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Effects of *Cudrania tricuspidata* Fruit Extract and Its Active Compound, 5,7,3',4'-Tetrahydroxy-6,8-diprenylisoflavone, on the High Affinity IgE Receptor-Mediated Activation of Syk in Mast Cells

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ABSTRACT

Cudrania tricuspidata fruits extract contains a rich source of prenylated flavonoids with potential anti-atherosclerotic, hepatoprotective, and anti-inflammatory properties. However, the effect of *Cudrania tricuspidata* fruits extracts and its active compounds on the high affinity IgE receptor (FcεRI)-mediated signaling remains unknown. In the present study, the effect of methanol extract from the fruits of *Cudrania tricuspidata* (MFC) and its active compound, 5,7,3',4'-tetrahydroxy-6,8-diprenylisoflavone (THDPI), on FcεRI-mediated signaling in mast cells were investigated. MFC and THDPI suppressed mast cell degranulation and Ca²⁺ influx. MFC also interfered with IgE-FcεRI interaction and decreased FcεRIβ mRNA expression in mast cells. Furthermore, MFC and THDPI inhibited the phosphorylation of Syk, LAT, and PLCγ, and F-actin redistribution. These results indicate that MFC and its active compound, THDPI, inhibit mast cell activation through the inhibition of FcεRI-mediated Syk activation, suggesting a therapeutic potential for controlling mast cell activation in inflammatory and/or allergic processes.

KEYWORDS:

5,7,3',4'-Tetrahydroxy-6,8-diprenylisoflavone, *Cudrania tricuspidata*, FcεRI, Mast cell, Syk

INTRODUCTION

The binding of immunoglobulin E (IgE) to high affinity IgE receptor (FcεRI) on mast cells and basophils is central to the induction of allergic inflammatory responses.¹ These responses are mediated by cross-linkage of an antigen to IgE bound on FcεRI on the surface of mast cells and basophils. This binding of IgE to FcεRI cross-linked with an antigen causes the aggregation of the FcεRI receptor, which activates the Src family non-receptor tyrosine kinases such as Lyn and Fyn.² Activation of Lyn provokes phosphorylation of spleen tyrosine kinase (Syk) which induces a variety of cellular responses, including mobilization of Ca²⁺, F-actin polymerization, and activation of the signal transduction pathways, resulting in degranulation of mast cells and cytokine production that ultimately contribute to allergic inflammatory reactions.³ It has been demonstrated that Syk has a critical role in IgE-FcεRI-mediated mast cell responses using the overexpression of a mutant form of Syk constructs that abolished the release of inflammatory mediators.⁴ Therefore, the inhibition of Syk activation was considered as a therapeutic target in allergic inflammatory diseases.⁵

The rat basophilic leukemia mast cell line (RBL-2H3), a tumor analog of mast cells, shows phenotypic characteristics of mucosal mast cells and has been widely used as an *in vitro* model for IgE-FcεRI-mediated responses in mast cells.⁶ After stimulation with an allergen, cells release chemical mediators such as histamine and β-hexosaminidase which are considered to be parameters of mast cell degranulation.⁷ Mast cell degranulation results in an acute response, such as the contraction of smooth muscle, vasodilation, and increased vascular permeability.⁸

Cudrania tricuspidata (Moraceae) is a deciduous tree widely distributed in Asia. *Cudrania tricuspidata* have been used as a traditional herbal remedies for anti-tumor, gastritis, and anti-inflammation.⁹ Recent studies have reported that prenylated flavonoids and xanthones isolated from the root bark of *C. tricuspidata* have anti-atherosclerotic, anti-inflammatory,¹⁰ and hepatoprotective activities.¹¹ The fruits of *C. tricuspidata* have been used as traditional fermented jams, juices, and alcoholic beverages with sugar in Korea. The fruits of *C. tricuspidata* have also been reported to contain prenylated isoflavonoids and benzylated flavonoids with anti-inflammatory and monoamine oxidase inhibitory activities.^{12, 13} Recent studies have reported that fruits of *C. tricuspidata* show protective effects on oxidative stress-induced neurotoxicity¹⁴ and antibacterial effects through affecting membrane permeability¹⁵ and inhibit the development of atopic dermatitis (AD)-like skin lesions induced by repeated application of house dust mite.¹⁶ In this study, the inhibitory effects of *C. tricuspidata* fruit extract and its active compound, 5,7,3',4'-tetrahydroxy-6,8-diprenylisoflavone (THDPI), were investigated on mast cell activation through the FcεRI-mediated signaling pathways.

MATERIALS AND METHODS

Chemicals and reagents

HycloneTM Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA, USA). Monoclonal anti-dinitrophenyl (DNP) IgE mouse antibody, 4-nitrophenyl N-acetyl-β-glucosaminide, DNP-human serum albumin

(DNP-HSA), and 2-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Purified mouse anti-rat FcεRIα-antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA). *Prime* RT-PCR premixTM was purchased from Genet Bio (Seoul, Korea), and PRO-PREPTM protein extraction solution and easy-BLUETM total RNA extraction kits were purchased from iNtRON Biotechnology (Kyunggi, Korea). Anti-phospho-Lyn antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-Lyn, anti-phospho-Syk, anti-Syk, anti-phospho-LAT, and anti-LAT antibody, fluorescent Ca²⁺ probe, Fura 2/AM, FITC-avidin, and FITC-conjugated F(ab')₂ goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA).

Preparation of methanol extracts from the fruits of *C. tricuspidata* (MFC)

The fruits of *Cudrania tricuspidata* (Moraceae) were collected from the Korea Forest Research Institute, Southern Forest Research Center (Jinju, Korea). A voucher specimen (accession number: KH1-5-090904) was deposited at the Department of Biosystems and Biotechnology, Korea University (Seoul, Korea). Methanol extract from the fruits of *C. tricuspidata* (MFC) was prepared as follows: The fresh fruits of *C. tricuspidata* (10.7 kg) were sliced and extracted three times with MeOH (10 L × 3) at room temperature. The solvent was evaporated *in vacuo* to obtain a total MeOH extract (630.95 g).¹⁷

Ultra performance liquid chromatography (UPLC) analysis of MFC

The MFC was analyzed using an Acquity UPLC system (Waters, Millford, MA, USA) with an Acquity UPLC BEH C18 column (1.7 μ m, 2.1 $\times$ 150 mm i.d.). The mobile phase consisted of solvent A (0.05% trifluoroacetic acid in water) and solvent B (acetonitrile). The starting eluent was 40% B at 0 min. The proportion of B was increased linearly to 100% from 0 to 13 min, held constant at 100% until 14 min, returned to the initial composition (40% B) at 14 min, and then held constant 1 min to re-equilibrate the column. The flow rate was 0.3 mL/min and the sample injection volume was 2 μ L. The column and sample managers were maintained at 35 and 15 $^{\circ}$ C. The UV detection wavelength was monitored at 280 nm.

Isolation and Identification of compounds from MFC

The isolation and identification of ten isoflavones, alpinumisoflavone, 4'-O-methylalpinumisoflavone, 5,7,4'-trihydroxy-6,8-diprenylisoflavone, 5,7,3',4'-tetrahydroxy-6,8-diprenylisoflavone, derrone, erysenegalensein E, isoerysenegalensein E, gancaonin A, warangalone, and osajin from MFC were described in our previous report.¹⁷

Cell cultures

Rat basophilic leukemia (RBL-2H3) cells (ATCC No. CRL-2256) were cultured at 37 $^{\circ}$ C, 5% CO₂ in MEM supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin.

Measurement of the release of β -hexosaminidase

137

138 Mast cell degranulation was determined by β -hexosaminidase release based on the
139 previously described method.¹⁸ Briefly, RBL-2H3 cells (1×10^5 cells/100 μ L/well) were
140 cultured in 96-well plates and incubated with monoclonal mouse anti-DNP IgE antibody
141 (anti-DNP IgE) (1 μ g/mL) for 24 h at 37°C. The IgE-sensitized RBL-2H3 cells were washed
142 twice with extracellular Hepes buffer (5 mM KCl, 125 mM NaCl, 20 mM Hepes, 1.5 mM
143 $MgCl_2$, 1.5 mM $CaCl_2$). After washing, the cells were treated for 1 h with different
144 concentrations of MFC and isolated compounds in 50 μ L of Hepes buffer containing BSA
145 (1%) and glucose (1%) at 37°C and subsequently stimulated with DNP-HSA (500 ng/mL), a
146 multivalent antigen for an additional 1 h. Supernatants (40 μ L) were transferred to a 96-well
147 plate and incubated with 50 μ L of substrate solution (5 mM of 4-nitrophenyl N-acetyl- β -D-
148 glucosaminide in 50 mM of citric acid buffer, pH 4.5). for 1.5 h at 37°C. The reactions were
149 stopped by the addition of 50 μ L of stop solution (0.5 M $Na_2CO_3/NaHCO_3$, pH 10.0) and the
150 absorbance at 405 nm was measured using a microplate reader (SpectraMax[®] M5, Molecular
151 Devices, Sunnyvale, CA, USA). The activity value measured from cells treated with
152 IgE/DNP-HSA was considered to represent 100% degranulation.

153

154 **Measurement of intracellular Ca^{2+}**

155

156 RBL-2H3 cells (1×10^5 cells/100 μ L/well) were cultured in black 96-well plates and
157 incubated with anti-DNP IgE (1 μ g/mL) for 24 h at 37°C. Cells were washed with PBS buffer
158 and incubated with Fura 2/AM (20 μ M) in 100 μ L of Tyrode buffer (5 mM KCl, 130 mM
159 NaCl, 10 mM Hepes, 0.6 mM $MgCl_2$, 1 mM $CaCl_2$, 1% BSA, 1% glucose) for 1 h. The cells

were washed three times with PBS buffer and treated with different concentrations of MFC and an isolated compound in 100 μ L of Tyrode buffer for 15 min and subsequently stimulated with DNP-HSA (500 ng/mL). The fluorescence intensity was recorded for 20 min using a microplate reader (SpectraMax[®] M5, Molecular Devices, Sunnyvale, CA, USA) at an emission of 512 nm and excitation of 340 and 380 nm. The fluorescence ratio (F_{340}/F_{380}) was used as an indication of a rise in the intracellular concentration of Ca^{2+} .¹⁹

Measurement of biotinylated-IgE binding to Fc ϵ RI

IgE biotinylation (biotinylated-IgE) was carried out according to previously reported methods.²⁰ Briefly, A DNP-IgE (0.5 mg) was dissolved in 500 μ L of PBS, and incubated with 50 μ L of 10 mM of succinimidyl-6-(biotinamido) hexanoate (Thermo Fisher Scientific, Waltham, MA, USA) dissolved in DMSO. After 1 h of incubation at 4°C, 1 mL of Tris (50 mM, pH 8) was added to deactivate residual reactive groups. The biotinylated-IgE were dialyzed with PBS at 4°C, recovered, and stored frozen until use. For binding experiments, RBL-2H3 cells (1×10^6 cells/1 mL/well) were cultured for 24 h in 12-well plates and washed with PBS containing 0.2% bovine serum albumin. The cells were incubated with biotinylated-IgE (2 μ g/ml) and MFC and an isolated compound for 1 h at 37°C, detached by gentle scraping, centrifuged at 4,000 rpm for 5 min, suspended in PBS, and stained with 20 μ L of FITC-avidin at 4°C for 30 min. As a background control, untreated cells were stained with FITC-avidin alone. The cells were then washed twice with PBS and subjected to analysis of IgE binding to RBL-2H3 cells using a flow cytometer (FACSCalibur, BD

Biosciences, San Jose, CA, USA). Values of florescence intensity were obtained from histogram statistic of CellQuest software (BD Biosciences, San Jose, CA, USA).

