Mast Cell-Mediated Allergic Response Is Suppressed by *Sophorae Flos*: Inhibition of Src-Family Kinase

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Complementary and alternative medicines are considered as a promising direction for the development of anti-allergic therapies in oriental countries. We screened approximately 100 oriental herbal medicines for anti-allergic activity. *Sophorae flos* exhibited the most potent effect on degranulation in antigen-stimulated mast cells. We further investigated the effect of *Sophorae flos* on the IgE-mediated allergic response *in vivo* and its mechanism of action in mast cells. *Sophorae flos* exhibited a significant inhibitory effect on degranulation in antigen-stimulated mast cells with IC_{50} values of ~31.6 μg/mL (RBL-2H3 mast cells) and ~47.8 μg/mL (bone marrow-derived mast cells). *Sophorae flos* also suppressed the expression and secretion of TNF-α and IL-4 in the cells and IgE-mediated passive cutaneous anaphylaxis (PCA) in mice. *Sophorae flos* inhibited the activating phosphorylation of Syk and LAT in mast cells. Further downstream, activating phosphorylation of Akt and the prototypic MAP kinases, namely, p38, ERK1/2, and JNK, were also inhibited. These results suggest that *Sophorae flos* inhibits the Src family kinase-dependent signaling cascades in mast cells and may thus exert anti-allergic activity. Exp Biol Med 233:1271–1279, 2008

Key words: *Sophorae flos*; anti-allergic activity; mast cells; src-family kinase; syk kinase

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Introduction

It is becoming increasingly apparent that mast cells are the pivotal effector cells in the early events associated with several allergic inflammatory diseases, such as allergic rhinitis, mast cell-mediated asthma, atopic dermatitis, and atopic eczema (1–4). The stimulation of mast cells through the cross-linking of FcεRI with the antigen initially activates essential Src family kinases, such as Lyn and Fyn, for subsequent activation of mast cells, resulting in the phosphorylation of the two tyrosine residues in this immunoreceptor tyrosine-based activation motif (ITAM) consensus sequence of the receptor. This phosphorylation resulted in a *trans*-phosphorylation reaction mediated by Lyn or another Src family kinase that associates with the receptor (5). The tyrosine-phosphorylated ITAMs then function as scaffolds for the binding of additional signaling molecules, such as adapters and enzymes. The binding of Syk predominantly to the tyrosine phosphorylated ITAM of FcεRIγ results in its conformational change and an increase in its enzymatic activity (6). This leads to the downstream propagation of signals such as tyrosine phosphorylation of LAT, phospholipase Cγ, and Ca^{2+} influx. Subsequently, mast cells rapidly release various allergic mediators, including histamine, proteases, cytokines, and arachidonic derivatives, and then various acute and chronic allergic reactions are induced by these mediators (7, 8). Many therapies, such as treatment with antagonists to leukotriene and histamine receptors, allergen-specific immunotherapy, and humanized anti-IgE antibody administration, have been clinically tried to cure the allergic diseases. However, unwanted side effects and certain difficulties are associated with these therapeutic modalities (9, 10). Therefore, many other approaches have been explored to develop new remedies, and oriental herbal medicines have long been

Table 1. Effects of Oriental Herbal Medicines on the Antigen-Induced Degranulation in Mast Cells

