



Trigonelline: An alkaloid with anti-degranulation properties

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ABSTRACT

Trigonelline, one of the alkaloids contained in coffee, is important not only as one of the constituents of aroma and flavor in coffee but also as a useful source of nutrition. Its anti-microbial, anti-carcinogenic, and anti-hyperglycemic effects have been investigated in previous studies. However, there have not been any studies examining the anti-degranulation effect of trigonelline. In this study, the anti-degranulation effect of trigonelline was evaluated in *in vitro* and *in vivo* models using a rat basophilic leukemia cell line, RBL-2H3 cells, and a passive cutaneous anaphylaxis (PCA) reaction in mice, respectively. In the β -hexosaminidase release assay, trigonelline effectively suppressed antigen-induced degranulation of RBL-2H3 cells in a dose-dependent manner without cytotoxicity. Trigonelline also inhibited Fc ϵ RI-mediated intracellular signaling pathways, such as phosphorylation of PLC γ 1, PI3K, and Akt, in antigen-stimulated RBL-2H3 cells and suppressed the PCA response in mice. Moreover, trigonelline also inhibited the microtubule formation in RBL-2H3 cells, indicating that trigonelline could inhibit IgE-sensitized mast cell degranulation by attenuating both the intracellular calcium-dependent and independent pathways. These results revealed that trigonelline possesses the anti-degranulation effect against the development of allergic diseases.

1. Introduction

The type I allergy reaction is a rapid hypersensitivity reaction, which includes allergic rhinitis, allergic dermatitis, food allergy, bronchial asthma, and anaphylaxis (Vaillant and Zito, 2019). The reaction is caused by the entry of an antigen (known as an allergen), such as pollen, certain foods, and even drugs, from the environment into the body (Actor, 2012). Globally, between 10 and 30 % of the population suffers from allergic rhinitis, 300 million people have asthma, and about 240–550 million people suffer from a food allergy (Pawankar et al., 2013). Allergies have become a social problem because allergy symptoms can not only decrease the quality of life but also cause life-threatening reactions. Type I hypersensitivity reactions begin with a bond between antigens by at least two immunoglobulin E (IgE) molecules attached to specific high-affinity receptors (Fc ϵ RI) on the mast cell surface. The activation of mast cells involves the release of chemical mediators that have been formed and stored in granules. This process of releasing chemical mediators is called degranulation, which is a trigger for some allergy reactions (Moon et al., 2014). In the past 50 years, the

number of people suffering from allergies, especially type I allergies, has been increasing dramatically throughout the world. Hence, the discovery of drugs and functional food sources with anti-allergic activity is necessary.

Among the compounds contained in coffee, caffeine along with chlorogenic acids and diterpenes has received attention related to its potency in the field of human health and disease prevention, but researchers began to focus on trigonelline (Cano-Marquina et al., 2013). Trigonelline, or *N*-methyl nicotinic acid, is contained in coffee beans at 1–3 % of dry weight (Ashihara, 2015), which indirectly contributes to the formation of flavor-forming compounds in coffee (Wei and Tanokura, 2015). Aside from being one of the flavor-forming compounds, trigonelline is also partially converted to nicotinic acid during the roasting process (Ashihara, 2015). Nicotinic acid, also known as niacin, is an essential human nutrient in the form of vitamin B₃, making coffee beverages a significant source of the vitamin. Trigonelline also has several health benefits; it can be used to inhibit the invasion of liver cancer cells *in vitro*, to prevent dental caries, and to treat hyperglycemia, hyperlipidemia, and liver/kidney dysfunctions (Hirakawa et al.,

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2005; Daglia et al., 2002; Hamden et al., 2013). Although there have been reports on the health benefits of trigonelline, there have not been any studies examining the anti-degranulation effect of trigonelline. In this study, we investigated the mechanism of the degranulation-suppressive effect of trigonelline on RBL-2H3 cells stimulated with antigens. We also evaluated the inhibition of *in vivo* degranulation by trigonelline using a passive cutaneous anaphylaxis (PCA) mouse model.

2. Materials and methods

2.1. Reagents

Mouse anti-dinitrophenol (DNP) monoclonal IgE, DNP-human serum albumin (HSA), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, bovine serum albumin (BSA), wortmannin, Triton X-100, and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Sigma (St. Louis, MO, USA). *p*-Nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside and Immunostar LD were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). Biotinylated rat anti-mouse IgE was purchased from BD Pharmingen (San Diego, CA, USA). Aprotinin and pefabloc SC were obtained from Roche Applied Science (Basel, Switzerland). A phosphatase inhibitor cocktail was purchased from Nacalai Tesque (Kyoto, Japan). The following antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA): anti-Lyn, anti-phosphorylated Lyn, anti-Syk, anti-phosphorylated Syk, anti-Bruton's tyrosine kinase (Btk), anti-phosphorylated Btk, anti-phosphatidylinositol 3-kinase (PI3K) p55, anti-phosphorylated PI3K p85/p55, anti-phospholipase C- γ 1 (PLC γ 1), anti-phosphorylated PLC γ 1, anti-Akt, anti-phosphorylated Akt, anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), horseradish peroxidase-labeled (HRP-labeled) anti-rabbit IgG antibody, and Alexa Fluor 488-labeled anti- α -tubulin antibody.

2.2. Sample preparation

Trigonelline (Sigma-Aldrich, Buchs, Switzerland; purity $\geq 98\%$) was dissolved in distilled water (DW). The pH of the solution was adjusted to 7.4, and the concentration was adjusted at 20 mmol L^{-1} . The trigonelline solution was subsequently sterilized by filtration using a $0.22\text{ }\mu\text{m}$ filter.

2.3. Cell seeding

RBL-2H3 cells were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in DMEM supplemented with 100 U mL^{-1} of penicillin, $100\text{ }\mu\text{g mL}^{-1}$ of streptomycin, and 10% FBS. For cell seeding, the cells were detached from culture dishes with phosphate-buffered saline (PBS) containing 0.02% ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA) and 0.25% trypsin. After terminating trypsinization with 10% FBS-DMEM, the cell suspension was centrifuged at $190 \times g$ for 5 min at room temperature. The supernatant was removed, and the cells were centrifuged in 10% FBS-DMEM at $190 \times g$ for 5 min at room temperature. Furthermore, the cell density was adjusted at $2.0 \times 10^5\text{ cells mL}^{-1}$ with 10% FBS-DMEM, inoculated at $200\text{ }\mu\text{L well}^{-1}$ in a 96-well culture plate (Corning, Corning, NY, USA), and incubated for 18 h at 37°C under humidified 5% CO_2 – 95% air.

