doi: 10.1111/j.1365-2222.2008.02936.x
Clinical and Experimental Allergy, **38**, 794–804

ORIGINAL PAPER **Basic Mechanisms in Allergic Disease**

Camellia japonica suppresses immunoglobulin E-mediated allergic response by the inhibition of Syk kinase activation in mast cells

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Clinical and Experimental Allergy

Summary

Background Novel approaches are being explored to develop new therapies for various allergic diseases. Complementary and alternative medicines are considered to be promising avenues for the development of such new therapies.

Objectives To investigate the effect of many Korean plants on the IgE-mediated allergic response in mast cells and *in vivo*, and its mechanism of action.

Materials and methods The anti-allergic activity was tested by evaluating effects on degranulation of mast cells in culture and passive cutaneous anaphylaxis (PCA) *in vivo*. Its mechanism of action was investigated by immunoblotting analysis, immunoprecipitation, RT-PCR, and other molecular biological approaches in mast cells.

Results We screened approximately 100 natural plant extracts collected in Korea for *in vitro* anti-allergic activity. The leaf extract of *Camellia japonica* (LECJ) exhibited the most potent effect on degranulation in antigen-stimulated rodent and human mast cells. LECJ reversibly inhibited degranulation in a dose-dependent manner, with IC_{50} values of ~50 μg/mL for the mast cells, and it also suppressed the expression and secretion of TNF-α and IL-4 in rat basophilic leukaemia-2H3 mast cells. In agreement with its *in vitro* activity, LECJ significantly inhibited mast cell-mediated PCA in an animal model. LECJ inhibited activating phosphorylation of tyrosine Y371 on Syk kinase, indicating that LECJ inhibits the activity of Src-family kinases in mast cells. In the *in vitro* kinase assay, LECJ directly inhibited Lyn kinase, the major Src-family kinase in the cells. It also suppressed Akt and MAP kinases, which are critical for the production of various pro-inflammatory cytokines in mast cells. In high-performance liquid chromatography analysis, quercetin-3-β-D-glucoside and eugenol were identified as the major active components.

Conclusion The present results strongly suggest that the anti-allergic activity of LECJ is mediated through inhibiting degranulation and allergic cytokine secretion by inhibition of Src-family kinase in mast cells and it may be useful for the treatment of mast cell-related immediate and delayed allergic diseases.

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Keywords allergy, *Camellia japonica*, mast cells, Src-family kinase, Syk kinase
Submitted 20 August 2007; revised 27 November 2007; accepted 2 December 2007

Introduction

Antigen-induced release of preformed inflammatory mediators from mast cells causes the immediate symptoms of IgE-mediated allergic diseases, including allergic rhinitis, asthma, atopic dermatitis, and atopic eczema [1, 2]. The activation of signalling pathways in antigen-stimulated mast cells depends initially on the interaction of FcεRI with the Src kinases, Lyn and Fyn, and subsequently on the downstream activation of Syk and other tyrosine kinases [3, 4]. The binding of Syk to the tyrosine phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) of FcεRIγ is mediated by its two SH2 domains, and results in a conformational change in Syk

that increases its enzymatic activity [5]. This leads to the downstream propagation of signals for the activation of LAT, SLP-76, Gab2, phospholipase Cγ, phospholipase D, and other signalling molecules. Subsequently, mast cells rapidly release various allergic mediators, including histamine, cytokines, and arachidonic acid derivatives [3] that mediate various acute and chronic allergic reactions [6, 7].

There are, however, many approaches to the treatment of allergic diseases, such as allergen-specific immunotherapy, DNA vaccination, administration of humanized anti-IgE antibody, soluble IL-4 receptor treatment, and treatment with antagonists to leukotriene and histamine receptors. Certain difficulties and side-effects, however, are associated with these therapies [8, 9]. Consequently, novel approaches are being explored to develop new therapies, including blocking mast cell activation with tyrosine kinase inhibitors [10]. Also, complementary and alternative medicines provide a promising avenue for the development of new therapies. Many herbal extracts are used as traditional folk remedies for treating diseases in Asian countries, including Korea, Japan, and China. In the course of continuing efforts to find active plant extracts to treat allergic diseases [11–14], we screened approximately 100 extracts of natural plants collected in Korea, and report for the first time that the leaf extract of *Camellia japonica* L. (LECJ) exhibits potent *in vitro* and *in vivo* anti-allergic activity through inhibiting mast cells. LECJ suppressed the activating phosphorylation of Syk kinase and mitogen-activated protein (MAP) kinases, most likely through the direct inhibition of Src-family kinase.

Materials and methods

Materials

Minimal essential medium (MEM) and other cell-culture reagents were purchased from GIBCO/Life Technologies Inc. (Rockville, MD, USA). DNP-BSA, DNP-specific monoclonal IgE, formamide, Arabic gum, and diphenylhydramine (DPH) were from Sigma Chemical Co. (St. Louis, MO, USA), and PP2 was obtained from Calbiochem (La Jolla, CA, USA). Monoclonal or polyclonal antibodies were purchased from the following sources: antibodies against the phosphorylated forms of Akt (polyclonal), ERK1/2 (monoclonal), JNK (polyclonal), p38 (polyclonal), and Syk (Y317 in murine) from Cell Signalling Technology Inc. (Danvers, MA, USA); antibodies against Syk (polyclonal) and Actin (monoclonal) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); and a monoclonal antibody against phosphotyrosine (4G10) and polyclonal antibodies against LAT, SLP-76, and Gab2 from Upstate Biotechnology (Lake Placid, NY, USA).

Animals

Male ICR mice (age 4 weeks) were purchased from the Dae Han Experimental Animal Center (Eumsung, Korea), and were housed in the animal facilities at the College of Medicine in Konkuk University. Ten mice were placed in a cage fitted with a laminar airflow cabinet. The mice were kept at 22 ± 1 °C and a relative humidity of $55 \pm 10\%$ in a 12 h light–dark cycle. This study was performed in accordance with institutional guidelines.

