Doctoral Thesis

Study on Biological Functions of *Echinacea Purpurea* Extracts

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Contents

Chapter 1

General introduction

Allergy is a disorder of immune response that is classified into several types according to the mechanism involved. Immediate hypersensitivities such as hay fever and food allergies are categorized as type-I allergies, in which mast cells and basophilic leukocytes play an important role in the allergic reaction. Allergens are presented by dendritic cells. These cytokines induce the differentiation and class switching of B cells, and they become IgE productive plasma cells. IgEs released from plasma cells and they bind to the surface of mast cells. When the allergen reacts with IgE, that is antigen-antibody reaction, mast cells immediately release the chemical mediators, which lead to allergic symptoms such as cough and runny nose. Mast cell is stimulated by the antigen-antibody reaction, signal transduction and the intracellular increase of Ca^{2+} are induced. Histamine stored in the cells is released by degranulation. Histamine causes smooth muscle contraction and the promotion of mucus secretion. On the other hand, when phospholipase A_2 is activated, arachidonic acid is released from the biological membrane, and leukotrienes (LTs) are produced by 5-lipoxygenase and released. LTs promote the chronicity of allergic symptoms by migrating eosinophils to allergic reaction sites.

Inflammation is a response of body tissues to pathogens, broken cells, or irritants, and is a protecting reaction related to immune cells, blood vessels, and molecular mediators. The chemical mediator launched in hypersensitivity or the like acts on blood vessels to boom blood glide and decorate vascular permeability. This promotes exudation of frame fluids and blood proteins, and neighborhood migration and infiltration of inflammatory cells which include neutrophils, lymphocytes, and macrophages. As a result, inflammatory signs and symptoms along with redness, heat, swelling, and pain are exhibited.

Echinacea was used at length by Native Americans and by traditional herbalists in the United States, and in Canada. Many different medical conditions such as sore throat, swollen gum, gastrointestinal disorder were treated with Echinacea roots long ago by native Americans. The term Echinacea refers to several plants in the genus Echinacea. While there are nine known species of Echinacea, only the three of them, *Echinacea purpurea, Echinacea angustifolia,* and *Echinacea pallida*, are used as medicinal plants for the prevention and treatment of the common cold and influenza. *Echinacea purpurea* is a kind of herbaceous perennial flowering plant of Asteracea family, and it is widely distributed all over the world. Native Americans utilize its roots as medicine and European people have it for herb tea, also known as the purple coneflower. The functions are Echinacea polyphenols such as caffeic acid derivatives including chicoric acid, which are abundant in the roots.

The objectives of this study are to elucidate the effects of anti-allergic and anti-inflammation of *Echinacea purpurea* extracts in mast cells and macrophages.

Keywords

Echinacea Purpurea, allergy, inflammation, mast cell, macrophage, polyphenols.

Abbreviations:

AA; arachidonic acid, DNP; 2,4-dinitrophenyl, DPPH; 2,2-diphenyl-1-picrylhydrazyl, EDTA; ethylenediaminetetraacetic acid, EP; *Echinacea purpurea*, FcεRI; Fcε receptor I, FBS: fetal bovine serum, GAE; gallic acid equivalent, IgE; immunoglobulin E, LOX; lipoxygenase, LT; leukotriene, OPA; *o*-phthalaldehyde, PBS; phosphate buffered saline, SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis, TBS; Tris buffered saline, TNF-α; and tumor necrosis factor-α, IL-6; interleukin 6, IL-1β, NO; nitric oxide, TLR 4; Toll-like receptor.

Chapter 2

Anti-allergic effect of *Echinacea purpurea* extracts in mast cells

Abstract:

Histamine and leukotrienes (LTs), the chemical mediators released from mast cells, play an important role in type-I allergies such as hay fever. *Echinacea purpurea* (EP) has traditionally been used for herbal tea and has been reported to show biological functions. We evaluated the inhibitory activity of water extracts of EP petals, leaves, and stems against the chemical mediators released from mast cell lines. Petal and leaf extracts exhibited a significant inhibitory effect on histamine release from the stimulated cells, while the stem extract did not exert any effect. Activity of the petal extract was much stronger than that of the leaf extract. All the extracts significantly suppressed LTB_4 production in the stimulated cells and displayed similar activities. The petal extract decreased Syk phosphorylation and Ca^{2+} influx associated with signal transduction in the stimulated cells. These results suggest that EP petal extract may have a relieving effect on allergic symptoms.

2.1. Introduction

Allergy is a disorder of immune response that is classified into several types according to the mechanism involved[1-3]. Immediate hypersensitivities such as hay fever and food allergies are categorized as type-I allergies, in which mast cells and basophilic leukocytes play an important role in the allergic reaction[4] (Fig.1). The specific binding of antigens, such as pollens, to immunoglobulin E (IgE) antibodies bound to Fcε receptor I (FcεRI) on the cell membrane induces cell stimulation by cross-linking of IgEs. This triggers the intracellular signal transduction, such as the phosphorylation of proteins, followed by Ca^{2+} influx into the cytoplasm. The increase in Ca^{2+} concentration causes the release of stored histamines from the granules through degranulation[5]. The influx of Ca^{2+} also induces the release of arachidonic acid (AA) from phospholipids of the cell membrane by activating phospholipase A_2. AAs are oxidized by 5-lipoxygenase (LOX), and leukotrienes (LTs) such as LTB_4 are then produced through cascade reactions[6], which are secreted into the extracellular space (Fig.2). Histamine and LTs act as chemical mediators in type-I allergies, causing bronchoconstriction, dilatation and hyperpermeability of blood vessels, leukocyte chemotaxis, and extension of inflammation involved in various allergic symptoms such as hypersecretion of mucus, sneezing, and cough[7-9].

Functional foods have received particular attention in recent years due to their potential in relieving allergic symptoms instead of using symptomatic drug therapy, because foods may have fewer side effects. Echinacea is a perennial flowering plant of the Asteraceae family native to North America, and is commonly called purple coneflower. Native Americans have used Echinacea as a traditional medicine for various diseases such as colds[10-12]. Today, Echinacea is widely cultivated all over the world and has been utilized for herbal tea that is made of hot water extracts of the whole powdered plant including petals, leaves, stems, and roots, and the extracts have been used to improve the respiratory and immune systems[13,14]. It has been suggested that the extracts of *Echinacea purpurea* (EP), a major species of

Echinacea, exert various biological functions such as antibacterial, antioxidant, and anti-inflammatory activities[15-17].

The substances responsible for these functions are polyphenols such as caffeic acid derivatives including chicoric acid, and hydrophobic alkylamides including isobutylamides which are abundant in the roots[18-20]. Recently, Gulledge et al. has reported that EP root extract and its alkylamide can suppress mast cell degranulation *in vitro*[21]. However, the effect of the aerial part extracts of EP on type-I allergies is unclear. In the present study, we investigated the inhibitory activity of water extracts prepared from EP petals, leaves, and stems against the release of histamine and LTB_4 from mast cells *in vitro*.

2.2 Objectives

In type I allergy reaction, IgEs on mast cells are cross-linked with allergens, which induces the release of chemical mediators such as leukotrienes (LTs) and histamine. EP contains polyphenols such as caffeic acid derivatives including chicoric acid, which may have bioactivities as a functional food. The objective of this study is to elucidate effects of EP extracts on type I allergic reaction in mast cells *in vitro*.

2.3 Experimental

2.3.1 Materials

Folin-Ciocalteu's reagent was purchased from Nacalai Tesque (Kyoto, Japan). Chicoric acid was obtained from Tokyo Chemical Industry (Tokyo, Japan). 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Fig.3) was supplied by Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of reagent grade.

2.3.2 Preparation of EP extracts

EP cultivated in Kumamoto, Japan was obtained from a market. Extracts were individually prepared from the petals, leaves, and stems of EP as follows. Fifty grams of chopped frozen petals, leaves, and stems were mixed with 500 mL of distilled

water at 80°C for 2 h, and then sonicated for 5 min (Scheme 1). The extracts were filtered with filter papers (No.1, 125 mm, Advantec, Tokyo, Japan). The extraction was carried out with the residues by the same procedure. The extraction liquids were combined and freeze-dried. The yields of EP petals, leaves, and stems, which yields were 5.58, 6.65, and 12.24%, respectively.

2.3.3 Histamine release assay

Inhibitory activity of the EP extracts against histamine release was evaluated according to the method of Byeon et al.[24]. and Matsuo et. al.[25]. RBL-2H3, a rat basophilic leukemia cell line, was obtained from JCRB Cell Bank (Tokyo, Japan). The cells were cultured in Minimum Essential Medium Eagle (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C under 5% CO_2-95% air. To sensitize RBL-2H3 cells with IgE, the cells suspended in the medium containing a mouse anti-2,4-dinitrophenyl (DNP) IgE monoclonal antibody (Yamasa, Choshi, Japan) were seeded on a 24-well cell culture plate at the density of 4 x 10^5 cells/well, and cultured for 20 h. The cells of each well were washed twice with phosphate-buffered saline (PBS, pH 7.4) at 37°C. The EP extracts dissolved in Tyrode buffer (450 μL) consisted of 137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, and 0.05% bovine serum albumin (BSA), pH 7.2, was added to each well. After the incubation at 37°C for 10 min, 50 μL of DNP-BSA (Calbiochem, Darmstadt, Germany) in Tyrode buffer (2 μg/mL) was added and stimulated for 20 min at 37°C. The reaction was terminated by cooling the plate on ice. Tyrode buffer (50 μL) without DNP-BSA was used for the measurement of spontaneous histamine release from the cells without the stimulation. For the determination of the total amount of histamine stored in the granules, 50 μL of 5% Triton X-100 was added to each well instead of Tyrode buffer, and the culture plate was placed on ice for 20 min and the cells were lysed by pipetting. The supernatants after the stimulation or the cell lysate

were transferred to microtubes and centrifuged at 1,700 rpm at 4°C for 5 min and at 14,000 rpm at 4°C for 10 min, respectively. Histamine contents in the supernatants were subjected to HPLC analysis as follows. The sample solution (100 µL) was mixed with 50 µL of 8 µM 1-methelhistamine as an internal standard and 50 µL of 200 mM *N*-acetyl-L-cysteine. Histamine in the solution was measured by reversed-phase HPLC with fluorescence detection (Vietinghoff et al. 2006). HPLC was performed on a polymer-based column (Shodex ODP-50-4E, 4.6 x 250 mm) maintained at 50°C. Five µL of sample solution was injected and eluted by methanol/water (35/65, v/v) containing 30 mM $Na_2B_4O_7$ and 0.2 mM *o*-phthalaldehyde (OPA) at a flow rate of 0.7 mL/min. Excitation and emission wavelength for the detection were 340 and 450 nm, respectively.

