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# Anti-Allergic Effect of Clove

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**Abstract:** Allergic rhinitis is a common disease which has affected more than 500 million people worldwide over the last 20 years. Though not a severe disease, it has significant impacts on lowering quality of life due to its co-occurring symptoms like headache, asthma and sinusitis. Faced with this problem, scientists all over the world are constantly in search of active substances from natural sources to find the candidates for functional food materials. In this study, we focused on the anti-allergic effect of cloves; one of the spices used worldwide. Clove extract in 70% ethanol suppressed degranulation of rat basophilic leukemia cell line, RBL-2H3 cells. In addition, the elevation of intracellular $Ca^{2+}$ concentration induced by antigen stimulation was obviously suppressed by clove extract. Cloves can thus be a good candidate for the food and pharmaceutics industries.

**Keywords:** clove; anti-allergic effect; degranulation

## 1. Introduction

The rapid growth in the prevalence, severity, and complexity of allergic diseases in modern population has been of significant health concern. This is due to the burden they impose on patient's quality of life by displaying various symptoms such as asthma, chronic urticaria, allergic rhinitis, conjunctivitis and angioedema to life-threatening anaphylaxis [1-3]. Allergic rhinitis is a type I allergic disease induced by an immunoglobulin E (IgE) that has affected more than 500 million people worldwide over the last 20 years. Development of the disease commences with sensitisation to allergen, which elicits the predominant production of IgE by B cells [4]. The high-affinity IgE receptor (FcεRI) is expressed on mast cells and basophils. An allergic reaction is provoked by cross-linking of allergens to IgE bound on FcεRI, leading to FcεRI aggregation. Aggregation of FcεRI is followed by a rise in intracellular $Ca^{2+}$ concentration and the degranulation of mast cells. Chemical mediators including histamine, β-hexosaminidase and inflammatory mediators such as prostaglandins are released from intracellular granules during mast cell degranulation [1,5]. Released chemical mediators are responsible for the nasal itching, and sneezing symptoms of allergic rhinitis. Thus prevention of degranulation of mast cells and basophils is one of the most effective approaches to attenuate allergic symptoms.

The drugs used to treat allergic diseases are usually, anti-histamine, mast cell stabilizer and leukotriene receptor antagonists. However, anti-histamine drugs have undesirable side effects, most notably drowsiness, dry mouth etc. The use of functional food has become more popular in recent years, a safe and effective management of allergic rhinitis and other atopic diseases through food resources has received much attention [6-8]. Grape seed, jujube fruits, citrus species, passion fruit seeds, mango peels [6,9-12] and many others, have been shown to possess anti-allergic and anti-anaphylactic activities. In addition, many other spices such as rosemary, cinnamon and ingredients such as Aster yomena and Ocimum tenuiflorum [3,5,13-14], have also been shown to stabilize mast cell and cause inhibition of the allergic markers such as histamine, IL-4, and β-hexosaminidase in IgE-mediated allergic reaction.

Clove (Syzygium aromaticum) is a spice widely used to add flavour to food preparations [15]. It is indigenous to east Africa and Asian countries like Indonesia, and it has been reported to have many therapeutic uses. Clove oil has been experimentally shown to have potent antimicrobial activity against dental caries-causing microorganisms promoting their use as antimicrobial agents in dentistry [16]. It is also used to relieve pain, to control nausea and vomiting, stomach distension and gastrointestinal spasm [16-18]. However the anti-allergic effects of cloves have not yet been revealed. This study was therefore aimed at analysing the potentials of clove in suppressing the development of allergic responses in mice cells.

## 2. Materials and Methods

### *2.1. Reagents*

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, bovine serum albumin (BSA), fetal bovine serum (FBS), mouse anti-dinitrophenol (DNP) monoclonal IgE, DNP-human serum albumin (HSA) conjugate, and Triton X-100 were products of Sigma-Aldrich (St. Louis, MO, USA).All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) unless otherwise noted.

### *2.2. Sample preparation*

Clove seed powder was provided by S&B Foods Inc. (Tokyo, Japan). The powder was suspended in 70% ethanol at 0.05g/ml and extracted at 12°C for 24 h. The solution was then centrifuged at 4°C, 15,000 × *g* for 20 min to remove insoluble materials**.** The supernatant was filtered through a 0.22 μm membrane filter, and concentrated using an evaporator and a freeze-drier. The final concentration was adjusted to 70mg/mL with 70% ethanol. It was sterilized by filtration through a 0.22 μm membrane filter and the ethanol extract of clove (EEC) was used for the experiments described below.