Name of herbal medicine	Plant name	Number of voucher specimen	Percent inhibition of degranulation[a]
Acanthopanacis cortex	*Acanthopanax sessiliflorus*	CA03–021	0
Acanthopanacis cortex	*Acanthopanax senticosus*	CA03–001	15
Achuranthis radix	*Achyranthes japonica*	CA03–028	21.9
Aconiti jaluencis tuber	*Aconitum jaluense*	CA04–059	16.6
Adenophorae radix	*Adenophora triphylla var. japonica*	CA03–014	0
Ailanthi radicis cortex	*Ailanthus altissima*	CA03–044	0
Alismatis rhizoma	*Alisma canaliculatum*	CA04–063	1.4
Alpiniae semen	*Alpinia oxyphylla*	CA03–040	14.6
Amomi cardamomi fructus	*Amomum cardamomum*	CA03–011	0
Anemarrhenae rhizoma	*Anemarrhena asphodeloides*	CA03–062	10.4
Arctii semen	*Arctium lappa*	CA03–027	0
Artemisiae apiaceae herba	*Artemisia annua*	CA03–074	0
Asteris radix	*Aster tataricus*	CA03–042	2.8
Aurantii fructus	*Citrus aurantium*	CA03–058	2.9
Aurantii immatri pericarpium	*Citrus unshiu*	CA04–056	73.1
Bambusae caulis in taeniam	*Phyllostachys nigra*	CA04–041	37.5
Bambusae caulis in taeniam	*Phyllostachys nigra*	CA03–055	21.2
Bambusae folium	*Sasa borealis var. gracilis*	CA03–056	7.1
Bletillae rhizoma	*Bletilla striata*	CA04–016	15.2
Borneolum	*Dryobalanops aromatica*	CA03–026	3.1
Broussonetiae fructus	*Broussonetia kazinoki*	CA03–045	0
Caryophylli cortex	*Syringa dilatata*	CA03–051	42.4
Celosiae semen	*Celosia argentea*	CA04–055	0
Cinnamomi cortex spissus	*Cinnamomum loureirii*	CA03–035	0
Cistanchis herba	*Cistanche deserticola*	CA03–037	15.1
Clematidis radix	*Clematis florida*	CA03–032	0
Cnidii rhizoma	*Cnidium officinale*	CA03–071	45.3
Cyperi rhizoma	*Cyperus rotundus*	CA03–089	6.8
Dioscoreae rhizoma	*Dioscorea oppostifolia*	CA04–022	17.1
Dioscoreae rhizoma	*Dioscorea tenuipes*	CA04–024	0.5
Drabae semen	*Lepidium apetalum*	CA03–050	0
Epimedii herba	*Epimedium koreanum*	CA03–038	0
Erycibae caulis	*Sorbus commixta*	CA04–039	7.2
Fagopyri semen	*Fagopyrum esculentum*	CA04–003	0
Gardeniae fructus	*Gardenia jasminoides*	CA04–061	0
Gastrodiae rhizoma	*Gastrodia elata*	CA04–048	5.3
Gentianae macrophyllae radix	*Aconitum pseudo-laeve var. erectum*	CA03–067	0
Gleditsiae fructus	*Gleditsia japonica*	CA03–054	0
Gleditsiae spina	*Gleditsia sinensis*	CA03–052	21.5
Hedyotidis diffusae herba	*Oldenlania diffusa*	CA04–019	0
Hoelen	*Polypori umbellati*	CA03–013	23.1
Hoelen rubra	*Poria cocos*	CA04–037	0
Hordei fructus germinatus	*Hordeum vulgare var. hexastichoi*	CA04–012	5
Hoveniae lignum	*Hovenia dulcis*	CA03–061	0
Inulae radix	*Inula helenium*	CA04–065	71
Kochiae fructus	*Kochia Scoparia*	CA03–064	0
Licii radicis cortex	*Lycium chinense*	CA03–060	5.2
Ligustici rhizoma	*Ligusticum chuanxiong*	CA04–047	20.2
Linderae radix	*Lindera strichnifolia*	CA03–022	0.3
Lini semen	*Linum usitatissimum*	CA03–020	0
Lithospermi radix	*Lithospermum erythrorhizon*	CA03–043	0
Longanae arillus	*Dimocarpus longan*	CA04–033	0
Lycii fructus	*Lycium chinense*	CA04–004	0
Malvae semen	*Malva vertisillata*	CA04–011	0
Massa medicata fermentata	*Tricticum sativum*	CA03–019	16.1
Maydis stigma	*Zea mays*	CA04–032	0
Melandrii herba	*Melandrium firmum*	CA03–024	0
Meliae fructus	*Melia azedarach*	CA03–072	0
Melonis calyx	*Cucumis melo*	CA03–003	6.7
Mori rructus	*Morus alba*	CA04–027	1.3
Mucunae caulis	*Spatholobus suberectus*	CA03–002	69
Myristicae semen	*Myristica fragrans*	CA03–036	76.4

Table 1. Continued.

Name of herbal medicine	Plant name	Number of voucher specimen	Percent inhibition of degranulation[a]
Nepetae spica	*Schizonepeta tenuifolia*	CA03–095	52.4
Olibanum	*Boswellia sacra*	CA03–034	23
Paeoniae radix rubra	*Paeonia lactiflora*	CA03–046	1.1
Perillae herba	*Perilla frutescens*	CA03–017	33.3
Perillae semen	*Perilla frutescens*	CA03–018	0
Peucedani radix	*Angelice decursiva*	CA03–049	21.5
Peucedani radix	*Anthriscus sylvestris*	CA03–048	0
Phaseoli angularis semen	*Phaseolus angularis*	CA04–038	2.9
Pinelliae tuber	*Pinellia ternate*	CA04–015	0
Pini ramulus	*Pinus densiflora*	CA04–030	69.1
Plantaginis semen	*Plantago asiatica*	CA04–044	0
Platycodi radix	*Platycodon grandiflorum*	CA04–005	0
Polygalae radix	*Polygala tenuifolia*	CA03–031	0
Potentillae radix	*Potentilla chinensis*	CA03–033	0
Prunellae spica herba	*Prunella vulgaris*	CA03–081	6.1
Pruni cortex	*Prunus yedoensis*	CA03–100	47.6
Pruni Nakaii semen	*Prunus nakail*	CA03–029	3.2
Psoraleae semen	*Psoralea corylifolia*	CA04–020	0
Raphani semen	*Raphanus sativus var.hortensis*	CA03–004	0
Rehmaniae radix crudus	*Rehmannia glutinosa*	CA04–042	0
Sanguisorbae radix	*Sanguisorba officinalis*	CA03–066	53.7
Santali alba lignum	*Santalum album*	CA04–017	3
Schizandrae fructus	*Schizandra chinensis*	CA04–031	0
Scirpi rhizome	*Sparganium erectum*	CA04–026	0
Scrophulariae radix	*Scrophularia buergeriana*	CA03–092	0.8
Smilacis rhizoma	*Smilax china*	CA04–066	32.4
Solani Nigri herba	*Solanum nigrum*	CA03–025	0
Sophorae flos	*Sophora japonica*	CA01–014	91.5
Sophorae subprostratae radix	*Sophora subprostrata*	CA03–015	7
Tetrapanacis medulla	*Tetrapanax papyriferus*	CA04–070	2.8
Thujae semen	*Thuja orientalis*	CA04–018	0
Trichosanthis radix	*Trichosanthes kirilowii*	CA04–002	0
Ulmi pasta semen	*Ulmus macrocarpa*	CA04–013	22.9
Uncariae ramulus et uncus	*Uncaria rhynchophylla*	CA03–053	0
Xanthii fructus	*Xanthium strumarium*	CA04–046	0
Zingiberis rhizoma	*Zingiber officinale*	CA04–001	21.2
Zizyphi spinosi semen	*Zizyphs jujuba*	CA03–016	0.1
0.1% DMSO			2.1
10 μM PP2			92.5