2.3.4 LTB_4 production assay

Inhibitory activities of the EP extracts against LTB_4 production were evaluated according to the method described previously[26] with some modification. PB-3c, a mouse mast cell line, received from JCRB Cell Bank was cultured in RPMI-1640 with 2 mM L-glutamine and 25 mM HEPES (Wako) containing 10% FBS (HyClone), 1% MEM non-essential amino acids (Gibco, NY, USA), 1 mM sodium pyruvate (Gibco), 0.0035 µl/mL 2-mercaptoethanol (Wako), 2 ng/mL interleukin (IL)-3 (PeproTech, London, UK), 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C under 5% CO_2-95% air. PB-3c (5 x 10^5 cells/mL) was pre-cultured in the culture media supplemented with 50 µM AA (Sigma-Aldrich) for 48 h. After washing the cells twice with PBS, 4 x 10^6 cells were placed in a microtube. The cells were re-suspended in 180 µL Tyrode buffer containing the EP extracts and incubated at 37°C for 10 min. Subsequently, the cells were stimulated with 10 µM calcium ionophore A23187 (Sigma-Aldrich) at 37°C for 20 min and the stimulation was terminated by adding 200 µL acetonitrile/methanol (30/25, v/v) containing 1 mM ascorbic acid, 2 mM ethylenediaminetetraacetic acid (EDTA), and 1 µM prostaglandin (PG) B_2 (Cayman) as an internal standard. The samples were stored at -80°C until HPLC analysis. The

sample was centrifuged at 14,000 rpm for 15 min at 4°C and the supernatant was filtrated. LTB_4 in the supernatant was measured by the reversed-phase HPLC on an ODS-A column (6.0 x 150 mm, 5 μm particle size, YMC, Kyoto, Japan) at 40°C. The injected samples (50 μL) were eluted with 5 mM CH_3COONH_4: acetonitrile: methanol (30/25/45, v/v/v) at a flow rate of 1.0 mL/min. LTB_4 was detected through the absorbance at 280 nm.

2.3.5 Polyphenol determination

Total polyphenol contents in the EP extracts were determined by the Folin-Ciocalteu method[27]. The EP extracts were appropriately diluted with water. One mL of the sample was mixed with 1 mL of 1 N Folin-Ciocalteu's reagent and stand at room temperature for 3 min. Thereafter, 1 mL of 10% Na_2CO_3 (w/v) was added to the solution and incubated at 30°C for 30 min. The absorbance of the reaction mixture was measured at 760 nm. The results were calculated by a standard curve obtained from gallic acid under the same conditions and expressed as mg gallic acid equivalent (GAE). Chicoric acid in the extracts was determined by reversed-phase HPLC using an ODS column with UV detection according to a previously described method[28]

2.3.6 Radical scavenging assay

To know the antioxidative properties of the EP extracts, DPPH radical scavenging abilities were measured by the Blois's method with some modification[21]. The EP extracts were mixed with 100 μM DPPH in 75% ethanol and incubated for 30 min at 30°C in the dark. After centrifugation at 14,000 rpm at 20°C for 5 min, the absorbance at 517 nm of the reaction mixture was measured. Reaction mixture without samples and DPPH was used as a control and a blank, respectively. Trolox, an α-tocopherol analog, was used as a reference antioxidant. The radical scavenging ability was calculated as follows:

DPPH radical scavenging ability (%) = 100 - (As-Ab)/(Ac-Acb) x 100

Where As is the absorbance of the sample, Ab is that of the blank, and Ac is that of the control, Acb is that of the control blank.

2.3.7 Analysis of tyrosine-phosphorylated proteins

RBL-2H3 cells on a 24-well culture plate were incubated with anti-DNP IgE for 20 h as described above. The IgE-sensitized cells were washed twice with PBS and suspended in 450 μL of Tyrode buffer containing 2.0 mg/mL of the petal extract. After the incubation at 37°C for 10 min, 50 μL of DNP-BSA in Tyrode buffer was added and then incubated for 20 min at 37°C. The reaction was terminated by washing the cells with cold Tris-buffered saline (TBS, pH 7.4) -1 mM EDTA twice. Cold lysis buffer (200 μL) consisted of TBS-EDTA containing 1% Triton X-100, 50 mM NaF, and 5 mM Na_3VO_4 was added to the cells on ice. After 5 min, the cell lysate was collected with a cell scraper and stored at -80°C. Laemmli sample buffer with dithiothreitol (Atto, Tokyo, Japan) was added to the same amount of the thawed sample and denatured at 95°C for 5 min. Proteins in the samples (30 μL) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an acrylamide slab gel system (AnykD TGX gel, Bio-Rad, Hercules, CA, USA) according to Laemmli's method[28]. Proteins in the gel were transferred to polyvinylidene difluoride (PVDF) membrane by general Western blotting procedure. The PVDF membrane was incubated with Odyssey blocking buffer (TBS) provided by LI-COR (Lincoln, NE, USA) for 1 h. After washing the membrane with 0.05% Tween-TBS, it was incubated with mouse monoclonal antibodies against phosphotyrosine (4G10, Millipore, Burlington, MA, USA) or α-tubulin (DM1A, Cell Signaling Technology, Danvers, MA, USA) for 1 h. After washing the membrane with 0.05% Tween-TBS, it was incubated with goat anti-mouse IgG conjugated with IRDye 800CW (LI-COR). The membrane used for detecting phosphotyrosine was treated with stripping buffer (LI-COR) to remove antibodies from the membrane according to the protocol of the provider. The membrane was reprobed with a primary antibody against Spleen tyrosine kinase (Syk, SYK-01, Thermo Fisher

Scientific, Waltham, MA, USA) and goat anti-mouse IgG conjugated with IRDye 680RD (LI-COR) as a secondary antibody. The immunoreactive substances were analyzed by the Odyssey CLx (LI-COR) at near-infrared fluorescence (800 nm and 680 nm).

2.3.8 Analysis of Ca^{2+} concentration in cytoplasm

Ca^{2+} concentration in the cytoplasm of RBL-2H3 was measured using Calcium Kit II-Fluo4 provided by Dojindo (Kumamoto, Japan). RBL-2H3 (3×10^4 cells/100 µL) was seeded on 96-well clear-bottom black microplate and incubated with anti-DNP IgE for 20 h. The further procedure was conducted according to the manufacturer's protocol. Briefly, RBL-2H3 cells sensitized with IgE were incubated with the Loading Buffer (5 µg/mL Fluo 4-AM, 0.04% Pluronic F-127, and 1.25 mM probenecid) in the presence of the petal extract (0.25 mg/mL, 0.5 mg/mL, and 1.0 mg/mL) at 37°C for 1 h. The cells were stimulated by adding DNP-BSA. The fluorescence intensity (excitation at 485 nm and emission at 520 nm) was continuously measured by a fluorescence plate reader (PerkinElmer WALLAC 1420 ARVOmx/Light, Waltham, MA, USA) at 37°C.

2.3.9 Statistical analysis

Data are expressed as the mean ± standard deviation (SD) (n = 3). The experiments were conducted several times to confirm the reproducibility of the results. Statistical significance of differences was analyzed using the Tukey-Kramer multiple comparison test. Differences with *p* values less than 0.05 were considered significant.

2.4 Results

2.4.1 Effect of EP extracts on histamine release from RBL-2H3

Inhibitory activities of the EP extracts (1.5 mg/mL) against release of histamine from RBL-2H3 stimulated by antigen-IgE cross-linking were evaluated (Fig.4). The petal extract significantly suppressed histamine release, which was 16% of the histamine release in the absence of the EP extract (control). The leaf extract appeared to suppress the release of histamine, although the activity was not so strong as the leaf extract. On the other hand, the stem extract did not affect the release of histamine. Chicoric acid, a major polyphenol in EP[29], did not exert significant inhibitory effect on the histamine release at 10 μg/mL (data not shown). The EP extracts did not exert cytotoxicity at a concentration of 2.0 mg/mL as determined by the Trypan blue assay (data not shown). The effect of the petal extract on the histamine release from RBL-2H3 at various concentrations. A dose-dependent tendency was observed at 0.5-2.0 mg/mL of petal extract, and the suppressive effect at 1.0 and 2.0 mg/mL were significant ($p < 0.01$) (Fig.5).

2.4.2 Effect of EP extracts on LTB_4 production in PB-3c

Inhibitory activities of the EP extracts (1.5 mg/mL) against LTB_4 production in PB-3c stimulated by calcium ionophore were evaluated (Fig.6). All the extracts significantly reduced LTB_4 production to approximately 50% compared with control. Although statistical differences in the inhibitory activities were not observed among the EP extracts, the inhibitory effect of the petal extract tended to be stronger than that of leaf and stem extracts. Chicoric acid did not exert significant inhibitory effect on the LTB_4 production at 10 μg/mL (data not shown). None of the EP extracts showed cytotoxicity at 2.0 mg/mL as determined by the Trypan blue assay (data not shown). The indicates effect of the petal extract on LTB_4 production in PB-3c at various concentration. A significant dose-dependent activities were demonstrated at 1.0-2.0 mg/mL of petal extract ($p < 0.01$) (Fig.7).

2.4.3 Polyphenol contents of EP extracts

The total polyphenol content of the EP extracts. The average amounts of petal, leaf, and stem extracts were 73.5, 33.1, and 15.4 mg GAE/g dried extract, respectively. Chicoric acid in petal, leaf, and stem extracts were 3.23, 4.58, and 1.77 mg/g dried extract, respectively (Fig.8).

2.4.4 Radical scavenging ability of EP extracts

DPPH radical scavenging abilities of EP extracts. All the extracts exhibited dose-dependent scavenging abilities. The half-maximal inhibitory concentrations (IC_{50}) of petal, leaf, and stem extracts were 24, 58, and 145 μg/mL, respectively (Fig.9).

2.4.5 Effect of EP petal extract on tyrosine phosphorylation of cell proteins

Western blot analysis using primary anti-phosphotyrosine (top left panel) and anti-β-actin antibody (bottom left panel) for RBL-2H3 cell lysate following stimulation by antigen-IgE cross-linking in the presence of the EP petal extract was illustrated. As compared to no stimulation (lane 1), an increase in phosphotyrosine residues of the cell proteins with a molecular weight of around 70 kDa (upper arrow) and 30 kDa (lower arrow) was observed (lane 2). On the other hand, 2.0 mg/mL of the petal extract suppressed the tyrosine phosphorylation (lane 3). The bands at 70 kDa were identified as Syk (middle left panel) by the detection using anti-Syk antibody after the stripping. There was no difference in Syk levels among no stimulation (lane 1), control (lane 2), and petal extract (lane 3). The fluorescence intensities of the bands at 70 kDa detected by anti-phosphotyrosine antibody were quantified and the values were normalized by each fluorescence intensity of Syk band (top right panel). The suppression of tyrosine phosphorylation at 70 kDa by the petal extract was significant ($p < 0.05$). There was no difference in b-actin levels at 42 kDa, a loading control of total cell proteins, among no stimulation (lane 1), control (lane 2), and petal extract (lane 3) (Fig.10).

2.4.6 Effect of EP petal extract on cytoplasmic Ca^{2+} concentration

Indicates the time-course analysis of cytoplasmic Ca^{2+} concentration in RBL-2H3 stimulated by antigen-IgE cross-linking in the presence of the EP petal extract. The relative fluorescence intensity of control was increased after the stimulation by adding DNP-BSA, whereas the intensity was constant without the stimulation. The relative fluorescence intensity was dose-dependently suppressed by the petal extract, in particular, 1.0 mg/mL of the petal extract exhibited strong inhibition (Fig11).

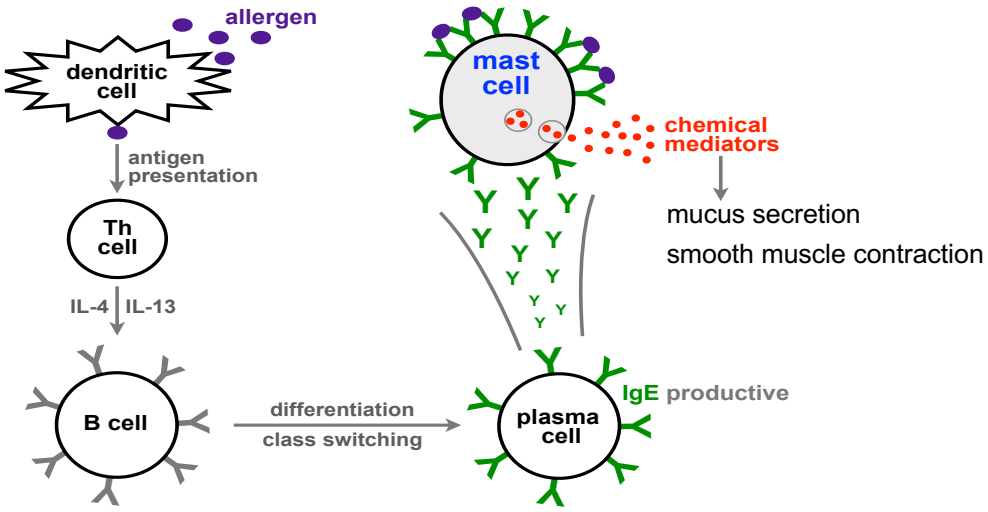


Figure 1. Onset mechanism of type I allergy.

