



Meliae cortex extract exhibits anti-allergic activity through the inhibition of Syk kinase in mast cells

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Abstract

The anti-allergic action of various Oriental medicinal herbs was investigated using in vitro and in vivo experimental models. Of these extracts, the ethanol extract of *Meliae cortex* (MC) exhibited the most potent activity in mast cells; its IC₅₀ values were 29±1.5 µg/ml for antigen stimulation and 57±3.4 µg/ml for thapsigargin stimulation. It inhibited compound-48/80-induced systemic anaphylaxis by 52.9% at a dose of 300 mg/kg in mice; it also inhibited the expression of the proinflammatory mediator TNF-α. With regard to its mechanism of action, MC suppressed the activating phosphorylation of Syk, a key enzyme in mast-cell signaling 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. Taken together, these results suggest that the anti-allergic activity of MC may be due to the inhibition of histamine secretion and cytokine expression through the Syk inhibition in mast cells.

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Keywords: *Meliae cortex*; Allergic reaction; Mast cells; Syk kinase; MAP kinase

Introduction

Allergic rhinitis, asthma, atopic dermatitis, and atopic eczema are among the common causes of chronic ill health. It is well known that IgE-dependent mast cell activation is associated with the allergic diseases. Upon IgE-dependent activation, mast cells rapidly secrete the preformed and de novo synthesized allergic mediators such as histamine, cytokines, proteases, and arachidonic derivatives. Consequently, various acute and chronic allergic responses are induced by these mediators (Church and Levi-Schaffer, 1997; Metcalfe et al., 1981). Although there are many approaches to the treatment of these diseases, such as allergen-specific immunotherapy, DNA

vaccination, monoclonal anti-IgE antibody administration, soluble IL-4 receptor treatment, and treatment with antagonists to leukotriene and histamine receptors, certain difficulties and side effects are encountered while employing these therapies (Kay, 2001a, 2001b). Hence, various approaches are being explored to develop new therapies. Among them, complementary and alternative medicine is one such promising field.

In Asian countries, many herb extracts have been used as traditional folk remedies for treating various diseases. However, the active components and mechanisms of action of most herbs are largely unknown, and few herbs have been screened for their in vitro and in vivo pharmacological activity. Therefore, a systematic study of these herbal remedies must be conducted. Recently, various medicinal herbs have been reported to exhibit in vitro and in vivo anti-allergic activities (Lee et al., 2006; Nagai et al., 2004; Makino et al., 2003; Kim et al., 2003; Lee

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Table 1
Effects of medicinal herb extracts on the antigen-induced degranulation in mast cells