Measurement of mRNA expression

RBL-2H3 cells (5×10^5 cells/500 μ L/well) were incubated for 24 h, treated with MFC and an isolated compound for 3, 6, 18, 24 h (time-dependent), and treated with various concentrations of MFC and an isolated compound for 24 h (dose-dependent). The cells were washed twice with PBS, and total RNA was isolated with easy-BLUETM (iNtRON Biotechnology, Korea), according to the manufacturer's instruction. cDNA synthesis was performed from 500 ng of total RNA by using ONE-STEP RT-PCR PreMix kit (iNtRoN Biotechnology, Korea). The following primer pairs were synthesized. (Bioneer, Korea).

Fc ϵ RI α , 5'-CATTGTGAGTGCCACCATTC-3', 5'-TTCTTCCAGCTACGGCATCT-3';
Fc ϵ RI β , 5'-TTGTCTGCTCCACACTCCAG-3', 5'-AGGCTGCCTCTCACCAGATA-3';
Fc ϵ RI γ , 5'-CACCTGTTCTCAGCTGCATT-3', 5'-GGATCAGGGAAGGAAAGAGG-3';
GAPDH, 5'-AGACAGCCGCATCTTCTTGT-3', 5'-CTTGCCGTGGGTAGAGTCAT-3'.

The denaturation, annealing, extension, and cycle conditions were performed as follows: 94°C for 45 s, 62°C for 45 s, 72°C for 45 s, and 26 cycles. The PCR reaction was performed with a 2720 Thermal Cycler (Applied Biosystems, USA). Aliquots of the PCR reactions were electrophoresed in 1.5% (w/v) agarose gels, and stained with SYBR Safe DNA gel stain (Invitrogen, USA). DNA bands were visualized with LAS 1000plus (FUJIFILM, Japan).

Measurement of p-Lyn, p-Syk, p-LAT, and p-PLC γ proteins

205

206 RBL-2H3 cells (2×10^6 cells/2 mL/well) were cultured in 6-well plates with IgE for 24 h.
207 Cells were then treated with different concentrations of MFC and an isolated compound for
208 24 h and subsequently stimulated with DNP-HSA (500 ng/ml) for 10 min. Cells were lysed
209 by PRO-PREPTM protein extraction solution (iNtRON Biotechnology, Korea) for 20 min at -
210 20°C. After incubation, lysates were centrifuged at 13,000 rpm for 10 min at 4°C for
211 clarification. Approximately 15 µL (30 µg of total protein) of supernatant was subjected to 10%
212 SDS-PAGE and transferred to PVDF membrane (Immobilon[®]-P, Millipore, USA). The
213 membrane was incubated with primary antibodies at 4°C overnight. After washing, the
214 membranes were probed with secondary antibodies for 1 h and developed with ECL western
215 blotting detection reagent, WEST-ZOL[®] plus (iNtRON Biotechnology, Korea). Bands were
216 visualized using an image analyzer, LAS 1000plus (FUJIFILM, Japan).

217

218 **Redistribution of F-actin using confocal microscopy**

219

220 RBL-2H3 cells (4×10^4 cells) were cultured in cell culture slide (SPL Lifescience, Korea)
221 and sensitized with IgE (2 µg/mL) for 3 h. Before antigen stimulation, RBL-2H3 cells were
222 treated with different concentrations of MFC, an isolated compound, or piceatannol for 1 h,
223 and then stimulated with DNP-HSA (500 ng/mL) for 15 min. Cell fixation was performed
224 with PBS containing 4% formaldehyde for 30 min and permeabilized twice with PBS
225 containing 0.1% Triton X-100 for 5 min. Staining for F-actin (green) was performed with
226 Alexa Fluor[®] 488 phalloidin (InvitrogenTM, USA) for 20 min in the dark. DAPI (4',6-
227 diamidino-2-phenylindole) staining was performed to identify all cell nuclei (blue) using a

mounting medium containing DAPI (VECTASHIELD[®] Mounting Medium with DAPI, VECTOR LABORATORIES, USA) prior to analysis by confocal fluorescent microscopy using a LSM 700 (Carl Zeiss, Germany) and ZEN software for the capture of images (Carl Zeiss, Germany).

Statistical analysis

Data obtained were expressed as the mean $\pm$ standard deviation (SD). Statistical significance was determined using GraphPad Prism (GraphPad Software, USA). The differences among the groups were evaluated by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison method. P value of less than 0.05 was considered to be statistically significant. All of the data were obtained from at least three independent experiments.

RESULTS AND DISCUSSION

Mast cells are crucial factors in the regulation of inflammatory and allergic responses due to their ability to produce pro-inflammatory mediators. The major mechanism for the activation of mast cells is the interaction of immunoglobulin E (IgE) with its high affinity receptor (Fc ϵ RI), on the cell surface, which initiates Fc ϵ RI signaling through Lyn and Syk to regulate degranulation.²¹ In this study, we investigated the inhibitory effects of methanol extract from the fruits of *C. tricuspidata* (MFC) on Fc ϵ RI-mediated mast cell activation and identified THDPI as an active compound responsible for the inhibitory action. β -Hexosaminidase has been commonly considered as an useful indicator for the evaluation of the activation of mast

cells in various allergic inflammation.²² As shown in Figure 1A, MFC significantly inhibited the release of β -hexosaminidase in Fc ϵ RI-mediated mast cells with an IC₅₀ value of 104.9 μ g/mL. Major constituents of MFC were identified using UPLC and ten compounds were isolated from MFC (Figure 2A and 2B). Among ten isolated compounds from MFC, THDPI attenuated the release of β -hexosaminidase with an IC₅₀ value of 20.4 μ M, while the other compounds revealed no significant effects at concentrations up to 40 μ M (Figure 1B and Table 1).

To investigate the mechanism underlying the inhibitory effects of MFC and THDPI on mast cell degranulation, free cytoplasmic calcium levels were examined. The movement of calcium across the membrane into the cytosol, either from an intracellular store such as the endoplasmic reticulum or from the external environment, plays a crucial role in IgE-mediated mast cell activation and degranulation.²² Therefore, many anti-allergic drugs are known to inhibit calcium influx into the cytosol, resulting in the suppression of the degranulation of mast cells.²³ After the addition of antigen (DNP-HSA) in IgE-sensitized RBL-2H3 cells, the rapid elevation of intracellular calcium influx was observed in the untreated group. However, the treatment of MFC and THDPI decreased the antigen-induced intracellular calcium levels in a dose-dependent manner (Figure 3). These data suggests that MFC and THDPI elicit its inhibitory effects on mast cell degranulation at least in part by the inhibition of calcium influx into the cytosol.

Binding of IgE to its receptor, Fc ϵ RI, on the surface of mast cells is one of the essential steps to initiate mast cell activation and degranulation. It has been reported that omalizumab, a recombinant DNA-derived humanized IgG1k monoclonal antibody, interferes with the IgE-Fc ϵ RI binding and inhibit mast cell degranulation,²⁴ and it is currently used for the control of

allergic asthma and atopic dermatitis.²⁵ Thus, interfering with IgE binding to FcεRI could be another cause to suppress IgE-FcεRI-mediated mast cell activation. When RBL-2H3 cells were treated with MFC at a fixed concentration of IgE, IgE binding levels were decreased to 86.9%, 79.3%, and 64.7% when treated with MFC at 100, 150, and 200 μg/mL, respectively, compared with control groups treated with IgE alone (Figure 4A). However, the treatment of THDPI showed no significant effects at concentrations up to 40 μM in IgE-FcεRI interaction (Figure 4B).

FcεRI are multimeric immune receptors consisting of one α-subunit, one β-subunit, and two γ-subunits. The α-subunit binds to the Fc portion of IgE, and β- and γ-subunits are thought to account for the cell-activation properties of FcεRI.²⁶ In FcεRI knockout mice, binding of IgE to mast cells was completely blocked causing no induction of degranulation following allergic reactions.²⁷ Therefore, modulation for FcεRI expression could be considered as a therapeutic target for preventing FcεRI-mediated allergic inflammatory diseases. Recently, epigallocatechin gallate (EGCG) has been reported as a possible inhibitor for FcεRI expression by inhibiting mRNA expression of γ-subunit in mast cells.²⁸ To examine whether MFC and THDPI have an effect on FcεRI expression in mast cells, RBL-2H3 cells were treated with MFC and THDPI for 24 h. MFC inhibited the only FcεRI β-subunit mRNA expression significantly, however mRNA expression of the α- and γ-subunits were not affected (Figure 5A and 5C). In the group treated with THDPI, however, no inhibitory effect on FcεRI subunits was observed (Figure 5B and 5D).

Cross-linking of the FcεRI on mast cells activates non-receptor type protein tyrosine kinases such as Lyn and Syk.²⁹ Lyn can be constitutively associated with FcεRI and is responsible for the phosphorylation of β- and γ-subunits. These phosphorylation events result in the

association of Syk with phosphorylated γ -subunit, thereby promoting the activation of Syk.³⁰ It has been reported that Syk-deficient mast cells completely abrogated the degranulation, calcium elevation, and activation of the ERK and JNK.²⁹ LAT is known to be phosphorylated by Syk and is important for activation of PLC γ , which is essential for increasing the intracellular calcium level in the calcium-dependent pathway.³¹ In LAT-deficient bone marrow-derived mast cells (BMMCs), tyrosine phosphorylation of Fc ϵ RI and Syk was intact following Fc ϵ RI engagement, however, activation of PLC γ and calcium mobilization were dramatically reduced.³² As demonstrated in Figure 6A and 6B, both MFC and THDPI showed no effect on the phosphorylation of Lyn, however, they suppressed the phosphorylation of Syk as well as its downstream effectors, LAT and PLC γ in IgE-sensitized RBL-2H3 cells stimulated by antigens. A previous study has reported that piceatannol, a protein kinase inhibitor, reduced the antigen-induced secretion in IgE-sensitized mast cells through the inhibition of phosphorylation of Syk without the inhibition of phosphorylation of Lyn.³³

The Fc ϵ RI-mediated signaling in mast cells leads not only to degranulation and cytokine production, but also to actin assembly and various morphological changes in the cells. These morphological changes involve the redistribution of F-actin and are in parallel with degranulation of mast cells.³⁴ Several studies have demonstrated that these morphological changes are down-stream of Syk and are Ca²⁺ dependent.^{35, 36} It has been also reported that activation of Fc ϵ RI induces no or minimal morphological change in Syk^{-/-} cells, but this is reconstituted by stable expression of Syk in these cells.³⁵ In addition, IgE-mediated redistribution of F-actin is observed only in Syk^{-/-} cells transfected with wild-type Syk.³⁷ In Figure 7, these morphological changes were observed in RBL-2H3 cells stained with fluorescent phalloidin to visualize F-actin. In unstimulated cells, F-actin (green) was at

steady state and concentrated at the cell membrane (Figure 7A). When stimulated with antigen (DNP-HSA), F-actin was redistributed and cells were spread (Figure 7B). However, this redistribution of F-actin was markedly inhibited in MFC-treated cells (Figure 7C and 7D) and in THDPI-treated cells (Figure 7E and 7F). These inhibitory effects on F-actin redistribution were compared with a Syk inhibitor, piceatannol, (Figure 7G) which has been reported to inhibit actin assembly, membrane ruffling, spreading, and degranulation in mast cells.³⁸

In conclusion, MFC and its active compounds, THDPI, inhibited FcεRI-mediated degranulation and the calcium influx. MFC also interfered with IgE-FcεRI interaction and decreased FcεRIβ mRNA expression. THDPI, however, did not show any significant effects on IgE-FcεRI interaction and FcεRI mRNA expression. It is possible that other minor compounds in MFC may be responsible for the inhibitory effects on the affinity of IgE to the receptors and the expression of FcεRIβ mRNA. Further study is required to identify other compounds in MFC responsible for these inhibitory effects. MFC and THDPI inhibited F-actin redistribution and downstream signaling by suppressing FcεRI-mediated Syk activation in mast cells (Figure 8).