First, allergens are presented by dendritic cells to Th cells. Next, these cytokines induce the differentiation and class switching of B cells, and they become IgE productive plasma cells. IgEs released from plasma cells bind to the surface of mast cells. When the allergen reacts with IgE, that is antigen-antibody reaction, mast cells immediately release the chemical mediators, which lead to allergic symptoms such as cough and runny nose.

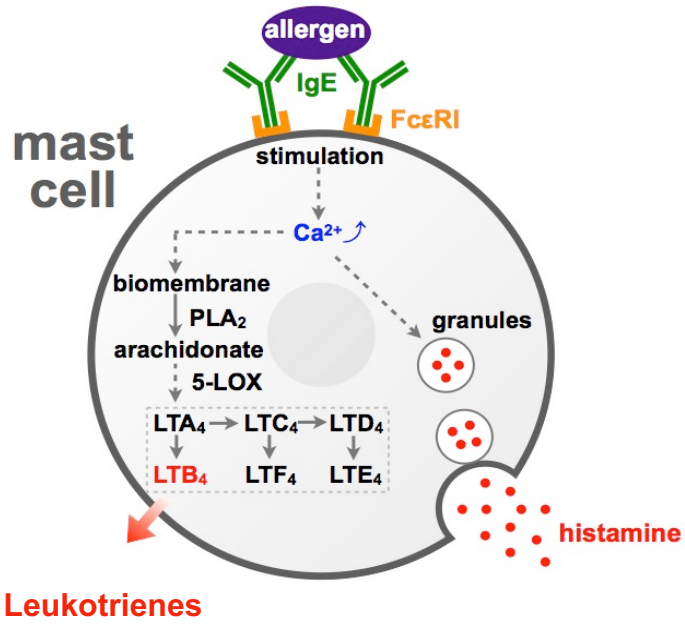


Figure 2. Mechanism of signal transduction mast cell

Mast cell is stimulated by the antigen-antibody reaction, signal transduction and the intracellular increase of Ca^{2+} are induced. As a result, the histamine stored in the cells is released by degranulation. Histamine causes smooth muscle contraction and the promotion of mucus secretion. On the other hand, when phospholipase A_2 is activated, arachidonic acid is released from the biological membrane, and leukotrienes (LT) are produced and released by 5-lipoxygenase and the like. LTs promote the chronicity of allergic symptoms by migrating eosinophils to allergic reaction sites.

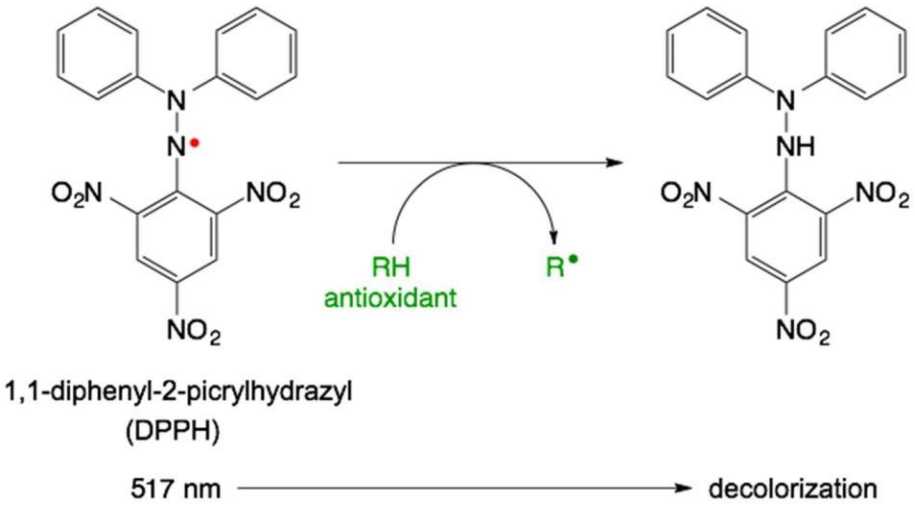
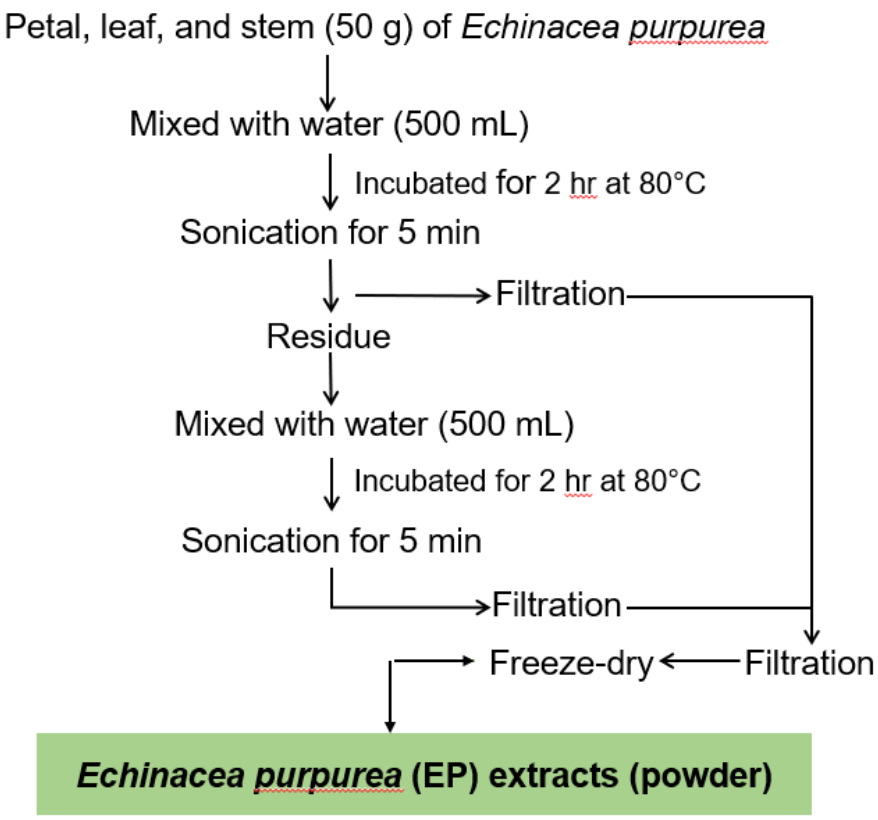


Figure 3. Structure of 1,1-diphenyl-2-picrylhydrazyl (DPPH).

The absorbance of the reaction mixture was measure at 517 nm. The reaction mixture without samples and DPPH was used as a control and a blank, respectively. The radical scavenging ability was calculated as follows:

DPPH radical scavenging ability (%) = 100 - (As-Ab)/(Ac-Acb) × 100

where As is the absorbance of the sample, Ab is the absorbance of the blank, Ac is the absorbance of the control, Acb is the absorbance of the control blank.



Scheme 1. Preparation of EP

Fifty grams of chopped frozen petals, leaves, and stems were mixed with 500 mL of distilled water at 80°C for 2 h. The extracts were then sonicated for 5 min and filtered. Extraction was carried out with the residues by the same procedure. Extraction liquids were combined and freeze-dried.

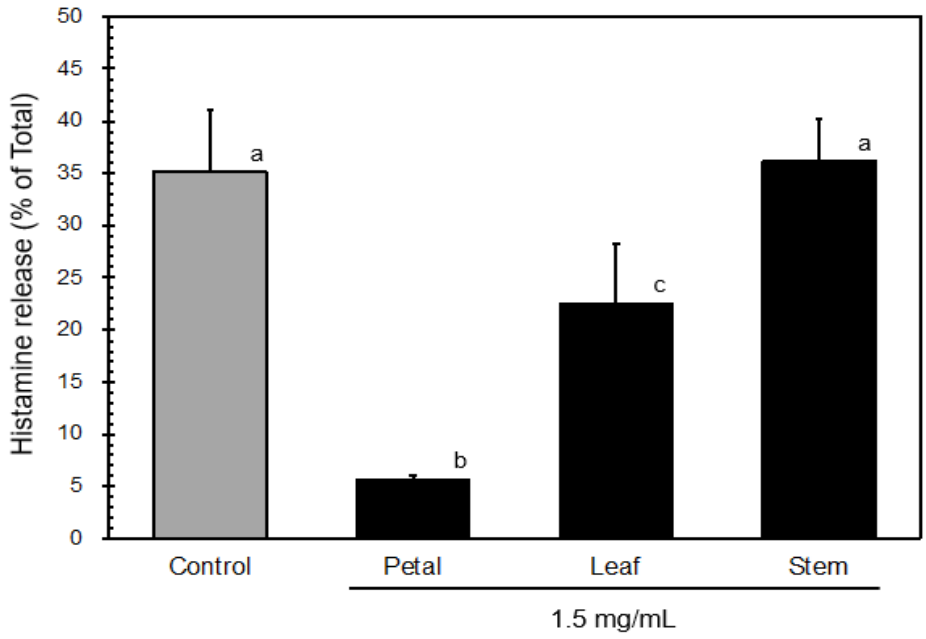


Figure 4. Effect of EP extracts on histamine release from RBL-2H3.

RBL-2H3 cells were pre-cultured with anti-2,4-DNP IgE for 20 h, and the cells were stimulated with DNP-BSA for 20 min at 37°C in the presence of EP extracts. The histamine in the supernatant was measured by HPLC. Data represent the mean ± SD (n = 3). Means without a common letter are significantly different ($p < 0.05$).

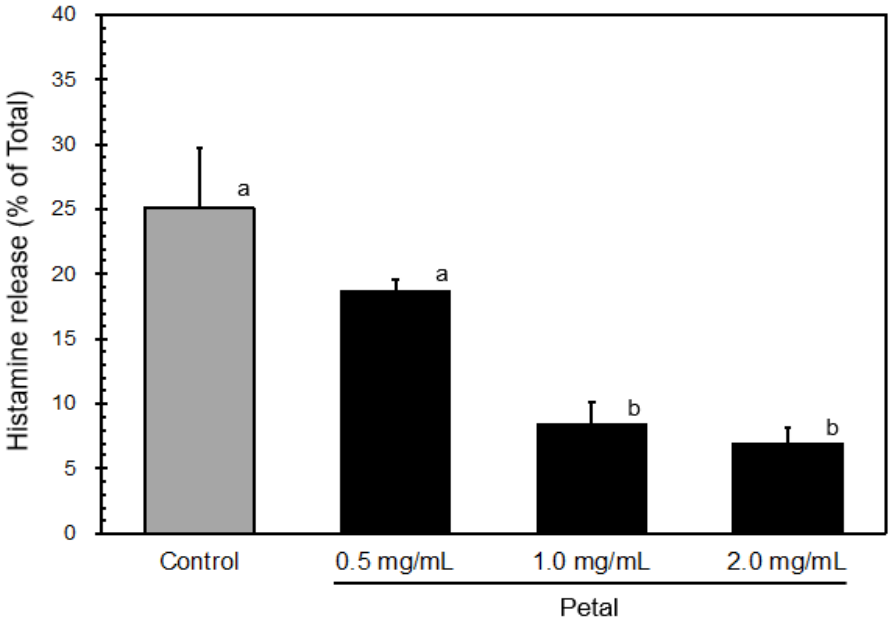


Figure 5. Dose-dependent suppressive effect of EP petal on histamine.