Name of the herbal drug	Plant name and authority	Voucher specimen number	Family	Percent inhibition of hexosaminidase ^a
Achyranthis bidentatae radix	<i>Achyranthes bidentata</i>	NP20-146	Amaranthaceae	26.5
Adenophorae radix	<i>Adenophora triphylla</i> var. <i>japonica</i>	NP20-179	Campanulaceae	18.8
Agrimoniae herba	<i>Agrimonia pilosa</i>	NP20-125	Rosaceae	8.3
Akebiae caulis	<i>Akebia quinata</i>	NP20-099	Lardizabalaceae	0.0
Albiziae cortex	<i>Albizia julibrissin</i>	NP20-170	Leguminosae	19.6
Alismatis rhizome	<i>Alisma plantago-aquatica</i> var. <i>orientale</i>	NP20-092	Alismataceae	9.5
Aloe	<i>Aloe vera</i>	NP20-070	Liliaceae	3.7
Ampelopsis radix	<i>Ampelopsis japonica</i>	NP20-056	Vitaceae	3.8
Belamcandae rhizome	<i>Belamcanda chinensis</i>	NP20-048	Iridaceae	71.7
Bitae cacumen	<i>Biota orientalis</i>	NP20-133	Cupressaceae	27.0
Bletillae rhizome	<i>Bletilla striata</i>	NP20-126	Orchidaceae	10.1
Sappan lignum	<i>Caesalpinia sappan</i>	NP20-149	Leguminosae	47.6
Carpesii fructus	<i>Carpesium abrotanoides</i>	NP20-122	Compositae	1.0
Cassiae semen	<i>Cassia tora</i>	NP20-174	Leguminosae	0.0
Celosiae semen	<i>Celosia argentea</i>	NP20-023	Amaranthaceae	8.9
Chrysanthemi flos	<i>Chrysanthemum morifolium</i>	NP20-005	Compositae	11.6
Cimicifugae rhizome	<i>Cimicifuga heracleifolia</i>	NP20-008	Ranunculaceae	18.9
Cirsii japonici herba	<i>Cirsium japonicum</i> var. <i>ussuriense</i>	NP20-129	Compositae	30.8
Aurantii immaturus fructus	<i>Citrus aurantium</i>	NP20-111	Rutaceae	3.7
Aurantii fructus	<i>Citrus aurantium</i>	NP20-112	Rutaceae	0.0
Clerodendri trichotomi folium	<i>Clerodendron trichotomum</i>	NP20-087	Verbenaceae	3.2
Daphnis genkwa flos	<i>Daphne genkwa</i>	NP20-075	Thymelaeaceae	82.6
Dendrobii herba	<i>Dendrobium nobile</i>	NP20-182	Orchidaceae	0.0
Dianthi herba	<i>Dianthus superbus</i>	NP20-106	Caryophyllaceae	0.4
Ecliptae herba	<i>Eclipta prostrata</i>	NP20-188	Compositae	12.8
Equiseti hiemalis herba	<i>Equisetum hiemale</i>	NP20-012	Equisetaceae	6.3
Eriobotryae folium	<i>Eriobotrya japonica</i>	NP20-162	Malaceae	1.5
Forsythiae fructus	<i>Forsythia koreana</i>	NP20-038	Oleaceae	4.4
Fraxini cortex	<i>Fraxinus rhynchophylla</i>	NP20-052	Oleaceae	1.5
Gentianae radix	<i>Gentiana scabra</i> var. <i>buergeri</i>	NP20-028	Gentianaceae	8.8
Ginkgo semen	<i>Ginkgo biloba</i>	NP20-167	Ginkgoaceae	10.9
Glycyrrhizae radix	<i>Glycyrrhiza uralensis</i>	NP20-177	Leguminosae	86.8
Hordei fructus	<i>Hordeum vulgare</i> var. <i>hexastichon</i>	NP20-117	Graminae	0.0
Imperatae rhizome	<i>Imperata cylindrica</i> var. <i>koenigii</i>	NP20-135	Gramineae	4.5
Laminariae thallus	<i>Laminaria japonica</i>	NP20-160	Laminariaceae	5.4
Leonuri herba	<i>Leonurus sibiricus</i>	NP20-144	Labiatae	2.0
Leonuri semen	<i>Leonurus sibiricus</i>	NP20-151	Labiatae	38.2
Lepidii semen	<i>Lepidium apetalum</i>	NP20-165	Cruciferae	1.3
Ligustri lucidi fructus	<i>Ligustrum lucidum</i>	NP20-189	Oleaceae	18.7
Lilii bulbis	<i>Lilium lancifolium</i>	NP20-185	Liliaceae	21.4
Malvae semen	<i>Malva verticillata</i>	NP20-107	Caryophyllaceae	0.5
Meliae cortex	<i>Melia azedarach</i> L. var. <i>japonica</i>	NP20-120	Meliaceae	85.1
Toosendan fructus	<i>Melia toosendan</i>	NP20-114	Meliaceae	0.0
Menthae herba	<i>Mentha arvensis</i> var. <i>piprerascens</i>	NP20-002	Labiatae	8.3
Oldenlandiae diffusae herba	<i>Oldenlandia diffusa</i>	NP20-054	Rubiaceae	5.0
Paeoniae radix rubra	<i>Paeonia lactiflora</i>	NP20-035	Paeoniaceae	17.2
Paeoniae radix alba	<i>Paeonia lactiflora</i>	NP20-178	Paeoniaceae	17.2
Moutan cortex	<i>Paeonia suffruticosa</i>	NP20-034	Paeoniaceae	0.0
Paridis rhizome	<i>Paris verticillata</i>	NP20-044	Liliaceae	3.6
Patriniae radix	<i>Patrinia villosa</i>	NP20-054	Valerianaceae	2.2
Peucedani radix	<i>Peucedanum decursivum</i>	NP20-153	Umbelliferae	0.0
Pharbitidis semen	<i>Pharbitis nil</i>	NP20-076	Convolvulaceae	4.7
Phaseoli semen	<i>Phaseolus calcaratus</i>	NP20-095	Leguminosae	8.2
Phaseoli radiate semen	<i>Phaseolus radiatus</i>	NP20-059	Leguminosae	5.9
Phellodendri cortex	<i>Phellodendron amurense</i>	NP20-027	Rutaceae	14.5
Phragmitis rhizoma	<i>Phragmites communis</i>	NP20-014	Graminae	2.3
Phyllostachys folium	<i>Phyllostachys nigra</i> M. var. <i>henonis</i>	NP20-016	Bambusaceae	14.3
Phyllostachys folium	<i>Phyllostachys nigra</i> M. var. <i>henonis</i>	NP20-016	Bambusaceae	0.0
Phytolaccae radix	<i>Phytolacca esculenta</i>	NP20-077	Phytolaccaceae	2.0
Picrorrhizae rhizoma	<i>Picrorrhiza scrophulariaeflora</i>	NP20-068	Scrophulariaceae	2.1
Plataginis semen	<i>Plantago asiatica</i>	NP20-098	Plantaginaceae	0.0
Rhei radix et rhizoma	<i>Rheum palmatum</i>	NP20-069	Polygonaceae	82.4
Rumexcis radix	<i>Rumex japonicus</i>	NP20-136	Polygonaceae	16.7
Scutellariae radix	<i>Scutellaria baicalensis</i>	NP20-025	Labiatae	80.5