2.4. β -Hexosaminidase release assay

Antigen-induced degranulation of RBL-2H3 cells was determined using a β -hexosaminidase release assay. After pre-incubation for 18 h, the cells were washed with PBS and sensitized with 50 ng mL^{-1} of anti-DNP IgE in 5% FBS-DMEM for 2 h. After IgE sensitization, the cells were washed twice with Tyrode's buffer composed of 20 mmol L^{-1} HEPES, 135 mmol L^{-1} NaCl, 5 mmol L^{-1} KCl, 1.8 mmol L^{-1} CaCl_2 ,

1 mmol L^{-1} MgCl_2 , 5.6 mmol L^{-1} glucose, and 0.05% BSA, at pH 7.4. Next, Tyrode's buffer containing various concentrations of trigonelline, $0.5\text{ }\mu\text{mol L}^{-1}$ wortmannin (positive control), or DW (control) was added at $120\text{ }\mu\text{L well}^{-1}$ and incubated at 37°C for 10 min. Then, $0.625\text{ }\mu\text{g mL}^{-1}$ of DNP-HSA was added at $10\text{ }\mu\text{L well}^{-1}$ and incubated for 30 min at 37°C . After incubation, the culture plate was placed on ice for 10 min to terminate antigen stimulation. The supernatant was collected from each well and transferred to another 96-well plate on ice. The cells were subsequently sonicated in $130\text{ }\mu\text{L}$ of Tyrode's buffer containing 0.1% Triton X-100 for 5 s well^{-1} on ice to prepare the cell lysates. Next, $50\text{ }\mu\text{L well}^{-1}$ of supernatant and cell lysate were transferred to another 96-well plate and pre-incubated at 37°C for 5 min. After pre-incubation, $100\text{ }\mu\text{L}$ of substrate solution containing 3.3 mmol L^{-1} *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside dissolved in 0.1 mol L^{-1} citrate buffer (pH 4.5) was added to each well and incubated at 37°C for 25 min. The enzymatic reaction was terminated by adding 2 mol L^{-1} glycine buffer (pH 10.4) at $100\text{ }\mu\text{L well}^{-1}$, and the absorbance was measured at 405 nm using a microplate reader (SH-8000Lab; Corona Electric, Hitachinaka, Japan). The percentage of β -hexosaminidase release was calculated according to the following formula:

$$\beta\text{-hexosaminidase release (\%)} = ((S-BS) / ((S-BS) + (C-BC))) \times 100\%$$

where S is the absorbance of the supernatant, BS is the absorbance of the blank used for the supernatant, C is the absorbance of the cell lysate, and BC is the absorbance of the blank used for the cell lysate.

2.5. Measurement of cell viability

Trigonelline cytotoxicity to RBL-2H3 cells was tested using a WST-8 solution (Nacalai Tesque). Anti-DNP IgE-sensitized cells were treated with various concentrations of trigonelline and stimulated with DNP-HSA as described in the β -hexosaminidase release assay. After washing the cells with PBS, as much as $100\text{ }\mu\text{L}$ of 10% FBS-DMEM containing $10\text{ }\mu\text{L}$ of WST-8 solution was added to each well, and the cells were incubated for 30 min at 37°C in the dark. After incubation, the absorbance of each well was measured at 450 nm and the reference at 655 nm using a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Measurement of antigen-antibody interaction

The antigen-antibody interaction was examined by enzyme-linked immunosorbent assay (ELISA). The 96-well plate was coated with $100\text{ }\mu\text{L well}^{-1}$ of DNP-HSA as antigen diluted at 250 ng mL^{-1} in 50 mmol L^{-1} carbonate buffer (pH 9.6) at 4°C overnight. This antigen would stick to the surface of the plate through adsorption. After that, the plate was washed 3 times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with $200\text{ }\mu\text{L well}^{-1}$ of PBS containing 5% skim milk at 37°C for 2 h. After washing 3 times with PBS-T, $50\text{ }\mu\text{L well}^{-1}$ of various concentrations of trigonelline was added and incubated for 20 min at 37°C . Then, 500 ng mL^{-1} of anti-DNP-IgE as primary antibody diluted in PBS was added at $50\text{ }\mu\text{L well}^{-1}$ and incubated at 37°C for 1 h (PBS alone was added as blank). After incubation, the plate was washed 3 times with PBS-T. Then, each well was treated with $100\text{ }\mu\text{L}$ of biotinylated rat anti-mouse IgE as secondary antibody diluted at 500 ng mL^{-1} in 5% skim milk-PBS at 37°C for 1 h. After washing 3 times with PBS-T, each well was treated with HRP-streptavidin conjugate (BioSource International, Camarillo, CA, USA) diluted 4,000 times in PBS containing 1% BSA at 37°C for 1 h. The streptavidin-HRP conjugate was bound to the secondary antibody that has been attached to the primary antibody. After incubation, the plate was washed 3 times with PBS-T, and 0.6 mg mL^{-1} of 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt dissolved in 50 mmol L^{-1} citrate buffer (pH 4.0) containing 0.03% H_2O_2 was added at $100\text{ }\mu\text{L well}^{-1}$ as substrate solution. The substrate produced a green color after reacting

with the bound enzyme. After the color was sufficiently developed, one hundred μL of 1.5 % oxalic acid was added to terminate the coloring reaction. The amount of primary antibody was estimated from the absorbance of the coloration by measurement at 415 nm using a microplate reader (SH-8000Lab).

2.7. Measurement of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)

$[\text{Ca}^{2+}]_i$ was measured using a Calcium kit-Fluo 3-AM (Dojindo Laboratories) according to the manufacturer's instructions. After pre-incubation, the RBL-2H3 cells were washed with PBS. Then, 120 μL of anti-DNP IgE in 5 % FBS-DMEM at 50 ng mL^{-1} was added to each well, and the plate was incubated for 2 h. Following the IgE-sensitization, the cells were washed twice with PBS and incubated with 100 μL of Fluo 3-AM for 1 h. After incubation, the cells were washed twice with PBS and incubated with 100 μL of trigonelline solution at 37 °C for 10 min. After that, the plate was measured using the microplate reader (SH-8000Lab) at 0 min. Then, 10 μL of DNP-HSA solution diluted in Tyrode's buffer at 0.625 $\mu\text{g mL}^{-1}$ was added to each well to stimulate the cells, and the fluorescence intensity was measured.

