μg/mL) or various flavonoids (20 μM) for 1 h and then stimulated for 4 h with antigen. (A,B) PGD₂ secretion was determined by enzyme-linked immunosorbent assay kit. (C,D) COX-2 protein level was determined with Western blot assay. PP2 (10 μM) is a general Src-family kinase inhibitor. Results are expressed as the mean ± SD of three independent experiments. (^{###}*p* < 0.01, ^{*}*p* < 0.05 and ^{**}*p* < 0.01).

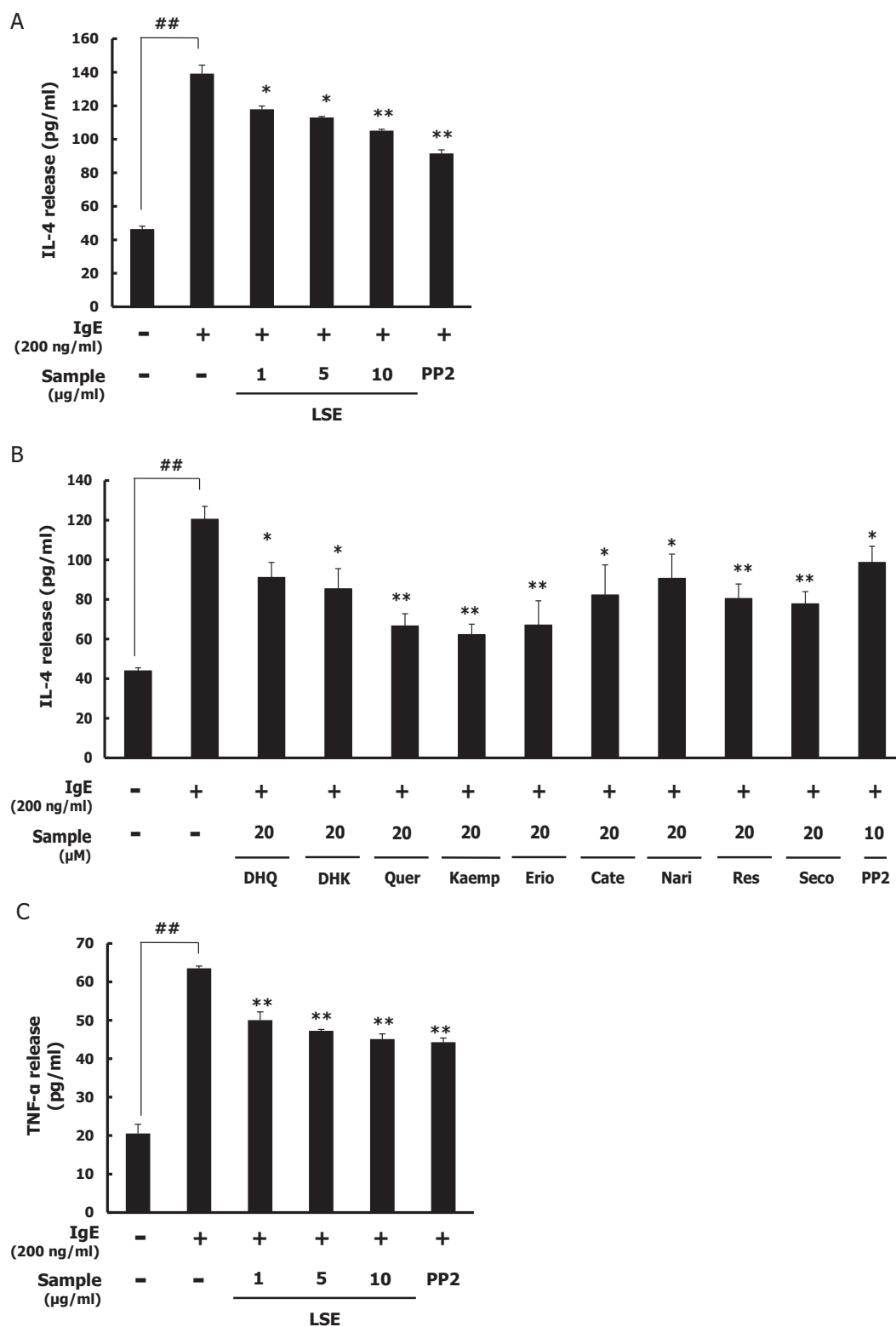


Fig. 4 – Effects of LSE and various flavonoids on IL-4 and TNF- α production and expression. RBL-2H3 cells were treated with LSE and various flavonoids for 1 h and then stimulated with DNP-BSA for 4 h. (A–D) IL-4 and TNF- α production was determined by enzyme-linked immunosorbent assay kit. (E,F) IL-4 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 ** $p < 0.01$).