These findings indicate that MFC and THDPI may have a potential therapeutic benefit for the controlling IgE-FcεRI-mediated mast cell activation in inflammatory and/or allergic process.

ABBREVIATIONS

THDPI, 5,7,3',4'-tetrahydroxy-6,8-diprenylisoflavone; DNP, dinitrophenyl; FcεRI, high-affinity IgE receptor; HSA, human serum albumin; IgE, immunoglobulin E; LAT, linker of activated T cells; Syk, spleen tyrosine kinase; MFC, methanol extracts from the fruits of *Cudrania tricuspidata*; PLCγ, phospholipase Cγ; UPLC, ultra performance liquid chromatography.

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Figure captions

Figure 1. Effect of MFC and THDPI on the mast cell degranulation in RBL-2H3 cells. Anti-DNP IgE-sensitized cells were treated with various concentrations of MFC (A) and THDPI (B) for 1 h followed by an antigen stimulation (DNP-HSA) for an additional 1 h. The release of β -hexosaminidase from cells treated with IgE/DNP-HSA was considered to represent 100% degranulation. Data represent the mean $\pm$ SD of three independent experiments, $^{###}p < 0.01$, compared with IgE-sensitized cells without DNP-HSA; $^{**}p < 0.01$, compared with IgE/DNP-HSA-treated cells. THDPI: 5,7,3',4'-tetrahydroxy-6,8-diprenylisoflavone.

Figure 2. Structures of principal compounds and UPLC chromatogram of MFC. (A) Chemical structures of principal compounds (B) UPLC chromatogram of MFC. The principal compounds are (1) derrone, (2) alpinumisoflavone, (3) erysenegalensein E, (4) 5,7,3',4'-tetrahydroxy-6,8-diprenylisoflavone, (5) isoerysenegalensein E, (6) gancaonin A, (7) 5,7,4'-trihydroxy-6,8-diprenylisoflavone, (8) 4'-O-methylalpinumisoflavone, (9) warangalone, and (10) osajin.

Figure 3. Effect of MFC and THDPI on Ca^{2+} influx in RBL-2H3 cells. Anti-DNP IgE-sensitized cells were incubated with Fura 2/AM for 1 h and thereafter treated with MFC (A) and THDPI (B) for 15 min and subsequently stimulated with an antigen (DNP-HSA, 500 ng/mL). Fluorescence intensity was measured for 20 min. (excitation; 340 and 380 nm, emission; 512 nm). Values were expressed as a ratio of the fluorescence at the two excitation wavelengths (F_{340}/F_{380}). The arrow represents the time at which antigen was added.

499

500 **Figure 4.** Effect of MFC and THDPI on IgE-FcεRI binding. RBL-2H3 cells were treated with
501 biotinylated-IgE (2 μg/mL) and various concentrations of MFC (A) and THDPI (B) for 1 h.
502 The cells were then stained with FITC-avidin for 30 min. IgE bound to FcεRI on RBL-2H3
503 cells was analyzed employing flow cytometry. Quantitative comparison of IgE-FcεRI binding
504 between IgE-treated cells and sample-treated cells. Values of fluorescence intensity were
505 obtained from histogram statistic of CellQuest software. Data represent the mean ± SD of
506 three independent experiments, ** $p < 0.01$, compared with the IgE-treated cells.

507

508 **Figure 5.** Effect of MFC and THDPI on FcεRI mRNA expression in RBL-2H3 cells. RBL-
509 2H3 cells were treated with MFC and THDPI for different time intervals (0, 3, 6, 18, and 24 h)
510 (A and B). RBL-2H3 cells were treated with various concentrations of MFC and THDPI for
511 24 h (C and D). The mRNA expression levels of FcεRI-subunit genes, α, β, and γ, were
512 determined by RT-PCR analysis. Quantitative representation of the expression of each
513 subunit of FcεRI and GAPDH mRNA using Image J software. Data represent the mean ± SD
514 of three independent experiments, * $p < 0.05$, ** $p < 0.01$, compared with the non-MFC-treated
515 cells.

516

517 **Figure 6.** Effect of MFC and THDPI on the FcεRI-mediated phosphorylation of Lyn, Syk,
518 LAT, and PLCγ in RBL-2H3 cells. Anti-DNP IgE-sensitized RBL-2H3 cells were cultured
519 with various concentrations of MFC (A) and THDPI (B) for 24 h followed by antigen
520 stimulation for 10 min. Then, each cell lysate was subjected to immunoblot analysis. The p-
521 Lyn, p-Syk, p-LAT, and p-PLCγ represent the phosphorylated Lyn, Syk, LAT, and PLCγ,

respectively. Data represent the mean $\pm$ SD of three independent experiments, ^{###} $p < 0.01$, compared with IgE-sensitized cells without DNP-HSA; ^{**} $p < 0.01$, compared with IgE/DNP-HSA-treated cells.

Figure 7. Effect of MFC and THDPI on F-actin assembly in RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were treated with MFC, THDPI, and piceatannol for 1h followed by antigen stimulation (DNP-HSA, 500 ng/ml) for 15 min. Cells were fixed and then double stained with fluorescent phalloidin for F-actin (green) and DAPI for cell nuclei (blue). Panels show representative images from (A) unstimulated cells, (B) cells stimulated with antigen, (C and D) MFC-treated cells stimulated with antigen, (E and F) THDPI-treated cells stimulated with antigen, (G) piceatannol-treated cells stimulated with antigen. Original magnification, x400.

Figure 8. A scheme of the inhibitory effects of MFC and THDPI on FcεRI-mediated signaling in RBL-2H3 cells. MFC and THDPI inhibited mast cell degranulation, calcium influx, and F-actin redistribution. The phosphorylation of Syk, LAT, and PLCγ was inhibited by MFC and THDPI. In addition, MFC inhibited IgE-FcεRI interaction and FcεRIβ subunit mRNA expression.

Table 1. Inhibitory effects of isolated compounds from MFC on the release of β -hexosaminidase from mast cells

Compound	β -hexosaminidase release (IC ₅₀ , μ M)
(1) Derrone	>40
(2) Alpinumisoflavone	>40
(3) Erysenegalensein E	>40
(4) 5,7,3',4'-tetrahydroxy-6,8-diprenylisoflavone	20.4
(5) Isoerysenegalensein E	>40
(6) Gancaonin A	>40
(7) 5,7,4'-trihydroxy-6,8-diprenylisoflavone	>40
(8) 4'-O-methylalpinumisoflavone	>40
(9) Warangalone	>40
(10) Osajin	>40
Ketotifen (positive control)	23.1

The IC₅₀ values were determined in a semilogarithmic graph with 4 different concentrations of compounds. At least 3 independent experiments were performed. Ketotifen, a mast cell stabilizer, was used as a positive control compound.