2.8. Immunoblot analysis

The steps in immunoblotting are protein extraction, electrophoresis, electrotransfer, and detection. For protein extraction, 2 ml of RBL-2H3 cell suspension was seeded into 3.5 cm dishes at 2.5×10^5 cells mL^{-1} and incubated at 37 °C for 18 h. After incubation, the cells were washed with PBS and treated with 1 ml dish $^{-1}$ of anti-DNP IgE (50 ng mL^{-1}) in 10 % FBS-DMEM. After incubation for 2 h, the dishes were washed twice with Tyrode's buffer, then 10 mmol L^{-1} trigonelline in Tyrode's buffer was added to the dishes at 980 $\mu\text{L dish}^{-1}$ and incubated for 10 min. For blank and control, Tyrode's buffer alone was used instead of 10 mmol L^{-1} trigonelline. The cells were then stimulated by adding DNP-HSA (2.5 $\mu\text{g mL}^{-1}$) in Tyrode's buffer at 20 $\mu\text{L dish}^{-1}$ and incubated for 5 min. After the incubation, the dishes were washed with sterilized PBS, and cell lysis buffer composed of 50 mmol L^{-1} Tris, 150 mmol L^{-1} NaCl, 50 mmol L^{-1} NaF, 30 mmol L^{-1} $\text{Na}_4\text{P}_2\text{O}_7$, 1 % Triton X-100, 2 $\mu\text{g mL}^{-1}$ of Pefabloc SC, Aprotinin, complete EDTA-free protease inhibitor cocktail, and a phosphatase inhibitor cocktail was added at 100 $\mu\text{L dish}^{-1}$. After that, the cell lysates were collected and put on ice for 30 min. They were centrifuged at 12,000 $\times g$ for 15 min at 4 °C, and each supernatant was equalized to the same protein concentration. The protein samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through electrophoresis method, transferred to polyvinylidene fluoride membranes (PVDF; GE Healthcare, Buckinghamshire, UK), blocked with 5 % skim milk-Tris-buffered saline containing 0.1 % Tween 20 (TBS-T) at room temperature for 1 h, and incubated overnight at 4 °C with the primary antibody in 5 % BSA-TBS-T. After incubation, the membranes were washed 3 times with TBS-T at room temperature for 5 min and then treated with the secondary antibody (HRP-labeled anti-rabbit IgG antibody) in 5 % skim milk-TBS-T at room temperature for 1 h. The signals were detected using ChemiDoc XRS Plus apparatus (Bio-Rad Laboratories) after the blots were developed with Immunosstar LD. The chemiluminescent intensity was then quantified using Quantity One software (Bio-Rad Laboratories).

2.9. Microtubule formation analysis

RBL-2H3 cells were seeded into a 35 mm culture dish at 1.5×10^5 cells mL^{-1} with 50 ng mL^{-1} of anti-DNP IgE and sensitized overnight at 37 °C. After washing with Tyrode's buffer twice, anti-DNP IgE-sensitized cells were treated with 980 μL of Tyrode's buffer containing trigonelline (2.5 and 10 mmol L^{-1}), 3 $\mu\text{mol L}^{-1}$ of nocodazole as positive control, or DW as a control at 37 °C for 10 min. Then cells were stimulated with 20 μL of 2.5 $\mu\text{g mL}^{-1}$ of DNP-HSA solution diluted in Tyrode's buffer

and incubated at 37 °C for 10 min. After washing twice with PBS, pre-treated cells were covered with 1 ml of 4 % paraformaldehyde at room temperature for 15 min with shaking. After washing with PBS for 5 min three times, the cells were blocked with 200 μL of PBS containing 0.3 % Triton X-100 and 5 % BSA for 1 h. After the blocking solution was aspirated, the cells were stained with Alexa Fluor 488-labeled anti- α -tubulin antibody diluted in PBS containing 0.3 % Triton X-100 and 1 % BSA at 4 °C overnight with shaking. After washing with PBS for 5 min three times, the cells were treated with 200 μL of DAPI solution diluted in PBS at room temperature for 15 min with shaking. After washing with PBS for 5 min three times, the cells were treated with an anti-fading agent SlowFade (Molecular Probes, Eugene, OR, USA) and observed with a confocal microscope (Fluoview FV1000, Olympus, Tokyo, Japan). A software FV10-ASW2.1 (Olympus) was used to analyze the images.

2.10. Passive cutaneous anaphylaxis (PCA)

This PCA experiment was performed according to the method by Yasunaga et al. (2016) with some modifications. Six-week-old female BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan) and maintained in a temperature-controlled room under a 12 h light/dark cycle at 24 ± 1 °C. The mice were given standard food and water *ad libitum*. This animal experiment was approved by the Animal Experiment Committee of Ehime University and was performed under the Guidelines of Animal Experiments of Ehime University. The mice were injected intradermally with 10 μL of anti-DNP IgE at 5 ng mL^{-1} into the right auricle and with 10 μL of PBS into the left auricle. After 23 h, the mice were orally administered 6 mg kg body weight (BW) $^{-1}$ or 30 mg kg BW $^{-1}$ of trigonelline. One hour later, the mice were challenged by injecting intravenously with 200 μL of PBS containing 200 μg of DNP-HSA and 0.5 % Evans blue through the tail vein. Thirty minutes after the challenge, the mice were sacrificed, and both auricles were photographed and collected. PCA reaction was estimated by quantifying the amount of Evans blue in the auricles. Evans blue pigment was extracted from each auricle with 600 μL of formamide at 70 °C overnight. After the extraction, the absorbance of the dye was measured at 620 nm using a spectrophotometer (Ultrospec 3000; Amersham Pharmacia Biotech, Uppsala, Sweden).

2.11. Statistical analysis

All results are represented as the mean $\pm$ standard deviation (SD) based on three separate experiments. Statistical significance among groups was evaluated using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. Statistical analysis was performed using GraphPad Prism version 7.02 (GraphPad Software, La Jolla, CA, USA). Values at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ against control were considered statistically significant.

3. Results

3.1. Effect of trigonelline on the antigen-mediated degranulation of RBL-2H3 cells

Trigonelline used in this experiment is a pure commercial reagent. The purpose of this experiment was to examine the inhibitory effect of trigonelline on β -hexosaminidase release from RBL-2H3 cells induced immunologically using the DNP-HSA antigen. This antigen binds to IgE antibody molecules that attach to the Fc ϵ RI receptor and stimulate mast cell activation. Activation of mast cells stimulates the release of chemical mediators such as histamine and leukotriene from inside granules in the mast cells, which is a process called degranulation. β -Hexosaminidase is one of the chemical mediators involved in allergic responses. Trigonelline inhibited the release of β -hexosaminidase from