Table 2 (continued)

Name of the herbal drug	Plant name and authority	Voucher specimen number	Family	Percent inhibition of hexosaminidase ^a
Sophorae fructus	<i>Sophora japonica</i>	NP20-024	Leguminosae	4.5
Sophorae tonkinensis radix	<i>Sophora tinkinensis</i>	NP20-049	Leguminosae	29.3
Sparganii rhizoma	<i>Sparganium stoloniferum</i>	NP20-141	Sparganiaceae	14.0
Spirodela herba	<i>Spirodela polyrhiza</i>	NP20-011	Lemnaceae	9.8
Stellariae radix	<i>Stellaria dichotoma</i> var. <i>lanceolata</i>	NP20-067	Caryophyllaceae	6.4
Sinomeniae tetrandrae radix	<i>Stephania tetrandra</i>	NP20-078	Menispermaceae	5.5
Strychni semen	<i>Strychnos nux-vomica</i>	NP20-082	Loganiaceae	1.7
Taraxaci herba	<i>Taraxacum mongolicum</i>	NP20-039	Compositae	6.9
Taxilli ramulus	<i>Taxillus chinensis</i>	NP20-089	Loranthaceae	3.0
Tetraoanacis medulla	<i>Tetrapanax papyriferus</i>	NP20-100	Araliaceae	0.0
Torreyae semen	<i>Torreya nucifera</i>	NP20-123	Torreyaceae	0.4
Viola herba	<i>Viola yedoensis</i>	NP20-040	Violaceae	33.6
Maydis stigmata	<i>Zea mays</i>	NP20-096	Graminae	0.0
0.1% DMSO				2.1
PP2				92.5

^a Percent inhibition of degranulation was determined by the measurement of the release of the granule marker β -hexosaminase 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 μ M PP2 were used as the negative or positive control, respectively.

et al., 2004a, 2004b). As a continuing effort, we aimed to screen extracts from various Oriental medicinal herbs that have been used as traditional folk remedies in Korea and China and have an in vitro and in vivo anti-allergic activity. *Meliae cortex*, also known as Go-Ryun-Pi in Korea, is a dried stem bark or root bark of *Melia azedarach* Linne var. *japonica* Makino (Meliaceae). The extract of the dried bark is known to have been traditionally used to treat ascariasis, oxyuriasis, abdominal pain due to intestinal parasitosis, scabies, tinea, and dermatosis. Although some active constituents of MC have been reported (Kim, 2001), limited information is available regarding its pharmacological activities in vitro and in animal models. From the present findings, it is evident that MC exhibits potent anti-allergic activity through the inhibition of mast cells by suppressing the activating phosphorylation of Syk and mitogen-activated protein (MAP) kinases. The results suggested that MC 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 cromolyn sodium were purchased from Sigma Chemical Co. (St Louis, MO). The minimal essential medium (MEM) and other cell-culture reagents were obtained from GIBCO/Life Technologies, Inc. (Rockville, MD). The antibodies were purchased from the following sources: antibodies against phosphotyrosine (pY) (4G10), Akt, ERK1/2, p38, and JNK from Upstate Biotechnology (Lake Placid, NY); antibodies against Syk, and TNF- α from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Animals. Male ICR mice (aged 4 weeks) were purchased from the Dae Han Experimental Animal Center (Eumsung, 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 $22 \pm 1^\circ\text{C}$ and a relative humidity of $55 \pm 10\%$ were maintained throughout the study. The animal study was done in accordance with the institutional guidelines and the protocol was approved by the Institutional Animal Care and Use Committee at Konkuk University before study.