Figure 1

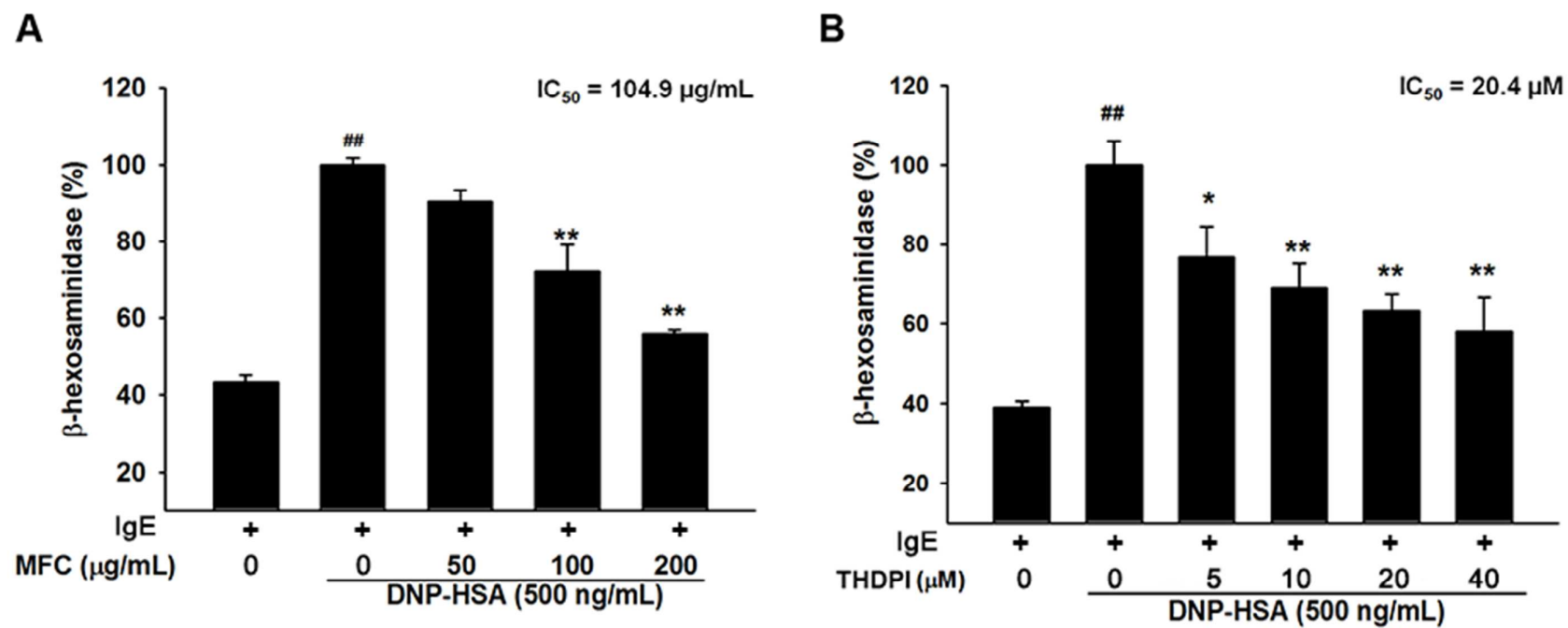


Figure 2

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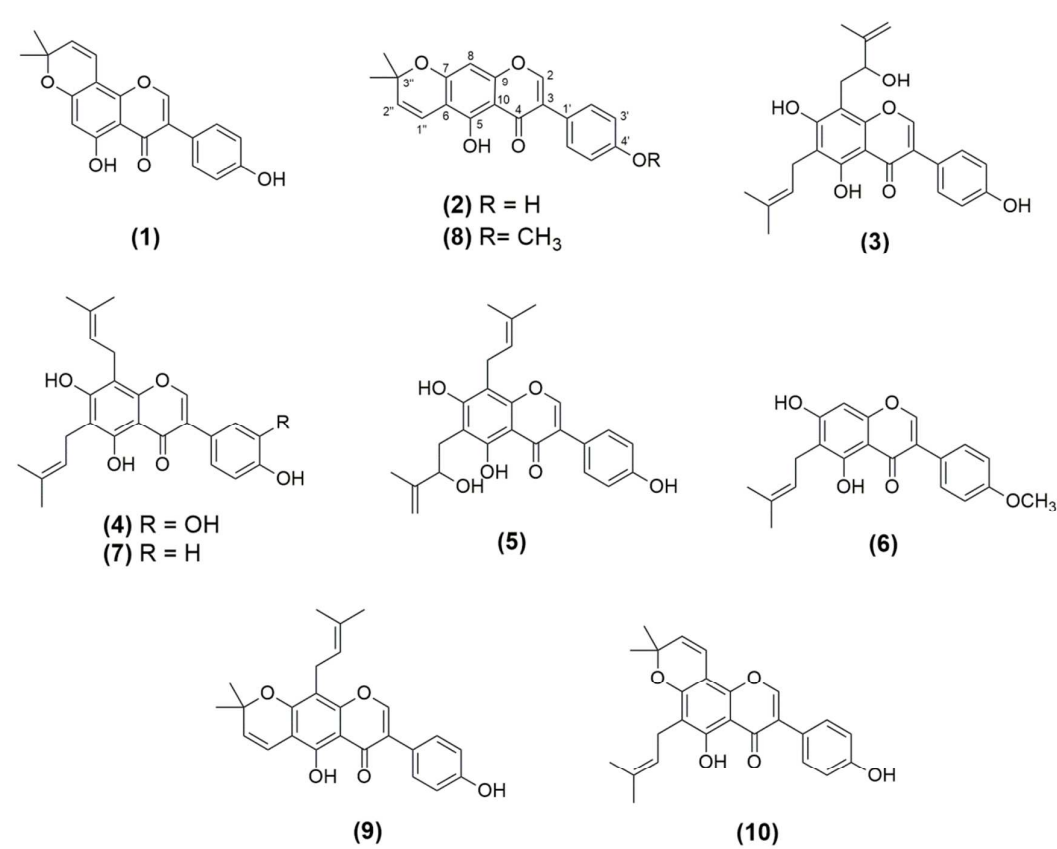


Figure 2

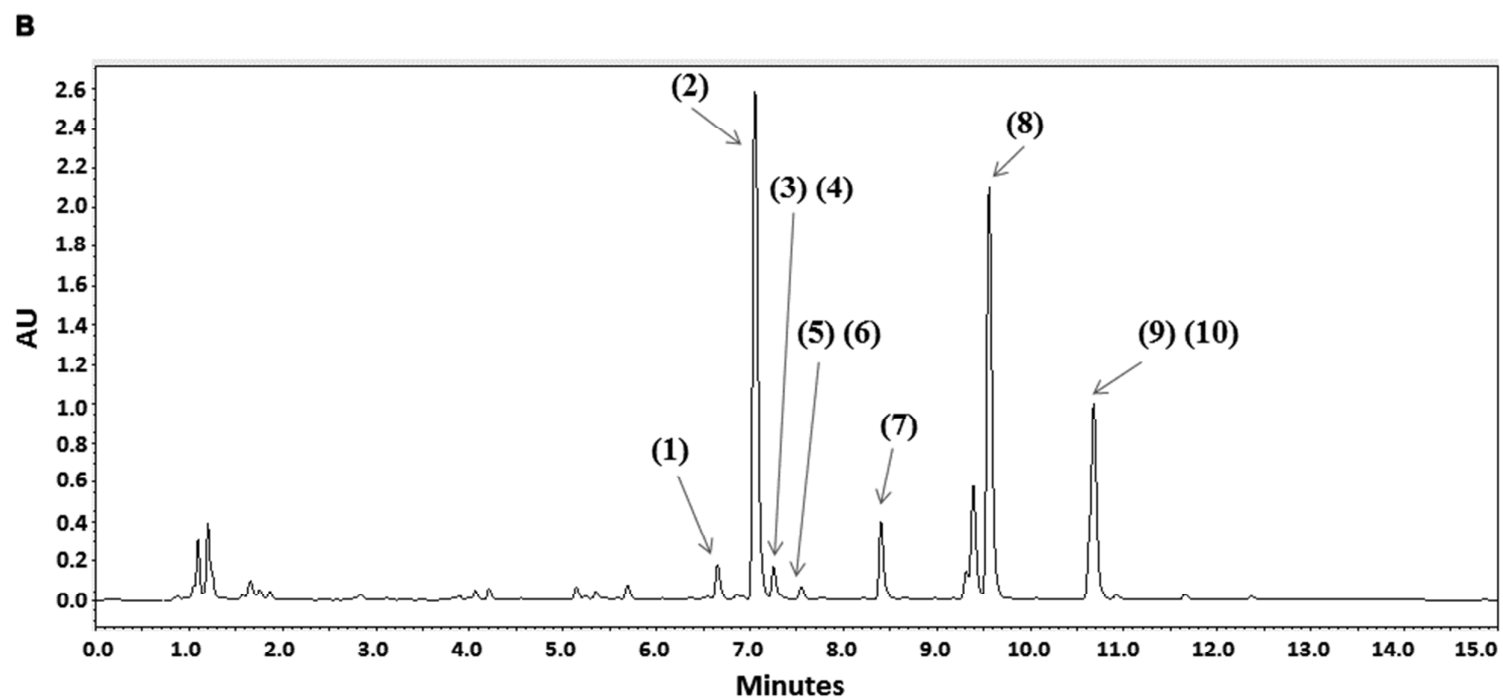


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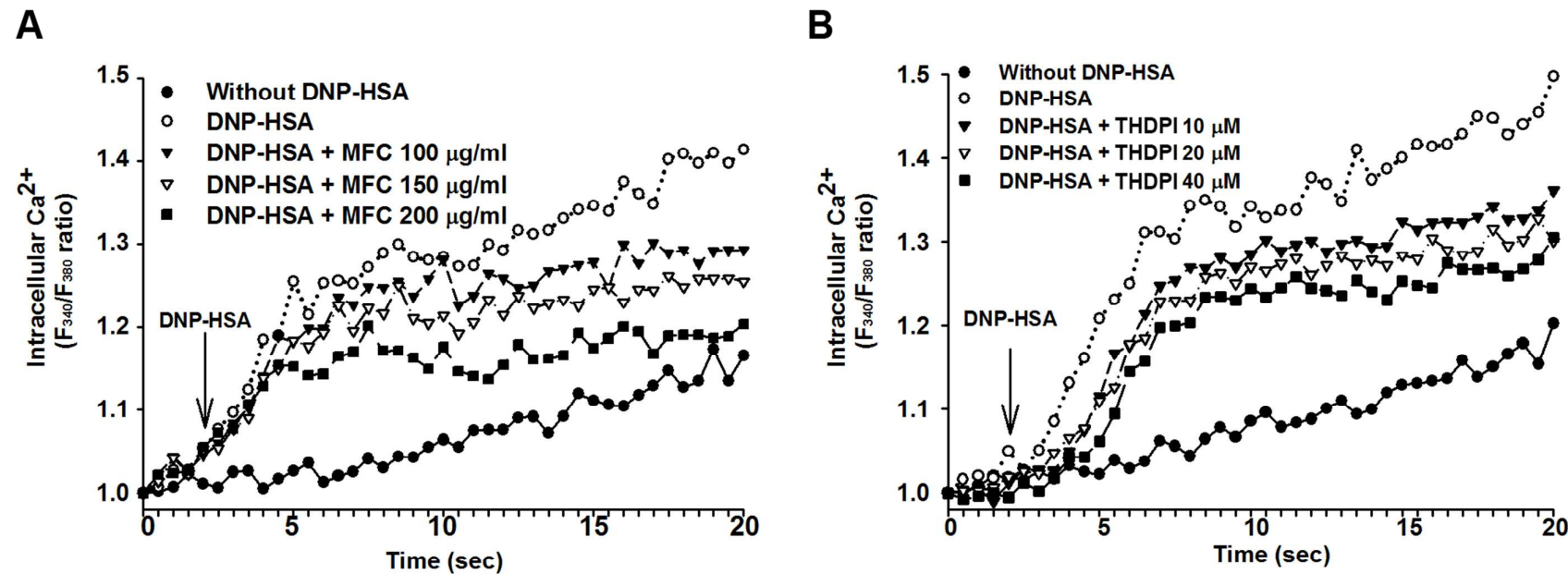