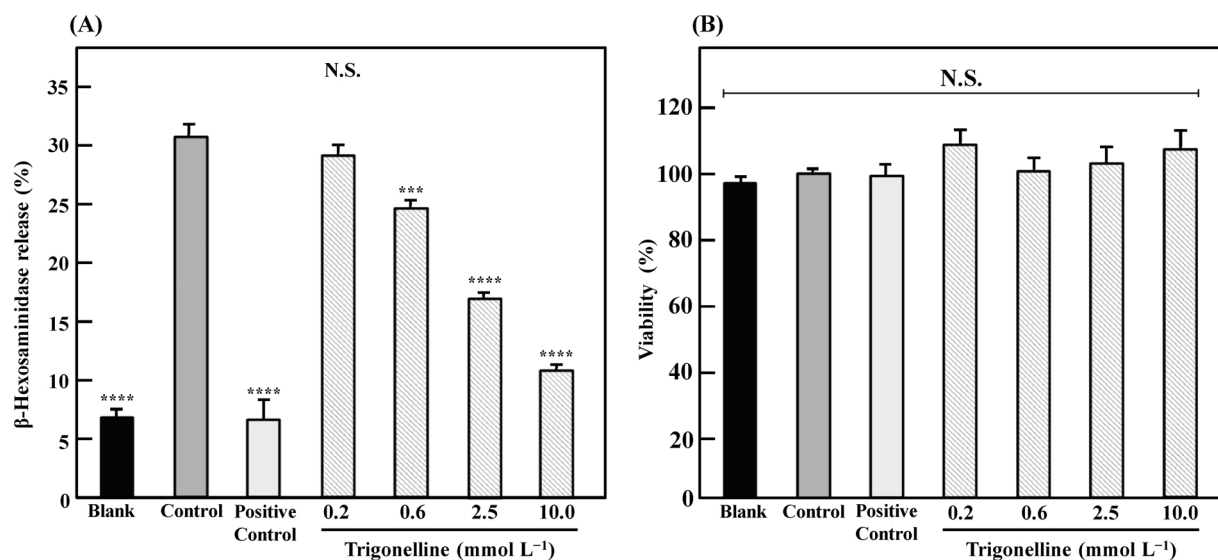


Fig. 1. Effect of trigonelline on RBL-2H3 cell degranulation. Effect of trigonelline on β -hexosaminidase release (A) and cell viability (B) in RBL-2H3 cells stimulated with IgE/antigen and treated with trigonelline at indicated concentrations, with $0.5 \mu\text{mol L}^{-1}$ wortmannin as positive control, or with DW as control. Data are represented as mean $\pm$ SD ($n = 3$). *** $p < 0.001$, **** $p < 0.0001$ against control. N.S. indicates not statistically significant.

antigen-induced RBL-2H3 cells (Fig. 1A). The concentrations of trigonelline were 0.2, 0.6, 2.5, and 10 mmol L^{-1} , which inhibited the release of β -hexosaminidase by 8.5, 20.4, 33.5, and 59.4 %, respectively. Trigonelline suppressed the degranulation in a dose-dependent manner as well as wortmannin as a positive control, without cytotoxic effects on the RBL-2H3 cells (Fig. 1B).

3.2. Effect of trigonelline on the antigen-antibody interaction

The interaction between antigen and antibody on the receptor triggers the activation of RBL-2H3 cells and their degranulation, therefore there was a possibility that trigonelline can interfere with this interaction. Furthermore, we examined whether the suppressive effect of trigonelline on RBL-2H3 cells degranulation was caused by inhibition of antigen-antibody binding using ELISA. Anti-DNP IgE and various concentrations of trigonelline were added to the 96-well microplate coated with DNP-HSA. The coloring reaction was performed after the addition of secondary antibody labeled with HRP-streptavidin conjugate. The result showed that the absorbance was not changed, regardless of the concentration variations of trigonelline (Fig. 2), showing that trigonelline inhibits the degranulation of RBL-2H3 cells without affecting antigen-antibody interaction.

3.3. Effect of trigonelline on $[\text{Ca}^{2+}]_i$ in the RBL-2H3 cells

The above findings confirmed that trigonelline reduces degranulation of IgE-Fc ϵ RI-mediated mast cells. Thus, we next investigated whether trigonelline affects the $[\text{Ca}^{2+}]_i$ level in mast cells. $[\text{Ca}^{2+}]_i$ increases when the RBL-2H3 cells induce degranulation by IgE-antigen stimulation. The pretreatment with trigonelline (at concentrations of 2.5 and 10 mmol L^{-1}) significantly reduced the $[\text{Ca}^{2+}]_i$ level in the RBL-2H3 cells induced by antigens in a dose-dependent manner (Fig. 3).

3.4. Effect of trigonelline on intracellular signaling pathways in antigen-stimulated degranulation

To further investigate whether trigonelline inhibits degranulation through regulating the signaling pathway, we examined the phosphorylation of some signaling molecules. As shown in Fig. 4, the phosphorylation of PLC γ 1, PI3 K, and Akt was significantly reduced by

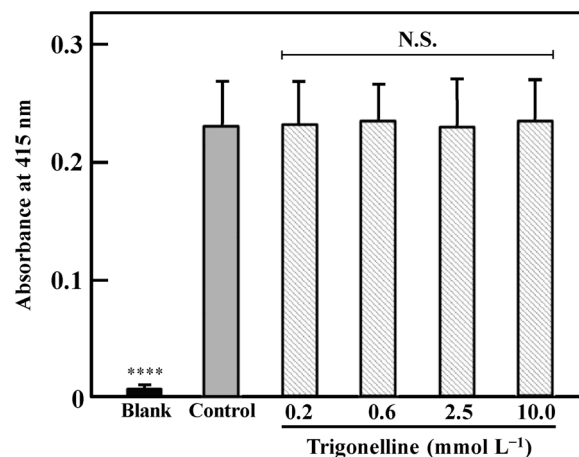


Fig. 2. Effect of trigonelline on the antigen-antibody interaction. Various concentrations of trigonelline and 500 ng mL^{-1} of anti-DNP IgE were added to each well of a 96-well microplate immobilized with DNP-human serum albumin. After washing with PBS-T, the rat anti-mouse IgE labeled with biotin and the streptavidin-HRP conjugate were added and washed out after the binding reaction. The green color was generated after the reaction of a chromogenic substrate with HRP. After sufficient color development, a stop solution was added and the absorbance was measured at 415 nm using a microplate reader. Data are represented as mean $\pm$ SD ($n = 3$). **** $p < 0.0001$ against control. N.S. indicates not statistically significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

trigonelline, while the phosphorylation of Lyn, Syk, or Btk did not suppressed by trigonelline. These results revealed that trigonelline suppresses antigen-induced degranulation in the RBL-2H3 cells through down-regulation of the signaling pathways involved in degranulation.

