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Aceriphyllum rossii Extract and Its Active Compounds, Quercetin and Kaempferol Inhibit IgE-mediated Mast Cell Activation and Passive Cutaneous Anaphylaxis

Myungsuk Kim, Sue Ji Lim, Suk Woo Kang, Byung-Hun Um, and Chu Won Nho

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9 Myungsuk Kim, Sue Ji Lim, Suk Woo Kang, Byung-Hun Um and Chu Won Nho *

10
11 Functional Food Center, Korea Institute of Science and Technology (KIST) Gangneung
12 Institute, Gangwon, Korea
13

14 *** Correspondence**

15 Chu Won Nho, Ph. D,
16 Functional Food Center,
17 Korea Institute of Science and Technology (KIST),
18 Daejeon-dong, Gangneung 210-340, Korea.
19 E-mail: cwnho@kist.re.kr,
20 Phone: +82-33-650-3651, Fax: +82-33-650-3679
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ABSTRACT: *Aceriphyllum rossii* contains an abundant source of natural flavonoids with potential antioxidant, anticancer and anti-inflammatory properties. However, the effect of *Aceriphyllum rossii* extract (ARE) on immunoglobulin E-mediated allergic responses remains unknown. In the present study, the effects of ARE and its active compounds, quercetin and kaempferol, on IgE-mediated rat basophilic leukemia mast cell activation and passive cutaneous anaphylaxis (PCA) were investigated. ARE, quercetin, and kaempferol inhibited secretion of β -hexosaminidase and histamine, and reduced the production and mRNA expression of interleukin-4 and tumor necrosis factor- α . ARE also decreased the production of prostaglandin E₂ and leukotriene B₄ and expression of cyclooxygenase 2 and 5-lipoxygenase. Furthermore, ARE, quercetin, and kaempferol inhibited IgE-mediated phosphorylation of Syk, phospholipase C γ , protein kinase C (PKC) μ , and the mitogen-activated protein kinases, extracellular signal-regulated kinase, p38, and c-Jun N-terminal kinase. ARE, quercetin, and kaempferol markedly suppressed mast cell-dependent PCA in IgE-sensitized mice. These results indicate that ARE and its active constituents, quercetin and kaempferol, may be a useful therapy for immediate-type hypersensitivity.

KEYWORDS: *Quercetin, kaempferol, Aceriphyllum rossii, IgE-mediated allergic diseases, RBL-2H3 mast cells*

INTRODUCTION

Allergic diseases such as atopic dermatitis, allergic rhinitis, and asthma are a major global public health concern. Allergic diseases are traditionally referred to as immediate or immediate-type hypersensitivity reactions, with immunoglobulin E (IgE) involved as a critical factor.¹

Mast cell activation mediated by the high affinity IgE receptor (FcεRI) is a key event in the allergic inflammatory response.² Upon allergen provocation, cross-linking of IgE molecules and their high affinity receptors triggers a complex series of biochemical reactions including calcium (Ca²⁺) influx, cytoskeletal reorganization, and the activation of protein tyrosine kinases, phospholipases, protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs).³ Following activation, mast cells secrete three classes of biologically active products, lipid-derived mediators, cytoplasmic granule-derived mediators, and growth factors and proinflammatory cytokines.⁴ Preformed granules contain histamine, which is a hallmark of allergic reactions and can induce vascular permeability. Lipid-derived mediators, including prostaglandins (PGs) and leukotrienes (LTs), promote vasodilation and erythema.^{5,6} Activated mast cells also secrete a broad range of growth factors, chemokines, and proinflammatory cytokines, which have the potential to recruit other immune cells either directly or indirectly. Thus, mast cells contribute to chronic inflammation and acute inflammation.⁴

There are a number of therapeutic agents available for the treatment of allergic diseases. However, they mostly act by modulating Th1/Th2 responses and by reducing the concentration of IgE. Glucocorticoids are the most commonly used therapy for allergic diseases, but long-term use leads to undesirable side-effects.⁵ Recently, natural products have been proposed as anti-allergic drug candidates due to their great immunomodulatory efficacy and established safety records.⁶ Some traditional medicinal plants have previously been identified for their anti-allergic effects by suppressing mast cell degranulation and activation

76 *in vitro* and *in vivo*.^{7,8}

77 *Aceriphyllum rossii* (*A.rossii*) Engler is a perennial herb that grows in damp rocky
78 conditions and is distributed throughout mid-northern Korea and China. The young leaves and
79 tender stems (before flowering) are used as a source of food in Korea.⁹⁻¹¹ *A. rossii* is also
80 known as *Mukdenia rossii* (Oliv.) Koidz, and is traditionally used in China as a diuretic or
81 heart stimulant. *Aceriphyllum acanthifolium* (Nakai) T. Lee, the other species of
82 *Aceriphyllum*, is used as an accompaniment to rice in Chinese and Korean cuisine.¹¹ Recently,
83 this plant was reported to contain several triterpenes and flavonol glycosides. These
84 compounds are known to inhibit acyl-CoA, and have antioxidant and antibacterial
85 properties.¹²⁻¹⁴ However, the effects of *A. rossii* and its constituents on allergic disease have
86 not yet been examined. In the present study, we evaluated the potential anti-allergic properties
87 of *A. rossii* extract (ARE) and its active compounds, quercetin and kaempferol, by
88 determining their effects on antigen-stimulated rat basophilic leukemia (RBL-2H3) cells *in*
89 *vitro* and mast cell-dependent allergic responses *in vivo*.

91 MATERIALS AND METHODS

92 **Reagents and Materials.** Reagents were obtained as follows: fetal bovine serum
93 (FBS; Hyclone, South Logan, UT, USA), penicillin (Invitrogen, Carlsbad, CA, USA),
94 Dulbecco's modified Eagle's medium (DMEM; Hyclone, South Logan, UT, USA),
95 antidinitrophenyl (DNP)-specific immunoglobulin E (IgE) (Sigma-Aldrich, St. Louis, MO,
96 USA), DNP-bovine serum albumin (DNP-BSA, Sigma-Aldrich, St. Louis, MO, USA), PIPES
97 (Sigma-Aldrich, St. Louis, MO, USA), PP2 (Sigma-Aldrich, St. Louis, MO, USA), and
98 cyclooxygenase 2 (COX-2) antibody, 5-lipoxygenase (5-LO) antibody, phospho-Lyn
99 (Tyr507) antibody, phospho-Syk (Tyr525/526) antibody, phospho-phospholipase C (PLC) γ
100 (Tyr783) antibody, phospho-PKD/PKC μ (Ser916) antibody, phospho-SAPK/JNK

(Thr183/Tyr185) antibody, SAPK/JNK (Thr183/Tyr185) antibody, phospho-p44/42 MAPK antibody (Erk1/2), p44/42 MAPK (Erk1/2) antibody, phospho-p38 antibody, p38 antibody, and β -actin antibody (all from Cell Signaling Technology Inc., Danvers, MA, USA).

Plant Material. *A. rossii* was collected in Pyeongchang, Gangwon Province, Korea, in August 2010. A voucher specimen (DGR-012) was deposited at the Korea Institute of Science and Technology (KIST), Gangneung Institute. Extraction was performed by sonication using an RK158S ultrasonic bath (Bandelin, Germany).

Extraction and Isolation of Flavones and Flavone Glycosides from *A. rossii*. One kilogram of the dried aerial parts of *A. rossii* was extracted with 5 L of 95% ethanol at room temperature for 4 h and filtered through Whatman No. 1 filter paper (GE Healthcare Life Sciences, PA, USA). The filtrate was concentrated under reduced pressure by rotary evaporation at 40°C and 120 g of EtOH extract (ARE) was obtained. ARE (100 g) was sequentially partitioned into hexane (17.3 g), methylene chloride (7.3 g), ethyl acetate (12.3 g), butanol (29.1 g), and water portions (31.9 g) for β -hexosaminidase assay. Among ARE partitioned into other solvents, ethyl acetate fraction (ARF) showed the highest inhibitory activity of mast cell degranulation (Figure S1), thus bioactivity-guided fractionation procedure was performed to obtain seven isolates. Ten grams of ARF was chromatographed on a silica gel column (Merck 230–400 mesh, 500 g, 5 cm diameter $\times$ 70 cm, Merck, Darmstadt, Germany) and successively eluted with a stepwise gradient of hexane/ethyl acetate [90:10 (3 L), 80:20 (2 L), 75:25 (2 L), 65:35 (2 L), 50:50 (2 L), 30:70 (2 L), 0:100 (1 L) by volume] to provide 5 fractions [ARF1 (168 mg), ARF2 (127 mg), ARF3 (150 mg), ARF4 (693 mg), ARF5 (790 mg)]. The sub-fractions were monitored by HPLC (Figure S2) and the ARF1–5 showed similar inhibitory activity of β -hexosaminidase release (Figure S3). Isolation of flavone and flavone glycosides (Figure 1) from *A.rossii* was done as previously described.¹⁴

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 75 cm² culture flasks at 37°C with 5% CO₂ in a humidified atmosphere.