### *2.3. Cell and cell culture*

RBL-2H3 cells (rat basophilic leukemia cells) were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 5% FBS at 37°C under humidified 5% $CO_2$.

### *2.4. β-Hexosaminidase release assay*

The assay was performed using the method of Nishi *et al*. and Hwang *et al.* [1,8] with some modifications. RBL-2H3 cells were seeded at 4.0 × $10^5$ cells/well into a 96-well plate (Corning, Corning, NY, USA) and sensitized with 50 ng/mL of anti-DNP IgE diluted in 5% FBS-DMEM for 24 h at 37°C. After washing with the modified Tyrode's (MT) buffer (20 mM HEPES, 135 mMNaCl, 5 mMKCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.05% BSA, pH 7.4), the cells were treated with 120 μL of various concentrations of EEC diluted in MT buffer for 10 min at 37°C. The cells were subsequently challenged for 30 min at 37°C by adding 10 μL of DNP-HSA diluted in MT buffer at the final concentration of 0.625 μg/mL. After the collection of supernatant, cells were sonicated in 130 μL of MT buffer containing 0.1% Triton X-100 for 5 sec on ice. Both supernatant and cell lysate were transferred into a new 96-well microplate at 50 μL/well and incubated for 5 min at 37°C. Then, 100 μL of 3.3 mM 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (Wako Pure Chemical Industries) dissolved in 0.1 M citrate buffer (pH 4.5) was added to each well and incubated at 37°C for 25 min. The enzyme reaction was terminated by the addition of 100 μL of 2 M glycine buffer (pH 10.4), and the absorbance was measured at 415 nm using a microplate reader. The β-hexosaminidase release rate was calculated as:

$$100 \times \left[ \frac{OD_{\text{supernatant}} - OD_{\text{blank of supernatant}}}{\{(OD_{\text{supernatant}} - OD_{\text{blank of supernatant}}) + (OD_{\text{cell lysate}} - OD_{\text{blank of cell lysate}})\}} \right] \quad (1)$$

*2.5. Cell viability assay*

The cytotoxicity of EEC to RBL-2H3 cells was examined using Cell Count Reagent SF (Nacalai Tesque). RBL-2H3 cells were seeded, sensitized with anti-DNP IgE, treated with various concentrations of EEC, and challenged with DNP-HSA as described above. After the cells were washed with phosphate-buffered saline (PBS, pH 7.4) once, 100 μL of DMEM containing 10 μL of WST-8 solution was added to each well of the cell culture plate and incubated for 45 min at 37°C. The absorbance was then measured at 450 nm and 655 nm using a microplate reader.

*2.6. Measurement of intracellular $Ca^{2+}$ concentration*

The intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]i$) was measured using a Calcium KitFluo 3(Dojindo Laboratories, Kumamoto, Japan) as reported by Nishi *et al*. [1], with some modifications. RBL-2H3 cells were seeded in a black 96-well culture plate and sensitized with anti-DNP IgE as described above for β-hexosaminidase assay. After washing with warm PBS twice, the IgE-sensitized cells were incubated with 100 μL of Fluo-3 AM for 1 h. The cells were then washed with PBS, and treated with 100 μL of various concentrations of EEC for 10 min. Basal reading was taken for 2 sec. Then, the cells were stimulated by the addition of 10μL of DNP-HSA diluted in MT buffer at the final concentration of 0.625 μg/mL, and the fluorescence intensity was immediately monitored for the next 120 sec with an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader.

*2.7. Statistical analysis*

Data obtained were expressed as mean ± standard deviation. One way ANOVA followed by Tukey-Kramer test as used to assess the statistical significance of the difference. Values with $^{*}p < 0.05$ or $^{**}p < 0.01$ were considered statistically significant.

**3. Results**

*3.1. Effect of EEC on the rate of β-hexosaminidase released*

Mast cells are known to undergo degranulation upon sensitization with IgE and activation with allergens. In order to determine the effect of EEC on mast cell degranulation, RBL-2H3 cells were sensitized with anti-DNP IgE and challenged with DNP-HSA. The rate of degranulation was measured by the percentage of β-hexosaminidase release with or without pre-treatment of the cells with different concentrations of EEC. As shown in Figure 1, treatment of RBL-2H3 cells with EEC suppressed the amount of β-hexosaminidase released in a dose dependent manner.