3.5. Effect of trigonelline on microtubule formation

Microtubule formation is a crucial process for degranulation, which is involved in the movement of intracellular granules and the fusion of cell membrane with granule membrane. Thus, the effect of trigonelline on microtubule formation was observed by fluorescence microscopy. As shown in Fig. 5, microtubules spread out to the plasma membrane after

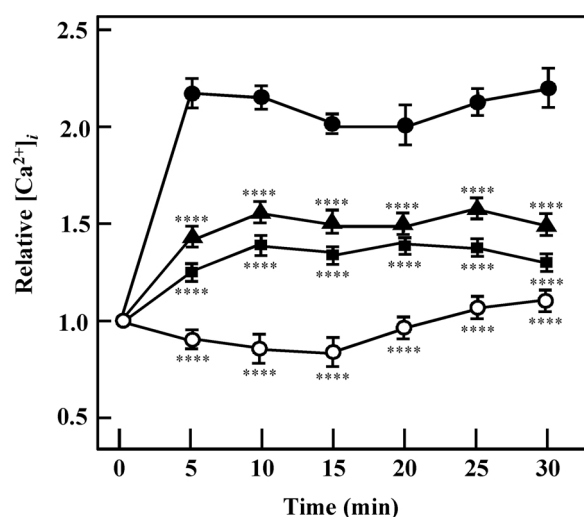


Fig. 3. Effect of trigonelline on IgE-mediated $[Ca^{2+}]_i$ in sensitized RBL-2H3 cells. The anti-DNP IgE-sensitized cells were loaded with Fluo 3-AM for 1 h. The cells were treated with trigonelline (2.5 and 10 mmol L^{-1}) or DW for 10 min and then stimulated by DNP-HSA. The fluorescent intensity was recorded every 5 min. Data are represented as mean $\pm$ SD ($n = 3$). ●, DW-treated cells stimulated with antigen; ▲, 2.5 mmol L^{-1} trigonelline-treated cells stimulated with antigen; ■, 10 mmol L^{-1} trigonelline-treated cells stimulated with antigen; ○, DW-treated cells not stimulated with antigen. **** $p < 0.0001$ against control.

antigen stimulation to build tracks for granule transport. On the other hand, the microtubule formation in RBL-2H3 cells stimulated with antigen was inhibited in the presence of trigonelline. These results suggest that trigonelline suppresses degranulation by affecting the microtubule formation occurring after the elevation in intracellular calcium concentration. In other words, trigonelline is able to inhibit the degranulation of mast cell by attenuating both the intracellular Ca^{2+} -dependent and independent pathways (Fig. 6).

3.6. Effect of oral administration of trigonelline on PCA model mice

Local extravasation was induced through a local injection of IgE antibodies followed by a DNP-HSA antigen challenge. IgE was injected intradermally into one ear of each mouse. Twenty-four hours after IgE administration, a DNP-HSA-Evans blue solution was injected into the tail vein of each mouse to induce a local allergic reaction. Oral administration of trigonelline 1 h before injection of antigens inhibited the mast cell-mediated PCA reaction in a dose-dependent manner (45.74 % at 6 mg $kg\ BW^{-1}$ and 59.53 % at 30 mg $kg\ BW^{-1}$), indicating that trigonelline exerts anti-allergic effects through the inhibition of mast cell degranulation (Fig. 7).

4. Discussion

Trigonelline is a pyridine derivative found in some plants and is less toxic to humans compared with chemical medicines (Qin et al., 2017). According to Tice (1997), trigonelline is found at high levels in fenugreek seeds (1,300 ppm) and coffee beans (3,000–13,000 ppm).

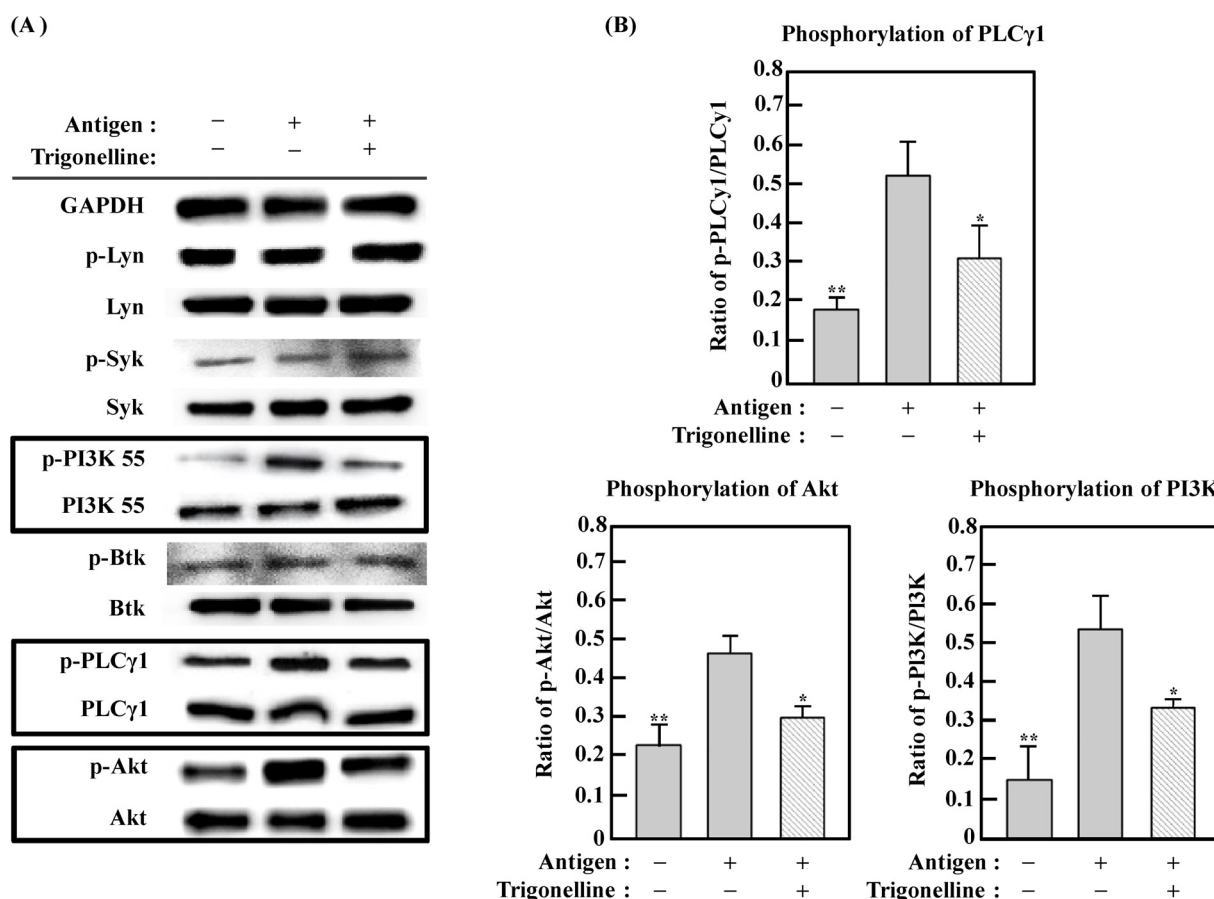


Fig. 4. Effect of trigonelline on signaling pathways involved in degranulation of RBL-2H3 cells stimulated by antigen. (A) Western blot diagram and (B) statistical analysis for the ratio of p-Lyn/Lyn, p-Syk/Syk, p-PI3K/PI3K, p-Btk/Btk, p-PLCγ1/PLCγ1, and p-Akt/Akt in each cell lysate. GAPDH was used as a loading control. The Western blot diagram represents three independent experiments with similar results. Results are expressed as mean $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$ against control.