Cell Viability Assay. Cell viability was checked 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 (2×10^5 cells/mL) for 24 h. After treating the cells with various concentrations of ARE for 6 h, the cells were washed and then treated with 200 μ L MTT (0.5 mg/mL) and cells were 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 by spectrophotometry at 550 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT).

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 ARE or compounds **1–7** at the indicated concentrations for 1 h at 37°C. After incubation, cells were challenged with 0.2 μ g/mL DNP-BSA for 1 h at 37°C. Histamine was then detected using an enzyme immunoassay kit (Oxford Biomedical Research, Rochester Hills, MI). Histamine release was expressed as a percentage of the total histamine produced by unstimulated cells. 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.

Measurement of Prostaglandin E₂ and Leukotriene B₄. Cell culture medium was collected, and the secretion of prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄) was determined using competitive radioimmunoassay kits (R&D Systems, MN, USA, and Cayman Chemical, MI, USA, respectively) according to the manufacturers' protocol.

Measurement of Cytokine Release. IgE-sensitized RBL-2H3 cells were incubated with ARE, quercetin, and kaempferol (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).

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. Amplification of the cDNA products (5 μ L) by PCR 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); 5-LO forward, 5'-TAC TCA TCA AGC GCT GCA CA-3', and 5-LO reverse, 5'-TGG CCA AAA GCC AGT CGT AT-3' (324 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), 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. Densitometry was performed using DIG chemiluminescent film (volume of all markers/volume of β -actin).

Generation of passive cutaneous anaphylaxis (PCA) in mice. A 0.5 mg of DNP-IgE was injected subcutaneously into the left ears of 4-week-old male BALB/c mice, 24 h before antigen challenge. After IgE-sensitization, the mice were orally administered with ARE (100, 300 mg/kg) or cetirizine (20 mg/kg). After 60 min, the mice were intravenously injected with 250 μ g of DNP-BSA in 250 μ L of 1 $\times$ phosphate-buffered saline (PBS) containing Evans Blue (5 mg/mL). After 1 h, mice were euthanized and ear tissue was collected. The ear tissue absorbed dye was extracted overnight in 700 μ L of formamide at 63°C. The absorbance was measured at 620 nm to calculate dye concentration. Procedures used in this study were approved by the Animal Care and Use Committee of KIST.

Western blot analysis. RBL-2H3 cells were lysed in RIPA buffer containing protease inhibitors and then placed 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) were loaded in each sample, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Blotted membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h, and then incubated with primary antibodies for 16 h at 4°C. After three washes in Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with 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).

Statistical Analysis. Each experiment was performed at least three times. Results are expressed as the mean $\pm$ standard deviation (SD). 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. $^{###}p < 0.01$ compared with the control group (DNP-IgE treatment alone); $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the DNP-IgE and DNP-BSA stimulated group.

RESULTS

Identification Flavone and Flavone-glycosides from *Aceriphyllum rossii*.

Seven flavones and flavone glycosides were identified in *A. rossii* using LC-NMR/MS, including gallic acid ethyl ester, quercetin, kaempferol, quercetin 3-O-(6"-galloyl)- β -D-glucopyranoside (QGG), quercetin 3-O- β -D-glucopyranoside (QG), kaempferol 3-O-(6"-galloyl)- β -D-glucopyranoside (KGG), and kaempferol 3-O- β -D-glucopyranoside (KG) (Figure 1).

Cytotoxicity of Test Substances. The effect of ARE on RBL-2H3 cell viability was evaluated by colorimetric MTT assay. Treatment with ARE (5–20 μ g/mL) had no effect on RBL-2H3 cell viability and no cytotoxicity was observed (data not shown).

Inhibitory Effects of ARE and Flavones and Flavone Glycosides from *A. rossii* on β -hexosaminidase and Histamine Release in IgE Stimulated RBL-2H3 Cells.

To determine the effect of ARE and its constituents on mast cell degranulation, its ability to inhibit the release of β -hexosaminidase was examined. ARE inhibited β -hexosaminidase release in RBL-2H3 cells treated with IgE at concentrations of 5, 10, and 20 μ g/mL in a dose-dependent manner. At a concentration of 20 μ M ARE, β -hexosaminidase

release was inhibited by 46% (Figure 2A). In addition, quercetin and kaempferol inhibited the degranulation of DNP-BSA-stimulated RBL-2H3 cells to a greater degree than gallic acid ethyl ester, QGG, QG, KGG, or KG (Figure 2B). Therefore, further studies were performed using quercetin and kaempferol. The effect of ARE, quercetin, and kaempferol on histamine release was also evaluated to determine potential anti-allergic activities. ARE, quercetin, and kaempferol inhibited histamine release in RBL-2H3 cells treated with IgE in a dose-dependent manner (Figures 2C and 2D).

Inhibitory Effects of ARE on PGE₂ and LTB₄ Production and COX-2 and 5-LO Expression in IgE Stimulated RBL-2H3 Cells. The FcεRI receptor activates the arachidonate cascade in antigen-activated mast cells.¹⁵ Thus, the effect of ARE on the production of proinflammatory lipid mediators (such as PGE₂ and LTB₄) was investigated. ARE significantly inhibited PGE₂ and LTB₄ secretion in IgE-stimulated RBL-2H3 cells in a dose-dependent manner. ARE (20 μM) inhibited PGE₂ and LTB₄ secretion by 5% and 53%, respectively (Figures 3A and 3B). The effect of ARE on the expression of cyclooxygenase 2 (COX-2) and 5-lipoxygenase (5-LO), enzymes that produce PGs and LTs from arachidonic acid, was also evaluated by Western blot assay and RT-PCR. IgE treatment induced COX-2 and 5-LO protein and mRNA expression in RBL-2H3 cells. However, treatment with 5, 10, or 20 μg/mL of ARE attenuated the IgE-induced upregulation of COX-2 and 5-LO (Figure 3C and 3D).

Inhibitory Effect of ARE, Quercetin, and Kaempferol on Proinflammatory Cytokine Production and Gene Expression in IgE Stimulated RBL-2H3 Cells. The proinflammatory cytokines, TNF-α and IL-4, are critical for allergic responses.¹⁶ We therefore examined whether ARE, quercetin, and kaempferol regulate the production and mRNA expression of TNF-α and IL-4 in IgE-sensitized RBL-2H3 cells. As shown, ARE and quercetin inhibited both TNF-α and IL-4 production (Figures 4A–D) and gene expression,

while kaempferol did not affect production and expression of TNF- α and IL-4 (Figures 4E and 4F).

Effect of ARE, Quercetin, and Kaempferol on Mast Cell-mediated PCA in Mice. PCA is widely used to determine local allergic reactions *in vivo*.¹⁷ To investigate whether ARE, quercetin, and kaempferol inhibited allergic responses, we used a mast cell-dependent PCA model. IgE was injected subcutaneously into mouse ear tissue, followed by injection of DNP-BSA containing 5% Evans Blue. Oral administration of ARE, quercetin, and kaempferol inhibited the antigen-induced PCA response in a dose-dependent (Figures 5A and 5B). The inhibitory effect of ARE, quercetin, and kaempferol was similar to that of 20 mg/kg cetirizine¹⁸, a typical anti-histamine drug, which was used as a positive control.

Effect of ARE, Quercetin, and Kaempferol on Fc ϵ RI Signaling in IgE Sensitized RBL-2H3 Cells. Fc ϵ RI signals through activation of Syk, Lyn, PKC, and PLC γ .¹⁹ PP2, a general Src-family kinase inhibitor, markedly suppressed phosphorylation of Syk.¹⁹ To investigate the mechanisms involved in ARE-, quercetin-, and kaempferol-mediated inhibition of degranulation, a number of intracellular signals were examined. DNP-BSA treatment of IgE-sensitized cells induced phosphorylation of Syk, PKD/PKC μ , and PLC γ at early time-points, and this phosphorylation was reduced by treatment with ARE, quercetin, or kaempferol (Figures 6A and 6B). During IgE-mediated activation of mast cells, three major subfamilies of MAPKs (extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38) can be activated, resulting in cytokine production.²⁰ To further identify the relevant downstream effectors that directly modulate cytokine production following ARE, quercetin, and kaempferol treatment, phosphorylation of ERK, JNK and p38 was examined. IgE-induced phosphorylation of ERK, JNK and p38 was inhibited by treatment with ARE, quercetin, or kaempferol (Figures 6C and 6D).