[a] RBL-2H3 mast cells were incubated for 30 min, with or without each extract at 100 μg/ml, before adding 25 ng/mL of the antigen DNP-BSA for 10 min. The degranulation was assessed through the measurement of the release of the granule marker β-hexosaminidase, using a colorimetric assay, through which the release of p-nitrophenol from p-nitrophenyl-N-acetyl-β-D-glucosaminide was measured as described in the "Materials and Methods" section. They are expressed as the mean values from two independent experiments. The degranulation by antigen was ~45% in the cells. PP2 is a general Src-family kinase inhibitor.

considered as one of the promising approaches for anti-allergic therapy.

Although many herbal extracts in Asian countries have been used as traditional folk remedies, their pharmacological activities and mechanisms of action, thus far, remain to be determined. One of these herbs, *Sophorae flos*, referred to as Goe-Hwa in Korea, has been used a long time in Korea, China, and Japan as a folk remedy for hemorrhage, allergic diseases, and hypertension (11). Only a few studies describing the pharmacological effects of *Sophorae flos*, however, have been performed thus far. This study is the first report on the anti-allergic activity of *Sophorae flos*; it exerts this activity by the inhibition of mast cells at the Src family kinase level and thereby exhibits potential as a therapeutic agent for IgE-mediated allergic diseases.

Materials and Methods

Reagents. Antibodies were purchased from the following sources: antibodies against the phosphorylated forms of ERK1/2, p38, JNK, and Akt, and against the phosphorylated form of Y317 Syk and Y191 LAT from Cell Signaling Technology Inc. (Danvers, MA); antibodies against Syk and Actin from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); and polyclonal antibodies against LAT and SLP-76 from Upstate Biotechnology (Lake Placid, NY). The HRP-linked antibody against mouse or rabbit IgG

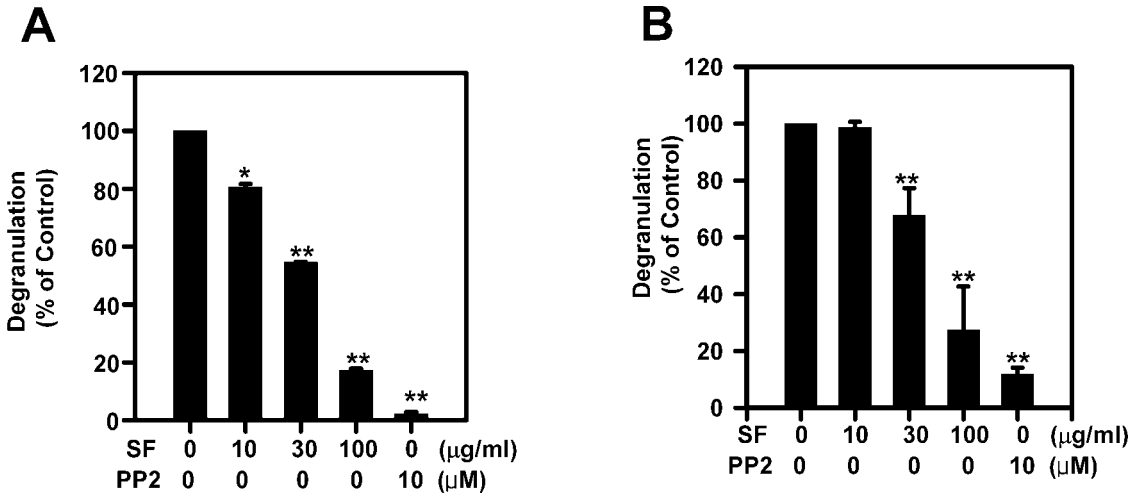

Figure 1. The effect of *Sophorae flos* on antigen-induced degranulation in RBL-2H3 cells and BMMCs. RBL-2H3 mast cells (A) and BMMCs (B) were incubated overnight in 24-well cluster plates with 50 ng/mL of DNP-specific IgE in a complete growth medium. The medium was replaced with a PIPES (for RBL-2H3 cells) or Tyrode buffer (for BMMCs) that contained the indicated concentrations of *Sophorae flos* (SF) before stimulation with 25 ng/mL of DNP-BSA for 10 min in order to measure the release of β-hexosaminidase. The values are expressed as the mean ± SEM from the three independent experiments. The asterisks indicate significant differences from the controls, * $P < 0.05$ and ** $P < 0.01$. PP2 is a general Src family kinase inhibitor.

was obtained from Cell Signaling Technology Inc. (Danvers, MA). Dinitrophenol (DNP)-BSA, DNP-specific monoclonal IgE, formamide, Arabic gum, Evans blue, and diphenylhydramine (DPH) were obtained from Sigma Chemical Co. (St. Louis, MO), and PP2 was obtained from Calbiochem (La Jolla, CA). The minimal essential medium (MEM) and other cell culture reagents were obtained from GIBCO/Life Technologies Inc. (Rockville, MD).

Animals. Male ICR mice (age, 4 weeks) were obtained from the Dae Han Experimental Animal Center (Eumsung, Korea) and were housed in the animal facilities at the Konkuk University College of Medicine. Ten mice were placed in each cage fitted with a laminar airflow cabinet. The mice were maintained at a temperature of 22 ± 1°C and at a relative humidity of 55 ± 10% throughout the study. This study was performed in accordance with the institutional guidelines.