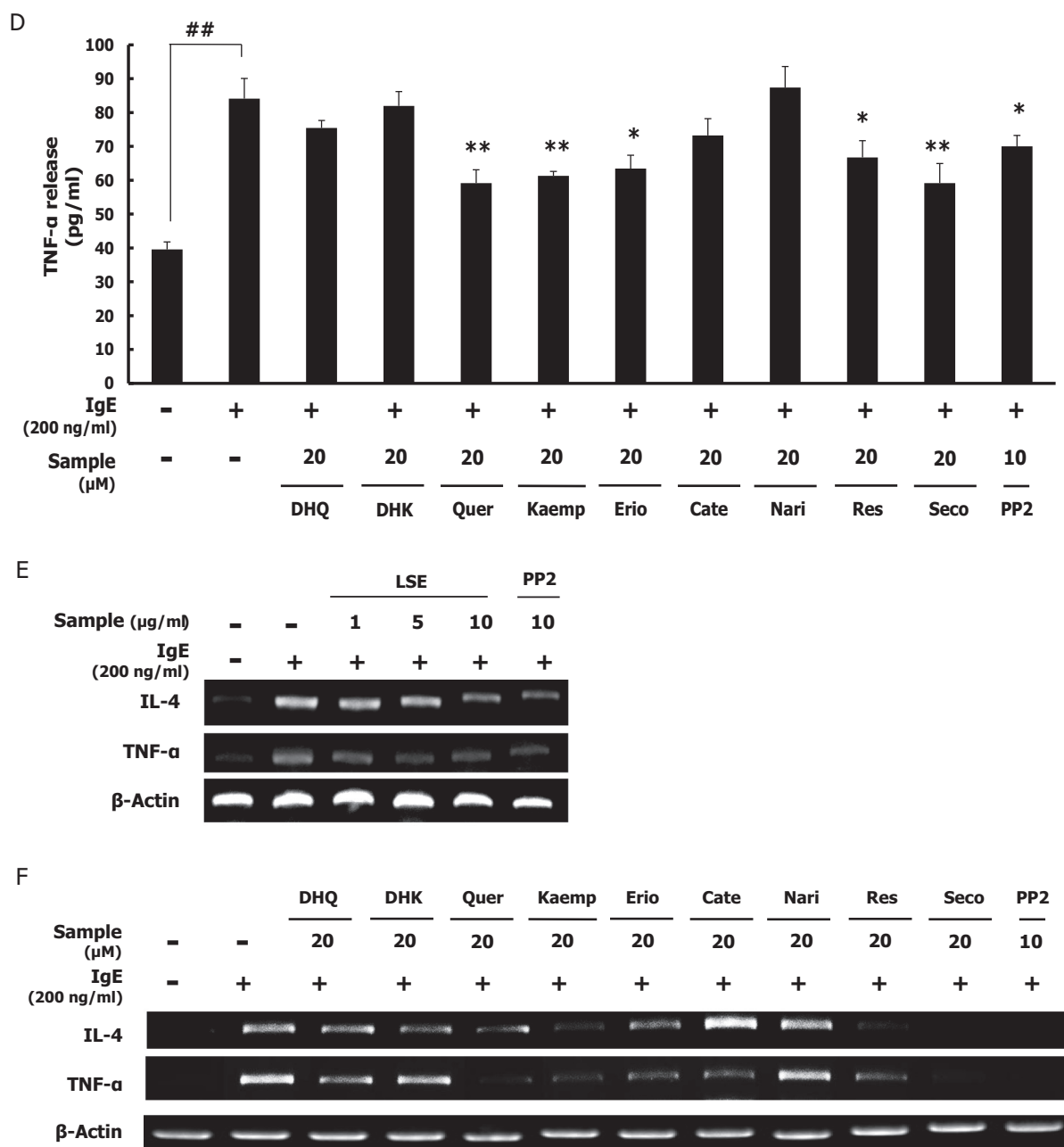


Fig. 4 – (continued)

The β -hexosaminidase assay has been commonly used to examine the degree of degranulation in mast cells and it is a useful method for investigating the potential of new drugs to inhibit the degranulation of mast cells (Aketani, Teshima, Umezawa, & Sawada, 2001; Pierini, Harris, Holowka, & Baird, 1997). Moreover, histamine, which is a chemical mediator in mast cells, plays an essential role in allergic diseases (Wershil, 2000). LSE and its active compounds suppressed degranulation in activated mast cells via decreasing the release of β -hexosaminidase (Fig. 2A and B) and histamine (Fig. 2C and D). LSE contains a large amount of dihydroquercetin (Ostroukhovaa et al., 2012) and various polyphenols, such as flavanonols (dihydrokaempferol), flavonols (quercetin and kaempferol), flavanones (naringenin and eriodictyol), flavanols ((+)-catechin), stilbenoids (resveratrol), and

lignans (secoisolaricresinol). Quercetin, kaempferol, and several other compounds are naturally occurring phytochemicals found in various fruits and vegetables and may have anti-allergic properties (Fujimura et al., 2002; Han et al., 2013; Kim et al., 2014; Koyama et al., 2006; Park et al., 2005; Yoo et al., 2012). For instance, quercetin, kaempferol, resveratrol, and eriodictyol from LSE inhibit degranulation by decreasing release of histamine, IL-4, and TNF- α (Kim et al., 2014; Shirley, McHale, & Gomez, 2016; Yoo et al., 2012). Furthermore, quercetin, kaempferol, and other compounds inhibit IgE-mediated allergic reactions *in vivo* (Han et al., 2013; Kim et al., 2014; Park et al., 2005; Shiozaki, Sugiyama, Nakazato, & Takeo, 1997; Yoo et al., 2012). For these reasons, we suggest that the effect of LSE on allergic reactions was influenced by these compounds.