Figure 4

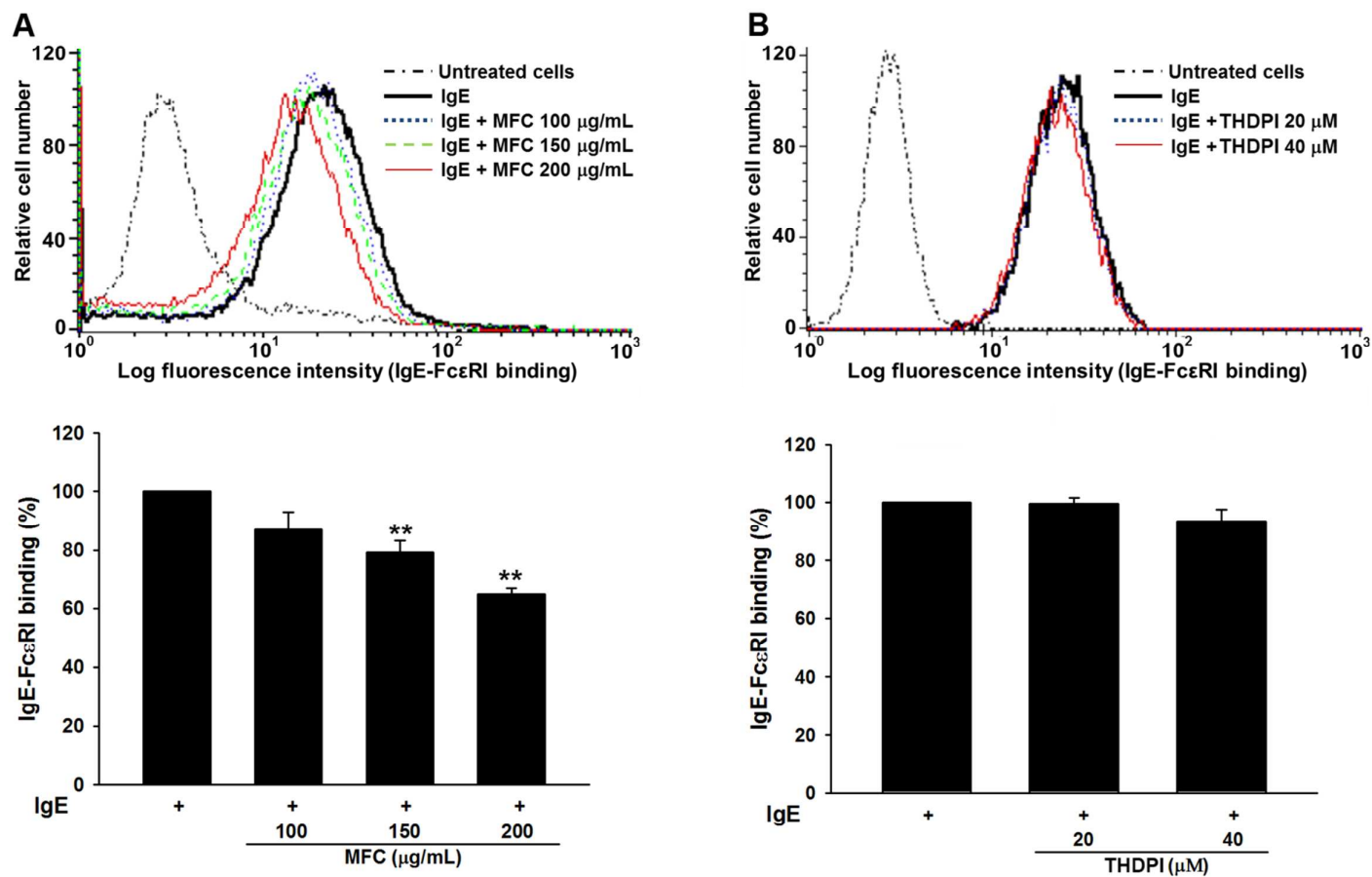


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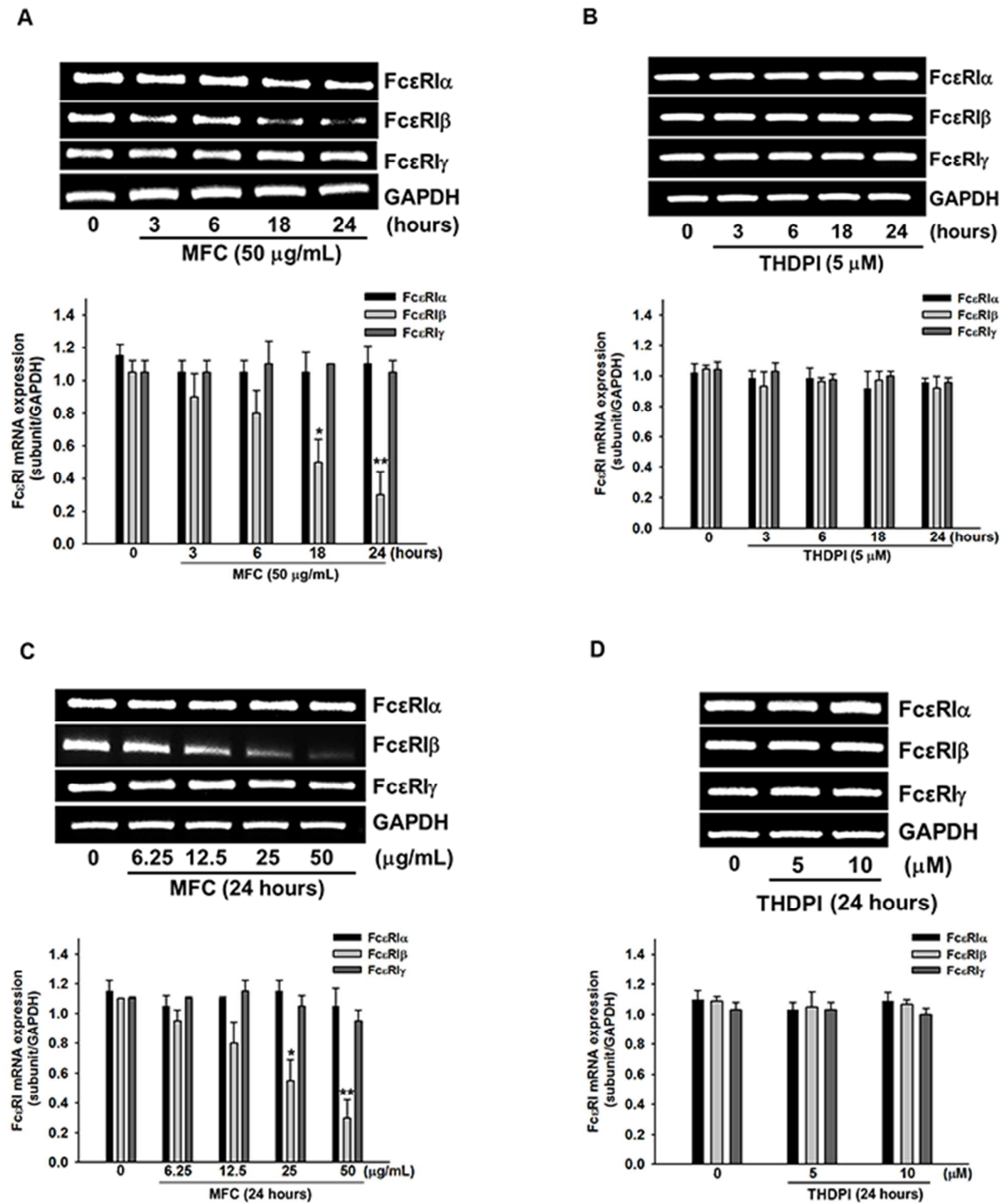


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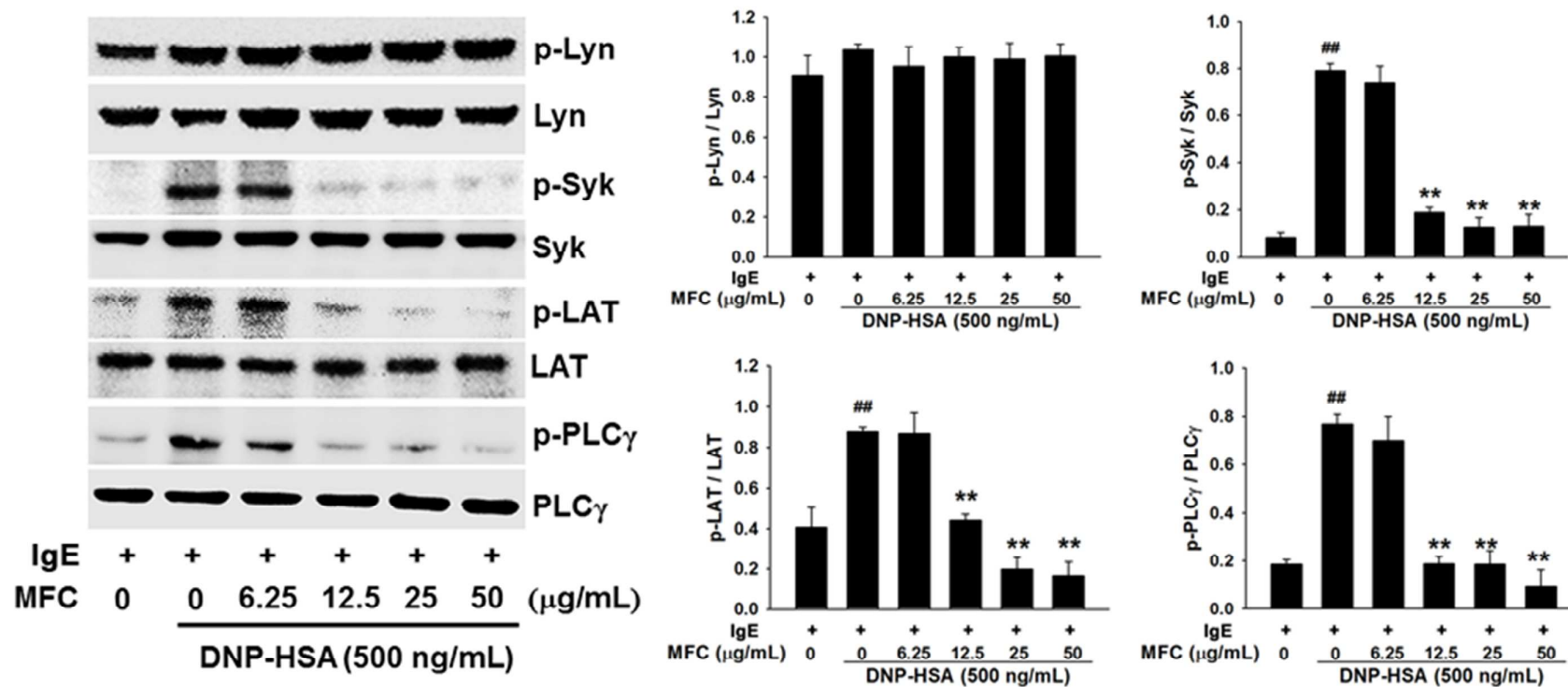
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Figure 6