Preparation of ethanol extract of *Meliae cortex* (MC). Various herbs, which are expected to be anti-inflammatory agents, were selected for screening on the basis of Kimi's classifications in "Bon-Cho-Hak," 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. 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% ethanol was added. After allowing the mixture to stand at room temperature for 7 days, the ethanol extracts were filtered and dried in a 50°C water bath with the use of a vacuum pump evaporator. The voucher specimens (numbered as in Table 1) were deposited at the College of Medicine in Konkuk University. The extracts were dissolved as 100 mg/ml in 100% DMSO and diluted to 100 μ g/ml in 0.1% DMSO with a PIPES-buffered medium (25-mM PIPES, pH 7.2, 159-mM NaCl, 5-mM KCl, 0.4-mM MgCl₂, 1-mM CaCl₂, 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 studies, we used 0.1% DMSO for in vitro assay and 5% Arabic gum for 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 15% fetal-bovine serum (FBS). In each experiment, the cells were harvested through trypsinization, were 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-ng/ml DNP-specific IgE to achieve 100% occupancy of Fc ϵ RI. 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-ng/ml 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*-nitrophenol from *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide was measured. The values were expressed as percentages of intracellular β -hexosaminidase that were released into the medium. The IC₅₀ values, the concentration necessary to obtain 50% inhibition of the response, were calculated through nonlinear regression analysis using the GraphPad software (San Diego, CA, USA).

Preparation and activation of BMNC. Bone marrow mast cells from male Balb/cJ mice were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics, and 10% fetal calf serum) and 50% WEHI-3 cell conditioned medium as a source of IL-3. After 3 weeks, >98% of the cells was found to be BMNC checked by the previously described procedure (Murakami et al., 1999). For cell stimulation, IgE-primed BMNC were washed and resuspended in Tyrode-BSA buffer (20 mM HEPES at pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.05% BSA) before stimulation with 20-ng/ml DNP-BSA for 10 min.

Compound-48/80-induced systemic anaphylaxis. Systemic anaphylaxis was induced by the mast-cell degranulator compound 48/80 (Lee et al., 2006; Alfonso et al., 2000). Each mouse was given an intraperitoneal injection of 8 mg/kg of compound 48/80 in saline. MC was suspended in 5% Arabic gum and were administered orally in doses of 30–300 mg/kg 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 a 25-ng/ml antigen (DNP-BSA) for 10 min or as indicated, were chilled with ice to terminate the stimulation, and were then washed two times with ice-cold PBS and lysed in 0.5 ml with 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- $\mu\text{g/ml}$ pepstatin, and a protease-inhibitor cocktail tablet). The lysates were kept on ice for 30 min and were then centrifuged $15,000\times g$ for 15 min at 4 °C. The supernatant fraction was “precleared” through the addition of 50- μl 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 (at 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_3VO_4 , 1-mM PMSF, 2.5-mM nitrophenylphosphate, 0.7- $\mu\text{g/ml}$ pepstatin, and a protease-inhibitor cocktail tablet) before being loaded onto a polyacrylamide gel.

Immunoblot analysis. The cell lysates or immunoprecipitated were denatured by boiling at 95 °C for 5 min in a $2\times$ Laemmli buffer (Laemmli, 1970). The proteins were separated with the use of SDS-PAGE and were transferred to nitrocellulose membranes (Schleicher and Schuell, BA85). After blocking in 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 individual monoclonal or polyclonal antibodies. The immunoreactive proteins were detected with the use of horseradish 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-ng/ml DNP-specific IgE to achieve 100% occupancy of Fc ϵ RI. The cells were washed and medium replaced with a PIPES-buffered medium and stimulated by 25-ng/ml DNP-BSA for 15 min without or with various concentrations of MC 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 TNF- α forward 5'-CACCACGCTCTCTGTCTACTGAAC-3', reverse 5'-CCGGAATCCGTGATGTCTAAGTACT-3'; rat GAPDH forward 5'-GTGGAGTCTACTGGCGTCTTC-3', reverse 5'-CCAAGGCTGTGGGCAAGTCA-3'.

Statistical analysis. The data were presented as the mean $\pm$ 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, USA).

Results

Effects of MC and other Oriental medicinal herbs on antigen- or thapsigargin-induced degranulation in mast cells