Data represent the mean ± SD (n = 3). Means without a common letter are significantly different ($p < 0.05$).

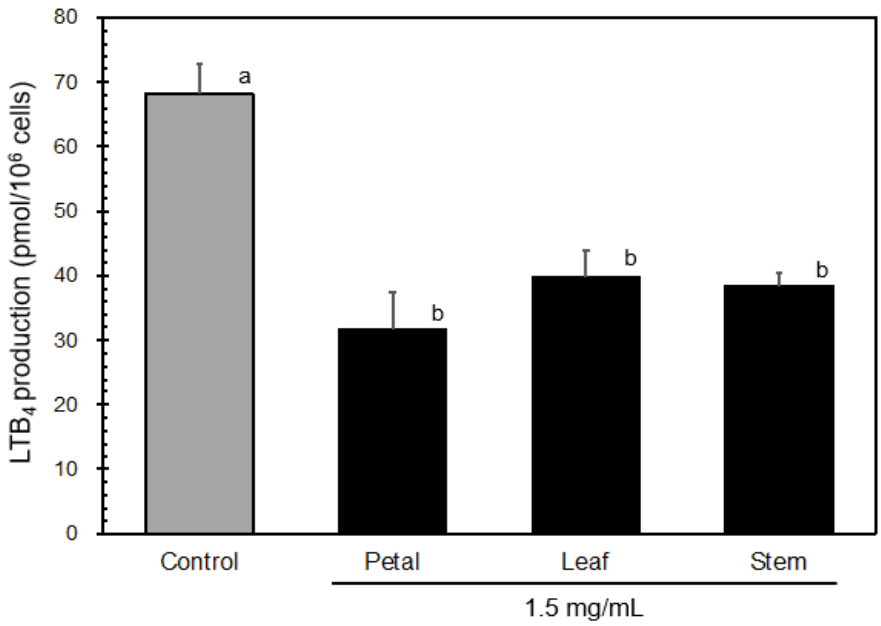


Figure 6. Effect of EP extracts on LTB_4 production in PB-3c.

PB-3c was pre-cultured with arachidonic acid for 48 h, and the cells were stimulated with calcium ionophore for 20 min. LTB_4 in the cell lysate was measured by HPLC. Data represent the mean ± SD (n = 3). Means without a common letter are significantly different ($p < 0.05$).

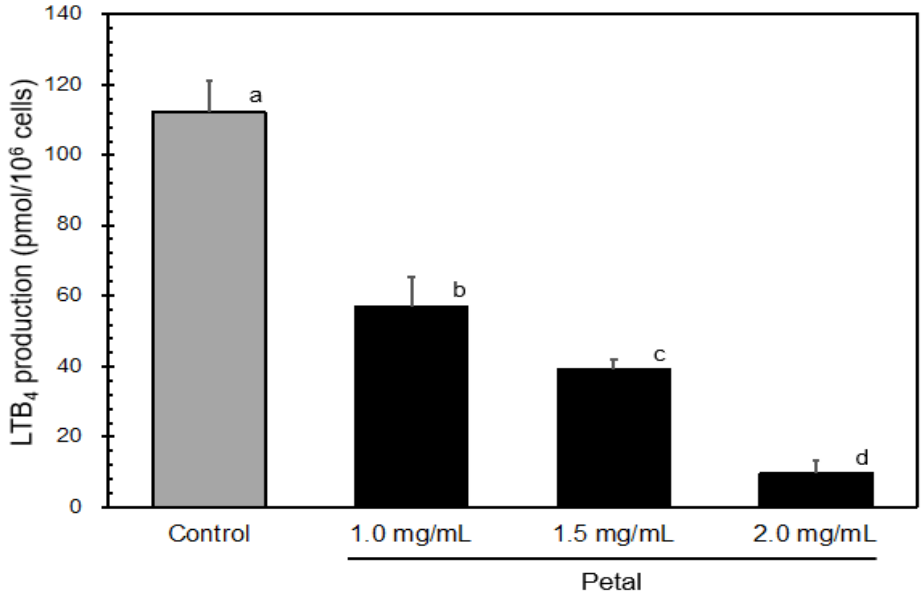


Figure 7. Dose-dependent suppressive effect of EP petal extract on LTB_4 production in PB-3c.

Data represent the mean ± SD (n = 3). Means without a common letter are significantly different ($p < 0.05$).

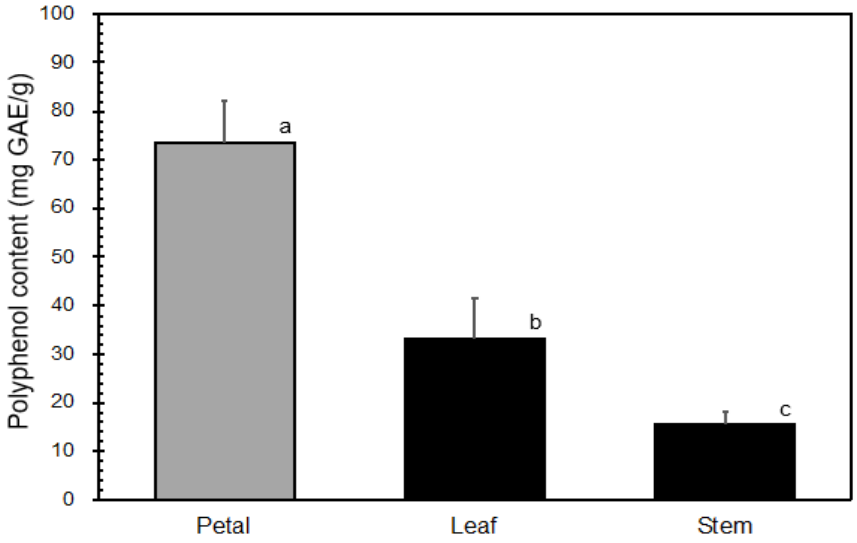


Figure 8. Polyphenol contents of EP extracts.

The EP extracts were mixed with Folin-Ciocalteu's reagent and incubated at room temperature for 3 min. After incubation with Na_2CO_3 at 30°C for 30 min, the absorbance at 760 nm was measured. The amounts were calculated in terms of mg gallic acid equivalent (GAE). Data represent the mean ± SD (n = 3). Means without a common letter are significantly different ($p < 0.05$).

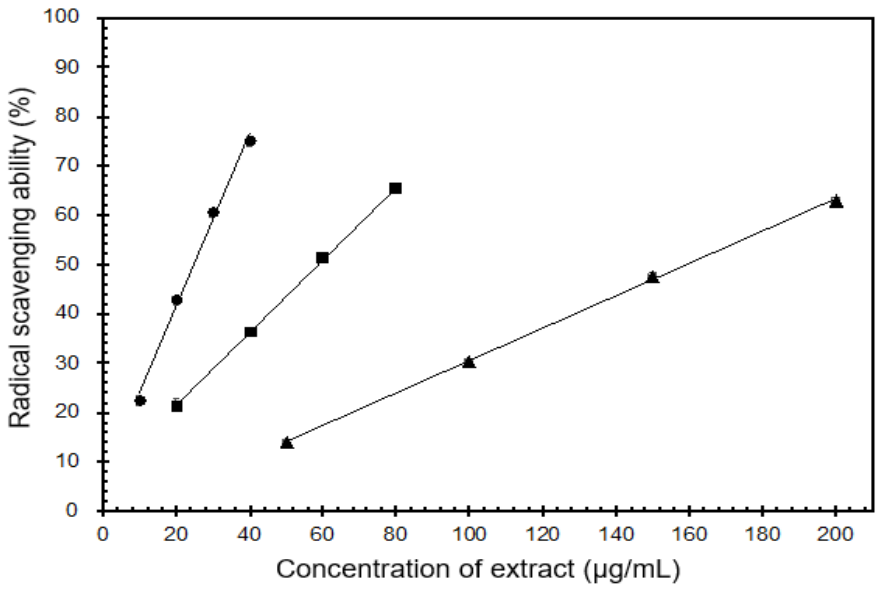


Figure 9. Radical scavenging ability of EP extracts.

EP extracts were mixed with DPPH in ethanol/water and incubated for 30 min at 30°C in the dark. The absorbance of the reaction mixture at 517 nm was measured. Data represent the mean ± SD (n = 3). Means without a common letter are significantly different ($p < 0.05$).

Petal extract (●), leaf extract (■), stem extract (▲).

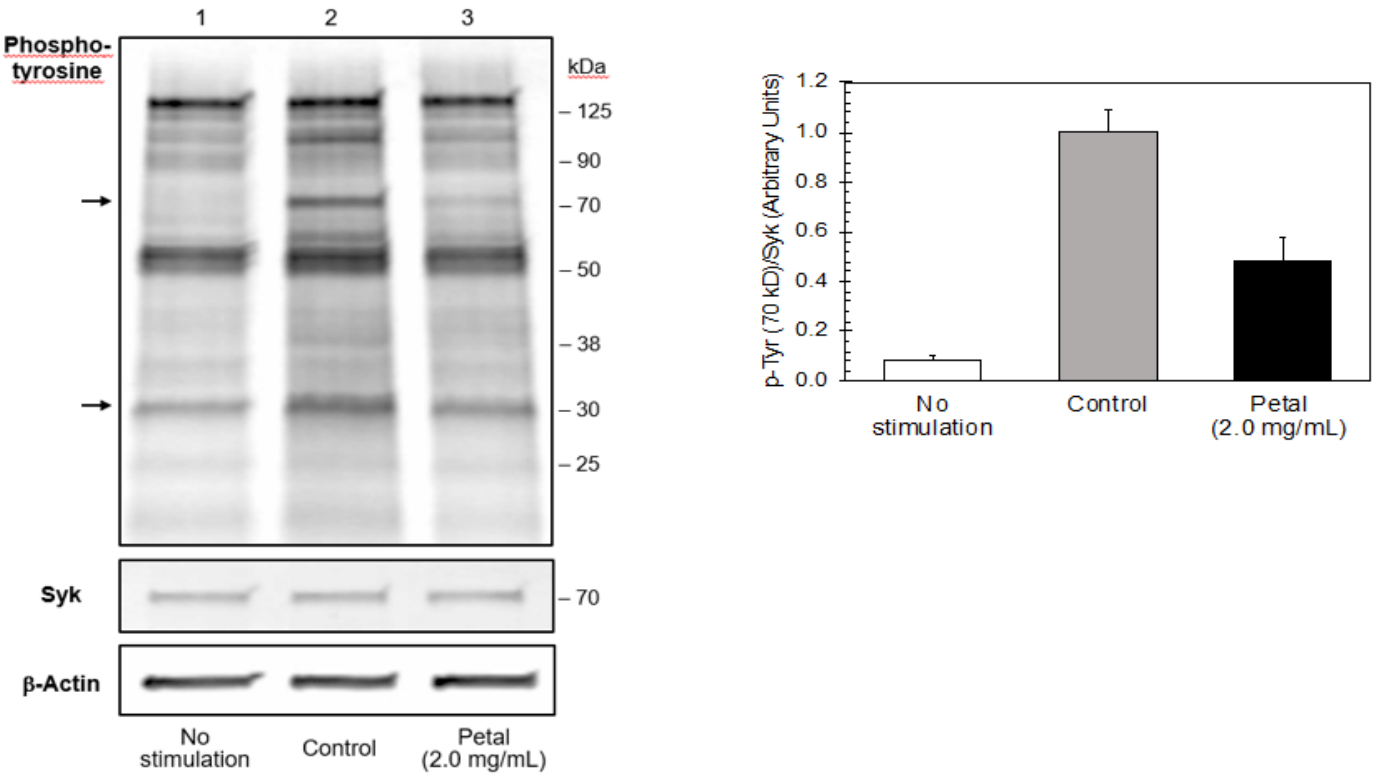


Figure 10. Effect of EP petal extract on tyrosine phosphorylation.