DISCUSSION

Immediate-type hypersensitivity is initiated and amplified by inflammatory mediators (histamines, LTs, and PGs) and cytokines (TNF- α and IL-4) released from immune cells such as mast cells, eosinophils, and basophils. In particular, mast cells are crucial players in the regulation of allergic inflammatory responses due to their ability to produce proinflammatory mediators including histamines, PGE₂, and LTB₄.²¹ A recent study in mast cell-deficient mice demonstrated that mast cell-derived mediators (histamines, cytokines, and proteases) play an important role during allergic inflammation.²² Therefore, a number of studies have identified specific mediators of mast cell activation, and reported novel compounds targeting these inflammatory mediators.^{3,23,24}

Quercetin and kaempferol are naturally occurring plant flavonoids found in many vegetables and fruits, and may possess anticancer, antioxidant, and anti-inflammatory properties.²⁵⁻²⁷ Moreover, quercetin and kaempferol are also known to suppress various mediators of allergic responses. For example, quercetin, kaempferol, and several other flavonoids suppress degranulation by inhibiting β -hexosaminidase release and production of TNF- α and IL-4.²⁸ In addition, quercetin and kaempferol suppress IgE-mediated allergic inflammation in RBL-2H3 and Caco-2 cells.²⁹ However, the molecular mechanisms by which quercetin and kaempferol act on allergic disease are not fully understood. In the present study, we evaluated the inhibitory effects of ARE and its active compounds, quercetin and kaempferol, on allergic mediators in RBL-2H3 cells and in a murine PCA model.

Activation of Fc ϵ RI receptors present on mast cells through high affinity binding and aggregation of IgE initiates a signaling cascade that results in degranulation and secretion of inflammatory mediators, including histamine, lipid-derived mediators, and inflammatory cytokines.³⁰ We observed that ARE, quercetin, and kaempferol inhibited the release of both β -hexosaminidase (Figures 2B and 2D) and histamine (Figures 2C and 2E) in a dose-dependent

manner. These results indicate that the anti-allergic effects of ARE, quercetin, and kaempferol are due to the suppression of histamine release, known to cause vascular permeability, smooth-muscle contraction, and inflammation.

The FcεRI receptor signals through Lyn, Syk and PLCγ to regulate degranulation.¹⁶ 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 the FcεRI β and γ chains. In turn, Syk induces phosphorylation of PLCγ and PKC.³¹ Notably, ARE, quercetin, and kaempferol also decreased the phosphorylation of Syk, PLCγ, and PKD/PKCμ (Figures 6A and 6B). These results suggest that ARE, quercetin, and kaempferol 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.

ARE also reduced the secretion of the inflammatory mediators PGE₂ and LTB₄, which are arachidonic acid metabolites, via inhibition of the COX and LO pathways (Figures 3A and 3B).³² Furthermore, ARE inhibited COX-2 and 5-LO expression, key enzymes in these pathways (Figures 3C and 3D). These results indicate that ARE inhibits synthesis of inflammatory mediators by suppressing the COX and LO pathways.

We also found that ARE and quercetin significantly reduced both the production and mRNA expression of the proinflammatory cytokines TNF-α and IL-4 (Figure 4). TNF-α and IL-4 play a crucial role in triggering and sustaining allergic reactions in mast cells. IL-4 induces T-cell development and switches B-cells to the IgE isotype. TNF-α also activates physiological immune responses by promoting leukocyte infiltration and tissue fibrosis, and initiates cytokine-mediated inflammatory states via stimulation of cytokine production.³³ Although inflammation is a physiological defense mechanism against pathogens, overexpression of cytokines can also cause serious inflammatory diseases such as atopic

dermatitis and asthma. The results suggest that these compounds may be useful for the treatment of allergic conditions.

MAPK pathways are a major therapeutic target in allergic disease.³⁴ Therefore, to investigate the mechanism by which ARE, quercetin, and kaempferol inhibit the activation of RBL-2H3 cells, MAPK (ERK, p38, and JNK) phosphorylation was evaluated. In this study, ARE, quercetin, and kaempferol inhibited IgE-induced phosphorylation of ERK, p38, and JNK, suggesting that these compounds attenuate mast cell activation by regulating MAPK signaling (Figures 6C and 6D).

In conclusion, ARE and its active compounds, quercetin and kaempferol, inhibited degranulation and the release of histamine, lipid mediators, and proinflammatory cytokines in IgE-stimulated RBL-2H3 cells, and had anti-inflammatory effects in a murine PCA model. ARE, quercetin, and kaempferol also inhibited downstream signaling from the FcεRI receptor by suppressing the phosphorylation of Syk, PLCγ, PKC, and MAPK, all of which mediate allergic responses in IgE-sensitized RBL-2H3 cells (Figure 7). These findings indicate that ARE, quercetin, and kaempferol may together be a useful therapeutic agent for preventing or treating IgE-mediated allergic diseases.

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ABBREVIATIONS USED

DMSO, dimethyl sulfoxide; FcεRI, Fc receptor for IgE; IL, interleukin; MAPK, mitogen-activated protein kinases; RBL, rat basophilic leukemia; TNF, tumor necrosis factor. IgE, immunoglobulin E; PLC, phospholipase; protein kinase C, PKC; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PGs, prostaglandins; LTs, leukotrienes;

351 COX-2, cyclooxygenase 2; 5-LO, 5-lipoxygenase.

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REFERENCES

- (1) Rosenwasser, L. J. Mechanisms of IgE Inflammation. *Curr. Allergy Asthma Rep.* **2011**, *11*, 178-183.
- (2) Gilfillan, A. M.; Tkaczyk, C. Integrated signaling pathways for mast-cell activation. *Nat. Rev. Immunol.* **2006**, *6*, 218-230.
- (3) Itoh, T.; Ohguchi, K.; Inuma, M.; Nozawa, Y.; Akao, Y. Inhibitory effects of polymethoxy flavones isolated from *Citrus reticulata* on degranulation in rat basophilic leukemia RBL-2H3: enhanced inhibition by their combination. *Bioorg. Med. Chem.* **2008**, *16*, 7592-7598.
- (4) Galli, S. J.; Tsai, M.; Piliponsky, A. M. The development of allergic inflammation. *Nature* **2008**, *454*, 445-454.
- (5) Oppenheimer, J. J.; Casale, T. B. Next generation antihistamines: therapeutic rationale, accomplishments and advances. *Expert Opin. Investig. Drugs.* **2002**, *11*, 807-817.
- (6) Chan, B. C.; Hon, K. L.; Leung, P. C.; Sam, S. W.; Fung, K. P.; Lee, M. Y.; Lau, H. Y. Traditional Chinese medicine for atopic eczema: PentaHerbs formula suppresses inflammatory mediators release from mast cells. *J. Ethnopharmacol.* **2008**, *120*, 85-91.
- (7) Ho-sub, L.; Kwan-Ha, P.; Kang-Beom, K.; Byeong-Sun, M.; Cheol-Min, S.; Yong-Seon, S.; Eun, A. S.; Young-suk, K.; Kang-Jeon, K.; Do-Gon, R. Anti-allergic activity of the *Sophorae radix* water extract in experimental animals. *Am. J. Chin. Med.* **2001**, *29*, 129-139.
- (8) Knipping, K.; van Esch, E. C.; Wijering, S. C.; van der Heide, S.; Dubois, A. E.; Garssen, J. *In vitro* and *in vivo* anti-allergic effects of *Arctium lappa* L. *Exp. Biol. Med.* **2008**, *233*, 1469-1477.

- (9) Lee, C. B. Illustrated Flora of Korea; Hyangmunsa: Seoul, Korea, 1989; p 409.
- (10) Lee, W. C. Coloured Standard Illustrations of Korean Plants; Academybook: Seoul, Korea, 1996; p 152.
- (11) Lee, Y. N. Flora of Korea; Kyohak Publishing Co., Ltd.: Seoul, Korea, 2002; p 280.
- (12) Han, J.-T.; Kim, H.-Y.; Park, Y.-D.; Lee, Y.-H.; Lee, K.-R.; Kwon, B.-M.; Baek, N.-I. Aceriphylllic acid A, a new ACAT inhibitory triterpenoid, from *Aceriphyllum rossii*. *Planta Med.* **2002**, *68*, 558–561.
- (13) Zheng, C. J.; Sohn, M. J.; Kim, K. Y.; Yu, H. E.; Kim, W. G. Olean-27-carboxylic acid-type triterpenes with potent antibacterial activity from *Aceriphyllum rossii*. *J. Agric. Food. Chem.* **2008**, *56*, 11752–11756.
- (14) Han, J. T.; Bang, M. H.; Chun, O. K.; Kim, D. O.; Lee, C. Y.; Baek, N. I. Flavonol glycosides from the aerial parts of *Aceriphyllum rossii* and their antioxidant activities. *Arch. Pharm. Res.* **2004**, *27*, 390–395.
- (15) Roth, K.; Chen, W. M.; Lin, T.J. Positive and negative regulatory mechanisms in high-affinity IgE receptor-mediated mast cell activation. *Arch. Immunol. Ther. Exp.* (Warsz). **2008**, *56*, 385–399.
- (16) Theoharides, T. C.; Kalogeromitros, D. The critical role of mast cells in allergy and inflammation. *Ann. N.Y. Acad. Sci.* **2006**, *1088*, 78–99.
- (17) Levy, L. Simultaneous passive cutaneous anaphylaxis and arthus reactions in mouse, rat and guinea-pig. *Immunology* **1964**, *7*, 91–96.
- (18) Chodaczek, G.; Bacsı, A.; Dharajiya, N.; Sur, S.; Hazra, T. K.; Boldogh, I. Ragweed pollen-mediated IgE-independent release of biogenic amines from mast cells via induction of mitochondrial dysfunction. *Mol. Immunol.* **2009**, *46*, 2505–2514.
- (19) Lee, J. H.; Kim, J. W.; Ko, N. Y.; Mun, S. H.; Her, E.; Kim, B. K.; Han, J. W.; Lee, H. Y.; Beaven, M. A.; Kim, Y. M.; Choi, W. S. Curcumin, a constituent of curry,