Preparation of leaf extract of Camella japonica

Fresh leaves of *Camellia japonica* L. (Theaceae) were collected from the Ulleung-do island of Korea in February or October of 2005–2006 and were authenticated by Dr Hyung Kyu Lee at the Korea Research Institute of Bioscience and Biotechnology in Korea. The extract was prepared as follows: dried leaves (100 g) from *C. japonica* L. were extracted with 1000 mL of ethanol at 50 °C, using an ultrasonic cleaner (Branson Ultrasonics Corporation, Danbury, CT). The extracted materials were concentrated with a speed bag (Biotron Corporation, Puchon, Korea) at 40 °C, for 24 h, with an extraction yield of approximately 10% (w/w). A voucher specimen (001-056) was deposited at the College of Medicine, Konkuk University.

Preparation and stimulation of bone-marrow-derived mast cells and rat basophilic leukaemia-2H3 cells

Bone marrow mast cell (BMMC) cultures were isolated from 5-week-old male Balb/cJ mice essentially as described [15]. BMMCs were cultured for up to 6 weeks in complete medium (RPMI 1640, containing 2 mM L-glutamine, 0.1 mM non-essential amino acids, antibiotics, and 10% fetal calf serum) containing 10 ng/mL IL-3. After 3 weeks, >98% of the cells were verified as BMMCs through the procedure described previously [15] and the FcεRIβ subunit was subjected to immunoblot analysis for simple verification. For cell stimulation, BMMCs were primed for 4 h or overnight with 20 ng/mL of DNP-specific IgE. BMMCs were washed and re-suspended 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) before stimulation with 20 ng/mL of the antigen DNP-BSA. Rat basophilic leukaemia (RBL)-2H3 cells were grown as monolayers in MEM with Earle's salts, supplemented with glutamine, antibiotics, and 15% fetal bovine serum (FBS).

Preparation of human mast cells and stimulation

Human mast cells (HMs), derived from $CD34^+$ pluripotent peripheral blood progenitor cells, were cultured as described [16] and were used after 6–9 weeks. $CD34^+$-

peripheral blood-derived HMs were sensitized overnight with 100 ng/mL biotinylated human myeloma IgE. The next day, the cells were pre-incubated for 1 h with LECJ (3–100 μg/mL) or the equivalent concentrations of carrier [dimethyl sulphoxide (DMSO)] and then challenged with streptavidin (1 ng/mL) for 30 min.

Measurement of degranulation in bone-marrow-derived mast cells and rat basophilic leukaemia-2H3 mast cells

Cells were transferred to 24-well (2×10^5 cells/0.4 mL/well) cluster plates and were incubated overnight in complete growth medium with 20 ng/mL DNP-specific IgE. The cultures were washed, and the required buffer solution was added (0.2 mL/well). Experiments on intact cells were performed in a PIPES-buffered medium for RBL-2H3 cells and in a Tyrode buffer for BMMCs. Unless otherwise stated, the cultures were incubated for 30 min, with or without LECJ, before adding 20 ng/mL of the antigen DNP-BSA for 10 min. Degranulation was assessed by measuring the release of the granule marker, β-hexosaminidase, as described previously [12]. IC_{50} values were evaluated by non-linear regression analysis using GraphPad software (San Diego, CA, USA).

Immunoblotting analysis

Mast cells in six-well plates were stimulated with 25 ng/mL antigen (DNP-BSA) for 7 min, and then chilled on ice to terminate the stimulation. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with 0.25 mL of 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 phenylmethylsulphonyl fluoride, 2.5 mM nitrophenylphosphate, 0.7 μg/mL pepstatin, and a protease-inhibitor cocktail tablet). The lysates were kept on ice for 30 min, and then spun down at 15 000 g for 15 min at 4 °C. The cell lysates were denatured at 95 °C for 5 min in a 2× Laemmli buffer [17]. The proteins were separated using SDS-PAGE, and were then transferred to nitrocellulose membranes (Schleicher and Schuell, BA85). Subsequent to blocking in TBS-T buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% skim milk powder, the membrane was incubated with individual primary antibodies. The primary antibodies were diluted 1 : 1000-fold, unless otherwise noted, and were incubated at 4 °C overnight. The membranes were washed three times, for 5 min each, with a TBS-T buffer, and then incubated with horseradish peroxidase (HRP)-coupled secondary antibodies diluted 1 : 2000-fold for 1 h at room temperature, washed five times (for 5 min each) with a TBS-T buffer, and visualized with enhanced chemoluminescence according to the manufacturer's protocols (Amersham Biosciences, Piscataway, NJ, USA).

Reverse transcription-polymerase chain reaction

The RBL-2H3 cells were harvested by trypsinization and were transferred to six-well (1×10^6 cells/3 mL/well) cluster plates, which were incubated overnight in complete growth medium containing 20 ng/mL DNP-specific IgE. The cells were washed, followed by medium replacement with a PIPES-buffered medium, and were stimulated with 25 ng/mL DNP-BSA for 15 min, with or without LECJ, and were washed twice with ice-cold PBS. The total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), and was reverse-transcribed using the Superscript first-strand synthesis system (Invitrogen), according to the manufacturer's protocol. PCR was performed at 94 °C for 45 s, at 55 °C for 45 s, and at 72 °C for 60 s, for 30 cycles. The following primers were used: rat TNF-α forward 5′-CACCACGCTCTTCTGTCTACTGAAC-3′, reverse 5′-CCGGACTCCGTGATGTCTAAGTACT-3′; rat IL-4 forward 5′-ACCTTGCTGTCACCCTGTTC-3′, reverse 5′-TTGTGAGCGTGGACTCATTC-3′; and rat GAPDH forward 5′-GTGGAGTCTACTGGCGTCTTC-3′, reverse 5′-CCAAG GCTGTGGGCAAGGTCA-3′.

Enzyme-linked immunosorbent assay of tumour necrosis factor-α and interleukin-4

The RBL-2H3 cells were stimulated with 25 ng/mL DNP-BSA for 4 h, with or without LECJ. TNF-α and IL-4 concentrations in the culture supernatant were determined using commercial ELISA kits according to the manufacturer's instructions (Invitrogen-Biosource Cytokine & Signalling, Camarillo, CA, USA).