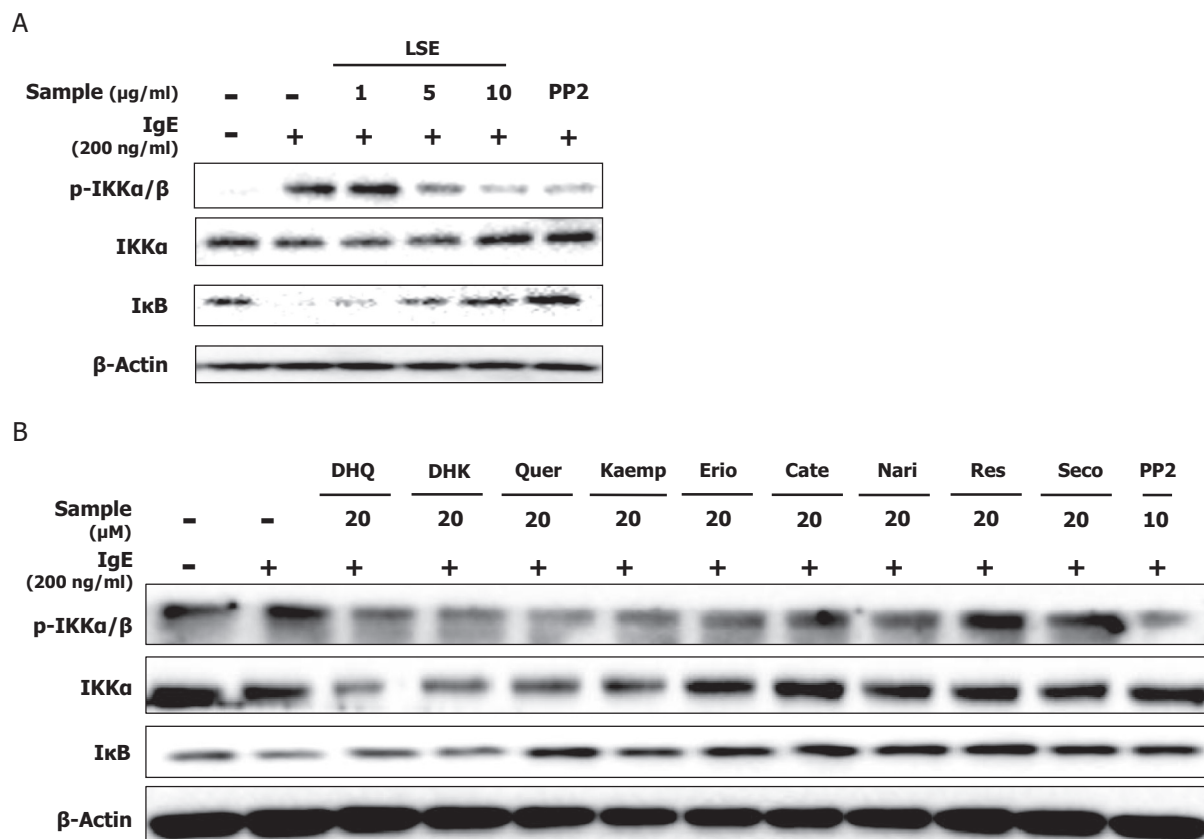


Fig. 5 – Effect of LSE and various flavonoids on NF-κB pathway. (A,B) RBL-2H3 cells were treated with LSE and various flavonoids for 1 h and then stimulated with DNP-BSA for 30 min. Phosphorylation of IKKα/β, IKKα and IκB was determined with Western blot analysis. PP2 (10 μM) is a general Src-family kinase inhibitor.

LSE and its active compounds strongly reduced the release and gene expression of IL-4 and TNF-α (Fig. 3). Pro-inflammatory cytokines, including TNF-α and IL-4, are crucial for allergic inflammatory diseases (Theoharides & Kalogeromitros, 2006). In particular, TNF-α is an important inflammatory mediator among various cytokines and chemokines. TNF-α is secreted by activated T cells and macrophages in response to infection and is produced upon antigen stimulation in mast cells (Gordon & Galli, 1990). IL-4 is an important cytokine in allergic responses. IL-4 is important in IgE production (Wershil, 2000) and stimulates the switch from naive T cells to allergic type Th2 cells (Huels et al., 1995; Lopez, Hines, Palmer, Alperin, & Hines, 2002). Thus, these results suggest that the anti-allergic activity of LSE and its compounds is due to decreases in IL-4 and TNF-α release in RBL-2H3 cells.