Preparation of Ethanol Extracts of *Sophorae Flos* and Other Herbal Medicines. The dried *Sophorae flos* and other herbal medicines were imported from China and were authenticated by the Korea Research Institute of Bioscience and Biotechnology in Korea. Each extract was prepared according to the process described in our previous report (12). Briefly, dried flower buds (100 g) of the *Sophora japonica* L. (Leguminosae) and other dried herbs were subjected to extraction with 1000 mL of ethanol at 50°C using an ultrasonic cleaner. The extracted materials were concentrated for 24 h in a speed bag at 40°C, and the yield of extracts was approximately 15–20% (w/w). To evaluate the effects of the extracts on Ag-mediated degranulation, mast cells were incubated with 100 μg/mL of each extract before stimulating with the DNP-BSA antigen. These voucher specimens (designated in Table 1) were deposited at the College of Medicine, Konkuk University, and at the Korea Research Institute of Bioscience and Biotechnology in Korea.

Preparation and Stimulation of Mast Cells. Bone marrow-derived mast cells (BMMCs) were isolated from male Balb/cJ mice according to the previously reported protocol (13) and were cultured for up to 10 weeks in a 50% enriched medium (RPMI 1640 containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics, and 10% fetal calf serum) containing 10 ng/mL of IL-3. After 3 weeks, the cells were used for the study after verifying that they expressed the FcεRIβ subunit and degranulated in response to IgE/antigen (12). The BMMCs were stimulated as according to the protocol described in our previous report (12). DNP-specific IgE-primed BMMCs were stimulated with 25 ng/mL of the antigen, i.e., DNP-BSA, for 10 min in a Tyrode-BSA buffer (20 mM HEPES at pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.05% BSA). RBL-2H3 cells were cultured in MEM with Earle's salts supplemented with glutamine, antibiotics, and 15% fetal bovine serum (FBS) and were then stimulated in a PIPES-buffered medium (25 mM PIPES at pH 7.2, 159 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, and 0.1% fatty-acid-free fraction V from bovine serum).

Ag-Stimulated Degranulation in Mast Cells. Degraulation of mast cells was measured according to the procedure described in a previous report (12). Briefly, the cells on 24-well (2×10^5 cells/0.4 mL/well) cluster plates were primed overnight with 50 ng/mL of DNP-specific IgE. The cultures were washed, and a PIPES-buffer (for RBL-2H3 cells) or a Tyrode buffer (for BMMCs) was added (0.2 mL/well). The cultures were then incubated for 30 min with or without *Sophorae flos* or other herbal extracts before the addition of 25 ng/mL of DNP-BSA for 10 min. The release