In vitro Lyn *kinase assay*

The final reaction mixture (25 μL), which consisted of 10 mU Lyn kinase (Millipore Corp., Billerica, MA, USA), was incubated with 50 mM Tris pH 7.5, 0.1 mM EGTA, 0.1 mM Na_3VO_4, 0.1% β-mercaptoethanol, 0.1 mg/mL poly (Glu, Tyr) 4 : 1, 10 mM Mg acetate, and [γ^{33}P]-ATP. The reaction was initiated through the addition of the Mg acetate and [γ^{33}P]-ATP. After incubation for 40 min at room temperature, the reaction was stopped through the addition of 5 μL of a 3% phosphoric acid solution. Ten microlitres of the reaction mixture was then spotted onto a Filtermat A (Millipore Corp.) and washed three times for 5 min in 75 mM phosphoric acid and once in methanol before drying and scintillation counting.

Passive cutaneous anaphylaxis

An anti-DNP-BSA-specific IgE (0.5 μg) was intradermally injected into the mouse ear, followed by LECJ treatments ranging from 100 to 1000 mg/kg or 50 mg/kg DPH as a reference drug, 24 h later. One hour after the oral

treatment with either vehicle or LECJ, the mice were challenged with an i.v. injection of a 250 μg antigen (DNP-BSA) in 250 μL PBS containing 4% Evans blue (Sigma-Aldrich, MO, USA). The mice were then euthanized an hour after the challenge of antigen, followed by the removal of the ear for the measurement of the amount of dye extravasated by the antigen. The dye was extracted overnight from the ear in 700 μL formamide at 63 °C, as described previously [18]. The absorbance intensity was measured at 620 nm.

High-performance liquid chromatography analysis of leaf extract of Camellia japonica

For the analysis of the composition of LECJ, the ethanol extract was dissolved in 50% methanol. Before it was injected onto the HPLC, all the samples were filtrated using of a 0.2 μm microspin polyvinylidenefluoride (PVDF) filter. The components of LECJ were determined through an HPLC system (Gilson Unipoint Chromatography Data System, Gilson Inc., Middleton, WI, USA) equipped with 321 pumps and a UV/Vis-151 detector. The constituents of the extract were separated using Shiseido CAPCELL PAK C18 MG 5 μm (4.6 × 150 mm), at a flow rate of 1.0 mL/min. The mobile phase was acetonitrile, with the following gradient programmes: isocratic at 25% acetonitrile for 10 min; a linear gradient to 80% acetonitrile for 30 min; and isoclatic at 80% acetonitrile for 45 min. The analytes were detected through UV absorption at 254 nm.

Statistical analysis

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

Results

Effects of Korean natural plant extracts and leaf extract of Camellia japonica *on antigen-stimulated degranulation and reversibility of inhibition in mast cells*

We used three types of mast cells to investigate the effects of Korean natural plant extracts on degranulation: human mast cells, RBL-2H3 cells, which are an analogue of rat mucosal mast cells [19] and primary mouse BMMCs, which express the IgE high-affinity receptor, FcεRI, at equivalent levels to RBL-2H3 cells (data not shown) following continuous efforts to find anti-allergic agents from natural plant extracts [11–14]. The effects of approximately 100 Korean plant extracts on degranulation of mast cells were assessed in RBL-2H3 mast cells. The plants were collected in Korea and prepared at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). Plant extracts from *Alnus maximowiczii* (stem), *Lindera erythrocarpa* (stem), *Rosa multiflora* (leaf and stem), *Vitis coignetiae* (stem), and *C. japonica* (leaf) inhibited degranulation (over 70%) at 100 μg/mL in RBL-2H3 cells (Table 1), with the LECJ being the most potent. LECJ suppressed antigen-induced degranulation in a dose-dependent manner in all three mast cells (Fig. 1a), with IC_{50} values of ~50 μg/mL. However, they showed no activity on ionomycin- or thasigargin-induced degranulation (Fig. 1b). To investigate the reversibility of LECJ, RBL-2H3 cells were washed five times with PIPES buffer after incubation with LECJ for 1 h, and the residual activity was measured. Mast cells recovered their degranulation response (Fig. 1c), indicating that LECJ inhibition was reversible.

Effect of leaf extract of Camellia japonica *on the antigen-stimulated expression and secretion of tumour necrosis factor-α and interleukin-4 in mast cells*

Cytokines such as TNF-α and IL-4 are critical for allergic inflammation mediated by mast cells [20]. In this study, we investigated whether or not LECJ suppressed the expression and secretion of TNF-α and IL-4 in the cells. LECJ inhibited antigen-stimulated expression and secretion of TNF-α and IL-4 in a dose-dependent manner (Figs 2a and b). The degree of inhibition of 100 μg/mL LECJ was similar to that of 20 μM PP2, a typical Src-family kinase inhibitor.

Mechanism of inhibition of leaf extract of Camellia japonica *in mast cells*

We examined the sequential activation of signalling molecules in antigen-stimulated mast cells. LECJ significantly and dose-dependently inhibited the activating phosphorylation of Syk kinase and its downstream molecule, LAT (Fig. 3a). Three major MAP kinases, Erk1/2, p38, and JNK, are phosphorylated by antigen stimulation, leading to the production of cytokines such as TNF-α and IL-4. LECJ dose-dependently inhibited p38, Erk1/2, and JNK phosphorylation (Fig. 3b). Syk kinase phosphorylation is mediated by Src-family kinases, initially Lyn and possibly other Src kinases [3]. We tested whether LECJ inhibition of Syk phosphorylation occurred via inhibition of Lyn, the first kinase to be activated by receptor aggregation in mast cells. Lyn kinase activity was determined using a specific antibody against phosphorylation on tyrosine 317 of murine Syk kinase (Y352 in human Syk kinase), which is primarily dependent on Lyn [21, 22]. LECJ and PP2, an Src-family kinase inhibitor, potently inhibited the phosphorylation of Y371 of Syk kinase, as well as the Y191 tyrosine residue of LAT in BMMCs

Table 1. Effects of Korean natural plant extracts on the antigen-induced degranulation in mast cells