Activation of mast cells also induces the production of PGD₂, a COX metabolite, which acts as a mediator of allergic and inflammatory diseases (Razin et al., 1983; Shirley et al., 2016). Moreover, it has been reported that the MAPK and NF-κB pathways are important for production of PGD₂, TNF-α, and IL-4 in mast cells (Blackwell & Christman, 1997; Zhang et al., 1997). LSE and its active compounds inhibited the production of the lipid mediator PGD₂ (Fig. 4A and B), an arachidonic acid metabolite (Roth et al., 2008), and protein expression of COX-2, which influences the generation of prostaglandins from arachidonic acid (Fig. 4C and D). LSE and its active compounds also inhibited phosphorylation of IKKα/β, degradation of IκB,

and MAPK activation in antigen-stimulated RBL-2H3 cells (Figs. 5, 6C and D). These results indicate that LSE and various compounds suppress production of inflammatory mediators in antigen-stimulated mast cells by attenuating NF-κB and MAPK activation.

Mast cells are activated by cross-linking of FcεRI, which successively phosphorylates receptor-associated tyrosine kinases including Lyn and Syk (Costello, Turner, & Walters, 1996). Cross-linking of FcεRI to antigens also induces the activation of other downstream molecules, such as PLCγ and PKC. The Syk pathway is crucial for activating downstream effectors (Costello et al., 1996; Zhang et al., 1997), such as MAPKs, which induce the secretion of pro-inflammatory cytokines (Rivera & Gilfillan, 2006). Therefore, Syk is a therapeutic target for improving allergic disorders. To examine the mechanisms of action of LSE and the compounds, we measured the phosphorylation of FcεRI. LSE and the compounds inhibited the phosphorylation of several downstream effectors, including Syk, PLCγ, and PKCμ (Fig. 6A and B). These results suggest that LSE and the compounds have anti-allergic activities by blocking the FcεRI signalling pathway.

Among various compounds isolated from LSE, the flavanonol group (dihydroquercetin and dihydrokaempferol) did not inhibit IgE-mediated allergic reactions and flavonol group (quercetin and kaempferol) was most active followed by almost indistinguishably flavanone group (naringenin and eriodictyol), flavanol group ((+)-catechin), stilbenoid group (resveratrol), and lignan group (secoisolaricresinol). According to previous reports,