- 426 suppresses IgE-mediated allergic response and mast cell activation at the level of Syk.
427 *J. Allergy Clin. Immunol.* **2008**, *121*, 1225–1231.
- 428 (20)Zhang, C.; Baumgartner, R. A.; Yamada, K.; Beaven, M. A. Mitogen-activated
429 protein (MAP) kinase regulates production of tumor necrosis factor- α and release of
430 arachidonic acid in mast cells: indications of communication between p38 and p42
431 MAP kinases. *J. Biol. Chem.* **1997**, *272*, 13397–13402
- 432 (21)Galli, S. J.; Kalesnikoff, J.; Grimaldeston, M. A.; Piliponsky, A. M.; Williams, C.
433 M.; Tsai, M. Mast cells as "tunable" effector and immunoregulatory cells: recent
434 advances. *Annu. Rev. Immunol.* **2005**, *23*, 749-786.
- 435 (22)Wershil, B.K. Mast cell-deficient mice and intestinal biology. *Am J Physiol*
436 *Gastrointest Liver Physiol.* **2000**, *278*, G343-G348.
- 437 (23)Tanifuji, S.; Aizu-Yokota, E.; Funakoshi-Tago, M.; Sonoda, Y.; Inoue, H.; Kasahara,
438 T. Licochalcones suppress degranulation by decreasing the intracellular Ca^{2+} level
439 and tyrosine phosphorylation of ERK in RBL-2H3 cells. *Int Immunopharmacol.* **2010**,
440 *10*, 769-776.
- 441 (24)Westcott, J.Y.; Wenzel, S.E.; Dreskin, S.C. Arachidonate-induced eicosanoid
442 synthesis in RBL-2H3 cells: stimulation with antigen or A23187 induces prolonged
443 activation of 5-lipoxygenase. *Biochim Biophys Acta.* **1996**, *1303*, 74-81.
- 444 (25)Kang, J. W.; Kim, J. H.; Song, K.; Kim, S.H.; Yoon, J.-H.; Kim, K.-S. Kaempferol
445 and quercetin, components of *Ginkgo biloba* extract (EGb 761), induce caspase-3-
446 dependent apoptosis in oral cavity cancer cells. *Phytother. Res.* **2010**, *24*, S77-S82.
- 447 (26)Kanakakis, C. D.; Tarantilis, P. A.; Polissiou, M. G.; Diamantoglou, S.; Tajmir-Riahi, H.
448 A. DNA interaction with naturally occurring antioxidant flavonoids quercetin,
449 kaempferol, and delphinidin. *J. Biomol. Struct. Dyn.* **2005**, *6*, 719-724.
- 450 (27)García-Mediavilla, V.; Crespo, I.; Collado, P.S.; Esteller, A.; Sánchez-Campos, S.;

- 451 Tuñón, M.J.; González-Gallego, J. The anti-inflammatory flavones quercetin and
452 kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and
453 reactive C-protein, and down-regulation of the nuclear factor kappaB pathway in
454 Chang Liver cells. *Eur. J. Pharmacol.* **2007**, *557*, 221-229.
- 455 (28)Mastuda, H.; Morikawa, T.; Ueda, K.; Managi, H.; Yoshikawa, M. Structural
456 requirements of flavonoids for inhibition of antigen-Induced degranulation, TNF-
457 alpha and IL-4 production from RBL-2H3 cells. *Bioorg. Med. Chem.* **2002**, *10*, 3123-
458 3128.
- 459 (29)Lee, E. J.; Ji, G. E.; Sung, M. K. Quercetin and kaempferol suppress immunoglobulin
460 E-mediated allergic inflammation in RBL-2H3 and Caco-2 cells. *Inflamm.Res.* **2010**,
461 *10*, 847-854.
- 462 (30)Beaven, M. A.; Metzger, H. Signal transduction by Fc receptors: the Fc epsilon RI
463 case. *Immunol. Today* **1993**, *14*, 222-226.
- 464 (31)Siraganian, R. P.; de Castro, R. O.; Barbu, E.A.; Zhang, J. Mast cell signaling: the
465 role of protein tyrosine kinase Syk, its activation and screening methods for new
466 pathway participants. *FEBS Lett.* **2010**, *584*, 4933-4940.
- 467 (32)Gonzalez-Periz, A.; Claria, J. New approaches to the modulation of the
468 cyclooxygenase-2 and 5-lipoxygenase pathways. *Curr. Top. Med. Chem.* **2007**, *7*,
469 297-309.
- 470 (33)Bradding, P. Human mast cell cytokines. *Clin Exp Allergy.* **1996**, *26*, 13-9.
- 471 (34)Abdel-Raheem, I.T.; Hide, I.; Yanase, Y.; Shigemoto-Mogami, Y.; Sakai, N.; Shirai,
472 Y. Protein kinase C-alpha mediates TNF release process in RBL-2H3 mast cells. *Br J*
473 *Pharmacol.* **2005**, *145*, 415-23.

FIGURE CAPTIONS

Figure 1. Chemical structure of flavones and flavone glycosides from *A. rossii*.

Figure 2. Effects of ARE, quercetin, and kaempferol on β -hexosaminidase and histamine release. (A) The degranulation was determined by measuring β -hexosaminidase release in DNP-IgE-sensitized RBL-2H3 cells. The RBL-2H3 cells were treated in the presence or absence of ARE (5, 10 or 20 $\mu\text{g/mL}$) for 1 h and then stimulated for 1 h with DNP-BSA. (B) The RBL-2H3 cells were treated in the presence or absence of gallic acid ethyl ester (1), quercetin (2), kaempferol (3), quercetin 3-O-(6"-galloyl)- β -D-glucopyranoside (4), quercetin 3-O- β -D-glucopyranoside (5), kaempferol 3-O-(6"-galloyl)- β -D-glucopyranoside (6), or kaempferol 3-O- β -D-glucopyranoside (7) (20 μM) 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 ARE (5, 10 or 20 $\mu\text{g/mL}$), quercetin (10 or 20 μM), or kaempferol (10 or 20 μM) for 1 h and then stimulated for 1 h with antigen. PP2 (10 μM) 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 $^{**}p < 0.01$)

Figure 3. Effects of ARE on PGE_2 and LTB_4 secretion. RBL-2H3 cells were treated with ARE (5, 10 or 20 $\mu\text{g/mL}$) for 1 h and then stimulated for 4 h with antigen. (A,B) PGE_2 and LTB_4 secretion was determined by enzyme-linked immunosorbent assay kit. (C,D) COX-2 and 5-LO protein and mRNA level was 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 $\pm$ SD of three independent experiments. ($^{##}p < 0.01$, $^*p < 0.05$ and $^{**}p < 0.01$)

Figure 4. Effects of ARE, quercetin, and kaempferol on TNF- α and IL-4 production and expression. RBL-2H3 cells were treated with ARE, quercetin, and kaempferol for 1 h and then stimulated with DNP-BSA for 4 h. (A-D) TNF- α and IL-4 production was determined by enzyme-linked immunosorbent assay kit. (E,F) TNF- α and IL-4 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 $^{**}p < 0.01$)

Figure 5. Effect of ARE, quercetin, and kaempferol on IgE-mediated passive cutaneous anaphylaxis (PCA). (A) ARE (100 or 300mg/kg), quercetin (50 mg/kg), kaempferol (50 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 is 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. ($^{##}p < 0.01$, $^{*}p < 0.05$ and $^{**}p < 0.01$).

Figure 6. Effect of ARE, quercetin, and kaempferol on Fc ϵ RI Signaling. (A,B) RBL-2H3 cells were treated with ARE, quercetin, and kaempferol for 1 h and then stimulated with DNP-BSA for 10 min. Phosphorylation of Lyn, Syk, PLC γ , and PKD/PKC μ was determined with Western blot analysis. (C,D) RBL-2H3 cells were treated with ARE, quercetin, and kaempferol 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 μ M) is a general Src-family kinase inhibitor.

Figure 7. ARE, quercetin, and kaempferol inhibited FcεRI signaling pathways. The level of phosphorylated Syk proteins were reduced by ARE, quercetin, and kaempferol. ARE, quercetin, and kaempferol inhibited other downstream signaling molecules, including PKD/PKCμ and PLCγ. The activation of MAPK were also suppressed by ARE, quercetin, and kaempferol.