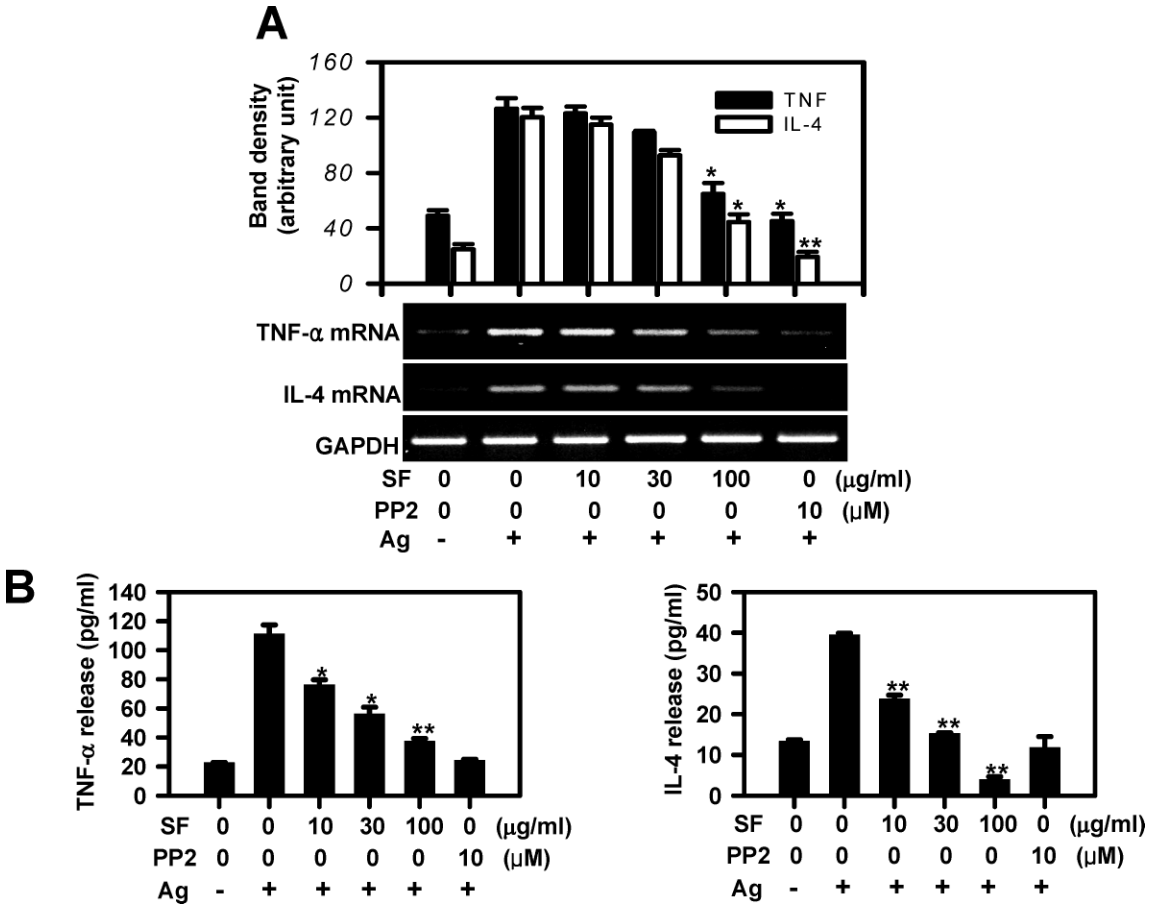

Figure 2. The effects of *Sophorae flos* on the expression and secretion of TNF-α and IL-4 in mast cells. (A) The indicated amounts of *Sophorae flos* (SF) or PP2 were added to the RBL-2H3 cells 30 min before the addition of 25 ng/mL of DNP-BSA (+) or were left unstimulated (–) after overnight incubation with 50 ng/mL of DNP-BSA-specific IgE. The cells were stimulated for 15 min for the assay of TNF-α and IL-4 mRNA by RT-PCR. The representative gel pictures (lower panel) and densitometric data (upper panel) from the three independent experiments are shown. (B) The IgE-primed RBL-2H3 cells were stimulated with 25 ng/mL of DNP-BSA for 4 h (+), or were left unstimulated (–) with or without *Sophorae flos* (SF). The concentrations of TNF-α and IL-4 released into the culture media were assessed by using commercial ELISA kits. The values are expressed as the mean ± SEM from the three independent experiments. Asterisks indicate significant differences from the controls, * $P < 0.05$ and ** $P < 0.01$. PP2 is a general Src family kinase inhibitor.

of p-nitrophenol from p-nitrophenyl-*N*-acetyl-β-D-glucosaminide by β-hexosaminidase secreted from cells was measured using a colorimetric assay (14). The values were expressed as the percentages of intracellular β-hexosaminidase released into the medium.

Immunoblotting Analysis of Cell Signaling Molecules. After stimulation of BMMCs by the antigen for 7 min, the cells were washed twice with ice-cold PBS and were lysed in 0.5 mL of an ice-cold lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet p-40, 10% glycerol, 60 mM octyl β-glucoside, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenylphosphate, 0.7 μg/mL pepstatin, and a protease inhibitor cocktail tablet). The cell lysates were placed on ice for 30 min, followed by centrifugation at 15,000 *g* at 4°C for 15 min. The loading samples were prepared by boiling at 95°C for 5 min in a 2× Laemmli buffer (15). The proteins were separated using SDS-PAGE and were then transferred onto nitrocellulose membranes. After blocking with TBS-T buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% skimmed milk powder, the membrane was incubated with each specific antibody against the target protein. The primary antibodies were diluted 1:2000-fold unless otherwise stated and were incubated overnight at 4°C. After washing the membranes 3 times for 5 min each with a TBS-T buffer, the immunoreactive proteins were labeled with HRP-coupled secondary antibodies diluted 1:2000-fold for 1 h, and were subsequently washed 5 times (for 5 min each) with a TBS-T buffer and enhanced chemoluminescence, according to the manufacturer's protocols (Amersham Biosciences, Piscataway, NJ).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for TNF-α and IL-4 mRNA. RT-PCR was performed as described in a previous report (12). Briefly, after stimulation of the IgE-primed RBL-2H3 cells (1×10^6 cells/3 mL/well) with 25 ng/mL of DNP-BSA for 15 min, total RNA was isolated and was reverse-transcribed

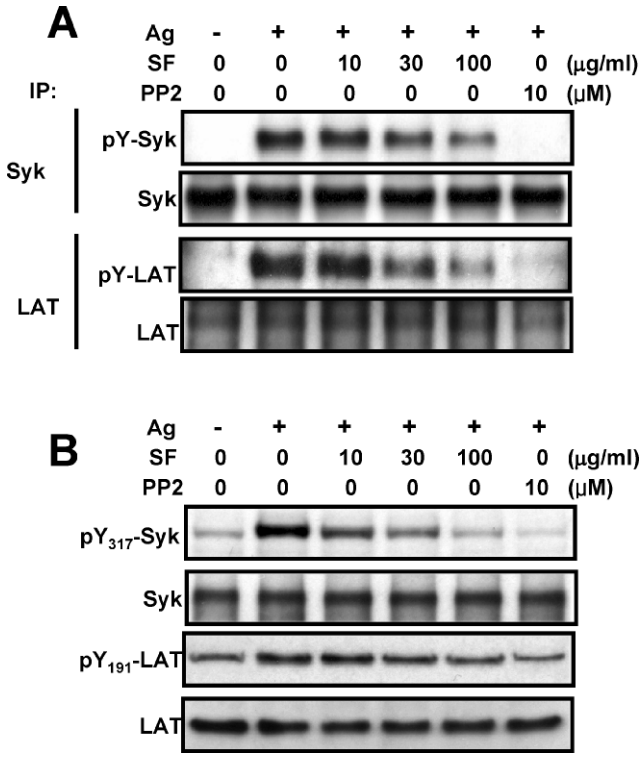

Figure 3. The effect of *Sophorae flos* on the activating phosphorylation of Syk and LAT. The RBL-2H3 cells or BMMCs were incubated overnight in 6-well plates with 50 ng/mL of DNP-specific IgE in a complete growth medium. The cells were stimulated with 25 ng/mL of the antigen (DNP-BSA) (+) or were left unstimulated (–) with or without *Sophorae flos* (SF) for 7 min. (A) In RBL-2H3 cells, Syk and LAT were immunoprecipitated by their respective specific antibodies, and the immunoprecipitated proteins were subjected to immunoblot analysis in order to detect phosphorylated or total proteins. (B) The proteins derived from the BMMC lysates were subjected to immunoblot analysis in order to detect the phosphorylated forms of Syk (Y317) or LAT (Y191). The representative blots from the three independent experiments are shown. PP2 is a general Src family kinase inhibitor.