IgE-sensitized RBL-2H3 cells were stimulated with DNP-BSA for 20 min at 37°C in the presence of EP extracts. The cells were treated with lysis buffer, and the cell lysates were subjected to SDS-PAGE followed by Western blotting using primary anti-phosphotyrosine (top left panel), Syk (middle left panel), or anti-b-actin (bottom left panel) antibodies. Lane 1, no stimulation; lane 2, control; lane 3, petal extract (2.0 mg/mL). The fluorescence intensities of Western blot bands at 70 kDa detected by anti-phosphotyrosine were quantified and the values were normalized by each Syk content (top right panel). Results are shown as mean ± SD of 3 independent experiments. Means without a common letter are significantly different ($p < 0.05$).

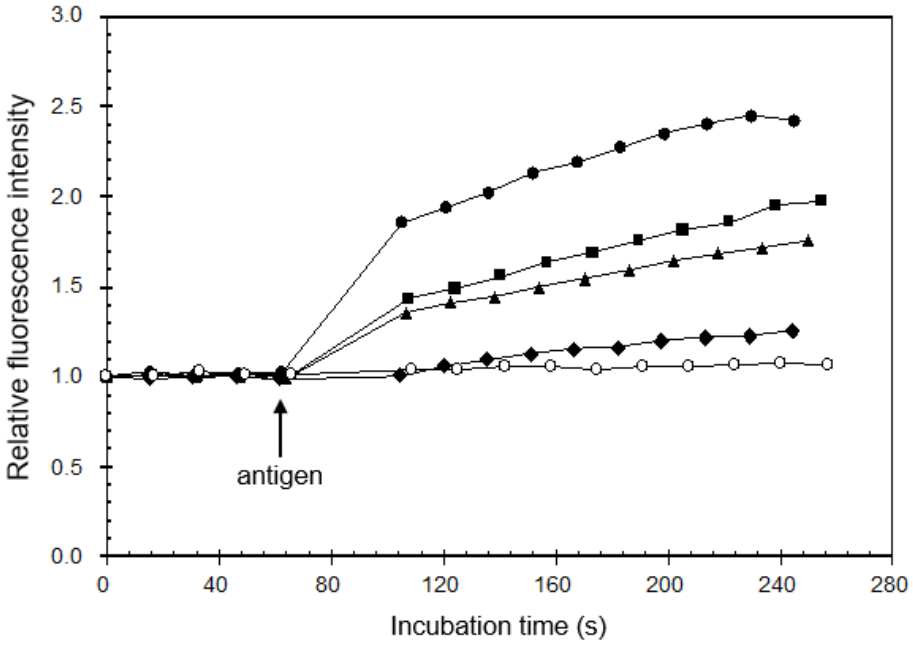


Figure 11. Effect of EP petal extract on cytoplasmic Ca^{2+} concentration.

RBL-2H3 cells with IgE were incubated with a fluorescent probe at 37°C for 1 h in the presence of petal extract. The cells were stimulated by adding DNP-BSA (antigen, ⟶). The fluorescence intensity was continuously monitored.

Control (●), 0.25 mg/mL (■), 0.5 mg/mL (▲), 1.0 mg/mL (♦), no stimulation (○).

2.5 Discussion

Suppression of the release of chemical mediators from basophilic leukocytes and mast cells is key to relieving the type-I allergic symptoms such as mucus hypersecretion. Most antiallergic drugs are designed to block histamine receptors, although they cause some side effects such as drowsiness. This has led to the search for antiallergic natural compounds without side effects, such as polyphenols, have been identified in various foods[30]. Echinacea has traditionally been used for herbal tea with safety food experiences and has been reported to have anti-inflammatory effects[12]. Gulledge et al. have reported the antiallergic effects of Echinacea roots, suggesting that dodeca-2E,4E-dienoic acid isobutylamide is an active component[21]. The objective of this study was to elucidate the antiallergic effects of the aerial part of EP using cultured cell lines, because the aerial part of the plant is also a major ingredient besides the root for herbal tea. We prepared the extracts from the aerial part of EP divided into petals, leaves, and stems using hot water as is done in making herbal tea. It has been reported that the amounts of alkylamides in EP aerial parts are very low as compared to the roots [29]. Accordingly, the extracts may contain hydrophilic substances such as polyphenols, although the amounts of hydrophobic substances such as alkylamides could be low in the extracts.

Histamine is a chemical mediator that is released by degranulation as a result of the cross-linking of IgEs with allergens on the surface of mast cells and basophilic leukocytes[5]. The inhibitory activity of the EP extracts against the release of histamine was evaluated on a rat basophilic leukemia cell line using a method previously described with some modifications[22,23]. Demonstrate that the petal extract significantly inhibits the release of histamine as compared to leaf and stem extracts (Fig.4 and 5). It is necessary to identify the responsible components in the hot water extracts of EP for this inhibitory activity. It has been suggested that polyphenols present in plants and foods can suppress the release of chemical mediators from mast cells[30-32]. In the present study, a positive correlation was observed between the inhibitory activity against the release of histamine (Fig.4) and

the total polyphenol content (Fig.8) of the extracts. Thus, the inhibitory effect of the petal extract on the release of histamine may be due to polyphenols, although the exact chemical compound is not clear. Chicory acid has been reported as a major polyphenol in EP, especially abundant in the leaves[33]. We have determined the chicoric acid in the EP water extracts and examined inhibitory effect on the histamine release from RBL-2H3 at 10 μg/mL, which corresponds to more than 2 mg/mL of the extracts, however, chicoric acid had no effect on the histamine release. This means that other polyphenols or hydrophilic compounds in the extract may inhibit the histamine release. Further experiments are needed to identify hydrophilic components other than chicoric acid involved in the inhibition of histamine release.

LTs are another type of chemical mediators produced through a pathway of the AA cascade mediated by 5-LOX in mast cells after stimulation by antigens[34]. The LOX reaction is a type of lipid peroxidation, in which lipid radicals are generated as the intermediate substances[35]. It has been suggested that antioxidants such as polyphenols can inhibit the inflammatory LOX reaction[36,37]. We have developed an experimental method to evaluate the inhibitory activity of food components such as polyphenols against LTB_4 production using a mast cell line[25], to which an assay was applied for the evaluation of the EP extracts. The petal, leaf, and stem extracts have similar activities in suppressing the LTB_4 production (Fig.6), in which the activity of petal extract seems to be stronger than the others, while their polyphenol amounts are not the same (Fig.8). The significant inhibitory effect of chicoric acid at 10 μg/mL corresponding to more than 2 mg/mL of the extracts on the LTB_4 production in PB-3c was not observed. Based on these results, we hypothesized that antioxidants other than polyphenols might be associated with the inhibition of LTB_4 production by leaf and stem extracts, and therefore, the antioxidant activity of the extracts was evaluated as DPPH radical scavenging ability, which is a widely used method to evaluate the antioxidant activity of food components. Results showed that antioxidant activity of the petal extract was the strongest, followed by the leaf and stem extracts (Fig.9), indicating that there is a positive correlation between the

polyphenol content and antioxidant activity of the extracts. This indicates that the antioxidant activities of the extracts are likely to be derived from polyphenols in the extracts. Consequently, any water-soluble substances other than antioxidants in the leaf and stem extracts could be involved in their ability to inhibit LTB_4.

After IgE-antigen stimulation, mast cells induce tyrosine phosphorylation of signaling molecules via a cascade reaction, followed by Ca^{2+} influx into the cytoplasm, which leads to the release of chemical mediators[5]. We focused on the petal extract because it exerted the strongest inhibitory activity on the release histamine as well on LTB_4 production, and we examined the effect on signal transduction in the stimulated mast cell line to elucidate the mechanisms underlying the inhibition of chemical mediators. The petal extract suppressed tyrosine phosphorylation of the signal molecule with a size of approximately 70 kDa, as illustrated (Fig.10). Syk is a 72 kDa-signaling molecule which mainly binds to the gamma chain of FcεRI, and the interaction results in its activation and phosphorylation, which in turn phosphorylate downstream proteins[38,39]. The signal molecule around 70 kDa was confirmed as Syk by reprobing the same membrane with a specific antibody to Syk. The EP petal extract also inhibited intracellular Ca^{2+} influx, as indicated (Fig.11), which may be due to the inhibition of upstream tyrosine phosphorylation, although more detailed mechanisms need to be elucidated. These data indicate that the EP petal extract can suppress histamine release and LTB_4 production, in which the inhibition of signal transduction of Syk and Ca^{2+} influx may be associated.

2.6 References

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Chapter 3

Anti-inflammatory effect of *Echinacea purpurea* extracts in macrophages

Abstract:

Inflammation is a response of body tissues to stimulations of pathogens, parasites, broken cells, or irritant chemicals, and is a protecting reaction related to immune cells, blood vessels, and molecular mediators.

We used RAW 264.7 macrophages to observe the results of EP extracts at the manufacturing of inflammatory mediators and the underlying mechanisms. Productions of nitric oxide (NO) have been decided via way of means of NO_2/NO_3 Assay kit-FX (Fluorometric)-2,3-Diaminonaphthalene Kit, tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), and IL-1β have been determined via way of means of ELISA assay. The alternate protein stage became evaluated via way of means of Western blot. NO manufacturing from the cells became suppressed where in the interest of the petal extract became more potent than that of the leaf extract. Petal extract became suppressed the TNF-α, IL-1β, and IL-6 manufacturing. And, petal extract considerably inhibited iNOS caused via way of means of LPS in a concentration-structured manner. These effects endorse that EP petal extract can also additionally have decreased the inflammatory response.

3.1 Introduction

The chemical mediator launched in hypersensitivity or the like acts on blood vessels to boom blood glide and decorate vascular permeability. This promotes exudation of frame fluids and blood proteins, and neighborhood migration and infiltration of inflammatory cells which include neutrophils, lymphocytes, and macrophages. As a result, inflammatory signs and symptoms along with redness, heat, swelling, and pain are exhibited (Fig.1.3). When lipopolysaccharide (LPS), a bacterial wall component, binds to Toll-like receptor (TLR) 4, a form of macrophage transmembrane receptor[1,2]. iNOS produces and releases huge quantities of NO from L-arginine and, reactive nitrogen species (peroxynitrite, $ONOO^-$) produced through the response of NO and superoxide (O) harm cells and exacerbate signs and symptoms (Fig.1.4)[3]. Inflammatory cytokines along with TNF-α, IL-6, and IL-1β also are produced and released, which once more stimulate macrophages and similarly sell the inflammatory response. TNF-α is an activator of the transcription element NF-κB, and the activated NF-κB induces the expression of iNOS and promotes the inflammatory response.

