Rubiae Radix suppresses the activation of mast cells through the inhibition of Syk kinase for anti-allergic activity

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Abstract

The effect of extracts from various Oriental medicinal herbs on mast-cell-mediated allergic reactions was investigated in this study. Of these extracts, the medicinal herb Rubiae Radix exhibited the most potent activity in the cells, with an IC50 value (concentration necessary to obtain 50% inhibition of the response) of approximately $35 \pm 2.1\ \mu g\, mL^{-1}$, and its inhibition of compound-48/80-induced systemic anaphylaxis by $48.6 \pm 8.5\%$ at $300\, mg\, kg^{-1}$ in mice. It also inhibited the expression of the pro-inflammatory mediator tumour necrosis factor-α (TNF-α). As for its mechanism of action, Rubiae Radix suppressed the activating phosphorylation of Syk, a key enzyme in mast-cell signalling processes, and that of Akt in a dose-dependent manner. It also inhibited the MAP kinase ERK1/2, which is critical for the production of inflammatory cytokines in mast cells, as indicated by the suppression of the activating phosphorylation of ERK1/2. These results suggest that Rubiae Radix suppresses the activation of mast cells through the inhibition of Syk for anti-allergic activity.

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Introduction

Mast cells and blood basophils are involved in a variety of allergic disorders, such as allergic rhinitis, dermatitis, asthma and food allergies, as well as catastrophic anaphylactic reactions to insect stings and some drugs. These cells respond to IgE-directed antigens via the high-affinity receptor for IgE, FcεRI, by releasing granules that contain preformed inflammatory mediators and by generating inflammatory lipids and cytokines (Kay 2001a, b). In the allergic condition, mast cells are activated by the multivalent binding of an antigen to immunoglobulin E (IgE) that is bound to its multimeric receptor, FcεRI. The ensuing aggregation of these receptors results in the rapid phosphorylation by Lyn kinase of the tyrosine residues in the immunoreceptor-tyrosine-based activation motifs (ITAMs) of the β- and γ-subunits of FcεRI, which in turn results in the recruitment and activation of the protein tyrosine kinase Syk. Syk is responsible for the activation of a large number of downstream-signalling molecules and, in this manner, regulates degranulation and the generation of inflammatory mediators (Rivera 2002). The Syk in the cells induces tyrosine phosphorylation of the adaptor proteins, the linker for the activation of the T cells (LAT) and the SH2-containing leucocyte-specific protein of 76 kDa (SLP-76), which provides essential docking sites for the assembly of signalling molecules for the further propagation of signals in activated mast cells (Rivera 2002).

Many herbs, including Rubiae Radix, are used as traditional folk remedies for various diseases in Asian countries. In addition, various medicinal herbs have been reported to have anti-allergic activity in-vitro and in-vivo (Kim et al 2003; Makino et al 2003; Lee et al 2004a, b; Nagai et al 2004). The active components and

mechanisms of action of most herbs, however, are largely unknown, and few have been screened for their pharmacological activity or in in-vivo animal models. Therefore, a systematic examination of these herbal remedies must be conducted.

Rubiae Radix, known as Chun-Cho-Keun in Korea and Qian Cao in China, is a dried root or rhizome of *Rubia cordifolia L.* (Rubiaceae). The extract of the dried root is known to have haemostatic, antibacterial, antitussive and expectorant activity (Huang 1993), and has been traditionally used for the treatment of haematemesis, epistaxis, haemofaecia, metrorrhagia, metrostaxis and other diseases. It has also been used in the treatment of allergic purpura, in combination with Radix Arnebiae and Radix Salaiae.

Some active constituents of Rubiae Radix have been identified (Huang 1993) and examined for pharmacological activity in-vitro and in animal models for anti-tumour activity (Itokawa et al 1983, 1984). However, the effects of Rubiae Radix on the degranulation of mast cells and on in-vivo anti-allergic activity have not yet been tested.

In this study, we aimed to screen extracts from various Oriental medicinal herbs for in-vitro and in-vivo anti-allergic activity and investigate these extracts' mechanisms of action. Rubiae Radix exhibited potent anti-allergic activity through the inhibition of mast cells by suppressing the activating phosphorylation of Syk and MAP kinases in the cells. The results suggested that Rubiae Radix has potential utility in the treatment of allergic diseases.

Materials and Methods

Reagents

DNP-specific monoclonal IgE and DNP-BSA, Arabic gum, Tween 80, compound 48/80, and sodium cromoglicate were purchased from Sigma Chemical Co. (St Louis, MO, USA). Minimal essential medium (MEM) and other cell-culture reagents were obtained from GIBCO/Life Technologies, Inc. (Rockville, MD, USA). Antibodies were purchased from the following sources: antibodies against phosphotyrosine (PY) (4G10), Akt, ERK1/2, p38 and JNK from Upstate Biotechnology (Lake Placid, NY, USA); antibodies against Syk and TNF-α from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Animals

Male ICR mice (aged 4 weeks) were purchased from the Dae Han Experimental Animal Center (Daejon, Korea), and they were housed in the animal facilities at the College of Medicine in Konkuk University. Ten mice were placed in each cage in a laminar airflow cabinet where a temperature of $22 \pm 1°C$ and a relative humidity of $55 \pm 10\%$ were maintained throughout the study. The study was carried out in accordance with the guidelines issued by the National Institutes of Health (NIH publication No. 85-23, 1985) and the protocol was approved before the study by the institutional Ethics Committee of Konkuk University.