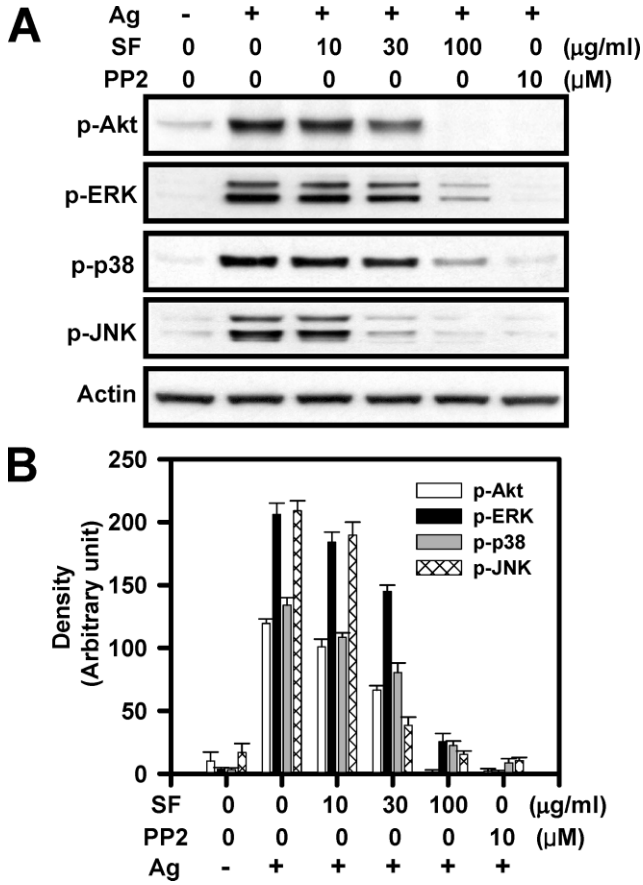

Figure 4. The effect of *Sophorae flos* on the activating phosphorylation of Akt and MAP kinases. (A) The RBL-2H3 cells were incubated overnight in 6-well plates with 50 ng/mL of DNP-specific IgE in a complete growth medium. The cells were stimulated with 25 ng/mL of DNP-BSA (+) or were left unstimulated (–) with or without *Sophorae flos* (SF) for 7 min. Activation of the phosphorylation of the indicated Akt and MAP kinases was determined by preparation of immunoblots from whole-cell lysates using specific antibodies for the phosphorylated forms of the signaling proteins. (B) Band densities are expressed as the mean ± SEM of the three independent experiments. PP2 is a general Src family kinase inhibitor.

using the Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA). Further, 30 cycles of polymerase chain reaction was performed at 94°C for 45 s, at 55°C for 45 s, and at 72°C for 60 s. The following primers were used: rat TNF-α: forward, 5′-CACCACGCTCTTCTGTCTACT GAAC-3′ and reverse, 5′-CCGGACTC CGTGATGTCT AAGTACT-3′; rat IL-4: forward, 5′-ACCTTGC TTCACCCTGTTC-3′ and reverse, 5′-TTGTGAGC GTGGACTCATTC-3′; and rat GAPDH: forward, 5′-GTG GAGTCTACTGGCGTCTTC-3′ and reverse, 5′-CCAA GGCTGTGGGCAAGGTCA-3′.

Enzyme-Linked Immunosorbent Assay (ELISA) for TNF-α and IL-4. The IgE-primed RBL-2H3 cells were stimulated with 25 ng/mL of DNP-BSA for 4 h. The level of TNF-α and IL-4 secreted from the cells was measured in the media by using an ELISA kit obtained from Invitrogen (Carlsbad, CA).

Mast Cell-Mediated Passive Cutaneous Anaphylaxis (PCA). The PCA model was induced in mice according to the procedure described in previous reports (12, 16). Briefly, an anti-DNP-BSA-specific IgE (0.5 μg) was intradermally injected into the right ear of a mouse, and *Sophorae flos* (100 to 1000 mg/kg, po) or diphenylamine (50 mg/kg, ip) was administered 24 h later. At 1 h after the treatment, the mice were then challenged with 250 μg of the antigen (DNP-BSA, iv) in 250 μL of PBS containing 4% Evans blue. The mice were then euthanized after 1 h, and the right ear was excised for assay of the extravasated dye. The dye was extracted overnight in 700 μL of formamide at 63°C, and the intensity was measured at 620 nm.

Statistical Analysis. The results have been presented as the mean ± SEM from three or more separate experiments. Statistical analysis was performed using one-way ANOVA and the Dunnett's test. All statistical calculations ($*P < 0.05$ and $**P < 0.01$) were performed using the SigmaStat software (Systat Software Inc., Point Richmond, CA).