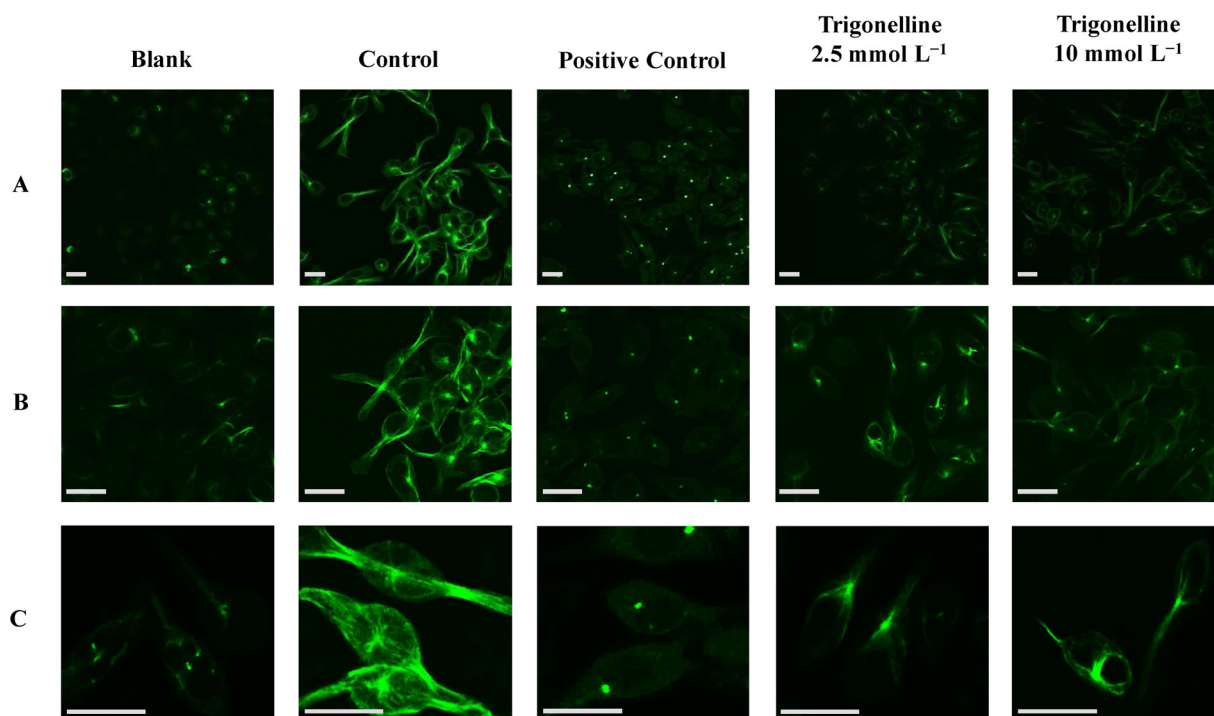


Fig. 5. Effects of trigonelline on microtubule formation in RBL-2H3 cells. Degranulation was induced with DNP-HSA as antigen after anti-DNP IgE-sensitized RBL-2H3 cells were treated with trigonelline (2.5 and 10 mmol L⁻¹), with 3 μmol L⁻¹ of nocodazole as a positive control, or with DW as control. After 30 min, cells were fixed with 4 % paraformaldehyde, permeabilized with PBS containing 0.1 % Triton X-100, and blocked with 5 % BSA-PBS. After staining with Alexa Fluor 488-labeled anti-α-tubulin antibody, the cells were observed under a confocal microscope with ×60 magnification of an objective lens (oil immersion). Three rows A, B, and C indicate the zoom factor of ×1, ×2, and ×4, respectively. These figures are typical images from three experiments. Scale bar, 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

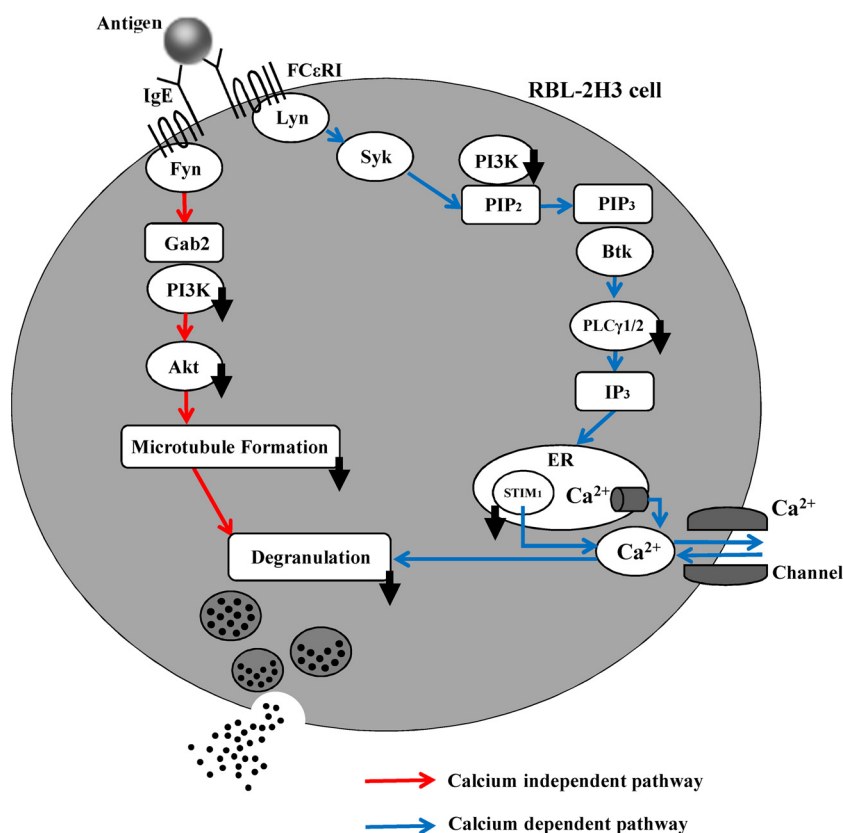


Fig. 6. A scheme showing the mechanism of action of the signaling pathways of trigonelline in inhibiting degranulation. This scheme describes the major molecules and activities in mast cell activation. It shows the inhibitory effect of trigonelline on the phosphorylation of PLCγ1, PI3K, and Akt, which affect the inhibition of antigen-stimulated degranulation of RBL-2H3 cells through both the intracellular Ca²⁺-dependent and independent pathways. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