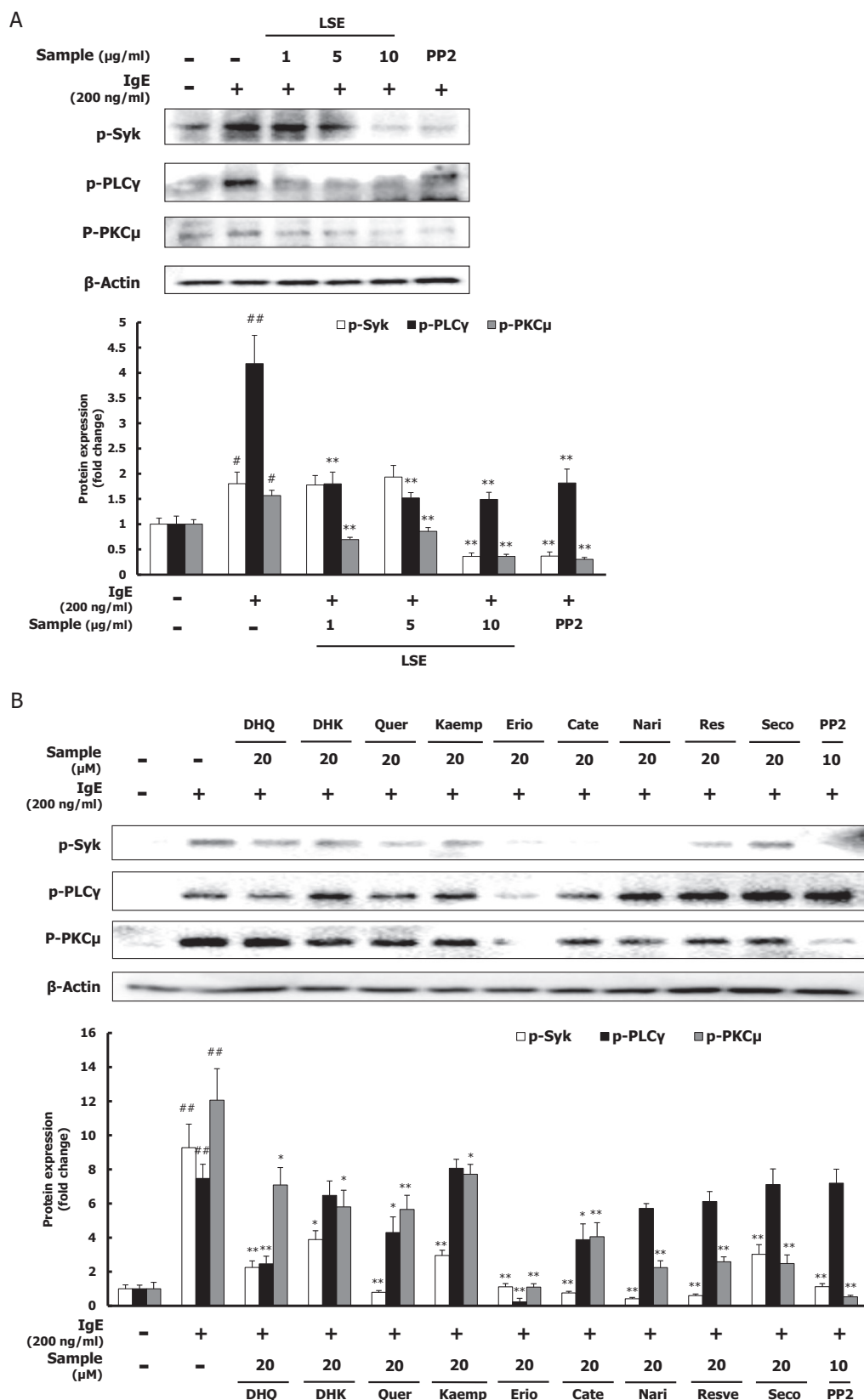
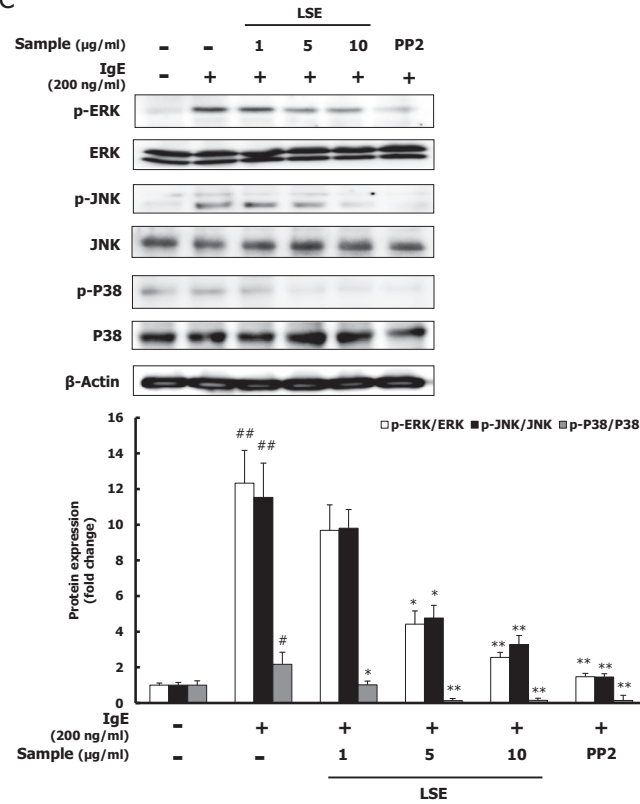


Fig. 6 – Effect of LSE and various flavonoids on FcεRI signalling. (A,B) RBL-2H3 cells were treated with LSE and various flavonoids for 1 h and then stimulated with DNP-BSA for 10 min. Phosphorylation of Syk, PLCγ, and PKD/PKCμ was determined with Western blot analysis. **(C,D)** RBL-2H3 cells were treated with LSE and various flavonoids 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.