Preparation of crude extract of Rubiae Radix

Various herbs, that were expected to be anti-inflammatory agents were selected for screening on the basis of Kimi's classifications in Bonchohak, the science of traditional medicinal plants. The herbs to be screened were purchased at the Kyong Dong medicinal-plants market, which specializes in the sale of traditional medicinal plants in Seoul, Korea. Their classification was carried out by Dr Keum Hee Hwang (Bio-Food and Drug Research Center, Konkuk University). The extracts were prepared using the following protocol. The dried medicinal plants were chopped with the use of a domestic mixer and 10 parts of 80% methanol was added. After allowing the mixture to stand at room temperature for 7 days, the methanol extracts were filtered and dried in a 50°C water bath with the use of a vacuum pump evaporator. Voucher specimens (Nos NP20-001 to 203) were deposited at the Bio-Food and Drug Research Center in Konkuk University. The extracts were dissolved as $100 \, \text{mg mL}^{-1}$ in 100% dimethylsulfoxide (DMSO) and diluted to $100 \, \mu\text{g mL}^{-1}$ in 0.1% DMSO with PIPES-buffered medium (25 mM PIPES, 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) as the final concentration for the in-vitro assay and were suspended in 5% Arabic gum for the in-vivo animal studies. As the control, we used 0.1% DMSO for the in-vitro assay and 5% Arabic gum for the in-vivo animal studies.

Cell culture and measurement of degranulation in RBL-2H3 mast cells

RBL-2H3 cells were grown as monolayers in MEM with Earle's salts, supplemented with glutamine, antibiotics and 10% fetal bovine serum (FBS). In each experiment, the cells were harvested through trypsinization, transferred to 24-well (2×10^5 cells/0.4 mL/well) cluster plates (Ali et al 1990), and were incubated overnight in a complete growth medium with $25 \, \text{ng mL}^{-1}$ DNP-specific IgE to achieve 100% occupancy of FcεRI. The cultures were washed and the required buffered solution was added (0.2 mL/well). Experiments on intact cells were performed in a PIPES-buffered medium. Unless stated otherwise, the cultures were incubated for 30 min with or without crude extracts before adding stimulants, $25 \, \text{ng mL}^{-1}$ antigen (DNP-BSA), or 150 nM thapsigargin for 10 min. The secretion of granules containing various allergic mediators was determined through the measurement of the release of the granule marker β-hexosaminidase with the use of a colorimetric assay,

through which the release of p-nitrophe-nol from p-nitrophenyl-N-acetyl-β-D-glucosaminide was measured (Ozawa et al 1993). The values were expressed as percentages of intracellular β-hexosaminidase that were released into the medium. The IC50 (concentration necessary to obtain 50% inhibition of the response) values were calculated through nonlinear regression analysis using the GraphPad software (San Diego, CA, USA).

Compound-48/80-induced systemic anaphylaxis

Systemic anaphylaxis was induced by the mast-cell degranulator compound 48/80 (Alfonso et al 2000; Hong et al 2003). Each mouse was given an intraperitoneal injection of $8 \, \text{mg kg}^{-1}$ of compound 48/80 in saline. Rubiae Radix extracts were suspended in 5% arabic gum and were administered orally in doses of $30–300 \, \text{mg kg}^{-1}$ 1 h before the injection of compound 48/80 to induce anaphylactic shock. The survival rate was monitored for 1 h after the injection of compound 48/80.

Cell stimulation and immunoprecipitation

The cells were washed and the medium was replaced with the PIPES-buffered medium. The cells were then stimulated with $25 \, \text{ng mL}^{-1}$ antigen (DNP-BSA) for 10 min or as indicated, were chilled with ice to terminate the stimulation and were then washed twice with ice-cold PBS and lysed in 0.5 mL 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$_3$VO$_4$, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenylphosphate, $0.7 \, \mu\text{g mL}^{-1}$ pepstatin, and a protease-inhibitor tablet). The lysates were kept on ice for 30 min and were then centrifuged $15\,000\,g$ for 15 min at 4°C. The supernatant fraction was precleared by the addition of $50 \, \mu\text{L}$ protein-G-agarose. The mixture was centrifuged after it was gently shaken for 1 h. Samples of the supernatant fraction with equal protein content were used for immunoprecipitation. Syk was immunoprecipitated through overnight incubation (4°C, with gentle shaking) with specific antibodies and, sequentially, with agarose. The agarose was washed five times with a washing buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Nonidet p-40, 10% glycerol, 10 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 2.5 mM nitrophenylphosphate, $0.7 \, \mu\text{g mL}^{-1}$ pepstatin, and a protease-inhibitor tablet) before being loaded onto a polyacrylamide gel.

Immunoblot analysis

The cell lysates or immunoprecipitated complex were denatured by boiling at 95°C for 5 min in a 2× Laemmli buffer (Laemmli 1970). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to nitrocellulose membranes (Schleicher & Schuell, BA85). After blocking in TTBS buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% skimmed milk powder, the membranes were incubated with individual monoclonal or polyclonal antibodies. The immunoreactive proteins were detected using horse-radish peroxidase-coupled secondary antibodies and through enhanced chemiluminescence, according to the manufacturer's (Amersham Pharmacia Biotech) instructions.