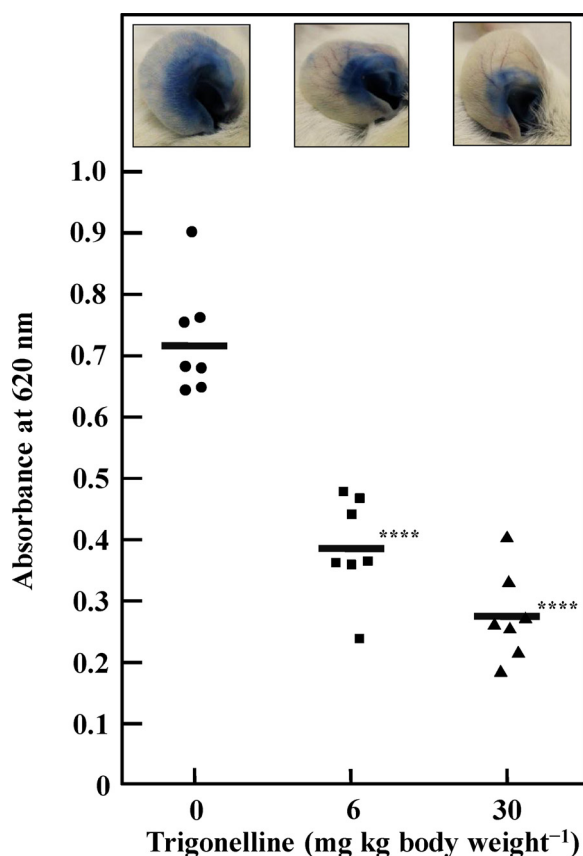


Fig. 7. Trigonelline inhibits the IgE-mediated PCA reaction. The blue dye extracted from each ear was detected using a spectrophotometer at 620 nm. There were three groups of treated mice: control (no trigonelline treatment), low trigonelline (6 mg kg BW⁻¹ trigonelline treatment), and high trigonelline (30 mg kg BW⁻¹ trigonelline treatment). Each group consisted of 7 female mice. Bars indicate the average of each group. **** $p < 0.0001$ against control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fenugreek extract is known to have anti-allergic activity derived from the bioactive compounds it contains, one of which could be trigonelline (Bae et al., 2012; Dar and Uddin, 2018). Coffee is also a source of trigonelline. The other compounds contained in coffee, such as caffeine, chlorogenic acid, and diterpene, have received considerable attention for their potential roles in human health and disease prevention, but researchers began investigating the potential of trigonelline related to health benefits (Zuo et al., 2015; Cardenas et al., 2015; Burdan, 2015). Even industry has become aware of the benefits of trigonelline to health. In Japan, there is a coffee shop that provides coffee rich in trigonelline (Ashihara, 2015). The importance of trigonelline in coffee is also related to nutrition. It has been revealed in recent studies that trigonelline has several biological activities beneficial for humans, including against dental caries, cancer cells, Alzheimer's disease, the hypocholesterolemic effect, and diabetes (Hirakawa et al., 2005; Daglia et al., 2002; Hamden et al., 2013; Tohda et al., 2005; Mawla and Osman, 2011). Trigonelline has many health benefits, but there have not been any studies that have examined the anti-allergic effect of trigonelline.

In type I allergic reactions, when allergens as antigens enter the human body for the first time, a type of white blood cell (B-cell) produces IgE antibodies, which attach to the surface of mast cells via a receptor molecule. There are two types of receptor molecules: those with high affinity for IgE (FcεRI) and those with lower affinity (FcεRII). IgE attaches to specific high-affinity receptors (FcεRI). Although the serum IgE concentration is very low compared with other

immunoglobulins, it is sufficient to bind to this receptor. Cell types that have high-affinity receptors (FcεRI) are mast cells and basophils. Mast cells are activated when there is a cross-linking of the FcεRI molecule by the antigen bond with IgE that occupies the molecule. Activated mast cells produce biological reactions. Mast cells synthesize lipid mediators enzymatically from precursors stored in cell membranes and secrete chemical mediators that have been formed and stored in the granules, which is a process called degranulation (Metcalf et al., 2010). On the degranulation of mast cells, the release of chemical mediators is associated with clinical manifestations of allergies. RBL-2H3 cell degranulation can be detected by releasing histamine and leukotriene as chemical mediators. However, β-hexosaminidase is more suitable as a biomarker for RBL-2H3 cell degranulation because it is easily, effectively, and efficiently detected (Moon et al., 2014; Naal et al., 2004). The release of β-hexosaminidase causes allergy symptoms, such as red and itchy eyes, hives, and a runny nose (Elshemy and Abobakr, 2013). Through an *in vitro* experiment, we have shown that trigonelline can inhibit antigen-stimulated degranulation of RBL-2H3 cells without cytotoxic effect (Fig. 1). The β-hexosaminidase activity (%) was suppressed by trigonelline in a dose-dependent manner. The release of β-hexosaminidase was significantly inhibited at trigonelline concentrations of 0.7, 2.5, and 10 mmol L⁻¹ without cytotoxic effect on the cells.

In order to examine which part of the degranulation mechanism is inhibited by trigonelline, an antigen-antibody interaction inhibition assay was performed using ELISA. If trigonelline inhibits the antigen-antibody interaction, the absorbance would be expected to decrease in a dose-dependent manner. The result showed that the absorbance of the supernatant did not decrease by trigonelline treatment (Fig. 2), which means that the degranulation inhibitory activity of trigonelline is not caused by the inhibition of antigen-antibody reaction. Therefore, further investigation was needed to find out the possible causes of degranulation inhibition by the suppression of signaling pathways activation.