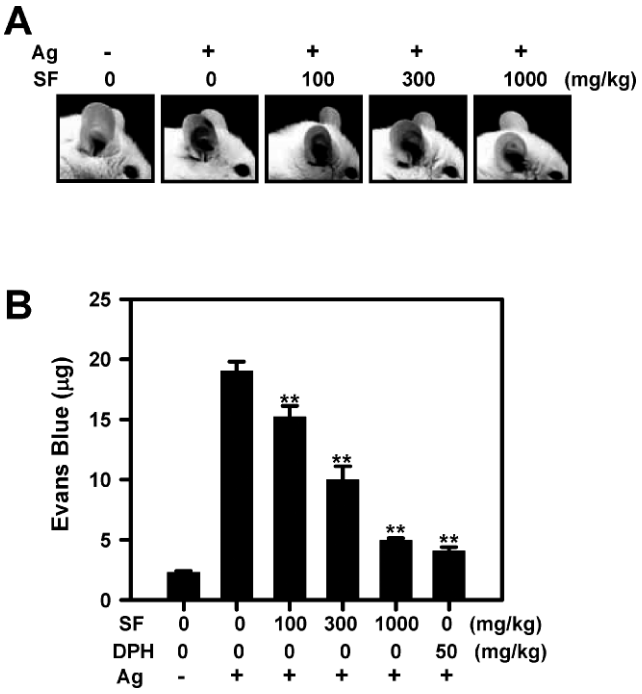

Figure 5. The effect of *Sophorae flos* on a mast cell-mediated local allergic reaction. An anti-DNP-specific IgE (0.5 μg) was intradermally injected into the ear of a mouse. The antigen, i.e., 250 μg of DNP-BSA (1 μg/mL in PBS containing 4% Evans blue) (+), or the vehicle (–) was administered 24 h later into the tail vein of the mouse. *Sophorae flos* (SF) was administered 1 h before administration of the antigen (DNP-BSA). The mice were then euthanized after 1 h, and the right ear was excised for assay of the extravasated dye. The dye was extracted overnight in 700 μL of formamide at 63°C, and the intensity was measured at 620 nm. (A) The representative pictures of the ears are shown. (B) The values are expressed as the mean ± SEM of the two independent experiments. Diphenylhydramine (DPH) has been used as the reference drug.

Results

The Effects of *Sophorae Flos* and Other Herbal Extracts on Antigen-Induced Degranulation in Mast Cells. During our continuous efforts to develop novel anti-allergic agents from natural products (12, 17–18), RBL-2H3 cells and bone marrow-derived mast cells (BMMCs) were used for the screening of potential inhibitors of degranulation in mast cells. RBL-2H3 mast cells were established from rats (19). The primary BMMCs were isolated from the bone marrow of mice according to a previously described protocol (13), and the expression level of FcεRI in the BMMCs was comparable to that in the RBL-2H3 cells (12). The effects of approximately 100 medicinal herbal extracts on the degranulation of mast cells have been measured in RBL-2H3 mast cells. The extracts from *Myristicae semen, Aurantii Immatri pericarpium, Inulae radix,* and *Sophorae flos* significantly inhibited degranulation (>70%) at a concentration of 100 μg/mL in RBL-2H3 cells (Table 1). Among them, *Sophorae flos* most potently suppressed antigen-induced degranulation in a dose-dependent manner in both RBL-2H3 cells (Fig. 1A) and BMMCs (Fig. 1B); its IC_{50} values were approximately 31.6 μg/mL and 47.8 μg/mL, respectively.

The Effect of *Sophorae Flos* on the Expression and Secretion of TNF-α and IL-4 in Antigen-Stimulated Mast Cells. It has been well established that various cytokines, such as TNF-α and IL-4, are critical for allergic response (20, 21). On the basis of previous reports, it was investigated whether *Sophorae flos* suppressed the expression and secretion of TNF-α and IL-4 in Ag-stimulated mast cells. *Sophorae flos* exhibited significant inhibition of the antigen-stimulated expression of TNF-α and IL-4 mRNA in the cells in a dose-dependent manner (Fig. 2A). In order to further prove this, we tested whether *Sophorae flos* suppressed the secretion of cytokines. Consistent with the above results (Fig. 2A), the secretion of TNF-α and IL-4 was significantly inhibited in a dose-dependent manner (Fig. 2B). The inhibitory effect of 100 μg/mL of *Sophorae flos* was similar to or more potent than that of 10 μM of PP2, a typical Src family kinase (SFK) inhibitor.

The Effects of *Sophorae Flos* on the Ag-Stimulated Signaling Pathway. Next, we measured the effects of *Sophorae flos* on the IgE-mediated signaling events, including the activating phosphorylation of Syk kinase and the downstream signaling molecules, which were examined to identify its mechanism of action. The activating phosphorylation of Syk kinase and LAT were significantly suppressed by *Sophorae flos* in RBL-2H3 mast cells (Fig. 3A). Several lines of evidence suggest that the Syk on tyrosine 317 (in mice) is initially phosphorylated by Lyn kinase or other Src family kinases in mast cells (22, 23). Based on these reports, we next determined whether the effect of *Sophorae flos* on Syk phosphorylation was mediated by the suppression of Lyn activity; this was achieved by using the specific antibody against phosphorylation on the tyrosine 317 of Syk. *Sophorae flos* and PP2, an Src family kinase inhibitor, attenuated the phosphorylation of Syk (Y317) in a dose-dependent manner (Fig. 3B). LAT, one of direct substrate molecules of Syk, was also significantly inhibited by *Sophorae flos* in both RBL-2H3 cells and BMMCs (Fig. 3A and 3B). This result strongly suggests that the effect of *Sophorae flos* on the activation of phosphorylation of Syk is mediated through the inhibition of Src family kinase in mast cells.