Echinacea is a perennial flowering plant of the Asteraceae family native to North America, and is commonly called purple coneflower. Native Americans have used Echinacea as a traditional medicine for various diseases such as colds[4-6]. Today, Echinacea is widely cultivated all over the world and has been utilized for herbal tea that is made of hot water extracts of the whole powdered plant including petals, leaves, stems, and roots, and the extracts have been used to improve the respiratory and immune systems[7,8]. It has been suggested that the extracts of *Echinacea purpurea* (EP), a major species of Echinacea, exert various biological functions such as antibacterial, antioxidant, and anti-inflammatory activities[9-11]. The substances responsible for these functions are polyphenols such as caffeic acid derivatives including chicoric acid, and hydrophobic alkylamides including isobutylamides which are abundant in the roots[12-14]. In this study, we investigated the effects of EP extracts on the production of inflammatory mediators and underlying mechanism.

3.2 Objectives

EP is crucial immunostimulatory and anti-inflammatory properties, is a natural perennial specifically with the comfort of the symptoms of the common cold. In this study, usage of the RAW 264.7 macrophages, we investigated the effect of EP extract the manufacturing of inflammatory mediators and to the mechanism which is in the underlying. The RAW 264.7 macrophage had been pre-treated with EP extract, observed through stimulation with lipopolysaccharide (LPS), which has induced an inflammatory reaction.

3.3 Experimental

3.3.1 Materials

The RAW 264.7 macrophages line was distributed from the RIKEN Bioresource. Lipopolysachharide (LPS) was obtained from Sigma-Aldrich. All other chemicals were of reagent grade.

3.3.2 Cell culture

The RAW 264.7 macrophages were distributed from the RIKEN Bioresource. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 1 mM sodium pyruvate, 100 μg/mL streptomycin, and 2 mM L-glutamine at 37ºC in an incubator containing 5% CO_2.

3.3.3 Measurement of NO production

RAW 264.7 macrophages were seeded in a 24-well plate at a density of 4×10^4 cells/400 μL/well, and cultured in a CO_2 incubator for 2 h. Then added EP extracts of different concentrations (0.1, 0.25, 0.5 mg/mL) and lipopolysaccharide 10 μg/mL to each cell suspension became incubated for 24 h. Gently take 450 μL of the supernatant of none, control, and EP extracts, transfer to a 1.5 mL microtube, and centrifuge at 1,000 × g for 15 min at 25ºC. This supernatant was taken of 150 μL,

transferred to a 0.5 mL microtube, and stored at -30ºC. As NO measurement reagent NO_2/NO_3 assay kit using 2,3-diaminonaphthalene Kit (Dojindo laboratory).

The first, preparation of NO_2/NO_3 standard curve and dilute 80 µL of 20 µmol/L $NaNO_2/NaNO_3$ standard solution by serial dilution with 80 µL of Buffer solution in 96-well plate as indicated. Next, added 20 µL of Buffer solution to each well (the total volume is 100 µl/well). Added 10 µL of Fluorescence Reagent (DAN) Solution to each well, and mixed. Then, leave the plate for 15 min at room temperature, and added 40 µL of Stop Solution to each well. Measure the fluorescence intensity of each well at 450 nm (excitation 360 nm) with a fluorescence microplate reader (Thermo Varioskan LUX). The second, added 80 µL of the EP extracts to a 96-well plate (Thermo 137101), 10 µL of NO_3 Reductase Solution, and 10 µL of Enzyme Cofactors Solution were added to each well, mixed well, and incubated at 37ºC for 30 min. Then, added 10 µL of DAN Solution to each well, mixed well, reacted at room temperature for 15 min, and added 40 µL of Stop Solution to stop the reaction. The fluorescence intensity at an excitation wavelength of 360 nm and a fluorescence wavelength of 450 nm was measured with a microplate reader.

3.3.4 ELISA assay

For TNF-α and IL-1β, RAW 264.7 macrophages have been seeded in 24-well culture plates at of 0.2×10^5 cells/400 µL, and cultured in a CO_2 incubator for 24 h. Petal extract and 10 µg/mL LPS were then added to each cell suspension and cultured for 1 h. For IL-6, RAW 264.7 macrophages were seeded in 24-well culture plates at of 1.0×10^6 cells/400 µL/well, and cultured in a CO_2 incubator for 2 h. Petal extract and 10 µg/mL LPS were then added to each cell suspension and cultured for 6 h. Gently take 450 µL of the supernatant of none, control, and petal extract, transfer to a 1.5 mL microtube, and centrifuge at 1,000 × g for 15 min at 25ºC. This supernatant was taken of 150 µL, transferred to a 0.5 mL microtube, and stored at -30ºC. Supernatant cytokines were measured by ELISA development kit according to the manufacturer's instructions. Coat high protein binding ELISA plate (Nunc

442404) with Monoclonal antibodies, diluted to 2 μg/mL in PBS, by added 100 μL/well and incubated at 4°C for 24 h. And, washed twice with PBS (200 μL/well). Then, block plate by added 200 μL/well of PBS with 0.05 % Tween 20 containing 0.1 % BSA (incubation buffer). Incubated for 1 h at room temperature. Next, washed five times with PBS containing 0.05 % Tween 20. Added 100 μL/well of EP petal extract and standards diluted in incubation buffer and incubated for 2 h at room temperature. Again, washed five times, and added 100 μL/well of Biotinylated monoclonal antibody and incubated for 1 h at room temperature. After, washed five times, and added 100 μL/well of Streptavidin-Horseradish Peroxidase diluted and incubated for 1 h at room temperature. Next, washed five times, and added 100 μL/well of appropriate substrate solution TMB and reacted for 4-10 min. Added 100 μL/well of the stop solution, and the absorbance at 450 nm was measured with a microplate reader.

3.3.5 Analysis of iNOS by western blot

RAW 264.7 macrophages were cultured in 24-well plates (2×10^5 cells/400 μL/well) for 2 h. Then, treated with 10 μg/mL LPS for 24 h except for the none and petal extract. The reaction was terminated by washing the cells with cold Tris-buffered saline (TBS, pH 7.4) -1 mM EDTA twice. Cold lysis buffer (200 μL) consisted of TBS-EDTA containing 1% Triton X-100, 50 mM NaF, and 5 mM Na_3VO_4 was added to the cells on ice. After 5 min, the cell lysate was collected with a cell scraper and stored at -80°C. Laemmli sample buffer with dithiothreitol (Atto, Tokyo, Japan) was added to the same amount of the thawed sample and denatured at 95°C for 5 min. Proteins in the samples (30 μL) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an acrylamide slab gel system (AnykD TGX gel, Bio-Rad, Hercules, CA, USA) according to Laemmli's method[28]. Proteins in the gel were transferred to polyvinylidene difluoride (PVDF) membrane by general Western blotting procedure. The PVDF membrane was incubated with Odyssey blocking buffer (TBS) provided by LI-COR (Lincoln, NE,

USA) for 1 h. After washing the membrane with 0.05% Tween-TBS, and it was incubated with anti-iNOS I I, NT rabbit antibody (ABN26, Millipore, Burlington, MA, USA) or beta-actin mouse antibody (ABM) for 1 h. After washing the membrane with 0.05% Tween-TBS, it was incubated with goat anti-rabbit IgG conjugated with IRDye 800CW (LI-COR) and goat anti-mouse IgG conjugated with IRDye 680RD (LI-COR). The immunoreactive substances were detected by Odyssey CLx (LI-COR) at near-infrared fluorescence (700 and 800 nm).

3.3.6 Cytotoxicity assay in RAW 264.7

Briefly, RAW 264.7 macrophages were seeded at a density of 2 x 10^4 cells per well in 96-well plates. After 2 h, EP extracts and 10 μg/mL LPS were then added to each cell suspension and cultured for 24 h 30 min. The supernatant was discarded and new DMEM medium was added. After that, added 10 μL each of Cell Counting Kit-8 (Dojin) to control and EP extracts, and the mixture was reacted in a CO_2 incubator for 1 h. Absorbance at 450 nm was measured with a microplate reader.

3.3.7 Statistical analysis

Data are expressed as the mean ± standard deviation (SD) (n = 3). The experiments were conducted several times to confirm the reproducibility of the results. Statistical significance of differences was analyzed using the Tukey-Kramer multiple comparison test. Differences with *p* values less than 0.01 were considered significant.

3.4 Results

3.4.1 Effect of EP extracts on NO production in RAW 264.7 macrophages

The effect of EP extracts on NO production in LPS-treated RAW 264.7 macrophage were measured to investigate its anti-inflammatory effects. The NO_2/NO_3 Assay kit-FX (Fluorometric) -2,3-diaminonaphthalene kit was used as the NO measurement reagent. Then added EP extracts of varying concentrations (0.1, 0.25, 0.5 mg/mL) and lipopolysaccharide 10 μg/mL to each cell suspension was incubated for 24 hr. NO production from the cells was suppressed by the EP extracts of 0.5 mg/mL, in which the activity of the petal extract was stronger than that of the leaf extract (Fig.3). The effect of the petal extract on NO production the from RAW 264.7 macrophages at various concentrations. A dose-dependent tendency was observed at 0.1-0.4 mg/mL of petal extract, and the suppressive effect at 0.2 and 0.4 mg/mL were significant ($p < 0.01$) (Fig.4).

3.4.2 Effect of petal extract on inflammatory cytokine TNF-α, IL-1β, and IL- 6 production in RAW 264.7 macrophages

The RAW 264.7 macrophage in the presence of petal extracts were stimulated with LPS, the TNF-α, IL-1β, and IL-6, which is released into the cell culture supernatant was measured by ELISA assay. As a result, treatment of RAW 264.7 macrophage with petal extract was 0.5 mg/mL significantly suppressed the IL-1β and IL-6 production (Fig.5 and 6), while TNF-α was only significantly reduced when the petal extract concentration was 2.0 mg/mL (Fig.7).

3.4.3 Effect of petal extract on iNOS expression in LPS-induced RAW 264.7 macrophages

The expression-suppressing impact of petal extract in iNOS in LPS-stimulated macrophages RAW 264.7 macrophages had been analyzed by Western Blotting. The expression levels of iNOS had been strongly induced by LPS, petal extract significantly inhibited iNOS induced by LPS in a concentration-dependent manner.

The detection of beta-actin became completed withinside the equal blot an inner control (Fig.8).

3.4.4 Effect of EP extracts on cytotoxicity in RAW 264.7 macrophages

All of the EP extracts, showed no cell toxicity at 0.5 mg/mL by cytotoxicity assay (data not shown).

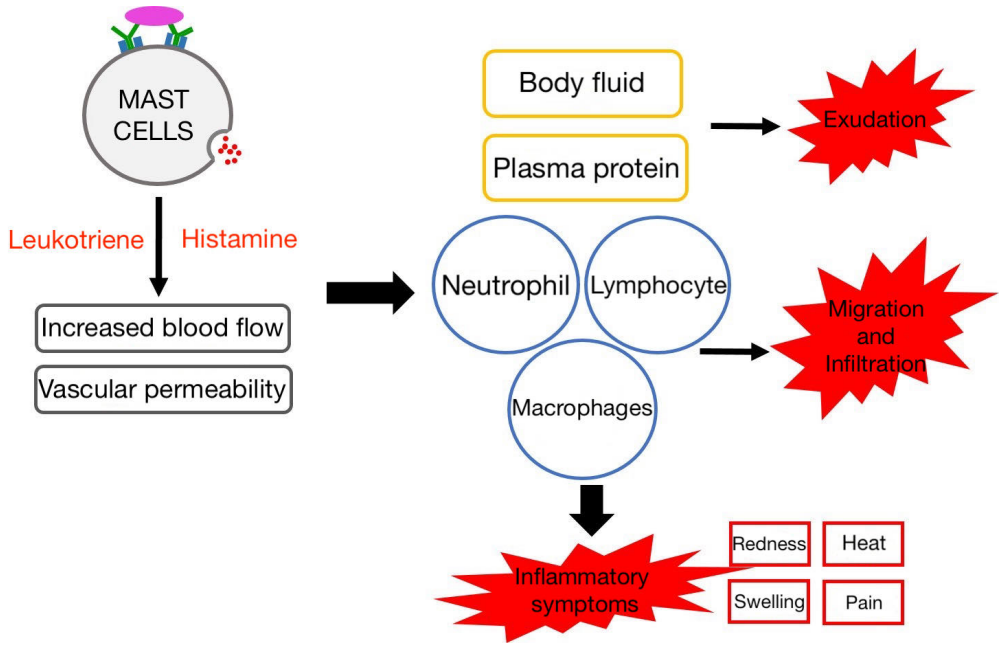

Figure 1. Induction mechanism of inflammation

Chemical mediators released in allergic reactions act on blood vessels to increase blood flow and increase vascular permeability. This promotes the exudation of body fluids and blood proteins, and the local migration and infiltration of inflammatory cells such as neutrophils, lymphocytes, and macrophages. As a result, inflammatory symptoms such as redness, heat, swelling, and pain are exhibited.