Reverse transcription–polymerase chain reaction (RT-PCR) for TNF-α mRNA

RBL-2H3 cells were harvested by trypsinization, transferred to 6-well (1×10^6 cells/3 mL/well) cluster plates, and incubated overnight in complete growth medium containing $25 \, \text{ng mL}^{-1}$ DNP-specific IgE to achieve 100% occupancy of FcεRI. The cells were washed and medium replaced with a PIPES-buffered medium and stimulated by $25 \, \text{ng mL}^{-1}$ DNP-BSA for 15 min without or with various concentrations of Rubiae Radix and then washed twice with ice-cold PBS. Total RNA was isolated by use of Trizol Reagent (Invitrogen) and reversed transcribed with the Superscript first strand synthesis system (Invitrogen) according to the manufacturer's protocol. PCR was performed at 94°C for 45 s, 55°C for 45 s and 72°C for 60 s for 30 cycles. The following primers were used: rat TNFα forward 5′-CACC ACGCTCTTCTGTCTACTGAAC-3′, reverse 5′-CCGGA CTCCGTGATGTCTAAGTACT-3′; rat GAPDH forward 5′-GTGGAGTCTACTGGCGTCTTC-3′, reverse 5′-CCA AGGCTGTGGGCAAGGTCA-3′.

Statistical analysis

The data were presented as the mean ± s.e.m. from three or more separate experiments. Statistical analysis was performed using one-way analysis of variance 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, USA).

Results

Effect of Rubiae Radix on antigen- or thapsigargin-induced degranulation in mast cells

Several studies have reported that inhibitors of mast cells exhibit anti-allergic activity in compound-48/80-induced anaphylactic allergic reactions (Kim et al 2003; Lee et al 2004a, b; Nagai et al 2004). In the continuing search for novel anti-allergic agents from natural products, assay systems for the screening of potential suppressors of degranulation have been conducted in cultured mast cells. In this study, approximately 100 natural products were primarily evaluated with antigen-stimulated degranulation in mast cells. As a result, the methanol extracts of Curcumae Radix, Glycyrrhizae Radix,

Polygoni Cuspidate Rhizoma, Rubiae Radix, Saururi Herba and Vaccariae Semen exhibited a potent inhibition of degranulation (more than 50% inhibition at the test concentration of $100 \mu g \, mL^{-1}$) in the cells (Table 1). Consistent with the previous report, the extract of Glycyrrhizae Radix significantly inhibited degranulation in the cells (Kim 2001). Among these medicinal herbs, Rubiae Radix suppressed antigen- or thapsigargin-induced degranulation in a dose-dependent manner (Figure 1). The IC50 values for the antigen or thaspsigargin, a well-known calcium ion inducer that blocks the uptake of Ca^{2+} into IP3-sensitive stores (Putney & Bird 1993), were approximately $35 \pm 2.1 \mu g \, mL^{-1}$ and $26 \pm 2.8 \mu g \, mL^{-1}$, respectively. These results suggested that Rubiae Radix may have a potent anti-allergic action in animal models.

Inhibition of TNF-α mRNA and protein expression

It was then determined whether or not Rubiae Radix inhibited the expression of TNF-α, a key mediator in mast-cell-dependent inflammatory events. Rubiae Radix significantly suppressed the expression of the TNF-α protein and of mRNA stimulated by antigens in a dose-dependent manner (Figures 2A, B).

Rubiae Radix inhibits compound-48/80-mediated anaphylaxis in mice

The above in-vitro results led us to measure the in-vivo anti-allergic activity of Rubiae Radix using a compound-48/80-mediated anaphylactic animal model. Compound 48/80 induced the anaphylactic death of ICR mice within 1 h after they were intraperitoneally injected with $8 \, mg \, kg^{-1}$ of the compound. As shown in Table 2, compound-48/80-induced anaphylactic death was dose-dependently inhibited by Rubiae Radix. Anaphylatic death was significantly inhibited by $48.5 \pm 8.5\%$ in mice that were given $300 \, mg \, kg^{-1}$ of Rubiae Radix.

Effects of Rubiae Radix on cellular-signalling molecules in an IgE-mediated signalling pathway

To investigate how Rubiae Radix suppressed degranulation in the mast cells, the effects of Rubiae Radix on the activation of cellular signalling molecules in the cells were tested. First, it was determined whether the antigen-induced activating phosphorylation of Syk, a key enzyme in mast-cell signalling processes in the cells, was suppressed by Rubiae Radix. As shown in Figure 3A, the activating phosphorylation of Syk was strongly inhibited in a dose-dependent manner. To further prove the inhibition of the pathway, it was also determined whether Rubiae Radix inhibited the downstream-signalling molecule phosphatidylinositol 3-kinase/Akt, as indicated by the activating phosphorylation of Akt. It was found that the activating phosphorylation of Akt was inhibited by Rubiae Radix

(Figure 3A). The results suggest that the major target of Rubiae Radix could be the activating phosphorylation of Syk in the cells. The herb's detailed mechanism of action, however, should be studied (i.e. whether Rubiae Radix directly inhibited Syk or upstream Src kinases, such as Lyn, Fyn, Fgr and Src in the mast cells).

Effects of Rubiae Radix on the activating phosphorylation of MAP kinases

The production of TNF-α, a major inflammatory mediator, was suppressed by Rubiae Radix (Figures 2A and 2B). Recently, ERK1/2 was reported as the signal for the production of interleukin (IL)-5, TNF-α, IL-3 and IL-13 in mast cells (Lorentz et al 2003). In addition, ERK2 activates phospholipase A2 (cPLA2), which results in the production of arachidonic acid, the precursor of various inflammatory mediators, such as leukotriene C_4/B_4 and PGD_2 (Hirasawa et al 1995a, b). To investigate the mechanism of action of Rubiae Radix, its effect on the activating phosphorylation of three MAP kinases – ERK1/2, p38, and JNK – in the cells was determined. As shown in Figure 3B, the phosphorylation of ERK1/2, p38 and JNK was significantly inhibited by the Rubiae Radix extract in a dose-dependent manner. These results strongly suggest that the inhibition of TNF-α was induced by the suppression of the IgE receptor $\rightarrow$ Syk $\rightarrow$ MAP kinase pathway.