In mast cells, phosphatidylinositol 3-kinase (24) and MAP kinases (25, 26) are important for the activation of transcription factors for the production of various cytokines, including TNF-α and IL-4. The phosphorylation of the MAP kinases, namely p38, ERK1/2, and JNK, and Akt, a surrogate for the activation of phosphatidylinositol 3-kinase were strongly suppressed by *Sophorae flos* (Fig. 4). The average IC_{50} value of *Sophorae flos* against the tested signaling molecules in Figure 4 was approximately 30 μg/mL.

The Effect of *Sophorae Flos* on Mast-Cell-Mediated PCA Reaction. Accumulating evidence in-

dicates that mast cells play a critical role in IgE-mediated allergic responses. The above results showed that *Sophorae flos* inhibited degranulation and secretion of proinflammatory cytokines, such as TNF-α and IL-4 in both RBL-2H3 cells and BMMCs. Next, we examined whether *Sophorae flos* inhibited the IgE-mediated local allergic response, a PCA reaction. The passive cutaneous anaphylaxis (PCA) was successfully induced by IgE and antigen injection in the mice. *Sophorae flos* significantly inhibited PCA in a dose-dependent manner to yield an ED_{50} dose of approximately 275 mg/kg (Fig. 5). The potency of the activity of *Sophorae flos* (1000 mg/kg) is similar to that of diphenylhydramine (DPH, 50 mg/kg), a typical anti-histaminic agent (Fig. 5).

Discussion

It is becoming increasingly apparent that mast cells are the central effector cells in the early events associated with allergic inflammatory responses induced by the rapid local and systemic release of allergic inflammatory mediators, such as histamine, serotonin, heparin, and various proinflammatory cytokines from the cells located throughout the human body (3, 4). In this study, for the first time, we demonstrated the anti-allergic activity of *Sophorae flos* in a mast cell-dependent animal model, which is most likely due to its ability to potently inhibit Src family kinase-dependent signals that lead to degranulation and cytokine secretion in antigen-stimulated mast cells.

Under allergic conditions, mast cells are activated by IgE-mediated antigens via the high-affinity receptor for IgE, namely FcεRI. The cross-linking of the IgE/FcεRI complex with the antigen firstly activates the Src family kinases, such as Lyn, Fyn, and possibly another isoform of Src family kinases, and subsequently, other signaling proteins, including Syk, LAT, SLP-76, Gab2 (6), and phospholipase (PL) D2 (27). It is well known that Src family kinases including Lyn play a key role in the activation of initiation of signaling in mast cells after receptor aggregation by the antigen and are the upstream kinases required for the activation of Syk (28). For this reason, many studies have been conducted to find therapeutically treatable tyrosine kinase inhibitors as mast cell stabilizers (29). This is the first report that identifies *Sophorae flos* as an inhibitor of the activating phosphorylation of Syk. As previously reported, Src family kinase functions as the upstream kinase for the direct or indirect activation of Syk in mast cells (22, 23). In this study, the phosphorylation of Syk was inhibited by *Sophorae flos* (Fig. 3A). Furthermore, the phosphorylation of tyrosine at 317 of Syk kinase, a direct phosphorylation site by Lyn kinase, was inhibited by *Sophorae flos* (Fig. 3B). Based on these results, it is possible that *Sophorae flos* is associated with the inhibition of Src family kinase, thus preventing the activating phosphorylation of Syk. The possibility, however, that *Sophorae flos* inhibited its autophosphorylation (30) and/or the complete activation of phosphorylation by PLD2 (31) in the cells could not be discounted.

Aggregation of IgE-occupied FcεRI by the antigen causes the production of IL-4, TNF-α, and many other cytokines (32, 33). The cytokines are known to induce pathogenic inflammatory symptoms in the later stages of an allergic reaction (21, 34). It has recently been reported that the MAP kinase ERK 1/2 is an essential signal in the production of several cytokines, including TNF-α and IL-13, in mast cells (25–26, 35). In this study, cytokine production and activation of three typical MAP kinases in the antigen-stimulated mast cells were suppressed by *Sophorae flos* (Fig. 4). The present results indicate that the inhibition of TNF-α and IL-4 production and secretion by *Sophorae flos* significantly correlated with the suppression of the MAP kinases in Ag-stimulated mast cells.

The passive cutaneous anaphylaxis (PCA) animal model used here is a well-established model for evaluating localized mast cell-mediated allergic reactions *in vivo* (12, 16). *Sophorae flos* effectively suppressed the PCA reaction in mice in a dose-dependent manner (Fig. 5). Substantial inhibition was observed with 100 mg/kg of *Sophorae flos* when administered orally. Near complete suppression of the allergic reaction was observed with 1000 mg/kg *Sophorae flos* (ED_{50}, 275 mg/kg), and this suppression was comparable to that achieved with 50 mg/kg of diphenylhydramine (DPH).

There have been many reports on the components and pharmacological activities of the extracts of the bark or pericarp of *Sophora japonica* L. (Leguminosae) (36–38). In contrast, only a few reports have thus far been published on the components of *Sophorae flos* and the pharmacological activity of these substances (39). Therefore, further studies are necessary to characterize the active components of *Sophorae flos* that exhibit anti-allergic activity for the therapeutic application.

In conclusion, this study is the first report indicating that *Sophorae flos* inhibits degranulation, cytokine secretion, and Syk phosphorylation in antigen-stimulated BMMCs and RBL-2H3 mast cells as well as a local allergic anaphylaxis in mice. The results strongly suggest that *Sophorae flos* exhibits anti-allergic activity through the inhibition of Syk activation in the cells, possibly through the inhibition of Src family kinase.

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