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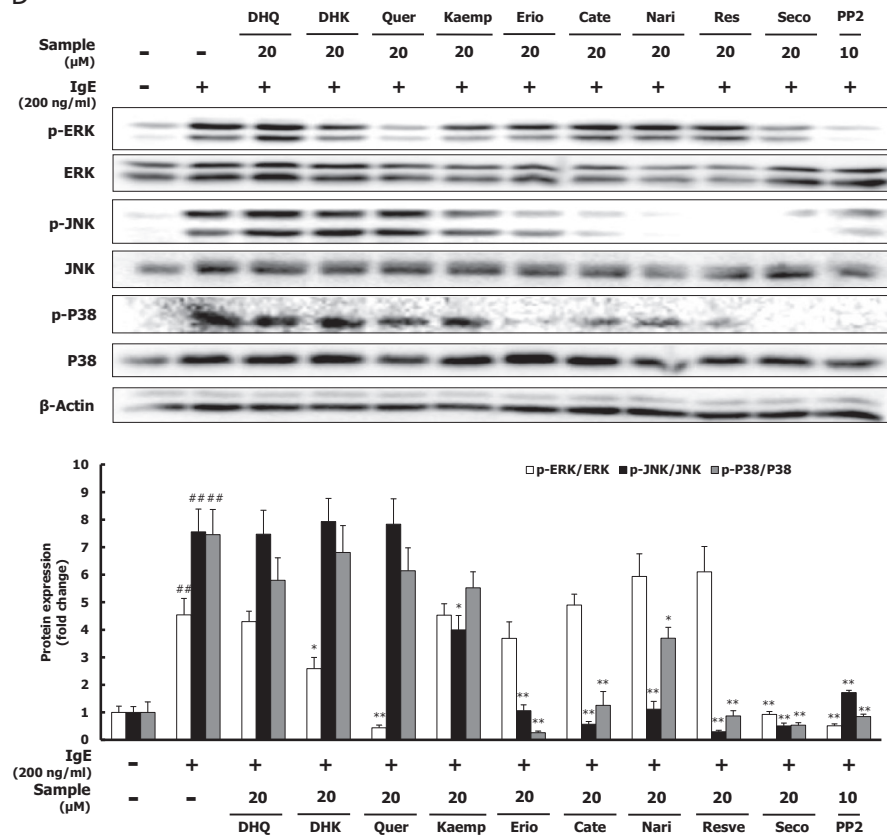


Fig. 6 – (continued)