B

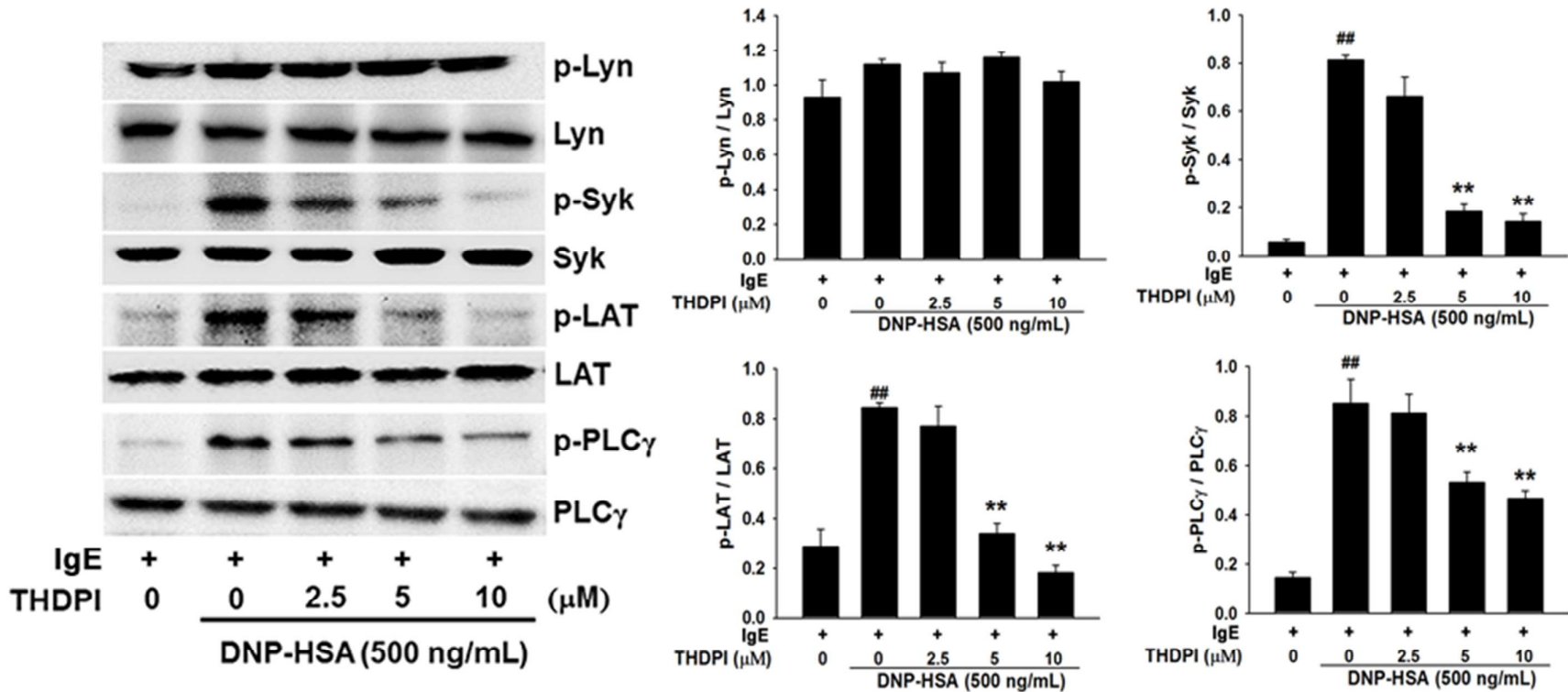


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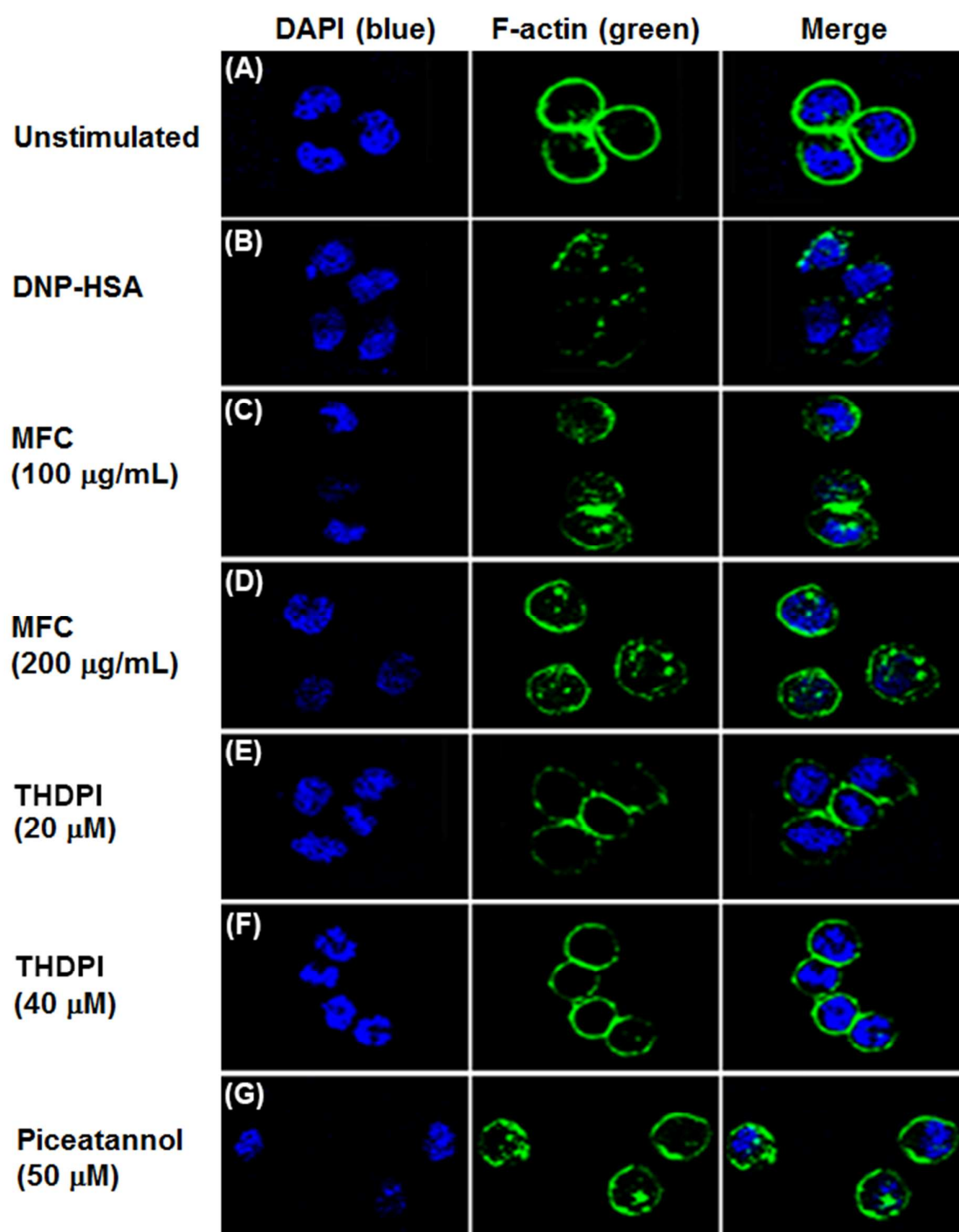
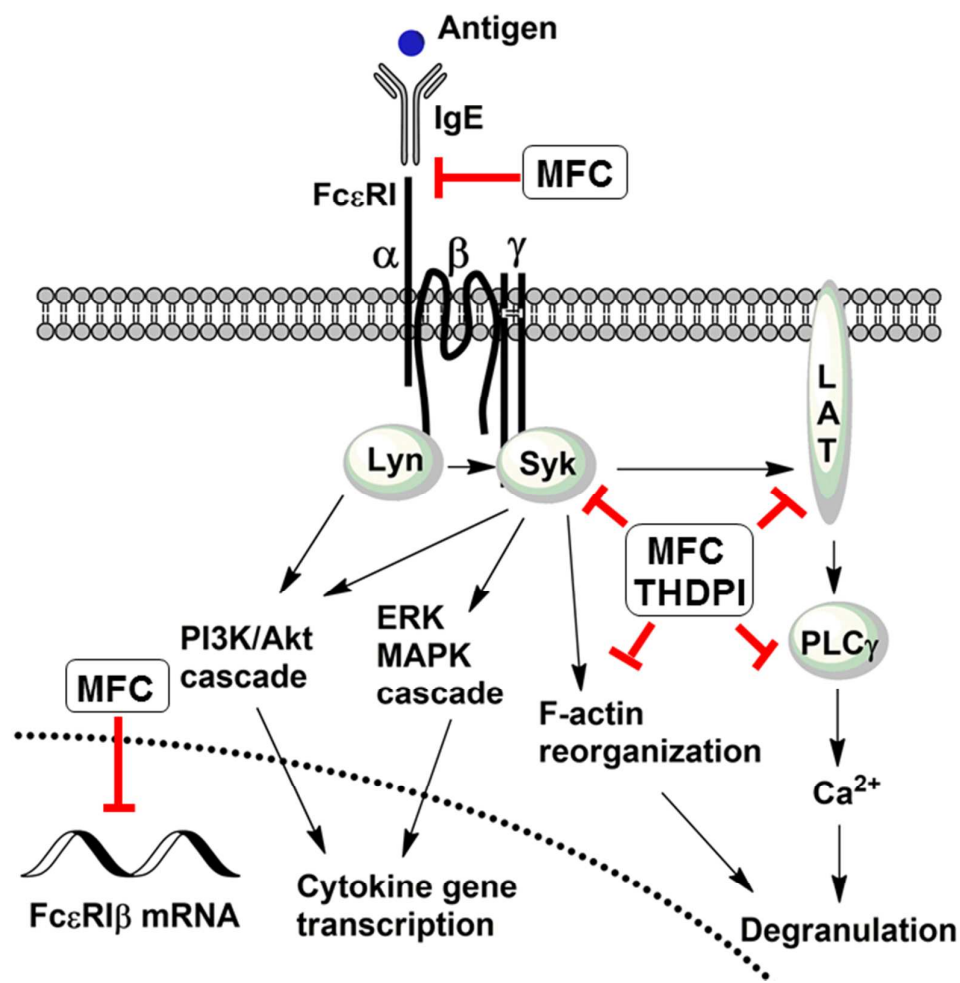
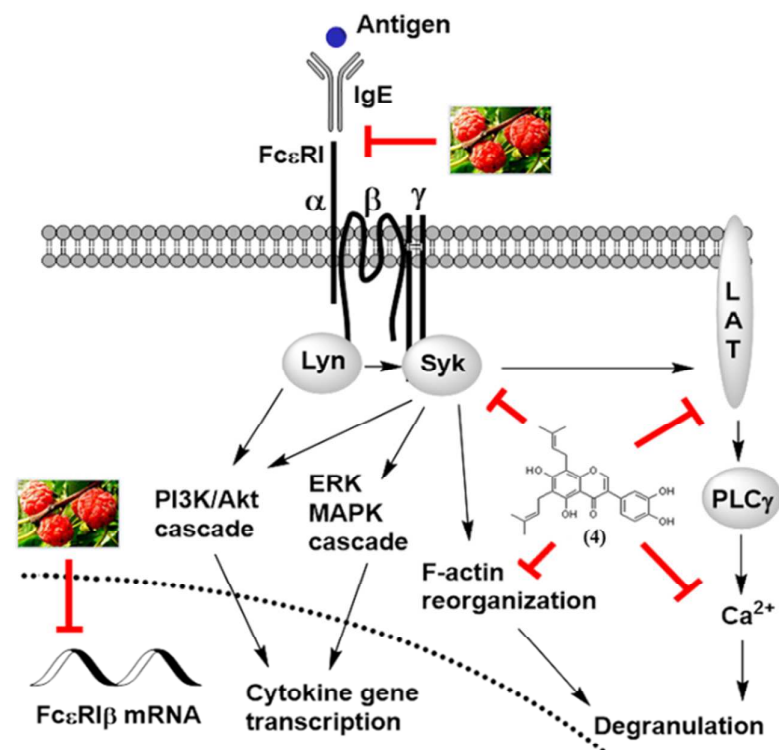
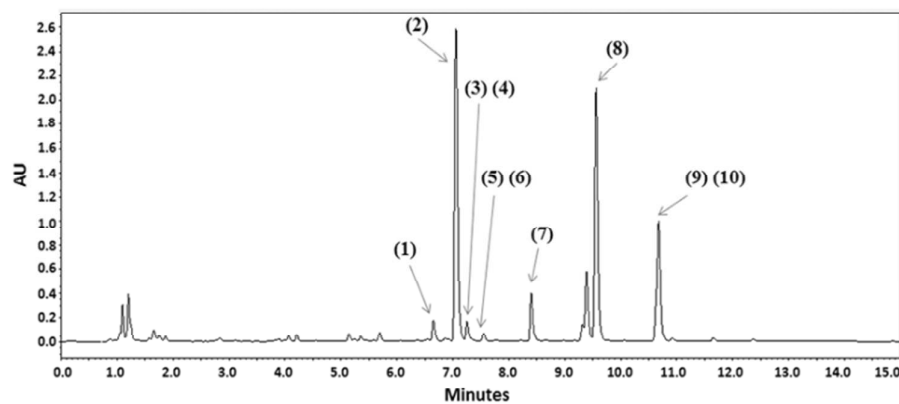
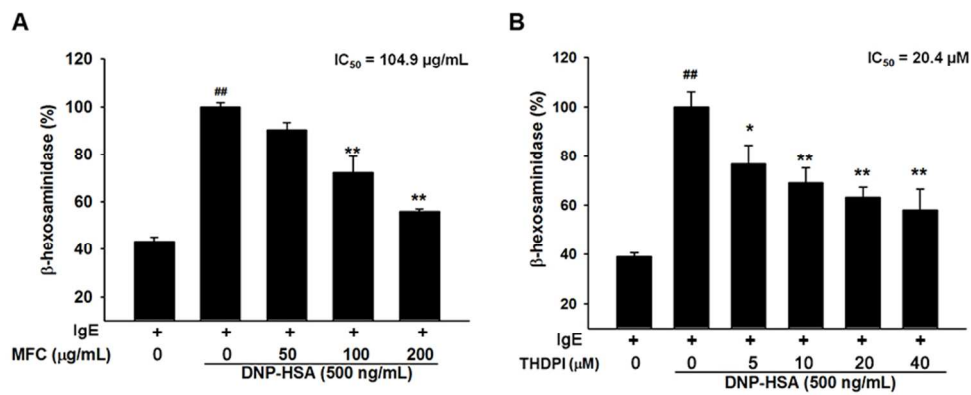