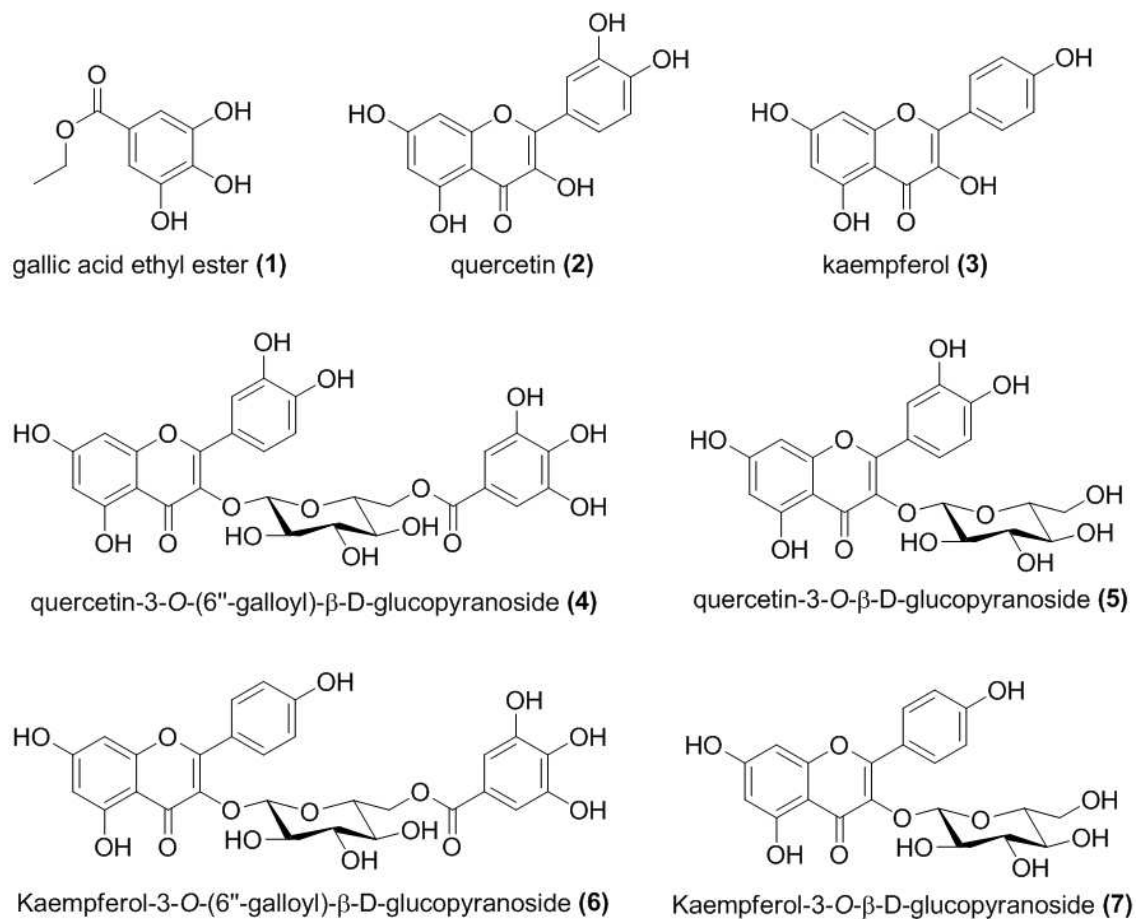
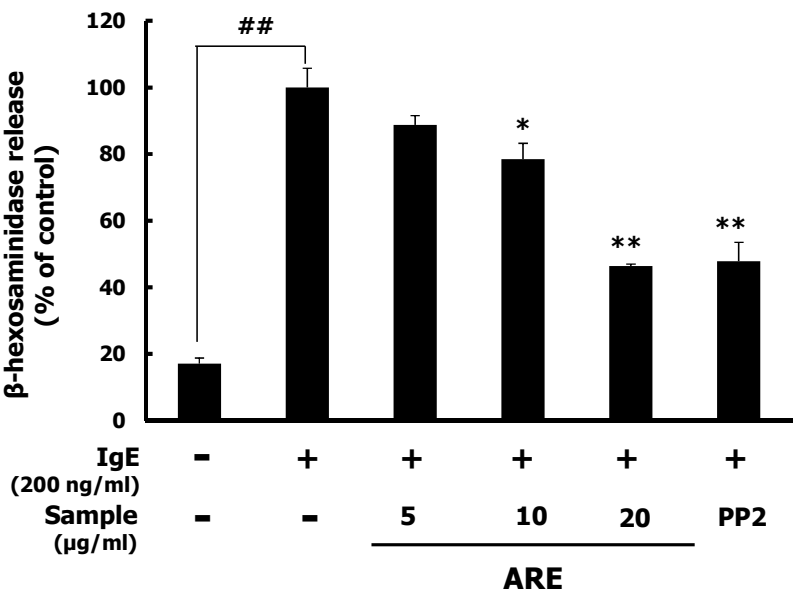