During the continuous search for novel anti-allergic agents from natural products (Lee et al., 2006, 2004a, 2004b; Kim

et al., 2003; Nagai et al., 2004), assay systems were employed for the screening of potential suppressors of degranulation in cultured mast cells. In this study, approximately 100 ethanol extracts of Oriental medicinal herbs were primarily evaluated for their effect on antigen-stimulated RBL-2H3 mast cells. We observed that the *Belamcandae rhizoma*, *Daphnis genkwa flos*, *Glycyrrhizae radix*, *Meliae cortex*, *Rhei radix et rhizoma*, and *Scutellariae radix* ethanol extracts exhibited potent inhibition of degranulation (more than 50% inhibition at the test concentration of 100 $\mu\text{g/ml}$) in the cells (Table 1). Consistent with a previous report, the *Glycyrrhizae radix* extract significantly inhibited degranulation in the cells (Lee et al., 2004a). Among these medicinal extracts, MC was subjected to further study because it has been used as a traditional folk remedy for dermatosis in Korea. MC significantly suppressed antigen- or thapsigargin-induced degranulation in a dose-dependent manner (Fig. 1). The IC_{50} values for the antigen and thapsigargin, a well-known calcium-ion inducer that blocks the uptake of Ca^{2+} into IP_3 -sensitive stores (Putney and Bird, 1993), were approximately 29 ± 1.5 $\mu\text{g/ml}$ and 57 ± 3.4 $\mu\text{g/ml}$, respectively.

Inhibition of TNF- α mRNA and protein expression

Next, we investigated whether or not MC inhibited the expression of TNF- α , one of key mediators in the mast-cell-dependent inflammatory events. MC significantly suppressed the antigen-stimulated expression of the TNF- α protein and mRNA in a dose-dependent manner (Figs. 2A and B).

Effects of MC on cellular signaling molecules in an IgE-mediated signaling pathway

To investigate how MC inhibited TNF- α expression and degranulation in mast cells, we measured the effects of MC on the activation of cellular signaling molecules in these cells. First, we tested whether MC suppressed the antigen-induced

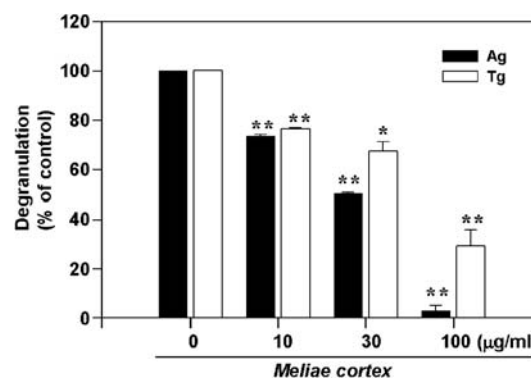


Fig. 1. MC inhibits antigen- or thapsigargin-induced degranulation. RBL-2H3 cells were incubated overnight in 24-well cluster plates with 20-ng/ml DNP-specific IgE in a complete growth medium. The medium was replaced with a PIPES-buffered medium that contained the indicated concentration of MC before stimulation with 25-ng/ml DNP-BSA (■) or 150-nM thapsigargin (□) to measure the release of β -hexosaminidase. The values are the mean $\pm$ SEM from the three independent experiments.

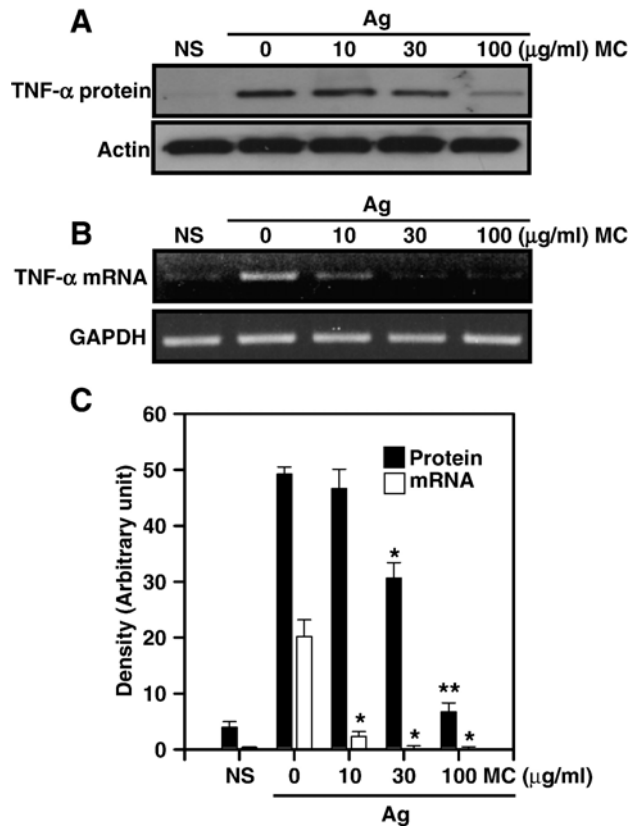


Fig. 2. Effects of MC on the expression of TNF- α in mast cells. The indicated amounts of MC were added to the RBL-2H3 cultures 30 min before the addition of 25-ng/ml DNP-BSA, or were left unstimulated (NS) after incubating overnight 20-ng/ml DNP-BSA-specific IgE. The cells were stimulated (A) for 4 h for the assay of the TNF- α protein by immunoblotting, and (B) for 15 min for the assay of TNF- α mRNA by RT-PCR. The results were representative immunoblots or gel pictures from the three independent experiments. (C) Band densities are presented as the mean $\pm$ SEM from the three independent experiments.