Figure 8

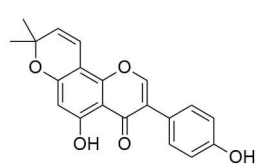
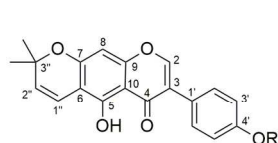
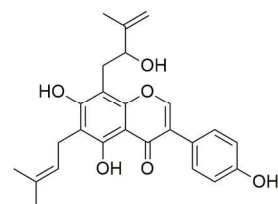
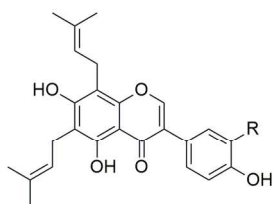
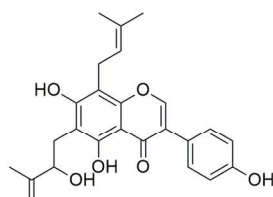
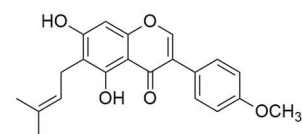
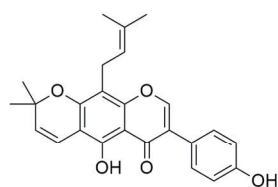
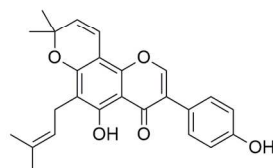


Graphic for table of contents

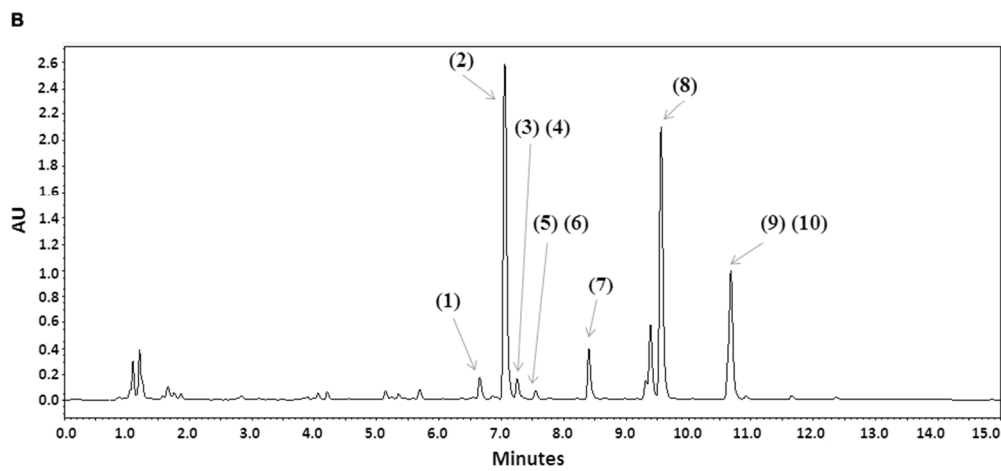
Fruits of *Cudrania tricuspidata*



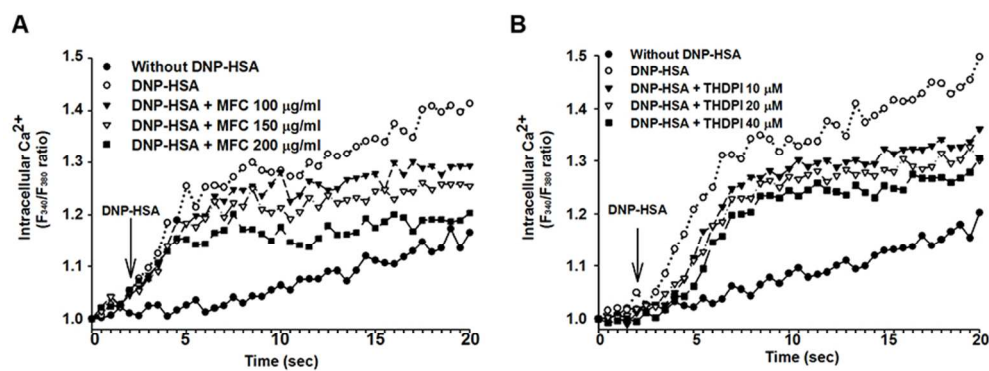
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(8) R = CH₃**(3)****(4)** R = OH
(7) R = H**(5)****(6)****(9)****(10)**