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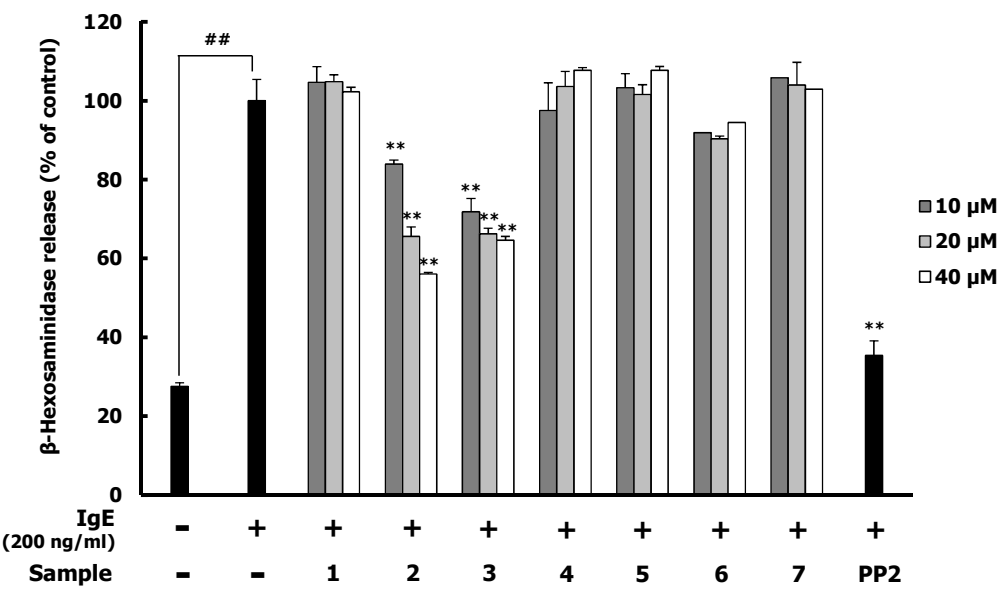
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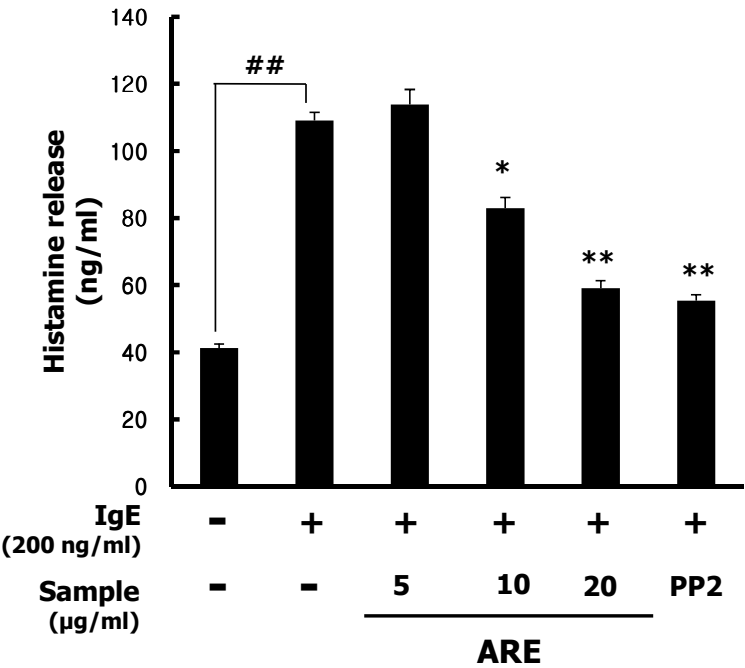
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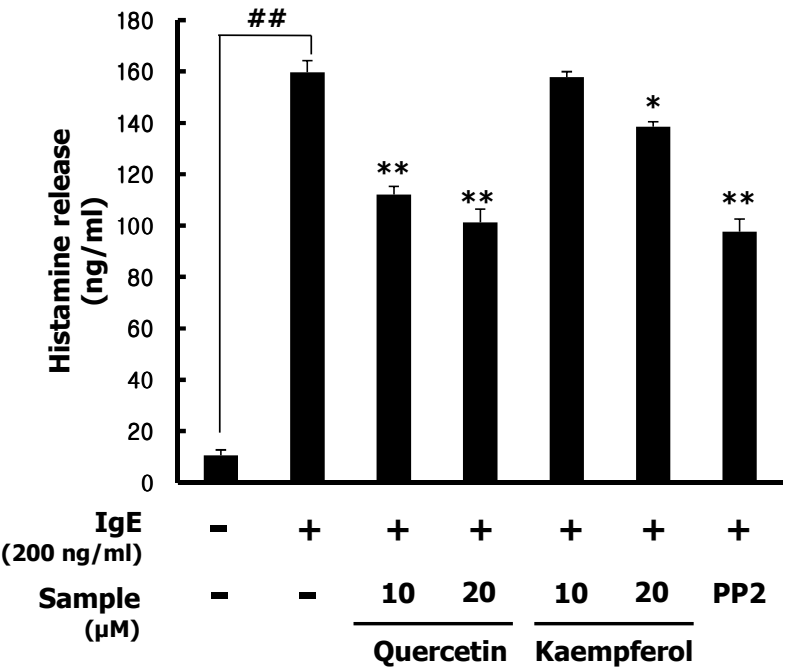
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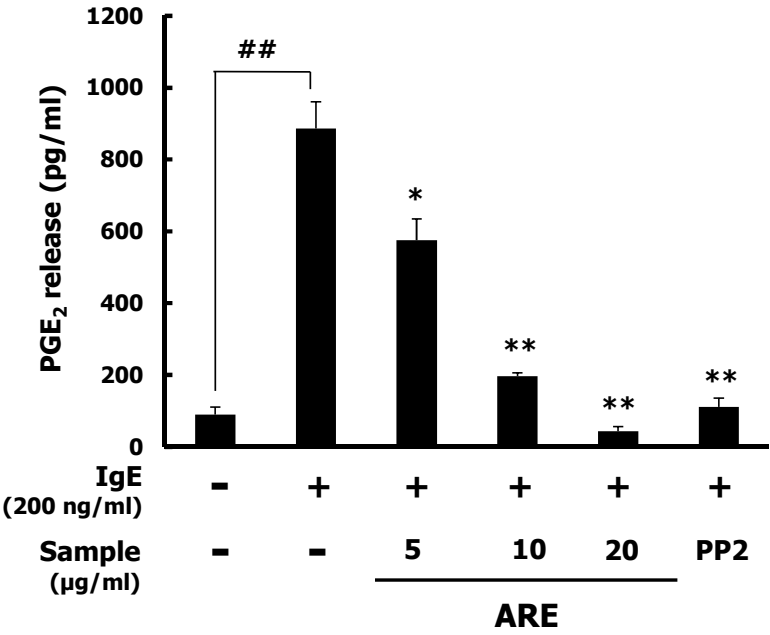
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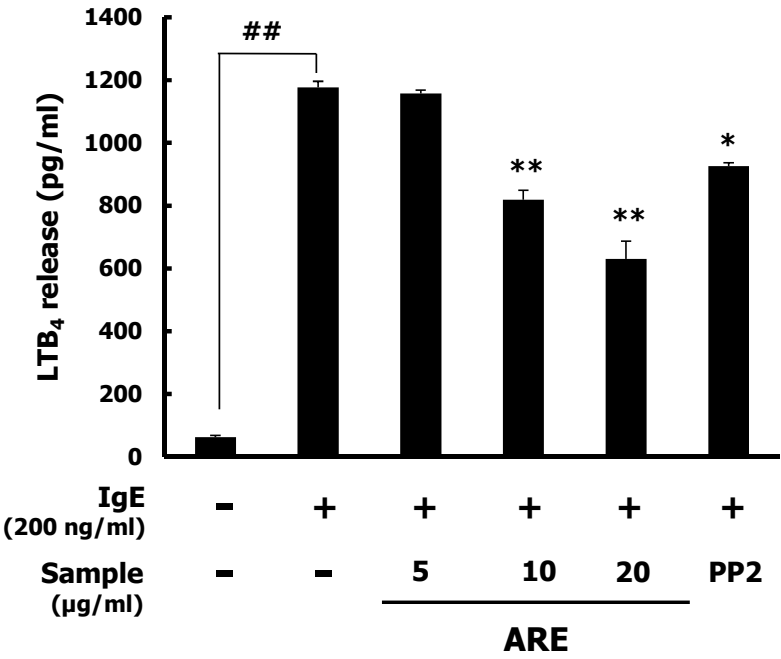
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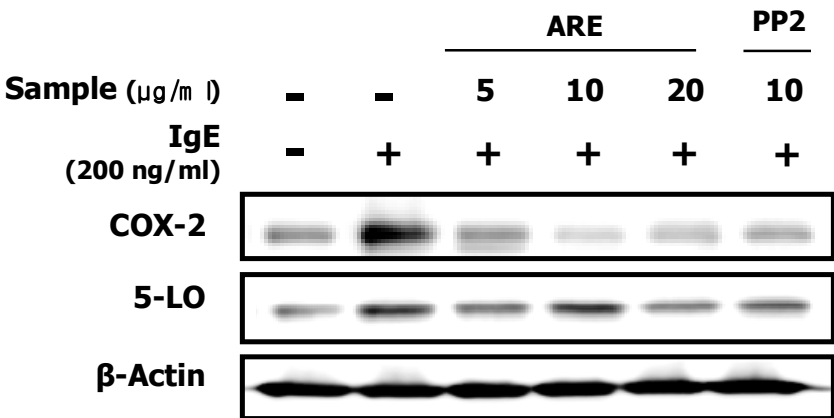


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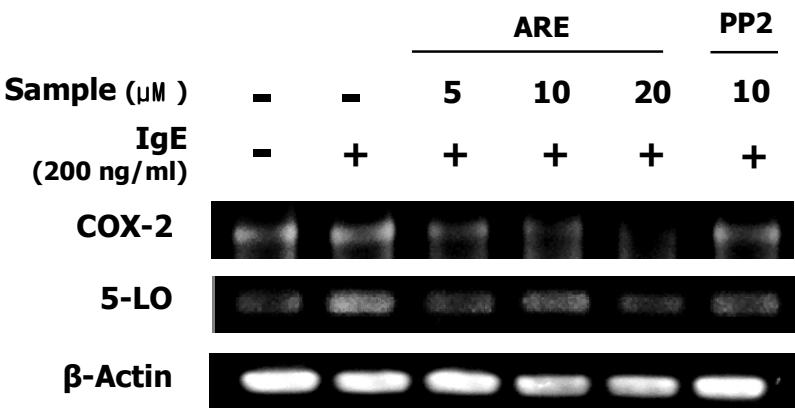
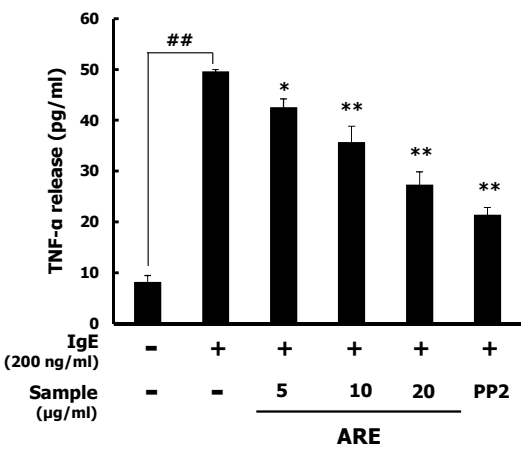