Inhibition of Syk by piceatannol suppresses degranulation and TNF-α expression

To prove the critical role of Syk for degranulation and TNF-α expression in mast cells, we utilized piceatannol, a specific inhibitor of Syk. As shown in Figure 4, degranulation and TNF-α expression were significantly suppressed in a dose-dependent manner.

Discussion

Allergic rhinitis, asthma, atopic dermatitis and atopic eczema are among the most common causes of chronic ill health. These diseases are becoming more prevalent and they increase the burden of health care costs in many countries. Although there are many approaches to the treatment of these illnesses, such as allergen-specific immunotherapy, DNA vaccination, monoclonal anti-IgE antibody, soluble IL-4 receptor treatment and antagonists to the receptors of leukotriene and histamine, these therapies still have many problems and side effects (Kay 2001a, b).

To determine the presence of anti-allergic agents in natural products, the in-vitro anti-allergic activity of many Oriental medicinal herbs, as well as in-vivo anaphylaxis in mice, was checked. Among them, Curcumae Radix, Glycyrrhizae Radix, Polygoni Cuspidate Rhizoma, Rubiae Radix, Saururi Herba and Vaccariae

Table 1 Effects of natural products on antigen-induced degranulation in mast cells

Name of the herbal drug	Plant name	Voucher specimen No.	Family	Percent inhibition of degranulation[a]
Anemarrhenae Rhizome	*Anemarrhena asphodeloides*	NP20-013	Liliaceae	0.0
Arctii Fructus	*Arctium lappa*	NP20-003	Compositae	15.9
Aristolochiae Fructus	*Aristolochia contorta*	NP20-166	Aristolochiaceae	0.0
Aristolochiae Radix	*Aristolochia contorta*	NP20-115	Aristolochiaceae	0.0
Artemisiae Annuae Herba	*Artemisia annua*	NP20-064	Compositae	3.9
Artemisiae Capillaris Herba	*Artemisia capillaries*	NP20-110	Compositae	0.2
Asparagi Radix	*Asparagus cochinchinensis*	NP20-181	Liliaceae	16.1
Broussonetiae Fructus	*Broussonetia papyrifera*	NP20-191	Moraceae	17.9
Buddlejae Flos	*Buddleja officinalis*	NP20-022	Loganiaceae	10.1
Bupleuri Radix	*Bupleurum falcatum*	NP20-007	Unbelliferae	14.0
Coicis Semen	*Coix lachryma-jobi var. mayueu*	NP20-093	Gramineae	3.6
Commelinae Herba	*Commelina communis*	NP20-020	Commelinaceae	6.5
Coptidis Rhizome	*Coptis chinensis*	NP20-026	Ranunculaceae	49.1
Cremastrae Appendiculatae Tuber	*Cremastra appendiculata*	NP20-058	Orchidaceae	2.8
Curcumae Radix	*Curcuma aromatica*	NP20-140	Zingiberaceae	69.7
Cynanchi Atrati Radix	*Cynanchum atratum*	NP20-065	Asclepiadaceae	3.5
Cyperi Rhizome	*Cyperus rotundus*	NP20-113	Cyperaceae	0.0
Dictamni Radicis Cortex	*Dictamnus dasycarpus*	NP20-030	Rutaceae	47.8
Dioscoreae Tokoro Rhizome	*Dioscorea tokoro*	NP20-103	Dioscoreaceae	2.3
Eriocauli Herba	*Eriocaulon sieboldianum*	NP20-021	Eriocaulaceae	20.6
Erythrinae Cortex	*Erythrina variegata var. orientalis*	NP20-080	Compositae	3.5
Euphorbiae Kansui Radix	*Euphorbia kansui*	NP20-073	Euphorbiaceae	2.0
Euphorbiae Pekinensis Radix	*Euphorbia pekinensis*	NP20-074	Euphorbiaceae	13.3
Fritillariae Cirrhosae Bulbus	*Fritillaria cirrhosa*	NP20-156	Liliaceae	0.0
Gardeniae Fructus	*Gardenia jasminoides*	NP20-017	Rubiaceae	0.2
Gastrodiae Rhizome	*Gastrodia elata*	NP20-173	Orchidaceae	27.4
Glycyrrhizae Radix	*Glycyrrhiza uralensis*	NP20-177	Leguminosae	80.5
Junci Medulla	*Juncus effusus var. decipiens*	NP20-108	Juncaceae	0.0
Kochiae Fructus	*Kochia scoparia*	NP20-104	Chenopodiaceae	0.0
Liriopis Tuber	*Liriope platyphylla*	NP20-180	Liliaceae	0.0
Lithospermi Radix	*Lithospermum erythrorhizon*	NP20-036	Boraginaceae	4.1
Lonicerae Caulis	*Lonicera japonica*	NP20-063	Caprifolaceae	1.7
Lonicerae Flos	*Lonicera japonica*	NP20-037	Caprifolaceae	7.6
Luffae Fructus Retinervus	*Luffa cylindrical*	NP20-086	Cucurbitaceae	4.5
Lycii radicis Cortex	*Lycium chinensis*	NP20-066	Solanaceae	16.5
Lygodii Spora	*Lygodium japonicum*	NP20-101	Pteridaceae	1.6
Mori Cortex	*Morus alba*	NP20-164	Moraceae	0.0
Mori Folium	*Morus alba*	NP20-004	Moraceae	13.8
Mori Ramulus	*Morus alba*	NP20-084	Moraceae	8.5
Persicae Semen	*Prunus persica*	NP20-145	Amygdalaceae	17.2
Platycodi Radix	*Platycodon grandiforum*	NP20-154	Campanulaceae	1.1
Polygonati Odorati Rhizoma	*Polygonatum odoratum var. pluriflorum*	NP20-183	Liliaceae	16.2
Polygoni Avicularis Herba	*Polygonum aviculare*	NP20-105	Polygonaceae	0.5
Polygoni Cuspidate Rhizoma	*Polygonum cuspidatum*	NP20-143	Polygonaceae	61.7
Polygoni Multiflori Ramulus	*Polygonum multiflorum*	NP20-171	Polyporaceae	12.3
Polyporus	*Polyporus umbellatus*	NP20-091	Polyporaceae	4.1
Poria	*Poria cocos*	NP20-090	Polyporaceae	2.7
Portulacea Herba	*Portulaca oleracea*	NP20-050	Polyporaceae	6.9
Prunellae Spica	*Prunella vulgaris var. lilacina*	NP20-018	Labiatae	0.0
Pruni Semen	*Prunus humilis*	NP20-072	Amygdalaceae	6.3
Puerariae Radix	*Pueraria thunbergiana*	NP20-006	Leguminosae	1.5
Pulsatillae Radix	*Pulsatilla koreana*	NP20-051	Ranunculaceae	0.5
Pyrrosiae Folim	*Pyrrosia lingua*	NP20-102	Polypodiaceae	7.8