activating phosphorylation of Syk, a critical signaling enzyme involved in the process of mast-cell activation (reviewed by Siraganian et al., 2002). As shown in Fig. 3A, the activating phosphorylation of Syk was significantly inhibited in a dose-dependent manner. To further prove the inhibition of the pathway, we also determined whether MC inhibited the downstream-signaling molecule phosphatidylinositol 3-kinase (PI-3K)/Akt, as indicated by the activating phosphorylation of Akt. Akt is activated through a pathway that requires PI-3K-dependent generation of phosphatidylinositol 3,4,5-trisphosphate and is thus sensitive to the inhibition of PI-3K. We found that the activating phosphorylation of Akt was inhibited by MC (Fig. 3A).

Effects of MC on the activating phosphorylation of MAP kinases

The production of TNF- α , a major proinflammatory cytokine, was inhibited by MC (Figs. 2A and B). Recently, extracellular signal-regulated kinase (ERK) 1/2 was reported to be the signal for the production of IL-5, TNF- α , IL-3, and

IL-13 in mast cells (Lorentz et al., 2003). In addition, ERK2 activates phospholipase A2 (cPLA2), resulting in the production of arachidonic acid, which is the precursor of various

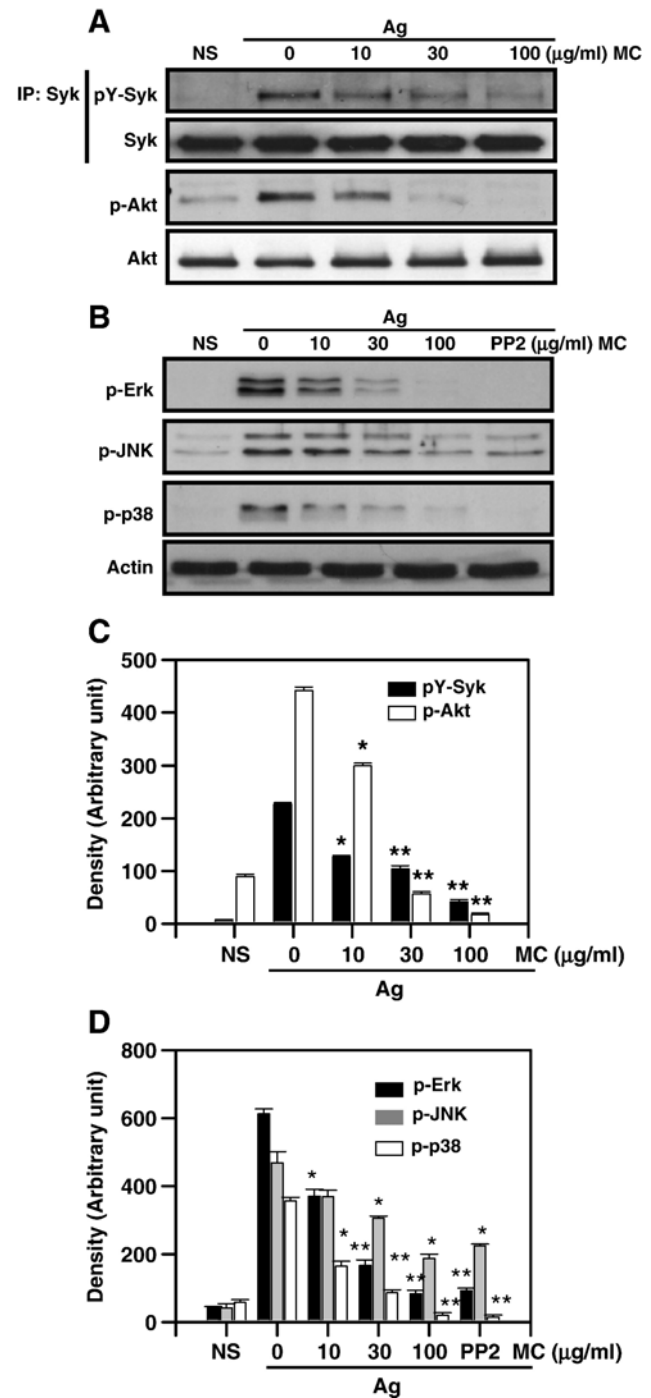


Fig. 3. Effect of MC on the activating phosphorylation of Syk, Akt, and MAP kinases. The RBL-2H3 cells were incubated overnight in 6-well plates with 25-ng/ml DNP-specific IgE in a complete growth medium. The cells were stimulated with 25-ng/ml DNP-BSA, with or without MC, for 15 min. The proteins derived from cell lysates were subjected to immunoblot analysis to detect phosphorylated forms of Syk, Akt (A), and MAP kinases (B) as described in Materials and methods. Representative blots from the three experiments are shown. Band densities for phospho-Syk, phospho-Akt (C), and phospho-MAP kinases (D) are presented as the mean $\pm$ SEM from the three independent experiments, respectively.

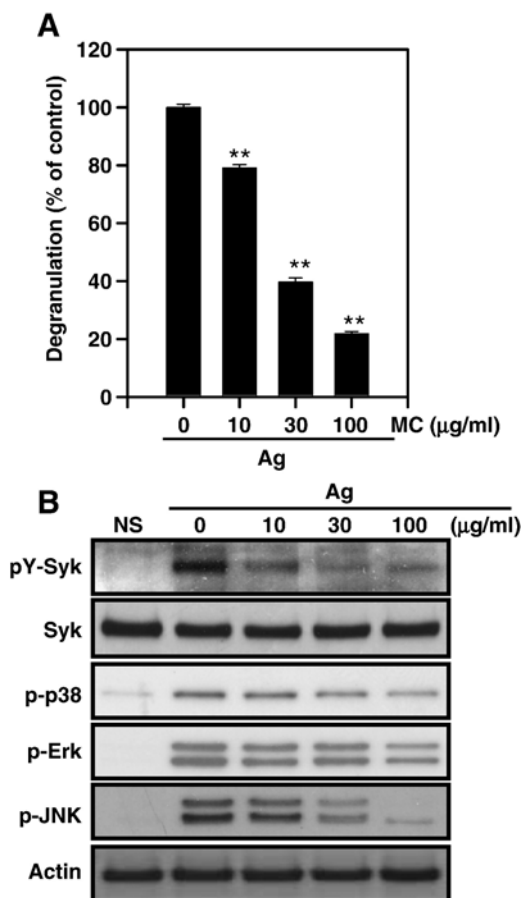