Plant name	Family	Part extracted	Voucher specimen number	Percent inhibition of degranulation*
Acer tegmentosum	Aceraceae	Stem	002-052	68
Acer truncatum	Aceraceae	Stem	002-035	67.7
Allium grayi	Liliaceae	Straw	002-053	65.1
Alnus maximowiczii	Betulaceae	Leaf	001-060	30.5
Alnus maximowiczii	Betulaceae	Stem	001-061	71.4
Aralia continentalis	Araliaceae	Stem	001-049	51.4
Aster spathulifolius	Compositae	Leaf	001-200	11.7
Aster spathulifolius	Compositae	Stem	001-201	29
Aster spathulifolius	Compositae	Flower	001-202	27.1
Aster spathulifolius	Compositae	Roots	001-203	18.9
Aucuba japonica	Cornaceae	Leaf	001-148	7.9
Aucuba japonica	Cornaceae	Stem	001-149	11.1
Aucuba japonica	Cornaceae	Leaf	001-150	21.7
Aucuba japonica	Cornaceae	Stem-heartwood	001-151	4.9
Aucuba japonica	Cornaceae	Stem-bark	001-152	9.4
Berberis koreana	Berberidaceae	Leaf, stem	002-036	0
Berberis amurensis var. *lactifolia*	Berberidaceae	Stem	001-157	80
Callicarpa dichotoma	Verbenaceae	Fruit	001-179	30.1
Callicarpa japonica var. *leucocarpa*	Verbenaceae	Fruit	001-225	3.5
Camellia japonica	Theaceae	Fruit	001-055	55.4
Camellia japonica	Theaceae	Leaf	001-056	94.5
Camellia japonica	Theaceae	Stem-bark	001-058	65.8
Campanula takesimana	Campanulaceae	Whole plant	002-063	66.2
Caragana sinica	Leguminosae	Stem	002-013	67.8
Carpesium abrotanoides	Compositae	Leaf	001-041	27.1
Carpesium abrotanoides	Compositae	Stem	001-042	19.4
Catalpa bignonioides	Bignoniaceae	Fruit	001-031	34.7
Cayratia japonica	Vitaceae	Leaf	001-008	11.6
Cayratia japonica	Vitaceae	Fruit	001-009	25.7
Celtis choseniana	Ulmaceae	Leaf	001-010	3.7
Celtis choseniana	Ulmaceae	Stem	001-011	4.4
Celtis choseniana	Ulmaceae	Fruit	001-012	10.8
Cinnamomum camphora	Lauraceae	Stem-bark	001-038	10.7
Cinnamomum japonicum	Lauraceae	Stem-bark	001-118	68.4
Clerodendrum trichotomum	Verbenaceae	Leaf	001-039	24.2
Clerodendrum trichotomum	Verbenaceae	Stem	001-040	34.2
Cleyera japonica	Theaceae	Stem-heartwood	001-100	62.6
Cocculus trilobus	Menispermaceae	Fruit	001-046	60.9
Daphniphyllum glaucescens	Euphorbiaceae	Leaf	001-176	6.5
Daphniphyllum glaucescens	Euphorbiaceae	Stem	001-177	17.4
Daphniphyllum macropodum	Euphorbiaceae	Leaf	001-024	7.3
Daphniphyllum macropodum	Euphorbiaceae	Twig	001-025	11.9
Distylium racemosum	Hamamelidaceae	Leaf	001-174	9.5
Elaeagnus glabra	Elaeagnaceae	Leaf	001-088	20.9
Elaeagnus glabra	Elaeagnaceae	Stem	001-089	9.4
Elaeagnus glabra	Elaeagnaceae	Stem-bark	001-092	63.4
Eurya emarginata	Theaceae	Leaf	001-162	50.7
Eurya emarginata	Theaceae	Stem	001-163	37.1
Hedera rhombea	Araliaceae	Leaf	001-147	26.8
Heloniopsis orientalis	Liliaceae	Straw	002-089	60.3
Ilex cornuta	Aquifoliaceae	Leaf	001-209	29.2
Ilex cornuta	Aquifoliaceae	Stem-heartwood	001-210	11.5
Ilex cornuta	Aquifoliaceae	Stem-bark	001-211	11.1
Ilex crenata var. *microphylla*	Aquifoliaceae	Leaf	001-178	24.1
Ilex integra	Aquifoliaceae	Leaf	001-002	33.5
Ilex integra	Aquifoliaceae	Stem-heartwood	001-003	9.1
Koelreuteria paniculata	Sapindaceae	Pericarp	001-079	58.6
Lindera erythrocarpa	Lauraceae	Stem	002-048	71.3
Lindera glauca	Lauraceae	Stem	002-004	27.3