Table 1   (*Cont.*)

Name of the herbal drug	Plant name	Voucher specimen No.	Family	Percent inhibition of degranulation[a]
Raphani Semen	*Raphanus sativa var. hortensis*	NP20-119	Cruciferae	0.0
Rehmannoae Radix	*Rehmannia glutinosa*	NP20-032	Scrophulariaceae	2.1
Rubiae Radix	*Rubia cordifolia*	NP20-138	Rubiaceae	81.5
Salviae Miltiorrhizae Radix	*Salvia miltiorrhiza*	NP20-142	Labiatae	13.7
Sanguisorbae Radix	*Sanguisorba officinalis*	NP20-131	Rosaceae	22.9
Saururi Herba	*Saururus chinensis*	NP20-109	Saururaceae	68.5
Scrophulariae Radix	*Scrophularia buergeriana*	NP20-033	Scrophulariaceae	8.2
Sesami semen Nigrum	*Sesamum indicum*	NP20-190	Pedaliaceae	14.9
Siegesbeckiae Herba	*Siegesbeckia glabrescens*	NP20-085	Compositae	23.3
Smilacis Glabrae Rhizoma	*Smilax glabra*	NP20-046	Liliaceae	6.3
Sojae Semen Praeparatum	*Glycine max*	NP20-010	Leguminosae	0.7
Sophorae Radix	*Sophora flavescens*	NP20-029	Leguminosae	33.4
Trachelospermi Caulis	*Trachelospermum jasminoides*	NP20-083	Apocyanaceae	5.8
Trichosanthis Fructus	*Trichosanthes kirilowii*	NP20-155	Cucurbitaceae	1.3
Trichosanthis Radix	*Trichosanthes kirilowii*	NP20-015	Cucurbitaceae	2.3
Tritici Immatri Semen	*Triticum aestivum*	NP20-192	Graminae	18.3
Typhae pollen	*Typha orientalis*	NP20-137	Typhaceae	16.5
Uncariae Ramulus et Uncus	*Uncaria rhynchophylla*	NP20-172	Rubiaceae	24.1
Vaccariae Semen	*Vaccaria segetalis*	NP20-148	Caryophyllaceae	55.4
Ziziphi Spinosae Semen	*Ziziphus spinosa*	NP20-168	Rhamnaceae	11.3
0.1% DMSO				2.1
PP2				92.5

[a]Percent inhibition of degranulation was determined by measuring the release of the granule marker β-hexosaminidase in the media as described in Materials and Methods. The degranulation by antigen was $32 \pm 2.3\%$ in the RBL-2H3 mast cells. 0.1% DMSO and 10 μM PP2 were used as the negative and positive control, respectively.
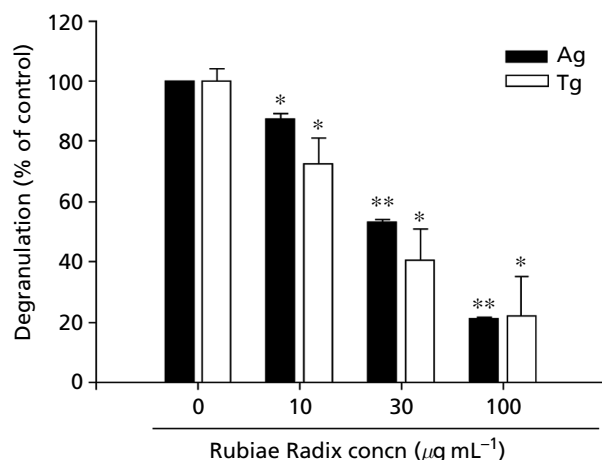

Figure 1   Rubiae Radix inhibits antigen (Ag)- or thapsigargin (Tg)-induced degranulation. RBL-2H3 cells were incubated overnight in 24-well cluster plates with 20 ng mL^{-1} DNP-specific IgE in a complete growth medium. The medium was replaced with a PIPES-buffered medium that contained the indicated concentration of Rubiae Radix extract before stimulation with 25 ng mL^{-1} DNP-BSA (■) or 150 nM thapsigargin (□) to measure the release of β-hexosaminidase. The values are the mean ± s.e.m. from three independent experiments.