Fig. 4. Effect of MC on degranulation, the activating phosphorylation of Syk and MAP kinases in BMMC. The bone marrow-derived mast cells were incubated with 50 ng/ml DNP-specific IgE in a complete growth medium. The cells were stimulated with 25-ng/ml DNP-BSA, with or without MC, for 10 min. β -Hexosaminidase was measured in medium and cell lysates as described in Materials and methods (A). The proteins derived from cell lysates were subjected to immunoblot analysis to detect the phosphorylated forms of Syk and MAP kinases as described in Materials and methods. Representative blots from the three independent experiments are shown (B).

inflammatory mediators such as leukotriene C_4/B_4 and PGD_2 (Hirasawa et al., 1995a,1995b). To investigate the mechanism of action of MC, its cellular effect on the activating phosphorylation of three MAP kinases – ERK1/2, p38, and c-Jun N-terminal kinase (JNK) – was determined. As shown in Fig. 3B, the activating phosphorylations of ERK1/2, p38, and JNK were significantly inhibited by MC in a dose-dependent manner.

Effects of MC on degranulation and cellular signaling stimulated by antigen in bone marrow-derived mast cells

To further confirm the activity of MC, we isolated the primary mast cells from bone marrow of Balb/cJ mice as described in the Materials and methods section. Consistent with the results from RBL-2H3 mast cells, MC significantly suppressed antigen-induced degranulation (Fig. 4A), the activating phosphorylation of Syk, and three MAP kinases (Fig. 4B) in a dose-dependent manner. These results suggested

that MC may have potent anti-allergic action in the animal models.

MC inhibits compound-48/80-mediated anaphylaxis in mice

The above in vitro results led us to measure the in vivo anti-allergic activity of MC by using a compound-48/80-mediated anaphylactic animal model. As the previous reports, compound 48/80 successfully induced anaphylactic death of ICR mice within 1 h of intraperitoneal injection of 8 mg/kg of the compound (Lee et al., 2006; Nagai et al., 2004). Consistent with the in vitro results, MC showed 52.9% survival rate at the dose of 300 mg/kg in the compound-48/80-induced anaphylactic animal model (Table 2).