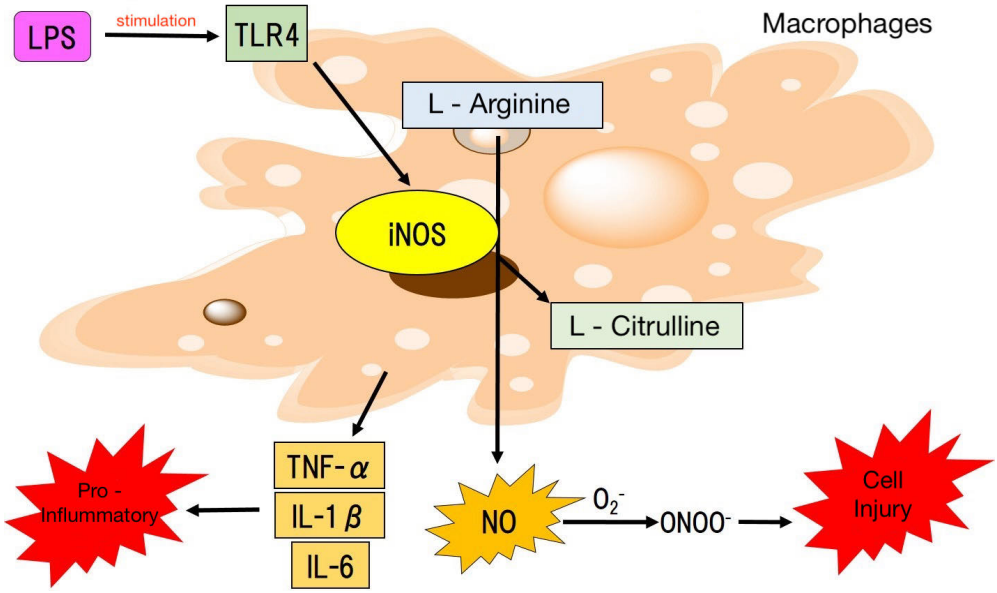

Figure 2. Inflammatory response mechanism in macrophages

LPS binds to Toll-Like Receptor 4 on the cell surface, signaling occurs and iNOS expression occurs. NO is produced using iNOS as a catalyst in the process of converting L-arginine to L-citrulline. Inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are also released, and these substances stimulate macrophages again, further promoting the inflammatory response.

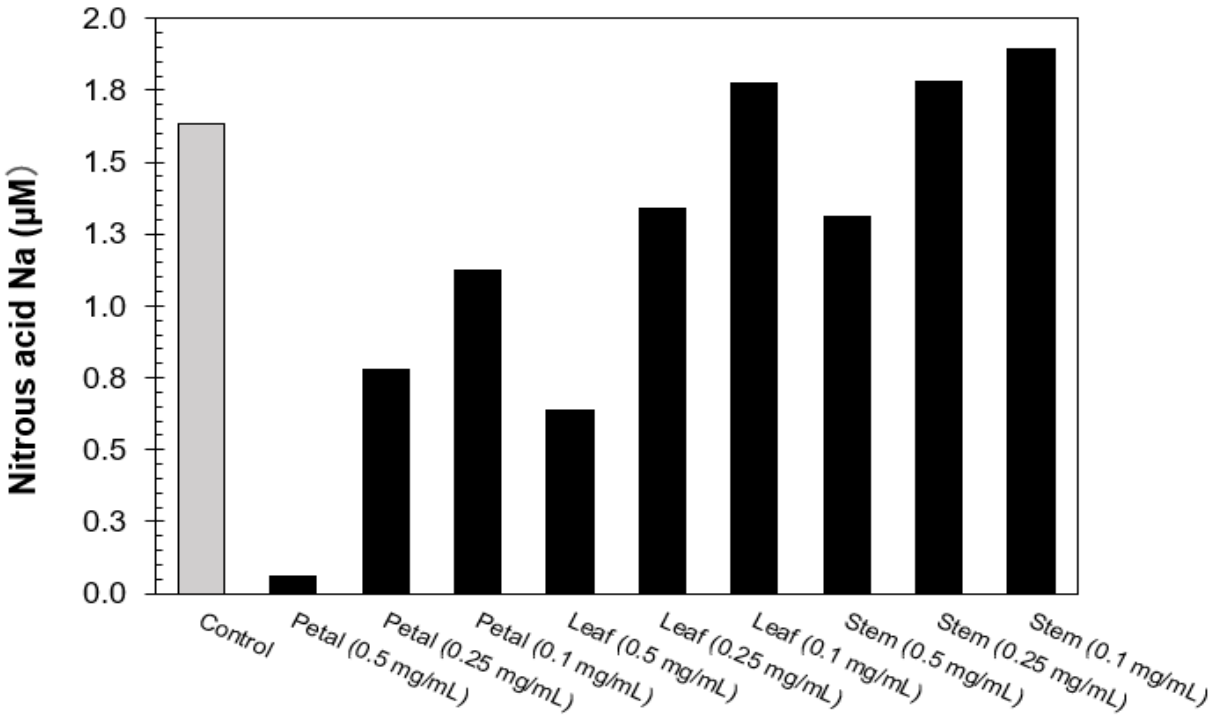

Figure 3. Effect of EP extracts on NO production by RAW 264.7 macrophages
The effect of the petal extract on NO production the from RAW 264.7 macrophages at various concentrations. NO production from the cells was suppressed by the EP extracts of 0.5 mg/mL, in which the activity of the petal extract was stronger than that of the leaf and stem extract.

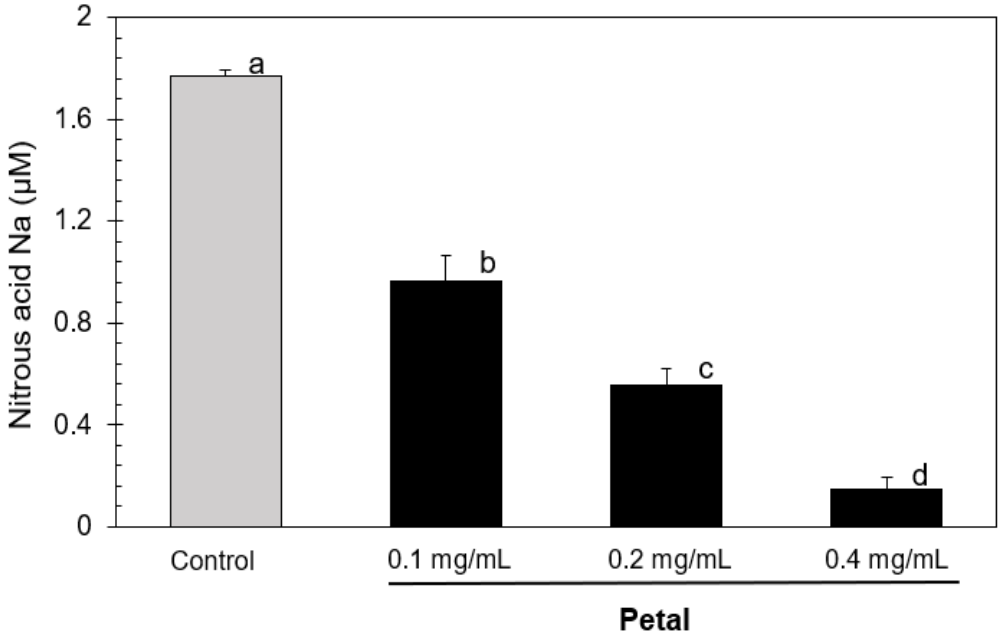

Figure 4. Dose-dependent suppressive effect of EP petal extract on NO production from RAW 264.7 macrophages

A dose-dependent tendency was observed at 0.1-0.4 mg/mL of petal extract, and the suppressive effect at 0.2 and 0.4 mg/mL. Data represent the mean ± SD (n = 3). Means without a common letter are significantly different ($p < 0.01$).

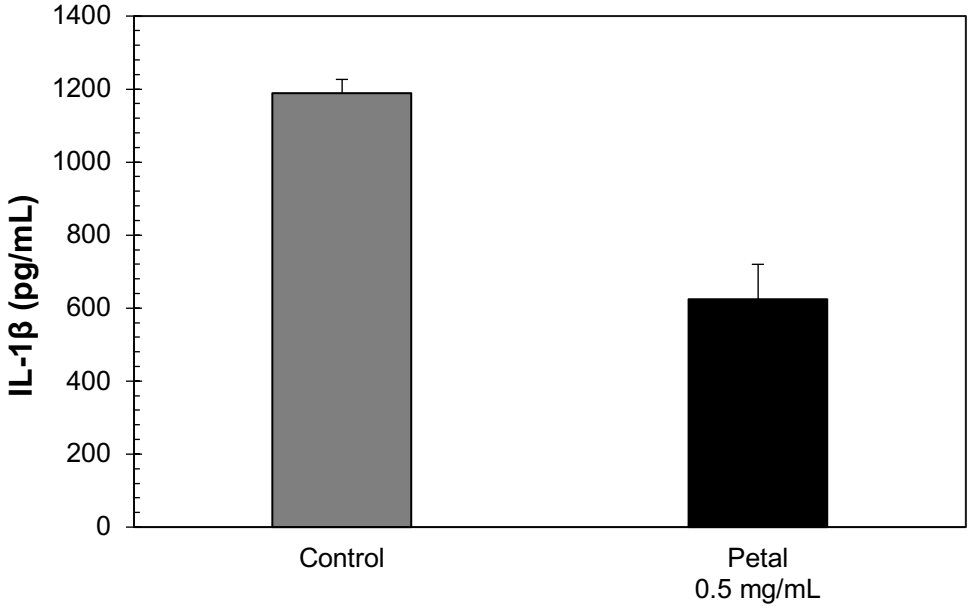

Figure 5. Effect of petal extract on inflammatory cytokine IL-1β production in RAW 264.7 macrophages

RAW 264.7 macrophages have been seeded in 24-well culture plates at of 0.2×10^5 cells/400 μL, and cultured in a CO_2 incubator for 24 h. Petal extract and 10 μg/mL LPS were then added to each cell suspension and cultured for 1 h. As a result, treatment of RAW 264.7 cells with petal extract was 0.5 mg/mL significantly suppressed the IL-1β production. Data represent the mean ± SD (n = 3). Means without a common letter are significantly different ($p < 0.01$).