Table 1. continued

Plant name	Family	Part extracted	Voucher specimen number	Percent inhibition of degranulation*
Litsea japonica	Lauraceae	Stem-heartwood	001-029	4.9
Litsea japonica	Lauraceae	Stem-bark	001-030	2.7
Lycoris squamigera	Amaryllidaceae	Leaf	002-058	68
Lycoris squamigera	Amaryllidaceae	Root	002-059	69.8
Machilus japonica	Lauraceae	Twig	001-141	59
Myrica rubra	Myricaceae	Leaf	001-142	40.5
Myrica rubra	Myricaceae	Stem-heartwood	001-143	60.4
Myrica rubra	Myricaceae	Stem-bark	001-144	61.8
Neolitsea aciculata	Lauraceae	Leaf	001-224	2.7
Osmanthus insularis	Oleaceae	Stem-heartwood	001-084	21.7
Osmanthus insularis	Oleaceae	Stem-bark	001-085	15.2
Pseudixus japonicus	Loranthaceae	Straw	001-059	53.3
Quercus glauca	Fagaceae	Leaf	001-180	6.7
Quercus glauca	Fagaceae	Stem-heartwood	001-181	4.7
Quercus glauca	Fagaceae	Stem-bark	001-182	44.8
Quercus salicina	Fagaceae	Leaf	001-183	9.1
Reynoutria sachalinensis	Polygonaceae	Stem	001-159	4.6
Rhodotypos scandens	Rosaceae	Fruit	001-087	32.1
Ribes fasciculatum var. *chinense*	Saxifragaceae	Fruit	001-026	38.2
Rosa multiflora	Rosaceae	Leaf, stem	002-085	73.3
Rubus takesimensis	Rosaceae	Leaf	001-123	16.4
Rubus takesimensis	Rosaceae	Roots	001-124	21.1
Rubus takesimensis	Rosaceae	Steam	001-125	3.2
Sedum takesimense	Crassulaceae	Leaf, Stem	001-121	31.5
Sedum takesimense	Crassulaceae	Roots	001-122	23.7
Silene takesimensis	Caryophyllaceae	Tree	001-168	66
Styrax japonica	Styracaceae	Leaf	001-062	60.1
Taxus cuspidata	Taxaceae	Leaf	002-081	67.7
Taxus cuspidata	Taxaceae	Stem	002-082	67.9
Thuja koraiensis	Cupressaceae	Leaf	002-024	63.6
Trichosanthes kirilowii var. *japonica*	Cucurbitaceae	Seed	001-034	39.7
Tsuga sieboldii	Pinaceae	Leaf	001-145	31.86
Tsuga sieboldii	Pinaceae	Stem	001-146	65.3
Vaccinium bracteatum	Ericaceae	Stem-heartwood	001-081	58.4
Vaccinium bracteatum	Ericaceae	Stem-bark	001-082	63.4
Valeriana officinalis var. *latifolia*	Valerianaceae	Leaf	001-032	22.1
Valeriana officinalis var. *latifolia*	Valerianaceae	Stem,Root	001-033	54.3
Viburnum dilatatum	Caprifoliaceae	Fruit	001-001	40.1
Viburnum erosum	Caprifoliaceae	Stem	002-027	61.2
Viburnum sargentii	Caprifoliaceae	Fruit	001-086	7.9
Vitis coignetiae	Vitaceae	Stem	001-073	70.2
0.1% DMSO				1.7
10 μM PP2				93.2

*Percent inhibition of degranulation was determined by the measurement of the release of the granule marker β-hexosaminidase in the media as described in 'Materials and methods'. They are expressed as the mean values from two independent experiments. The degranulation by antigen was ~45% in the RBL-2H3 cells. PP2 is a general Src-family kinase inhibitor.

DMSO, dimethyl sulphoxide; RBL, rat basophilic leukaemia.

(Fig. 4a). To further prove whether LECJ directly inhibits Lyn kinase, a major Src-family kinase in mast cells, we checked its inhibitory effect on Lyn kinase *in vitro*. As shown in Fig. 4b, LECJ suppressed Lyn kinase activity in a dose-dependent manner. Thus, the result strongly suggests that LECJ inhibits Syk phosphorylation via the inhibition of Src-family kinase in mast cells.

Effect of leaf extract of Camellia japonica *on mast-cell-mediated passive cutaneous anaphylaxis*

Next, we tested whether LECJ inhibited the allergic response in the passive cutaneous anaphylaxis (PCA) mouse model, a mast cell-mediated anaphylaxis model. As shown previously, IgE and antigen injection induced

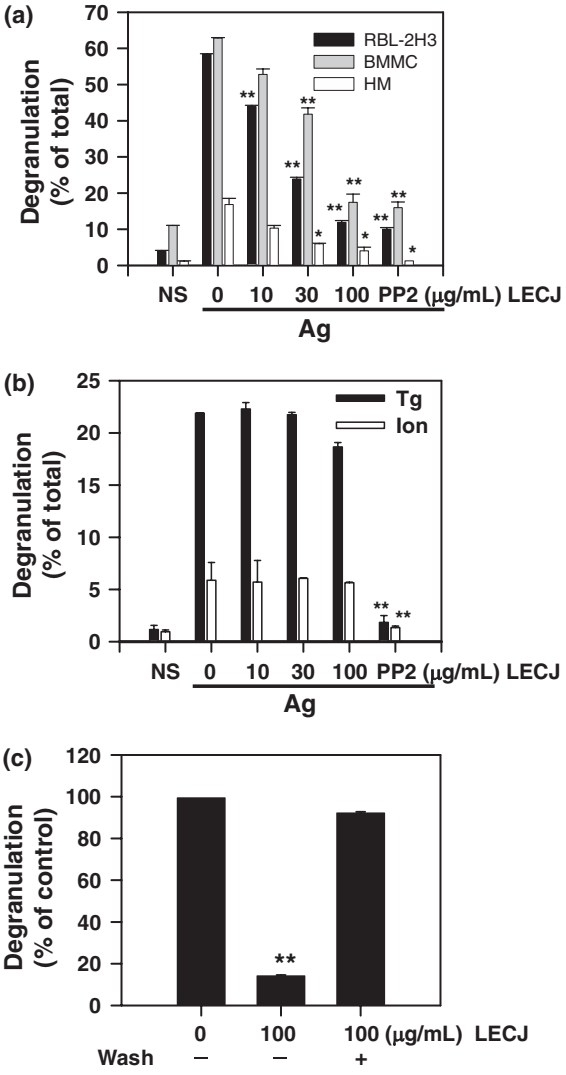

Fig. 1. Effect and reversibility of LECJ on antigen-stimulated degranulation in mast cells. (a) BMMCs or RBL-2H3 cells were incubated overnight in 24-well cluster plates with 20 ng/mL DNP-specific IgE in complete growth medium. The medium was replaced with Tyrode or PIPES buffer that contained the indicated concentration of LECJ before stimulation with 25 ng/mL DNP-BSA for 15 min, to measure the release of β-hexosaminidase. Human mast cells (HM) were sensitized overnight with 100 ng/mL biotinylated human myeloma IgE. The following day, the cells were pre-incubated for 1 h with or without LECJ then challenged with streptavidin (1 ng/mL) for 30 min. (b) RBL-2H3 cells were stimulated with thapsigargin (Tg, 300 nM) or ionomycin (Ion, 1 μM) with or without LECJ for 15 min. (c) RBL-2H3 cells were pre-incubated for 1 h with DMSO or 100 μg/mL LECJ. For recovery, LECJ-treated cells were washed five times with PIPES buffer before the treatment of antigen. The values are expressed as the mean ± SEM from three independent experiments. PP2 (10 μM) is a general Src-family kinase inhibitor. LECJ, leaf extract of *Camellia japonica*; BMMC, bone-marrow-derived mast cells; RBL, rat basophilic leukaemia; DMSO, dimethyl sulphoxide.