Semen showed potent activity in-vitro in the mast-cell assay system (Table 1). The extract of Curcumae Radix consists of multiple effective ingredients, such as β-elemene and curcumol. They are the main anti-neoplasm ingredient, which can be used for therapy against neoplastic disease, especially liver neoplasm (Deng et al 2004). Glycyrrhizae Radix is reported to have antispasmodic, carminative and antidote activity and is also taken for allergic-inflammatory disease, gastrointestinal problems, liver disorders and for bladder and kidney ailments. The main constituents of Glycyrrhizae Radix include triterpene saponins, flavonoids and coumarins. The oleanane-type saponins have been demonstrated to be the main bioactive principles of Glycyrrhizae Radix, which is bioactive in various ways, such as an antiviral and anti-inflammatory (reviewed by Gao et al 2004). Polygoni Cuspidate Rhizoma, a well known traditional Chinese medicine and officially listed in the Chinese Pharmacopoeia, has traditionally been used for the treatment of various inflammatory diseases, hepatitis tumours and diarrhoea in East Asian countries, such as China, Korea, Taiwan and Japan. Polygoni Cuspidate Rhizoma includes piceid, resveratrol, anthraglycoside B, emodin and physcion for its various pharmacological actions. Resveratrol and piceid inhibit the copper-catalysed oxidation of low-density lipoprotein and inhibit platelet clotting and arachido-
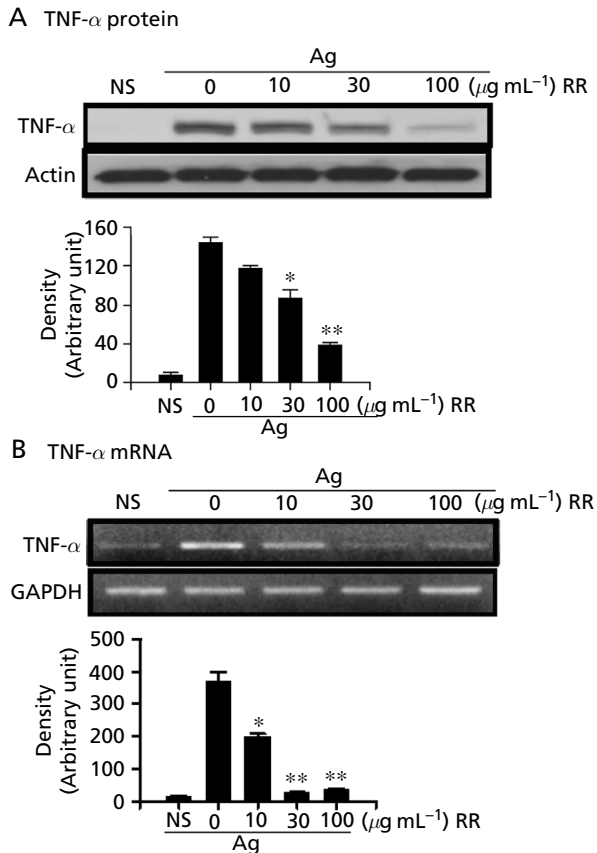
A TNF-α protein

B TNF-α mRNA

Figure 2 Effect of Rubiae Radix (RR) on the expression of TNF-α in mast cells. The indicated amounts of Rubiae Radix were added to the RBL-2H3 cultures 30 min before the addition of 25 ng mL^{-1} DNP-BSA, or were left unstimulated (NS) after incubating overnight with 20 ng mL^{-1} DNP-BSA specific IgE. The cells were stimulated for 4 h for the assay of the TNF-α protein by immunoblotting (A), or for 15 min for the assay of TNF-α mRNA by RT-PCR (B). The results were representative immunoblots or gel pictures from the three independent experiments.

Table 2 Protective activity of Rubiae Radix on compound 48/80-induced anaphylaxis in mice

Dose (mg kg^{-1})[a]	Compound 48/80 (8 mg kg^{-1})[b]	Survival rate (%)[c]
Vehicle	+	0.0
30	+	24.3 ± 4.3
100	+	31.5 ± 11.5
300	+	48.6 ± 8.5*
300	−	100.0
1000 (sodium cromoglicate)	+	75.0

[a]Administered orally in 200 μL/20 g mouse 1 h before the intraperitoneal injection of compound 48/80. Rubiae Radix extract was suspended in 5% Arabic gum. Eight mice were tested for each of the indicated doses. [b]Administered intraperitoneally in 200 μL/20 g mouse. [c]Measured 1 h after injection of compound 48/80, values are the means of three independent experiments.

were reported as the ingredients of Vaccariae Semen (Yun et al 1998).