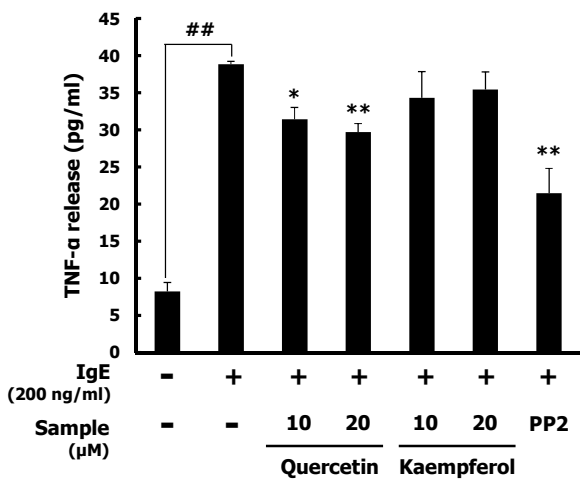
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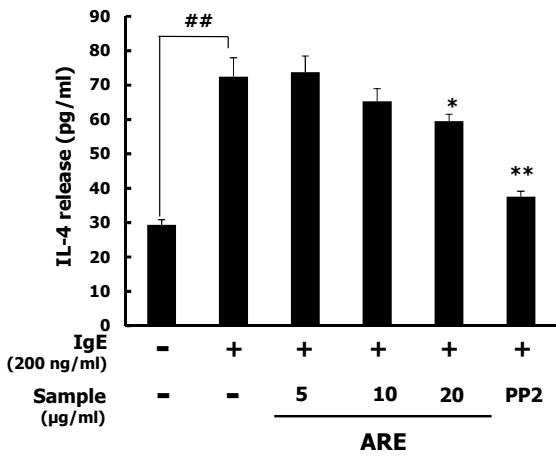
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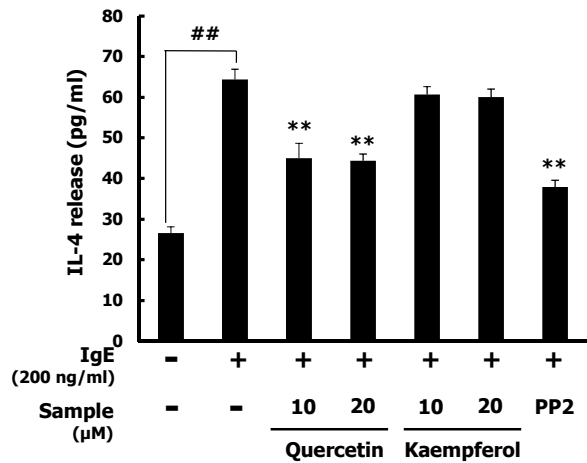
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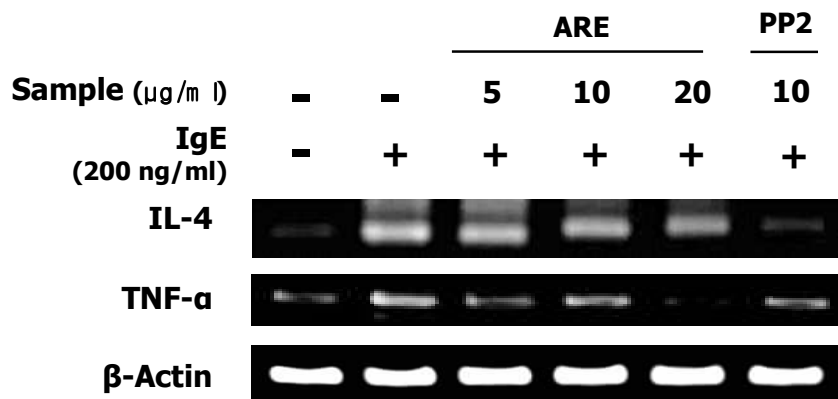
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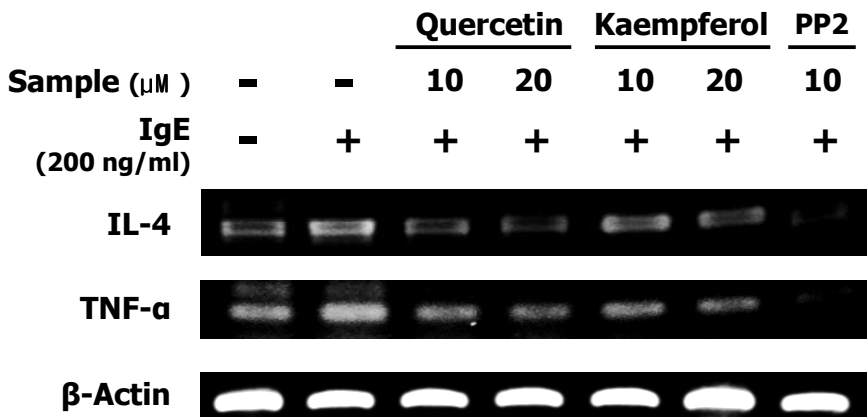
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Figure 4

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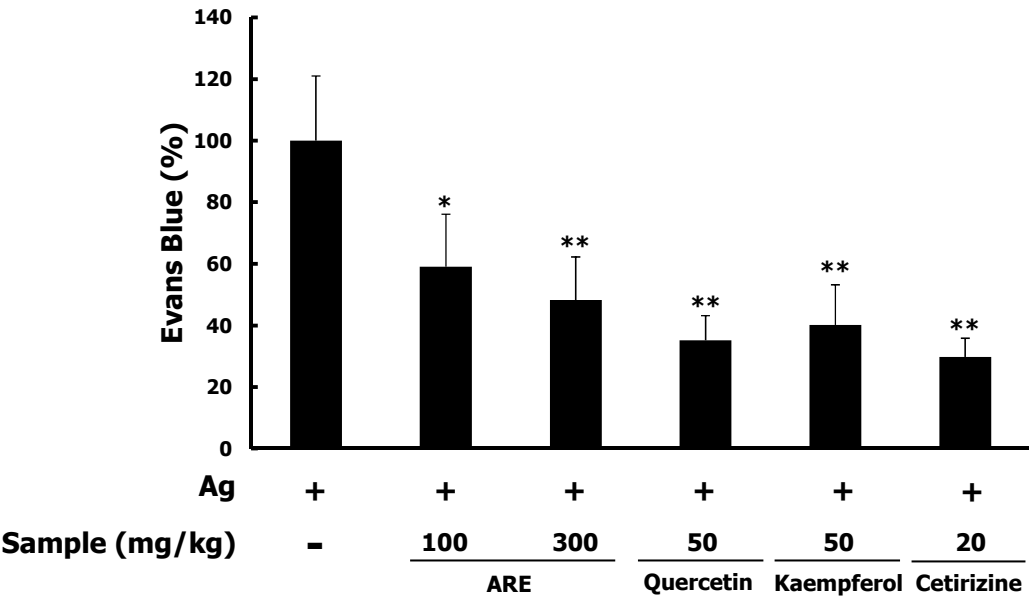
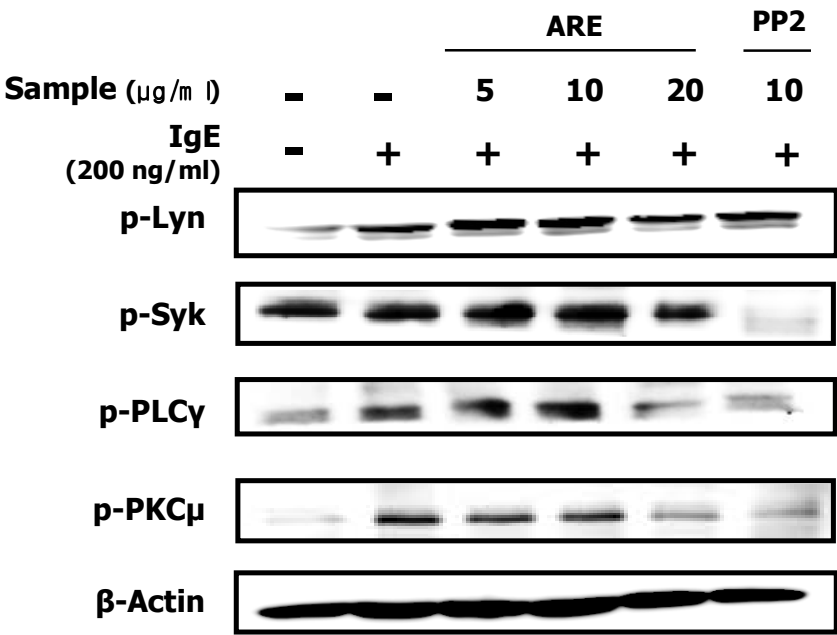