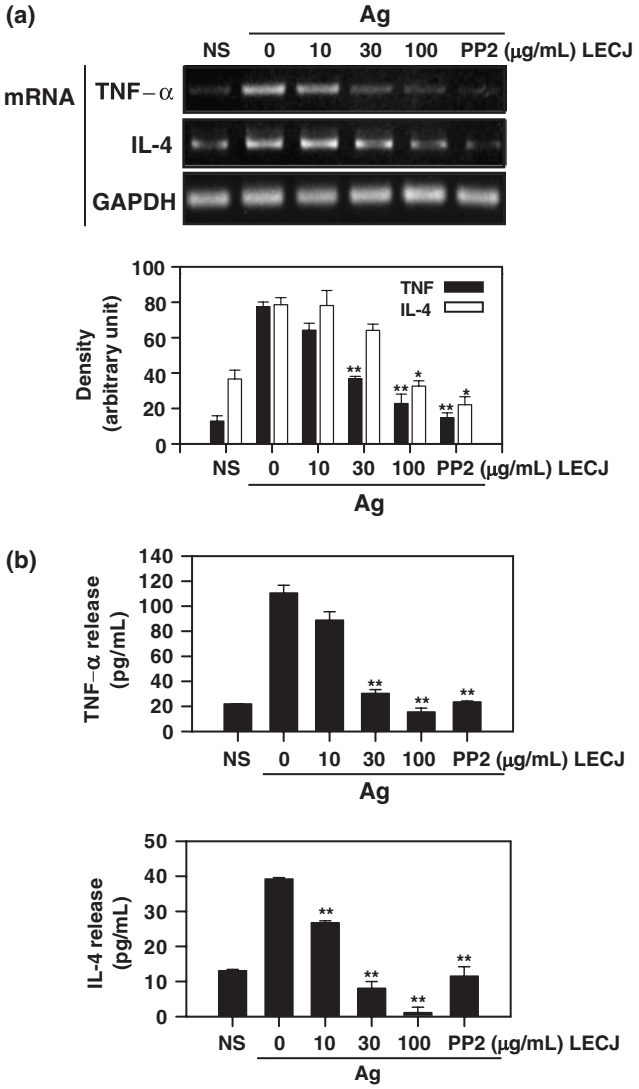

Fig. 2. Effects of LECJ on the expression and secretion of TNF-α and IL-4 in mast cells. (a) LECJ was added to the RBL-2H3 cultures 30 min before the addition of 25 ng/mL DNP-BSA, or cultures were left unstimulated (NS) for 4 h with 20 ng/mL DNP-BSA-specific IgE. The cells were stimulated for 15 min before assaying TNF-α or IL-4 mRNA by RT-PCR. The results are representative gel pictures from three independent experiments. (b) The secretion of TNF-α and IL-4 were measured by ELISA 4 h after stimulating RBL-2H3 cells with 25 ng/mL DNP-BSA. The values are expressed as mean ± SEM for the three independent experiments. PP2 (10 μM) is a general Src-family kinase inhibitor. LECJ, leaf extract of *Camellia japonica*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PCA in mice [11, 18] and LECJ significantly inhibited PCA in a dose-dependent manner with a similar degree as DPH, a typical anti-histamine drug (Fig. 5).

High-performance liquid chromatography analysis of leaf extract of Camellia japonica *and effects of the components on degranulation in mast cells*

To identify the active components of LECJ, it was firstly analysed using HPLC. We then identified the two major peaks: quercetin-3-β-D-glucoside (30.9% AUC, RT 4.6 min) and eugenol (17.8% AUC, RT 17.6 min), respectively (Fig. 6a). Next, we checked whether the components

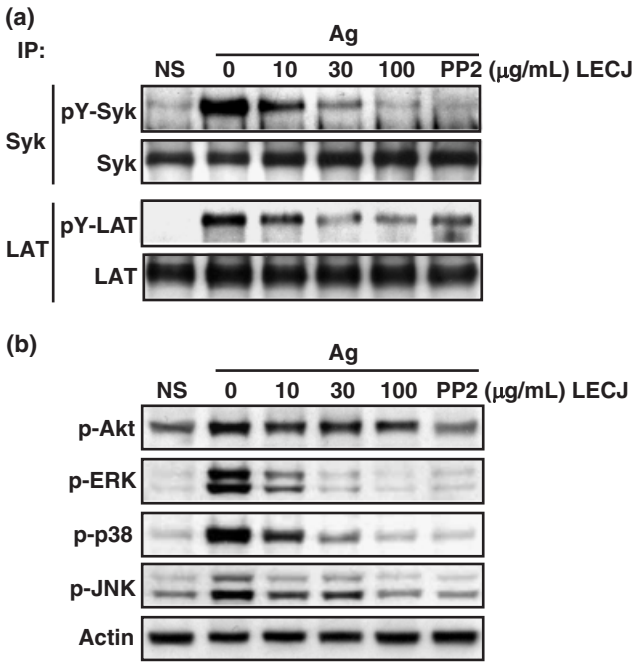

Fig. 3. Effect of LECJ on the activating phosphorylation of Syk, LAT, and MAP kinases in RBL-2H3 mast cells. (a) The RBL-2H3 cells were incubated overnight in six-well plates, with 20 ng/mL DNP-specific IgE, in a complete growth medium. The cells were stimulated with 25 ng/mL DNP-BSA, with or without LECJ, for 7 min. Syk kinase and LAT were immunoprecipitated with specific antibodies and subjected to immunoblot analysis to detect phosphorylated or total proteins. (b) The proteins derived from the RBL-2H3 cell lysates were subjected to immunoblot analysis to detect phosphorylated forms of MAP kinases. PP2 (10 μM) is a general Src-family kinase inhibitor. LECJ, leaf extract of *Camellia japonica*; BMMC, bone-marrow-derived mast cells; MAP, mitogen-activated protein; RBL, rat basophilic leukaemia.