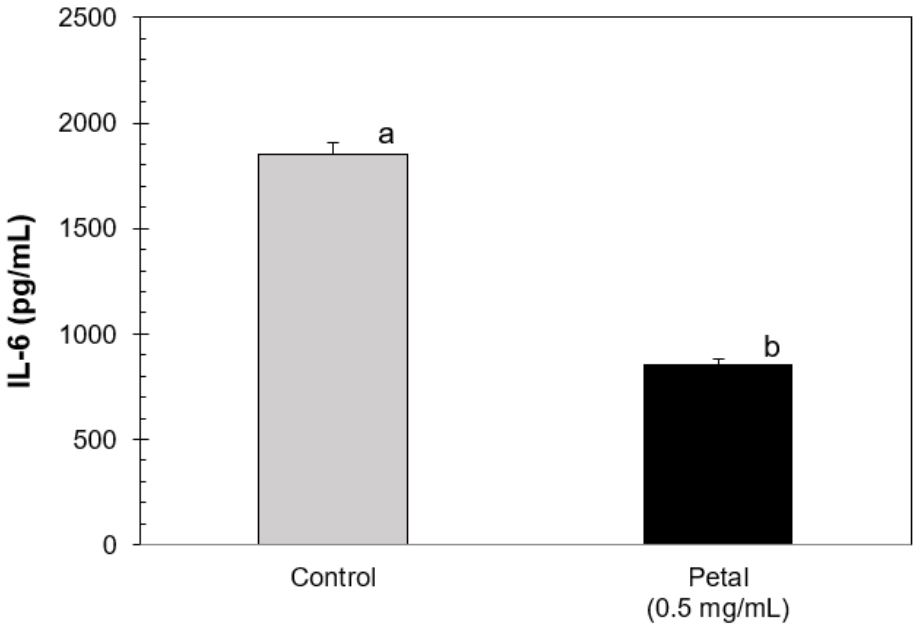

Figure 6. Effect of petal extract on inflammatory cytokine IL- 6 production in RAW 264.7 macrophages

IL- 6 production was RAW 264.7 macrophages have been seeded in 24-well culture plates at of 1.0 × 10^6 cells/well, and cultured in a CO_2 incubator for 2 h. Petal extract and 10 μg/mL LPS were then added to each cell suspension and cultured for 6 h. As a result, treatment of RAW 264.7 cells with petal extract was 0.5 mg/mL significantly suppressed the IL-6 production. Data represent the mean ± SD (n = 3). Means without a common letter are significantly different ($p < 0.01$).

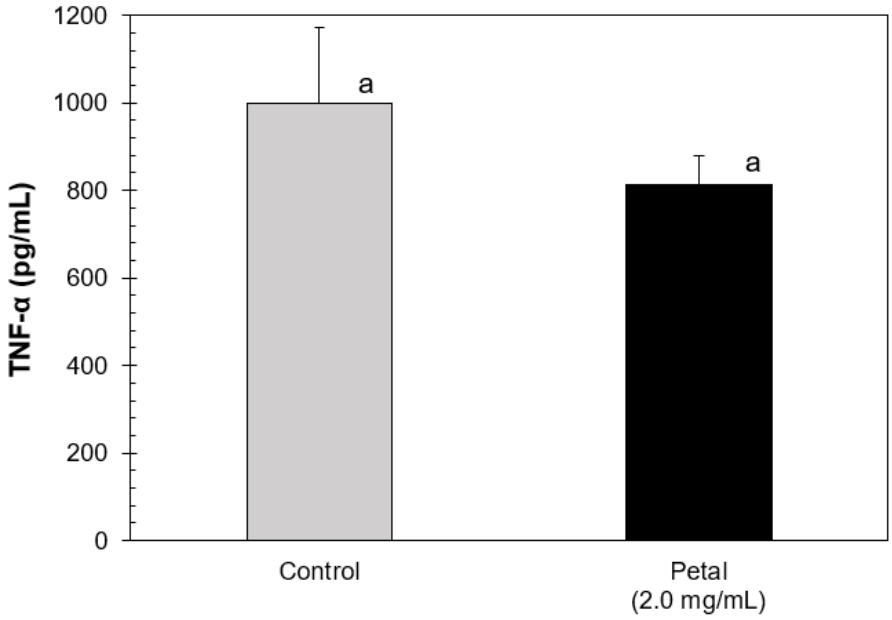

Figure 7. Effect of petal extract on inflammatory cytokine TNF-α production in RAW 264.7 macrophages

TNF-α production was RAW 264.7 macrophages have been seeded in 24-well culture plates at of 0.2×10^5 cells/well and 1.0×10^6 cells/well, and cultured in a CO_2 incubator for 2 h. Petal extract and 10 μg/mL LPS were then added to each cell suspension and cultured for 24 h. TNF-α was only significantly reduced when the petal extract concentration was 2.0 mg/mL.

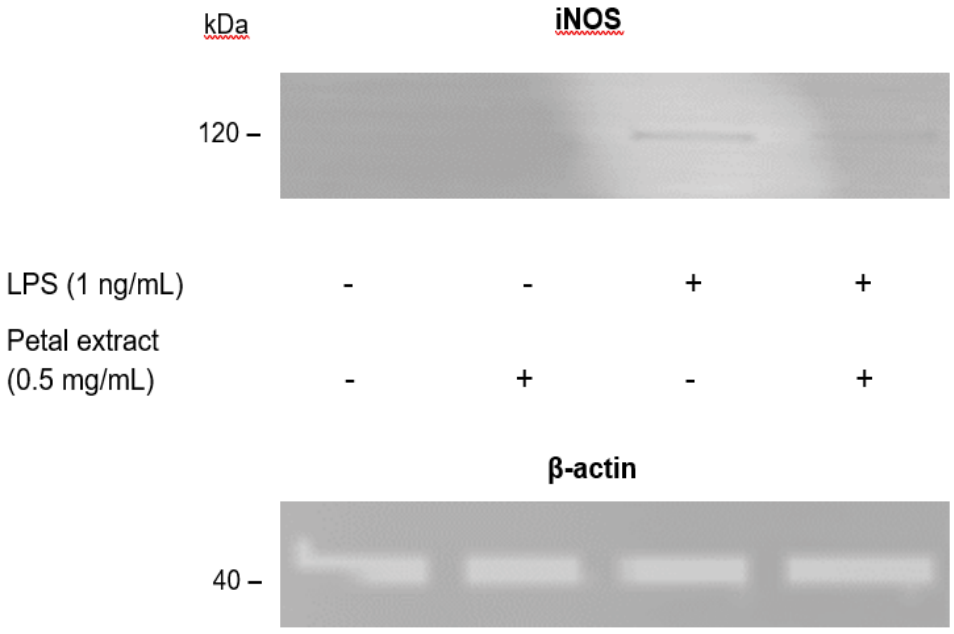

Figure 8. Effect of petal extract on iNOS expression in LPS-induced RAW 264.7 macrophages

The expression-suppressing effect of petal extract in iNOS in LPS-stimulated macrophages RAW 264.7 macrophages was analyzed by Western Blotting. The expression levels of iNOS become strongly induced by LPS, petal extract significantly inhibited iNOS induced by LPS in a concentration-dependent manner. The detection of beta-actin become completed withinside the identical blot an inner control.

3.5 Discussion

Chemical mediators released in allergic reactions act on blood vessels to increase blood flow and increase vascular permeability, and such allergic reactions and infectious diseases cause exudation of body fluids and plasma proteins and neutrophils and, promote local migration and infiltration of inflammatory cells such as macrophages. As a result, inflammatory symptoms such as redness, heat, swelling, and pain are exhibited. When LPS, a bacterial cell wall component[15], binds to TLR4, a type of macrophage transmembrane receptor, iNOS expression occurs via signal transduction. iNOS releases a large amount of NO from L-arginine, and $ONOO^-$, which is produced by the reaction between NO and O_2^-, damages cells and exacerbates the symptoms. And, inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are also produced and released[16], and these substances stimulate macrophages again to further promote the inflammatory response. TNF-α is an activator of the transcription factor NF-κB, and the activated NF-κB induces the expression of iNOS and promotes the inflammatory reaction[17,18].

Inflammation and intake of food are attracting interest as one of the approaches to mitigate on the grounds that there are little slow side effects than drugs, its use is expected. *Echinacea purpurea* is vital immunostimulatory and anti-inflammatory[19], particularly medicinal vegetation of perennial with a healing agent of the signs and symptoms of the common cold[20,21]. In previous studies, EP extracts, particularly in petals extract, have been shown to have anti-allergic property of inhibiting concentrations of leukotriene B_4 and histamine. In this study, we studied the inhibitory effect of petal extract on NO production by macrophages. NO production from RAW 264.7 macrophages by LPS stimulation was used as an indicator.

NO production from the cells was suppressed by the EP extracts, in which the activity of the petal extract was stronger than that of the leaf extract (Fig.3). The effect of the petal extract on NO production the from RAW 264.7 macrophages at various concentrations (Fig.4). A dose-dependent tendency was observed at 0.1-0.4 mg/mL of petal extract, and the suppressive effect at 0.2 and 0.4 mg/mL were

significant. In addition, RAW 264.7 after LPS stimulation was solubilized under the same conditions, and the iNOS expression level was examined by Western blotting using an anti-iNOS antibody. As a result, the petal extract was iNOS compared with control. There was a tendency to inhibit expression. From the results of NO release inhibitory activity and iNOS expression inhibition was not due to cytotoxicity.

Subsequently, the TNF-α, IL-1β, and IL-6 are an inflammatory cytokine was analyzed by ELISA assay. TNF-α macrophages produce and release has many effects such as induction of activation and apoptosis of macrophages and NK cells, it plays an important role in the inflammatory response. It also induces the production of IL-1β and IL-6. The IL-1β and IL-6 is produced from a variety of cells such as macrophages lymphocytes and fibroblasts and promotes the differentiation of B cells into antibody producing cells. As a result, the acute protein of RAW 264.7 macrophages, chemokines, the expression of cell adhesion molecules are induced[22]. Processing RAW 264.7 macrophages with petal extract was significantly inhibited IL-1β and IL-6 production by 0.5 mg/mL (Fig.5 and 6). On the other hand, TNF-α declined only significant if the petal extract concentration of 2.0 mg/mL (Fig.7). These results, petal extract inhibits the release of NO by inhibiting the expression of iNOS synthesis, inhibiting the excessive inflammatory response by inhibiting the production and release of TNF-α, IL-1β, and IL-6. The polyphenols were estimated was suggested as an active ingredient.

In conclusion, these data suggest that the hot water extract of EP petals may alleviate symptoms of inflammation.

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Chapter 4

Conclusions

In this study, we evaluated the effect of EP extracts on major mediators of allergic and inflammatory responses in mast cells and macrophages.

A positive correlation was observed between the inhibitory activity against the release of histamine and the total polyphenol content of the extracts. Antioxidant activity of the petal extract was the strongest, followed by the leaf and stem extracts, indicating that there is a positive correlation between the polyphenol content and antioxidant activity of the extracts. Consequently, any water-soluble substances other than antioxidants in the leaf and stem extracts could be involved in their ability to inhibit LTB_4. These data suggest that the hot water extract of EP petals may alleviate symptoms of type-I allergies by inhibiting the release of chemical mediators from basophilic leukocytes and mast cells.

The extract of EP petals has the most NO production suppression effect than the leaf and stem extracts. And petal extract suppressed the inflammatory cytokine TNF-α, IL-1β, and IL-6 production. The synthesis of iNOS was suppressed by petal extract. These results suggest that hot water extract of EP petals may alleviate inflammation by suppressing NO release by inhibiting iNOS expression.

Further studies will be required to clearly understand the uptake of the EP extracts into cells and their activity *in vivo* using allergic animal models.

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