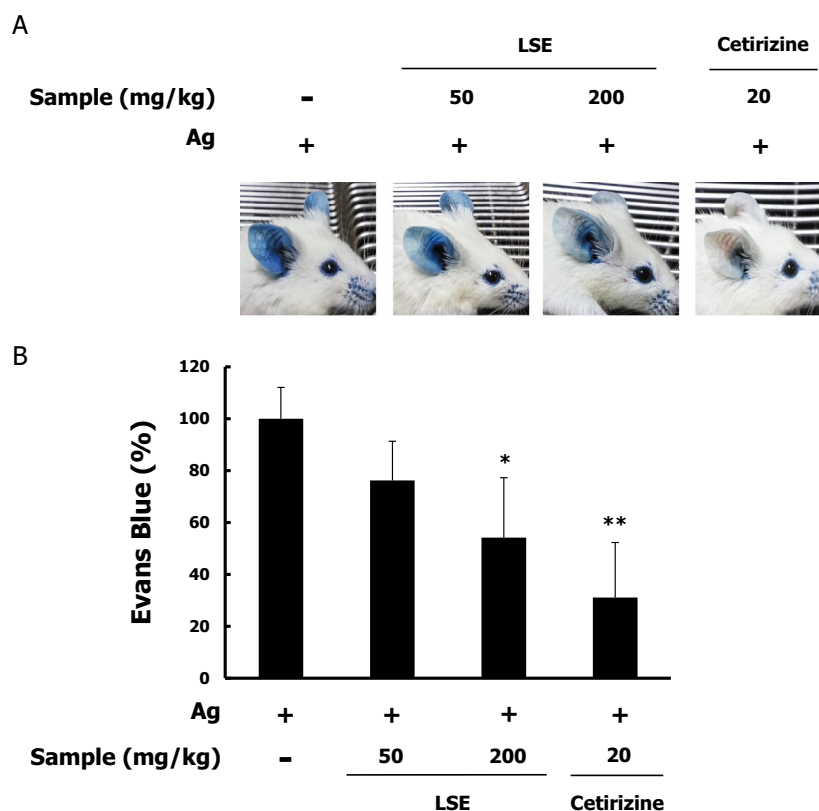


Fig. 7 – Effect of LSE on IgE-mediated passive cutaneous anaphylaxis (PCA). (A) LSE (50 or 200 mg/kg) or cetirizine (20 mg/kg) was orally administered to mice for 1 h before IgE sensitization. Evans blue stained ears were photographed. **(B)** The ear absorbed dye was extracted with formamide. The quantification data are presented as % of IgE-stimulated control. Cetirizine (20 mg/kg) is an anti-histamine reference drug. Results are expressed as the mean $\pm$ SD of three independent experiments (** $p < 0.01$).

quercetin, kaempferol, eriodictyol, (+)-catechin, naringenin, resveratrol, and secoisolariciresinol may have anti-allergic activities (Fujimura et al., 2002; Han et al., 2013; Kim et al., 2014; Koyama et al., 2006; Park et al., 2005; Yoo et al., 2012) while the inhibitory effects of flavanonol compounds such as dihydroquercetin and dihydrokaempferol on allergic responses have not been reported. In addition, quercetin and eriodictyol with more hydroxyl moieties showed a stronger anti-allergic effect than kaempferol and naringenin. Based on our experimental results, we could reach the following conclusions: (1) presence of a 2,3-double bond in dihydroquercetin or dihydrokaempferol apparently enhances the anti-allergic activity; (2) as the number of hydroxyl groups at the 3'-, 4'-, 5-, and 7-positions increased, the anti-allergic activities became stronger. These findings seem to correlate with previous studies that showed that flavones containing more 2,3-double bonds or hydroxyl groups have greater anti-allergic effects (Kim et al., 2014; Mastuda, Morikawa, Ueda, Managi, & Yoshikawa, 2002; Matsuda, Morikawa, Managi, & Yoshikawa, 2003; Park et al., 2008). Furthermore, quercetin and kaempferol have more potent antioxidant and anti-inflammatory activities than dihydroquercetin and dihydrokaempferol. For example, quercetin and kaempferol increase the antioxidant capacity more than dihydroquercetin and dihydrokaempferol by increasing the intracellular glutathione level, reducing reactive oxygen species (ROS) levels, and inhibiting DPPH radical-scavenging

activity (Kang et al., 2016; Kwon et al., 2004). Quercetin inhibits TNF-induced NF- κ B recruitment and pro-inflammatory mediators, but dihydroquercetin does not have any inhibitory effect (Ruiz, Braune, Hölzlzimmer, Quintanilla-Fend, & Haller, 2007).

5. Conclusion

The present study suggests that LSE and its various compounds are potent inhibitors of inflammatory mediator release in mast cells. The likely mechanism of action involves reducing degranulation and the generation of PGE₂, and pro-inflammatory cytokines and attenuating the activation of Fc ϵ RI, NF- κ B, and MAPKs. Based on these results, LSE and its constituents may be promising mast cell-blocking agents for improving IgE-mediated allergic disorders.

Conflict of interest

The authors declare that there are no conflicts of interest.

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