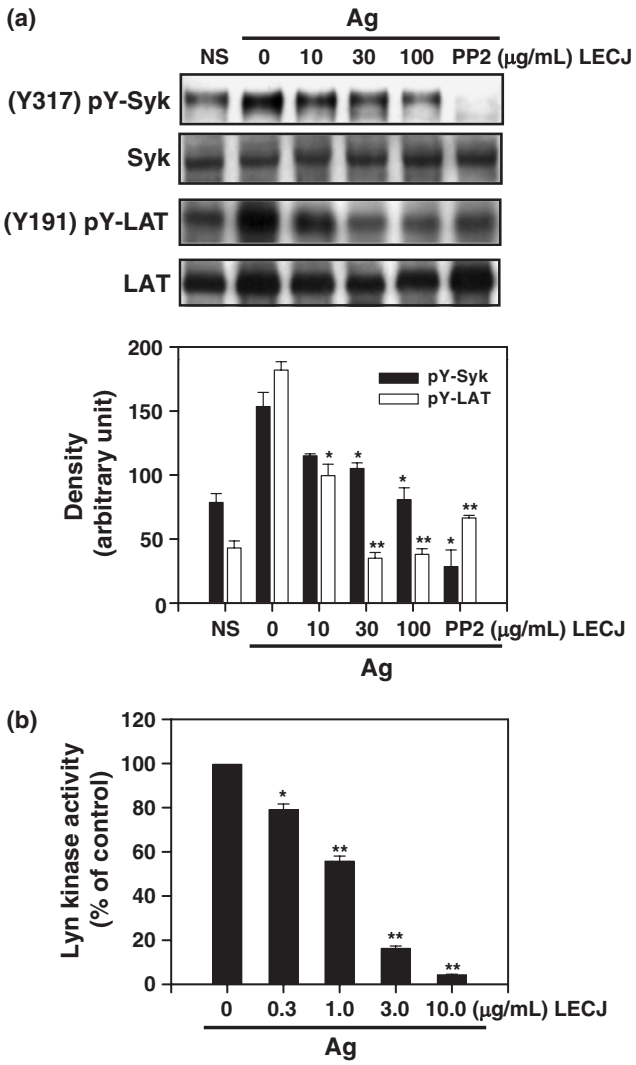

Fig. 4. Effect of LECJ on the phosphorylation of Y317 in Syk kinase in BMMCs and *in vitro* Lyn kinase assay. (a) The BMMCs were incubated with 20 ng/mL DNP-specific IgE in complete growth medium overnight. The cells were stimulated with 25 ng/mL DNP-BSA, with or without LECJ, for 7 min. The proteins derived from cell lysates were subjected to immunoblot analysis to detect the phosphorylated forms (Y317) of Syk. Representative blots are shown. Band densities are expressed as mean ± SEM for three independent experiments. (b) Lyn kinase (10 mU) was incubated in the kinase assay buffer at room temperature for 40 min and the activity was measured as described in 'Materials and methods'. The values are expressed as mean ± SEM for three independent experiments. PP2 (10 μM) is a general Src-family kinase inhibitor. LECJ, leaf extract of *Camellia japonica*; BMMC, bone-marrow-derived mast cells.

exhibited the inhibitory effect on degranulation in mast cells. The result was positive: the components manifested significant inhibitory effects on degranulation in mast cells, with IC_{50} values of ~65 μM for quercetin-3-β-D-glucoside (Fig. 6b) and ~263 μM for eugenol (Fig. 6c).

Discussion

C. japonica L. (Korean name, 'Dong-Baek-Na-Moo'; Japanese name, 'tsubaki') is a garden tree and its fruit is used as a source of oil in Korea and Japan. The leaf extract is also used as a tea in Korea. However, the anti-allergic activity of the LECJ has not been reported to date. In this study, we showed that the anti-allergic activity of LECJ was mediated by inhibition of Syk kinase activation in mast cells.

A 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 pro-inflammatory cytokines from mast cells located throughout the body [23]. Here, LECJ reversibly inhibited degranulation, the release of several allergic mediators in human and rodent mast cells (Fig. 1a). Mast cells are stimulated by IgE-mediated antigen via the high-affinity receptor, FcεRI. Following receptor aggregation, Src-family kinases such as Lyn and Fyn are activated, and subsequently phosphorylate the ITAM motifs of both the β and the γ subunits of FcεRI. The tyrosine-phosphorylated ITAMs then function as scaffolds for binding additional signalling molecules such as adaptors and enzymes [24, 25]. Syk kinase is recruited to the phosphorylated ITAM

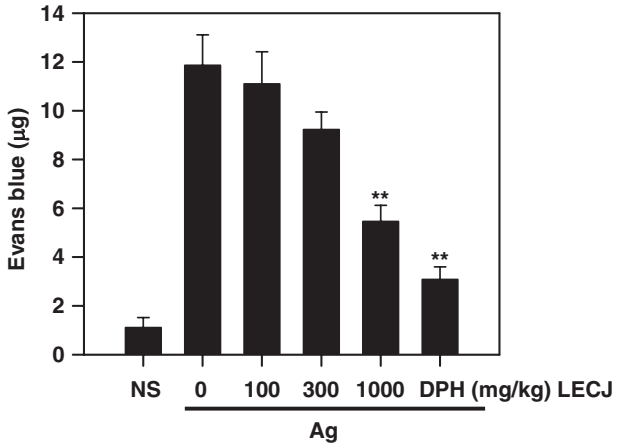

Fig. 5. Effect of LECJ on mast-cell-mediated passive cutaneous anaphylaxis. An anti-DNP-BSA-specific IgE (0.5 μg) was intradermally injected into the mouse ear. An injection of antigen, 250 μg DNP-BSA (1 μg/mL in PBS containing 4% Evans blue), was administered 24 h later into the mouse tail vein. The LECJ dose ranged from 100 to 1000 mg/kg, and was administered 1 h before the treatment of antigen. The mouse was euthanized 1 h after antigen challenge, and the ear was removed to measure the amount of dye extravasated by antigen treatment. The values are expressed as mean ± SEM from three independent experiments. Diphenylhydramine (DPH, 50 mg/kg) is a typical antihistamine drug. LECJ, leaf extract of *Camellia japonica*; PBS, phosphate-buffered saline.

motif of the FcεRIγ subunit and this leads to the downstream activation of signals such as the tyrosine phosphorylation of LAT, Gab2, phospholipase Cγ, and the influx of Ca^{2+} [5]. LECJ inhibited both the activating phosphoryaltion of Syk kinase (Fig. 3a) and a specific phosphorylation of tyrosine residue (Y317) on Syk kinase (Fig. 4a), which is primarily dependent on Lyn activity [21, 22]. Furthermore, LECJ directly inhibited Lyn kinase activity *in vitro* (Fig. 4c). The results strongly suggest that LECJ inhibited Syk kinase phosphorylation by directly inhibiting the Src-family kinase.