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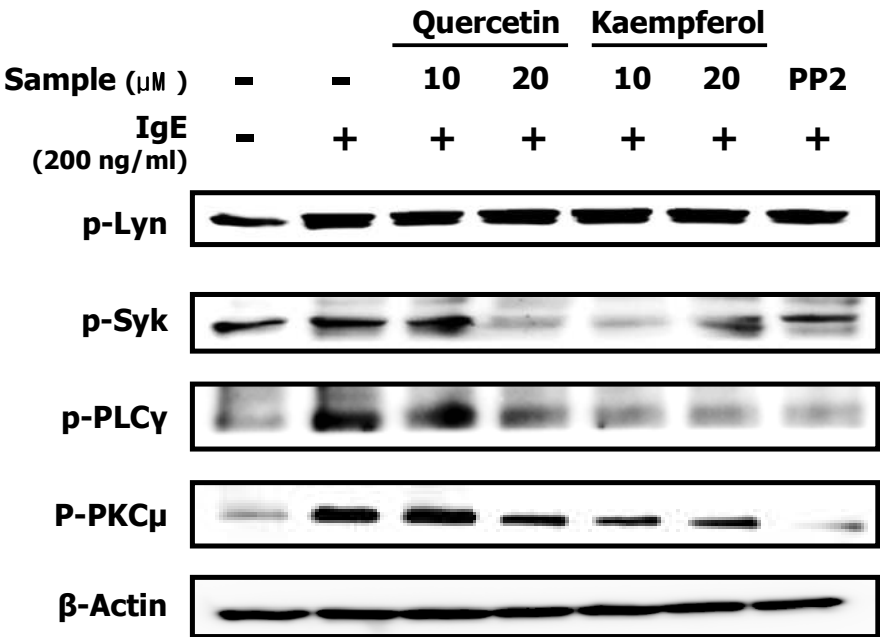
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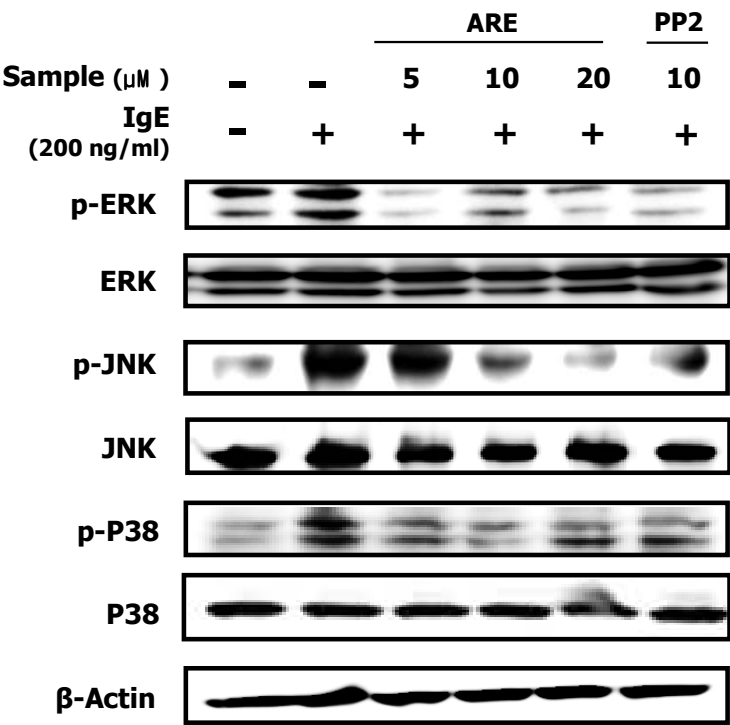
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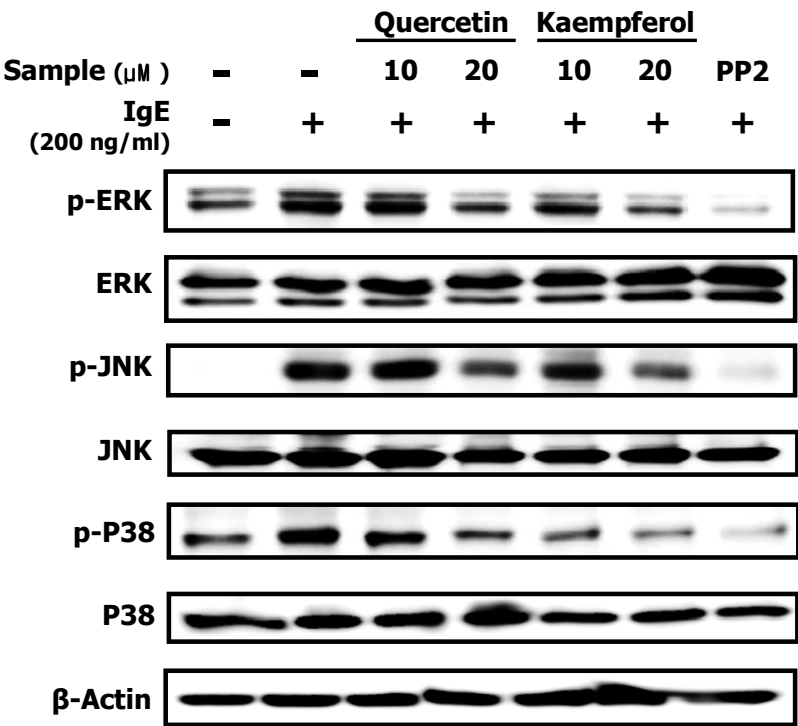
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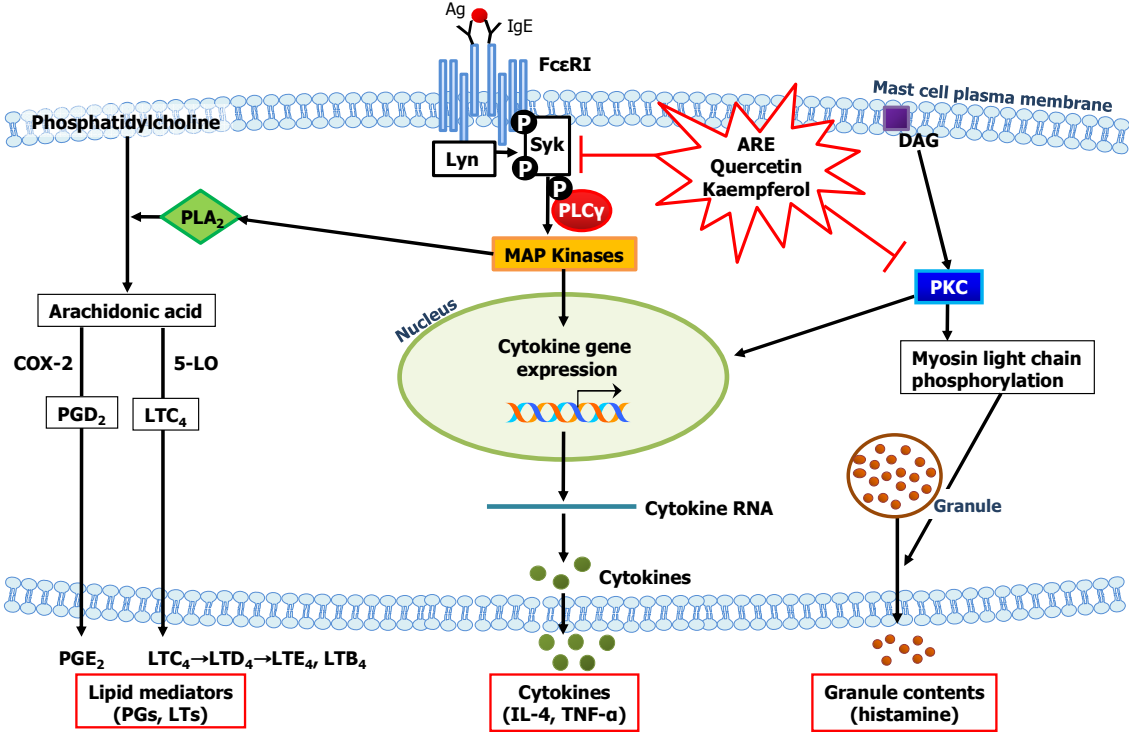


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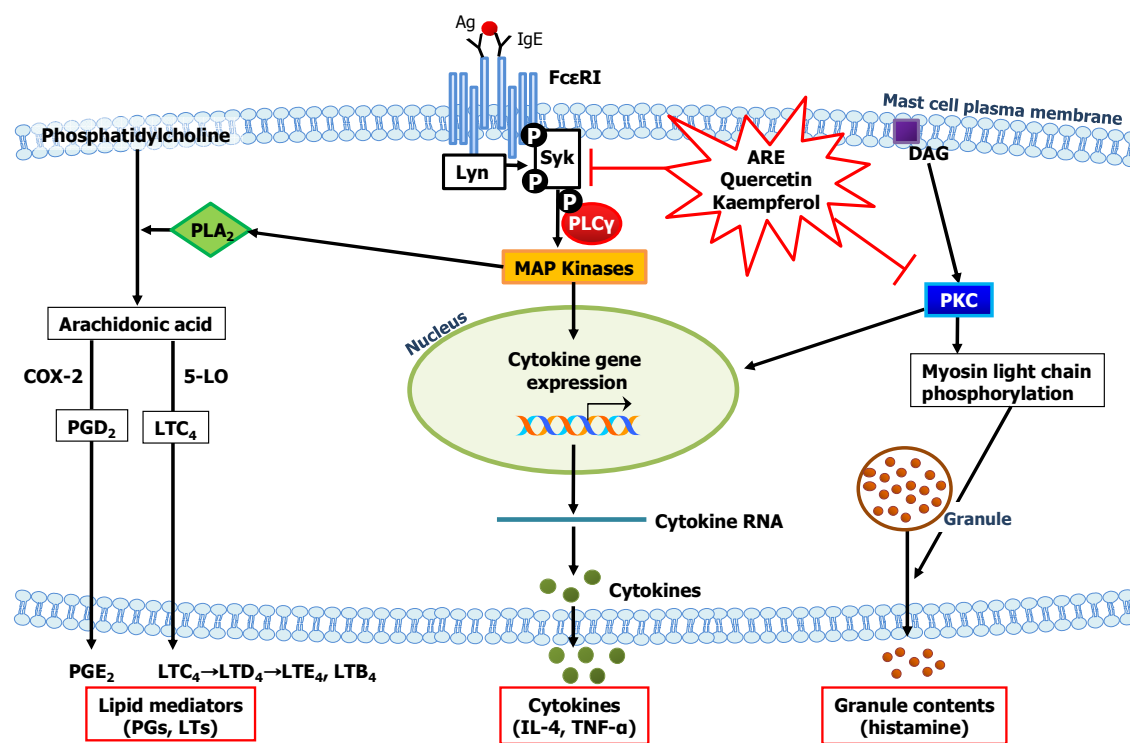


Table of Contents Graphic