Although the pharmacological activity and composition of the above extracts have been reported, less is known about the anti-allergic activity of the extracts. We focused on Rubiae Radix because it has been used as a folk remedy for allergic purpura and bronchitis. Although several active constituents of Rubiae Radix have been identified (Huang 1993) and examined for anti-tumour activity (Itokawa et al 1983, 1984), there has not been a systemic investigation of anti-allergic activity. In this study, we investigated whether Rubiae Radix has in-vitro and in-vivo anti-allergic activity. As expected, Rubiae Radix strongly inhibited antigen- or thapsigargin-stimulated degranulation in mast cells and in-vivo compound-48/80-induced anaphylaxis in mice (Figure 1, Table 2). Because we utilized the whole methanol extract of Rubiae Radix, the active ingredients that possessed the anti-allergic activity were not clear and the dose studied was relatively high for application in man. Therefore, further study to identify its active components should be undertaken.

Mast cells are major sources of inflammatory mediators, some of which are preformed and stored in secretory granules and others, such as the cytokines and lipid-derived eicosanoids, are generated de-novo. The release of inflammatory mediators can be stimulated through the Ag-induced aggregation of receptors with high affinity for IgE (FcεRI), which leads to the activation of the tyrosine kinase Syk and, ultimately, to the activation of phosphatidylinositol 3-kinase (PI-3K) (Turner & Kinet 1999) and the mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAP kinase (Hirasawa et al 1995b, 1998; Jabril-Cuenod et al 1996; Zhang et al 1997; Ishizuka et al 1999), in addition to the mobilization of calcium ions (Beaven et al 1996). To investigate the mechanism of action of Rubiae Radix, the kind of signals in the cells that were inhibited by Rubiae Radix to prevent
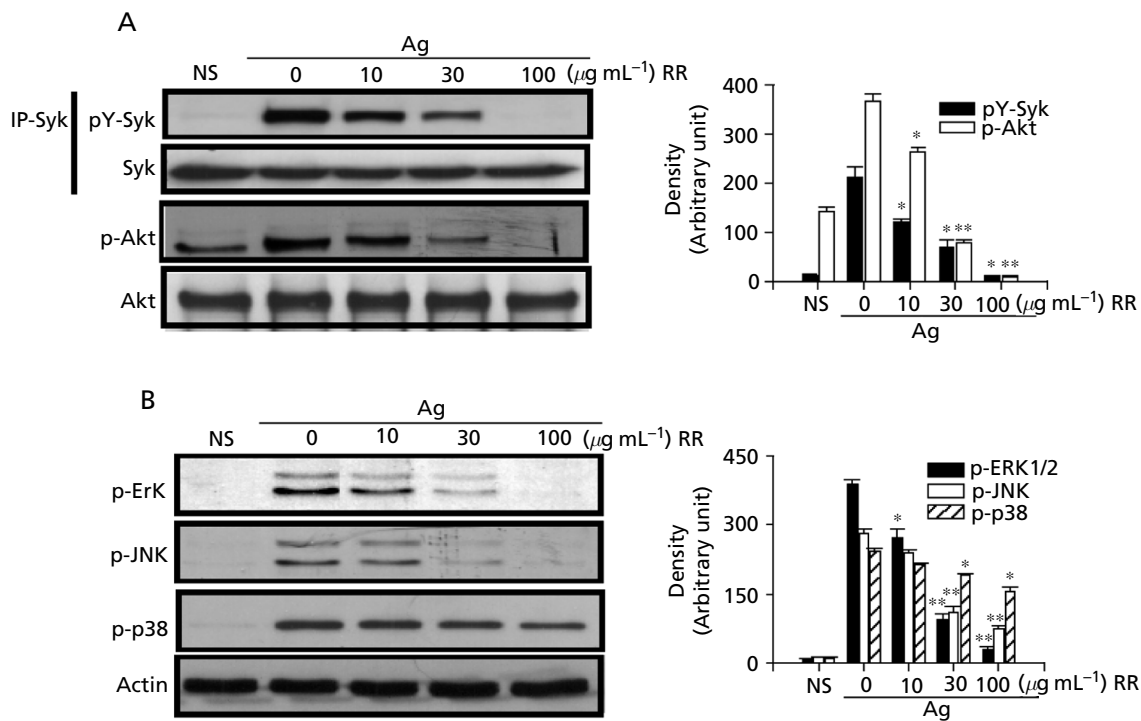
nate metabolism (Chu et al 2005). Many ingredients from Rubiae Radix have been reported. Specially, anthraquinones, including alizarin, purpurin, pseudopurpurin and cyclohexapeptide alkaloids have been identified as having anti-neoplastic actions (Itokawa et al 1983, 1984; Namba 1993). Although the extract of Rubiae Radix has been used as an anti-inflammatory folk remedy for treating allergic purpura and bronchitis, the active components are not identified at present. Extracts of Saururi Herba have been used for many years in oriental folk medicine to treat maladies such as oedema, gonorrhoea and jaundice. Recently, it has been reported that manassantin A and B suppress NF-κB activation by TNF-α and lipopolysaccharide (LPS) (Lee et al 2003). Vaccariae Semen (seed) has been used to activate blood flow and promote milk secretion, and also to treat amenorrhoea and breast infection in China. Eight triterpenoid saponins, vaccarosides A-H and two sapogenins, segetalic acid and vaccaric acid,

Figure 3 Effect of Rubiae Radix (RR) on the activating phosphorylation of Syk, Akt and MAP kinases. The RBL-2H3 cells were incubated overnight in 6-well plates with $25\,ng\,mL^{-1}$ DNP-specific IgE in a complete growth medium. The cells were stimulated with $25\,ng\,mL^{-1}$ DNP-BSA, with or without Rubiae Radix, for 15 min. The lysed proteins were subjected to immunoblot analysis to detect Syk, Akt, MAP kinases and phosphorylated proteins with specific primary antibodies or anti-phosphotyrosine antibodies, respectively, as described in Materials and Methods. Representative blots from three experiments are shown.