The release of chemical mediators is triggered by an influx of extracellular Ca²⁺ into the mast cells. This influx of Ca²⁺ results in an unstable mast cell membrane that is easily penetrated by chemical mediators (Gulledge et al., 2018). Trigonelline suppressed the increase in [Ca²⁺]_i in the RBL-2H3 cells. [Ca²⁺]_i in the mast cells increases by the activation of signaling pathways resulting from the interaction between antigen and antibody via FcεRI receptor bonds on mast cells. Fig. 3 shows that the level of [Ca²⁺]_i was suppressed by adding trigonelline in a dose-dependent manner. This suppression is along with the suppression of β-hexosaminidase release. This result indicates that the inhibitory effect of trigonelline on antigen-stimulated degranulation is due to suppressing the increase in intracellular Ca²⁺ concentration. Measuring [Ca²⁺]_i reveals that trigonelline suppresses degranulation of mast cells through inhibiting the calcium-dependent pathway.

Mast cells are activated through interactions between allergens and specific IgE antibodies that bind to FcεRI expressed on the surface of mast cells (Galli and Tsai, 2013). FcεRI is a multi-chain receptor that binds IgE with high affinity and is composed of IgE-binding α, β, and γ chains that are necessary for receptor expression and signal transduction. The α chain is located at the binding site for IgE that is responsible for ligand recognition; thus, β and γ subunits contain immunoreceptor tyrosine-based activation motifs (ITAMs). Phosphorylation of ITAMs plays a crucial role in propagating intracellular signaling (Zhang et al., 1996). β-Chain and γ-chain ITAMs are phosphorylated by activation of Lyn kinase. The phosphorylation of γ ITAMs activates Syk kinase (Metcalf et al., 2010; Kraft and Novak, 2006). Syk kinase activation appears to be necessary for the downstream signaling pathway. Several adaptor proteins have been identified in a molecular pathway following the activation of Syk kinase. Syk then phosphorylates the adaptor linker for activation of T cells (LAT), which is a membrane protein and activates the PI3 K pathway and the protein tyrosine kinase Btk. After Syk phosphorylation, this membrane protein forms a molecular complex that includes other adaptor proteins, including PLCγ1/2 (Siraganian

et al., 2010). Phosphorylated PLC γ 1/2 hydrolyzes phosphatidylinositol 4,5-bisphosphate into the second messengers, inositol 1,4,5-trisphosphate (IP $_3$) and diacylglycerol (DAG). IP $_3$ activates the IP $_3$ receptor on the endoplasmic reticulum and stimulates the release of Ca $^{2+}$, resulting in mast cell degranulation (Yang et al., 2012; Kania et al., 2017). Meanwhile, DAG activates protein kinase C (PKC), responsible for promoting the activation of various molecular pathway transcription factors, which results in pro-inflammatory cytokines and growth factor synthesis (Huang, 1989). In this study, the results of immunoblot analysis showed the suppression of phosphorylation of PLC γ 1, PI3 K, and Akt kinase in antigen-stimulated RBL-2H3 cells (Fig. 4). The phosphorylation of PLC γ 1, PI3 K, and Akt kinase was suppressed by adding trigonelline, which means that trigonelline inhibits degranulation of the cells (Fig. 4). PLC γ 1, a downstream target of Syk, was significantly inactivated by trigonelline. Suppressing the phosphorylation of PLC γ 1 inhibited the release of Ca $^{2+}$, which affected the suppression of mast cell degranulation; however, suppressing degranulation through the PKC pathway or independent calcium pathway also needs to be investigated.

The interaction between antigen and antibody on the surface of the mast cells triggers the activation of the pro-esterase enzyme. This enzyme causes the aggregation of microtubules in the mast cell cytoplasm. These microtubules are indispensable for the movement of granules containing chemical mediators towards the edge of the cells, so that it can be released out of the cells. From the results of intracellular signaling pathways in antigen-stimulated degranulation, it was shown that the phosphorylation of PI3 K and Akt kinase was suppressed by adding trigonelline. These signaling molecules are located on Fyn signaling pathway (the calcium-independent pathway) and Fyn–PI3 K pathway plays a key a critical role in the calcium-independent microtubule-dependent pathway (Nishida et al., 2011; Ogawa et al., 2014). The microtubule formation assay was conducted to prove that the suppression of degranulation might be caused by the inhibitory effect of trigonelline on the microtubule formation. Microtubule formation is crucial for degranulation process and it has been reported to occur after elevation in intracellular calcium concentration. Trigonelline was found to suppress degranulation by inhibiting microtubule formation (Fig. 5). These results indicate that trigonelline tends to affect the Fyn signaling pathway and thus inhibit the RBL-2H3 cells degranulation through prevention of microtubule formation (Fig. 6).

We examined the anti-allergic effect of trigonelline by *in vivo* experiment using PCA model mice. There were three groups of mice in this experiment: control (orally administered DW only), low trigonelline (6 mg kg BW $^{-1}$ of trigonelline), and high trigonelline (30 mg kg BW $^{-1}$ of trigonelline). The inhibitory effect of trigonelline against the PCA reaction induced in mice was measured. The intensity of the blue dye in the mice that were orally administered trigonelline decreased in a dose-dependent manner. Trigonelline at the dose of 6 mg kg BW $^{-1}$ inhibited the PCA reaction 45.7 % compared with the control group, while the dose of 30 mg kg BW $^{-1}$ inhibited the reaction 59.5 % compared with the control group.

5. Conclusions

In this study, we found that trigonelline inhibits mast cell degranulation, which was revealed through the PCA test using IgE-sensitized mice in an *in vivo* model. The *in vitro* study suggested that the degranulation-suppressive effect of trigonelline was not caused by inhibiting antigen-antibody interaction, but by modulating the intracellular signaling pathways involved in mast cell degranulation. Trigonelline inhibits IgE-sensitized mast cell degranulation by attenuating both the intracellular Ca $^{2+}$ -dependent and independent pathways. Trigonelline inhibited Fc ϵ R1-mediated intracellular signaling pathways by suppressing the phosphorylation levels of PLC γ 1, PI3 K, and Akt kinase in antigen-stimulated RBL-2H3 cells. These results suggested that trigonelline inhibits mast cell degranulation and may be

associated with reduced risk of allergic symptoms. Further, the results of this study can be the basis for developing trigonelline-rich products that provide health benefits, particularly those related to anti-allergy effects, as well as for developing drugs.

CRedit authorship contribution statement

Arita Dewi Nugrahini: Methodology, Investigation, Data curation, Formal analysis, Funding acquisition, Writing - original draft, Writing - review & editing. **Momoko Ishida:** Methodology, Validation. **Toshifumi Nakagawa:** Conceptualization, Investigation. **Kosuke Nishi:** Software, Supervision, Writing - review & editing. **Takuya Sugahara:** Supervision, Data curation, Formal analysis, Writing - review & editing.

Declaration of Competing Interest

All authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.molimm.2019.12.020>.

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