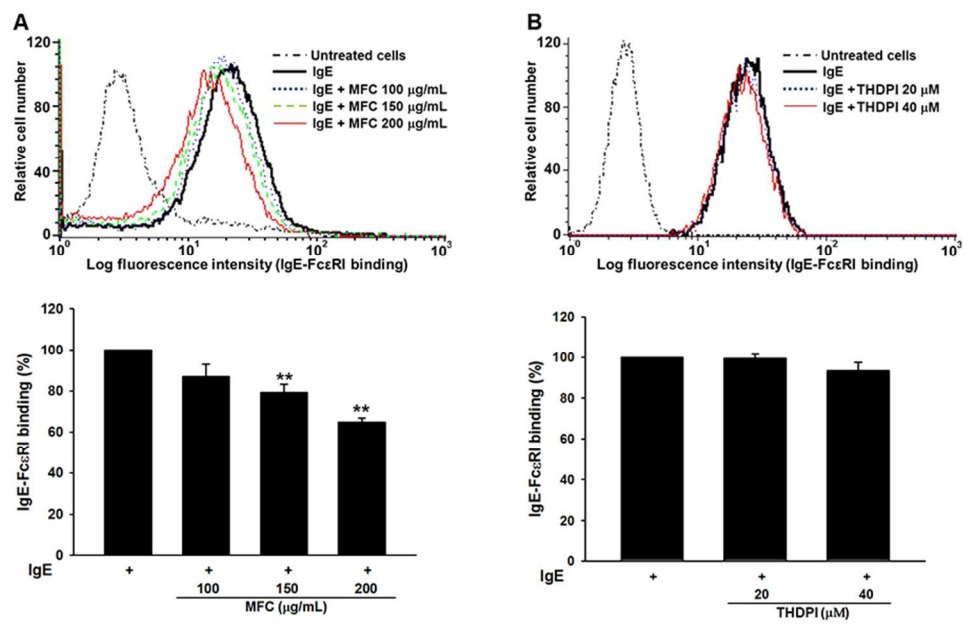
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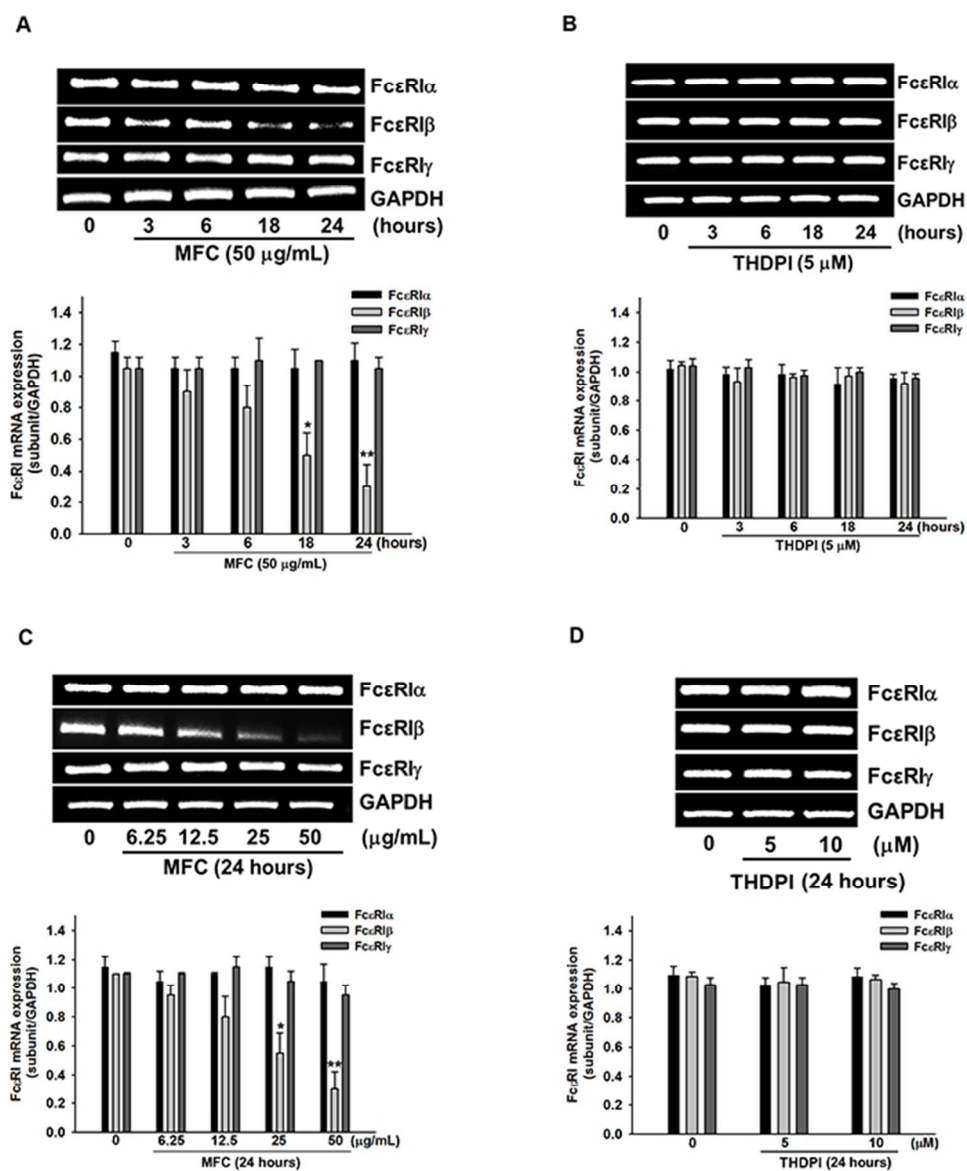
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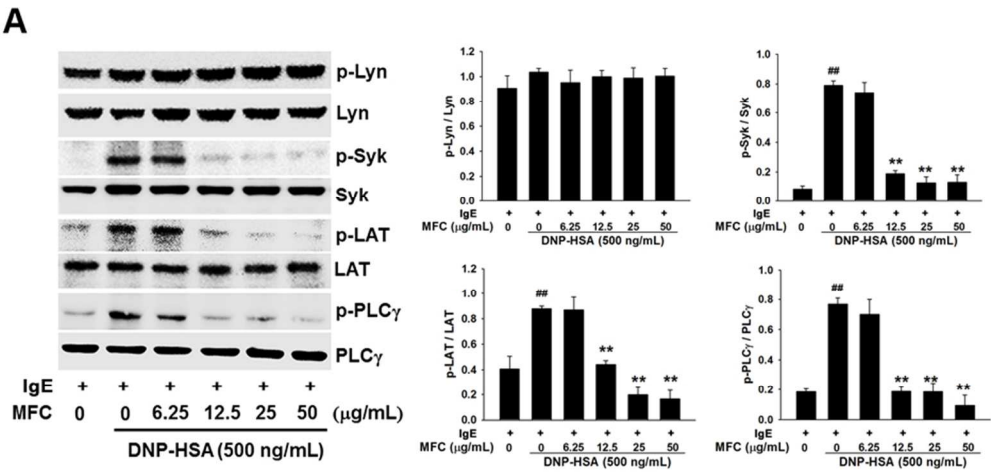
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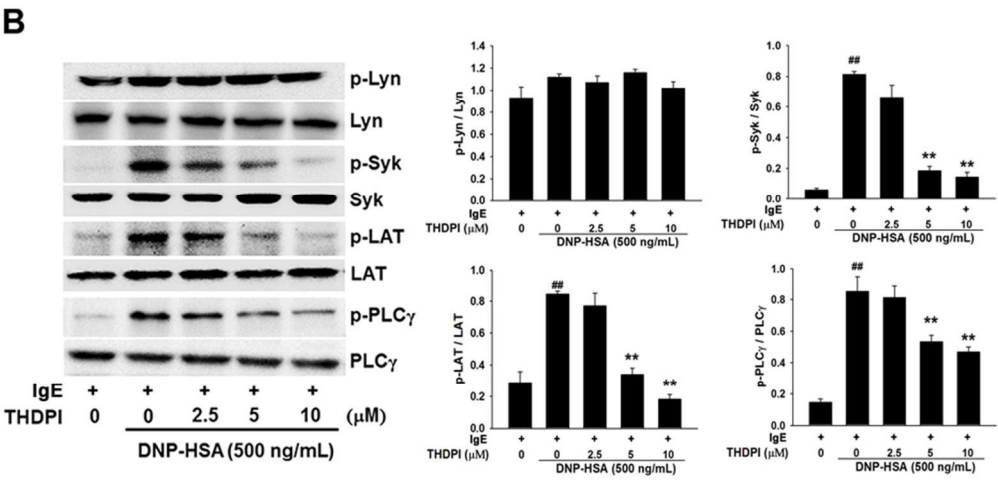
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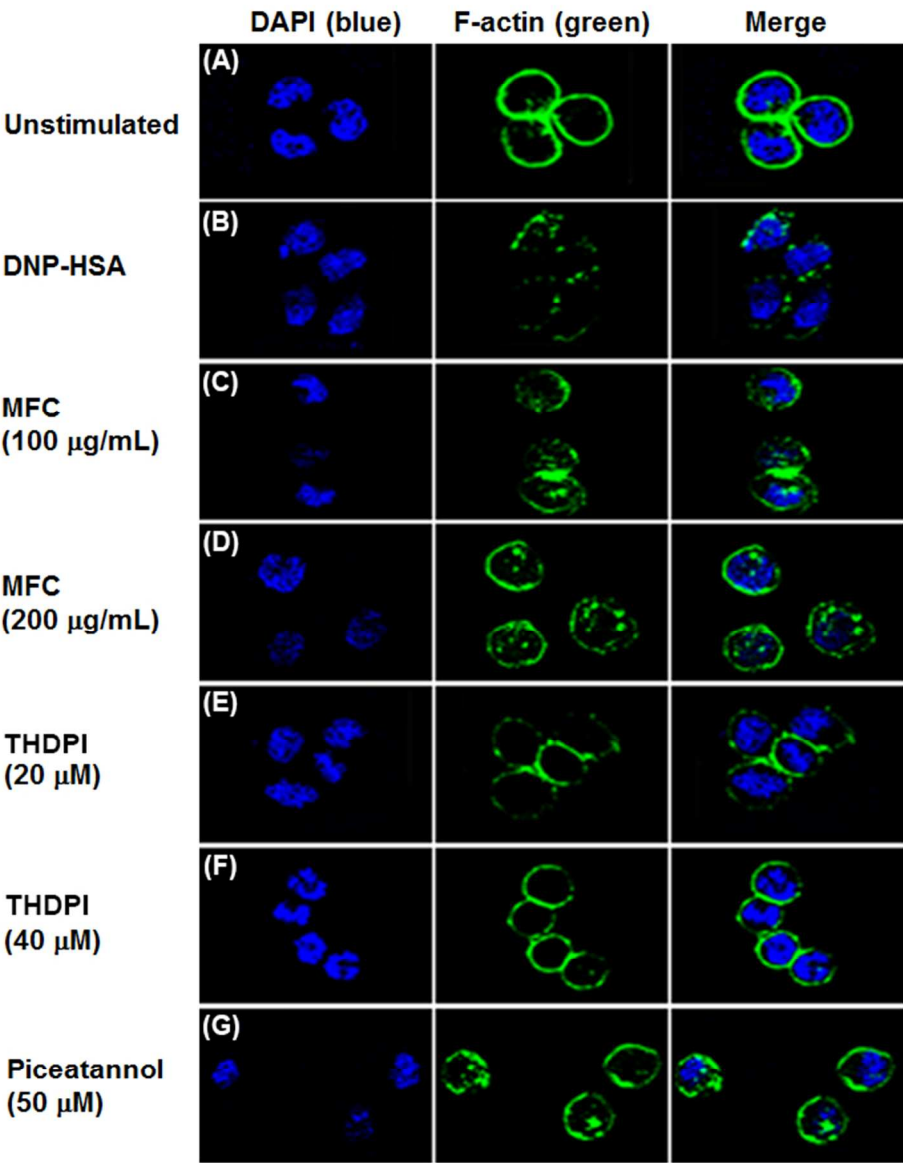
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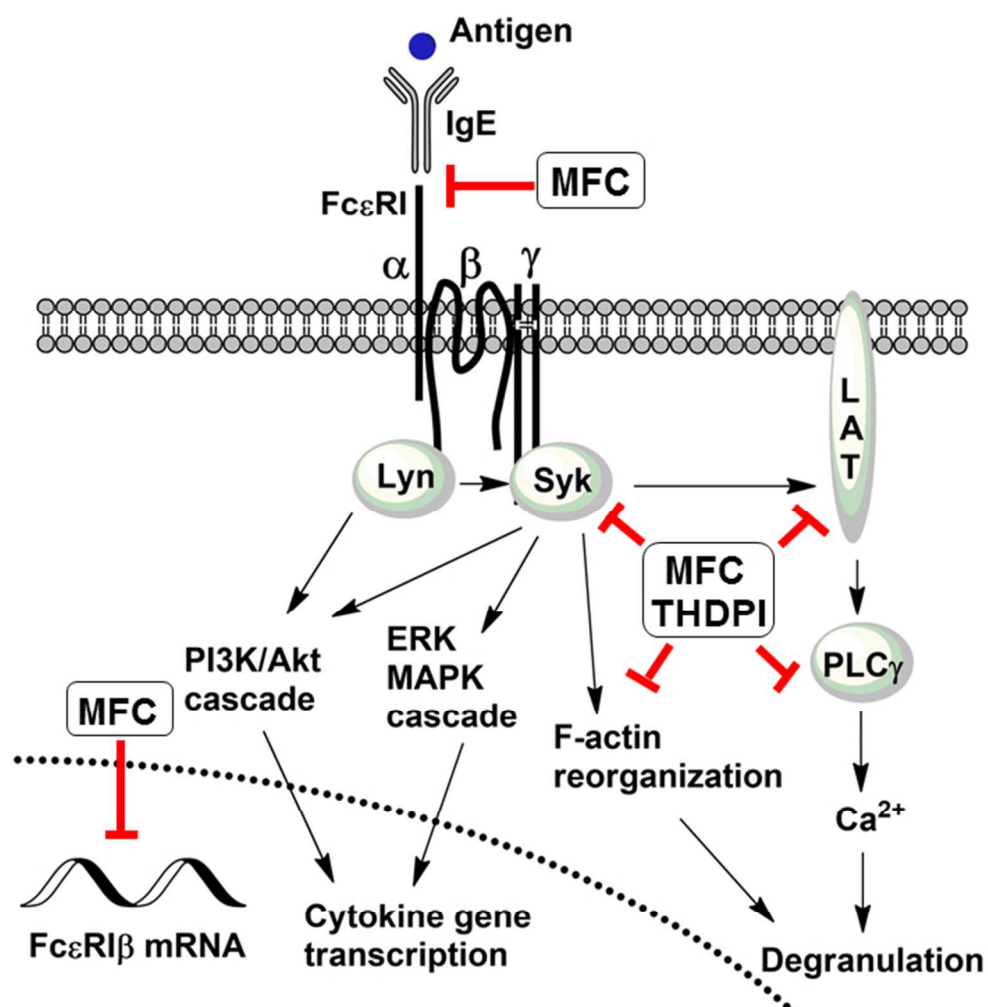
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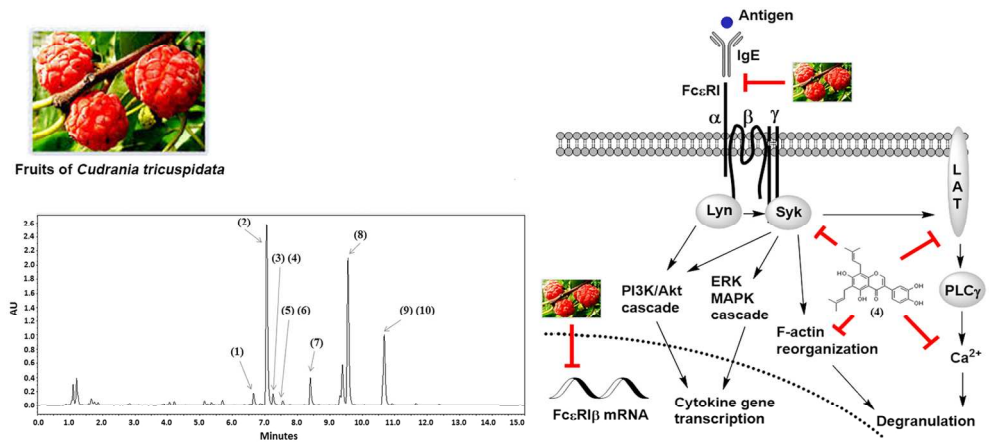
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