their activation was determined. As a result, the activating phosphorylation of Syk, a critical early-signalling protein in mast-cell signalling processes, was significantly inhibited by Rubiae Radix (Figure 3A). The cells have a unique signalling pathway: IgE receptor (FcεRI) →Lyn → Syk →LAT →SLP-76 → PLCγ → Ca^{2+}/PKCβ → degranulation. Many research groups are looking for new materials that could target early-signalling molecules, like Lyn or Syk, as anti-allergic agents (reviewed by Kovarova & Rivera 2004). The results in this study strongly suggest that Rubiae Radix targets the upstream-signalling molecules, such as Src-family kinases, which are critical for the phosphorylation of Syk in mast cells. The possibility, though, that Rubiae Radix inhibits autophosphorylation and other upstream-signalling molecules cannot be discounted.

Stimulated mast cells also produce a variety of cytokines, including interleukins 1, 3, 4, 5 and 6, as well as TNF-α and the granulocyte-macrophage colony-stimulating factor (Galli 1993; Baumgartner & Beaven 1996). Typically, the increased expression of cytokine mRNA and protein is detectable within 30 min and for several hours after the addition of a stimulant (Gordon et al 1990). Such cytokines, particularly TNF-α, are thought to mediate pathogenic inflammatory reactions at the later stages of the allergic reaction (Galli 1993). The activation of ERK1/2 was reported as being an essential signal for the production of IL-5, TNF-α, IL-3, and IL-13 in mast cells (Lorentz et al 2003), and the

production of TNF-α in the RBL-2H3 mast cells is dependent on the activation of the ERK2 cascade (Zhang et al 1997). In investigating the mechanism of action of Rubiae Radix, we focused on the ERK1/2 activation cascade because of the two aforementioned reports. Based on the findings that both the expression of TNF-α and ERK1/2 activation were suppressed by Rubiae Radix (Figures 2 and 3B), it can be concluded that the inhibition of TNF-α expression by Rubiae Radix is very much correlated with the inhibition of the ERK1/2 cascade. It can likewise be concluded that Rubiae Radix may be used to treat late-phase allergic symptoms, as well as reactions, as a challenge to antigens in allergic diseases.

Conclusions

In this study, it was reported for the first time that Rubiae Radix dose-dependently inhibited the degranulation of RBL-2H3 cells by DNP-BSA antigen and thapsigargin with IC50 values of $35 \pm 2.1\,\mu g\,mL^{-1}$ and $26 \pm 2.8\,\mu g\,mL^{-1}$, respectively, and that it effectively suppressed an in-vivo anaphylactic animal reaction. In addition, Rubiae Radix inhibited the expression of TNF-α in a dose-dependent manner as well as the activation of ERK1/2, which is a critical signalling event for the production of TNF-α. The results suggest that Rubiae Radix may be used to treat various IgE-
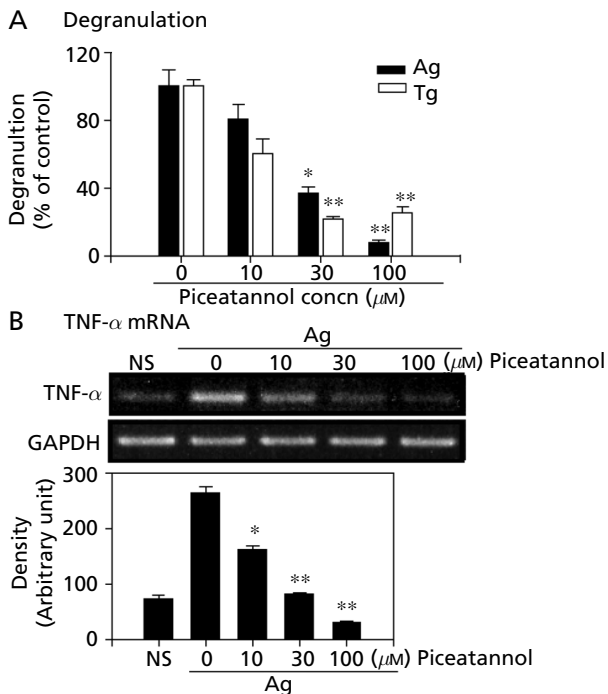
Figure 4 Effect of piceatannol, a Syk inhibitor, on degranulation and TNF-α expression. A. RBL-2H3 cells were incubated overnight in 24-well cluster plates with 20 ng mL^{-1} DNP-specific IgE in a complete growth medium. The medium was replaced with a PIPES-buffered medium that contained the indicated concentration of piceatannol as indicated before stimulation with 25 ng mL^{-1} DNP-BSA (Ag) ($\blacksquare$) or 150 nM thapsigargin (Tg) ($\square$) to measure the release of β-hexosaminidase. The values are the mean $\pm$ s.e.m. from three independent experiments. B. The indicated amounts of piceatannol were added to the cells 30 min before the addition of 25 ng mL^{-1} DNP-BSA, or were left unstimulated (NS) after incubating overnight with 20 ng mL^{-1} DNP-BSA specific IgE. The cells were stimulated for 15 min for the assay of TNF-α mRNA by RT-PCR. The results are representative gel pictures from three independent experiments.

mediated allergic diseases. More studies should be conducted, though, to identify the active components of Rubiae Radix.

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