Many inflammatory cytokines, including IL-4 and TNF-α, are produced by antigen stimulation in mast cells and mediate pathogenic inflammatory symptoms in later stages of an allergic reaction [20, 26]. ERK1/2 is an essential signal in the production of IL-5, TNF-α, IL-3, and IL-13 in mast cells [27] and in the production of TNF-α in RBL-2H3 mast cells, depending on the activation of the ERK2 cascade [28]. In addition, p38 MAP kinase stimulates IL-4 production in BMMCs [29]. LECJ inhibited TNF-α and IL-4 expression as well as activation of three typical MAP kinases (Fig. 3b), suggesting that these effects may be connected. In fact, MAP kinases are downstream signalling molecules of the Syk/LAT pathway [22, 30] that we showed and is inhibited by LECJ (via inhibition of Src kinase), as does PP2, an Src-family kinase inhibitor.

The pharmacological activities and constituents of *C. japonica*, such as saponins from fruits [31–33] and

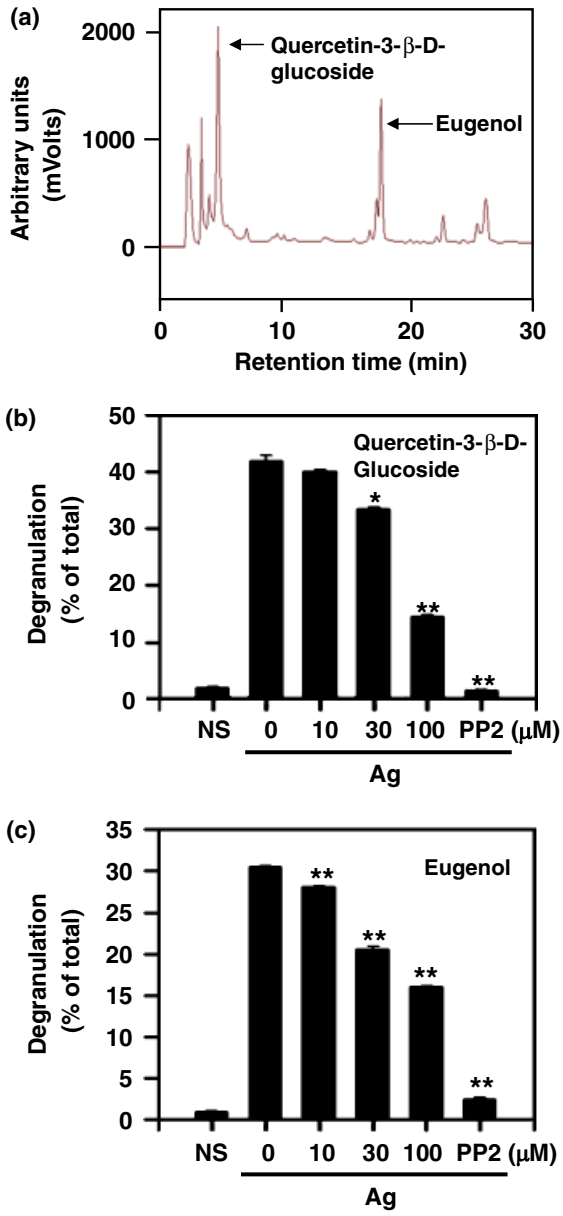

Fig. 6. HPLC chromatogram of LECJ and effects of isolated components on degranulation. (a) The components of LECJ were analysed using the HPLC system as described in 'Materials and methods'. The effects of quercetin-3-β-D-glucoside (b) and eugenol (c) on degranulation in RBL-2H3 cells were determined. The values are expressed as mean ± SEM from three independent experiments. PP2 (10 μM) is a general Src-family kinase inhibitor. LECJ, leaf extract of *Camellia japonica*; HPLC, high-performance liquid chromatography; RBL, rat basophilic leukaemia.

seeds [34, 35] and triterpenes of flowers [36, 37], are well characterized, but there are only a few reports on the activity of the leaf extract. Camellianoside and camelliatannin H are antioxidant glycosides [38] that inhibit human immunodeficiency virus type I protease [39]. In this study, we identified quercetin-3-β-D-glucoside and eugenol as the major components of LECJ. Eugenol was reported to be the major component of clove oil [40] and

its anti-allergic activity was also reported [41]. The effect of eugenol on degranulation in mast cells in our study was relatively minor because of its high IC_{50} value ($\sim$260 μM). Quecertin-3-β-D-glucoside was identified in this study as the most major component of LECJ, but its anti-allergic activity has not been reported to date. Quecertin-3-β-D-glucoside manifested much higher activity compared with eugenol (Figs 6b and c), and its quantity in the extract was higher (Fig. 6a). These results indicate that quecertin-3-β-D-glucoside is the main component of anti-allergic activity of LECJ. We believe, however, that other minor components including eugenol have some additional activity.

In this study, we showed for the first time that LECJ inhibited degranulation in human and rodent mast cells, the production of TNF-α and IL-4, as well as local allergic anaphylaxis. LECJ inhibited the activation of Syk kinase by the inhibition of Src-family kinase in mast cells for its anti-allergic activity.

Acknowledgements

This work was supported by the Regional Innovation Center Programme of the Ministry of Commerce, Industry and Energy in Korea, and by the Korea Research Foundation Grant funded by the Korea Government (MOEHRD) (The Regional Research Universities Programme/Chungbuk BIT Research-Oriented University Consortium), and in part by the second phase BK21 programme of the Ministry of Education and Human Resources Development. M. Radinger was supported by the Intramural Research Programme of NIAID, NIH, USA.

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