Discussion

Type I hypersensitivity allergic reaction is induced by the rapid local and systemic release of allergic inflammatory mediators such as histamine, serotonin, heparin, and various proinflammatory cytokines from mast cells that are located throughout the human body (Kemp and Lockey, 2002). In the present study, we aimed to identify the extracts from various Oriental medicinal herbs that have been used as traditional folk remedies in Korea and China and have in vitro and in vivo anti-allergic activities. We observed that the *Belamcandae rhizoma*, *Daphnis genkwa flos*, *Glycyrrhizae radix*, *Meliae cortex*, *Rhei radix et rhizoma*, and *Scutellariae radix* ethanol extracts exhibited potent inhibition of degranulation in mast cells (Table 1). We further studied the effects of MC because it has been used as a folk remedy for dermatosis. Although some of its pharmacological components have been previously identified (Kim, 2001), a systemic study for elucidating its anti-allergic activity has not been conducted. Hence, we investigated whether MC has in vitro and in vivo anti-allergic activities. As expected, MC strongly inhibited the activation of mast cells and in vivo compound-48/80-induced anaphylaxis in mice (Fig. 1 and Table 2).

Mast cells, a therapeutic target for the treatment of allergic diseases, are the major sources of inflammatory mediators; some mediators are preformed and stored in secretory granules, and others such as cytokines and lipid-derived eicosanoids are

Table 2

Protective activity of MC on compound 48/80-induced anaphylaxis

Dose (mg/kg) ^a	Compound 48/80 (8 mg/kg) ^b	Survival rate (%) ^c
Vehicle	+	0.0
30	+	17.2
100	+	21.4
300	+	52.9
300	–	100.0

^a Administered po by 200 μ l/20 g mouse 1 h before the injection of compound 48/80. MC was suspended in 5% Arabic gum. Eight mice were tested for each of the indicated doses.

^b Administered ip by 200 μ l/20 g mouse.

^c Measured 1 h after the injection of compound 48/80, values are the means of two independent experiments.

generated de novo. The release of inflammatory mediators can be stimulated through the antigen-induced aggregation of receptors with a high affinity for IgE (FcεRI); this leads to the activation of the tyrosine kinase Syk. Ultimately, it results in the activation of PI-3K (Turner and Kinet, 1999) and the MAP kinases, namely, ERK, 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, 1996). To investigate the mechanism of action of MC, we determined the kind of cellular signals that were inhibited by MC to prevent the activation of the mast cells. We observed that the activating phosphorylation of Syk, a critical early signaling protein in mast-cell signaling processes, was significantly inhibited by MC (Fig. 3A). Mast cells have the following unique signaling pathway: IgE receptor (FcεRI) → Lyn → Syk → LAT → SLP-76 → PLCγ → Ca²⁺/PKCβ → degranulation. Many research groups are exploring novel materials that could target early signaling molecules like Lyn or Syk in order to use them as anti-allergic agents (reviewed by Kovarova and Rivera, 2004). The results of this study may suggest that MC targets the upstream-signaling molecules such as the Src-family kinases that are critical for the phosphorylation of Syk in mast cells. However, the possibility that MC inhibits autophosphorylation of Syk and other upstream-signaling molecules could not be excluded.

Stimulated mast cells also produce a variety of cytokines, including IL-1, 3, 4, 5, and 6; TNF-α; and the granulocyte-macrophage colony-stimulating factor (Galli, 1993). Typically, the increased expression of cytokine mRNA and protein is detectable within 30 min and several hours after the addition of a stimulant (Gordon et al., 1990). Such cytokines, particularly TNF-α, are believed to mediate pathogenic inflammatory reactions at the later stages of the allergic reaction (Galli, 1993). The activation of ERK1/2 was reported to be 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 depends on the activation of the ERK2 cascade (Zhang et al., 1997). Because of the two aforementioned reports, the ERK1/2 activation cascade was focused on while investigating the mechanism of action of MC. Based on the findings that both TNF-α expression and ERK1/2 activation were suppressed by MC (Figs. 2 and 3B), it can be concluded that the inhibition of TNF-α expression by MC is highly correlated with the inhibition of the ERK1/2 cascade.

In conclusion, it was reported for the first time that MC dose-dependently inhibited the degranulation of RBL-2H3 cells and BMMC, and that it effectively suppressed an in vivo anaphylactic animal reaction. In addition, MC inhibited TNF-α expression and MAP kinase activation, the latter being a critical signaling event for TNF-α production. The results suggest that MC may be used to treat various acute and chronic IgE-mediated allergic diseases. However, since we utilized the entire extract, the active ingredients that exhibit the anti-allergic activity were not revealed, and the dose studied was relatively high for application in humans. Further studies are required to determine the components of MC that are responsible for the anti-allergic activity.

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