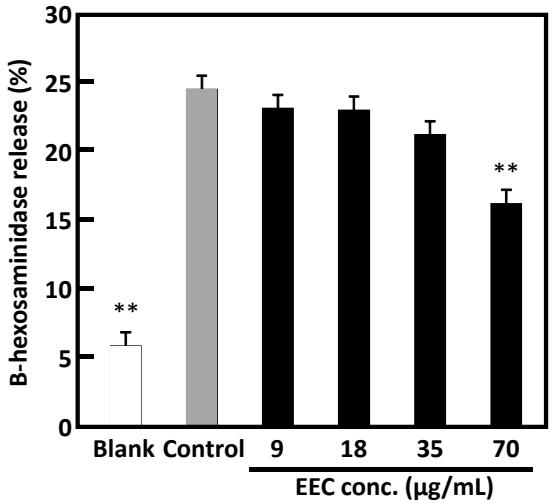


**Figure 1.** Effect of EEC on degranulation of RBL-2H3 cells stimulated with antigen. Anti-DNP IgE-sensitized RBL-2H3 cells were treated with various concentrations of EEC or 0.07% ethanol (blank and control). Degranulation was induced by the addition of DNP-HSA diluted in MT buffer. Blank cells were treated with MT buffer alone. Released β-hexosaminidase was used as a marker of degranulation. Experiments were performed in triplicate, and error bars indicate standard deviation. ** $p < 0.01$ against control by Tukey-Kramer test.

### *3.2. Effect of EEC on the cell viability of RBL2H3 cells*

Cell viability assay using the WST-8 kit (Cell Count Reagent SF) revealed that the relative viability of RBL-2H3 cells after pre-treatment with various concentrations of EEC was not significantly different from the control (Figure 2), indicating that EEC was not toxic to the cells.

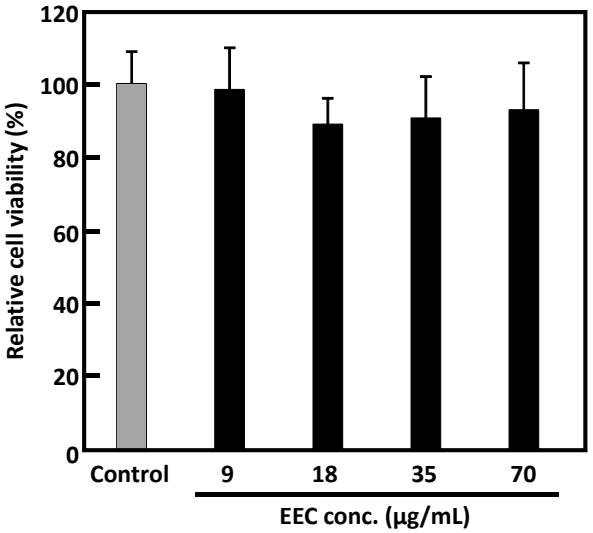


**Figure 2.** Effect of EEC on $[Ca^{2+}]i$ in RBL-2H3 cells stimulated with antigen. Anti-DNP IgE-sensitized RBL-2H3 cells were treated with various concentrations of EEC or 0.07% ethanol (control). Cell viability was measured using a WST-8 kit after stimulation with DNP-HSA. Experiments were performed in triplicate, and error bars indicate standard deviation. Tukey-Kramer test was used to assess the statistical significance of the difference against control.

*3.3. Effect of EECon the intracellular $Ca^{2+}$concentration*

Various cellular responses leading to mast cell degranulation are activated by the increase in intracellular $Ca^{2+}$ concentration ([$Ca^{2+}$]i). To find out if the suppressive effect of EEC on mast cell degranulation is due to an inhibition of the elevation levels of intracellular $Ca^{2+}$, the [$Ca^{2+}$]i in RBL-2H3 cells stimulated with antigen was measured using Fluo-3 AM. As shown in Figure 3, EEC inhibited the amount of intracellular $Ca^{2+}$ in stimulated RBL-2H3 cells as compared with the control.

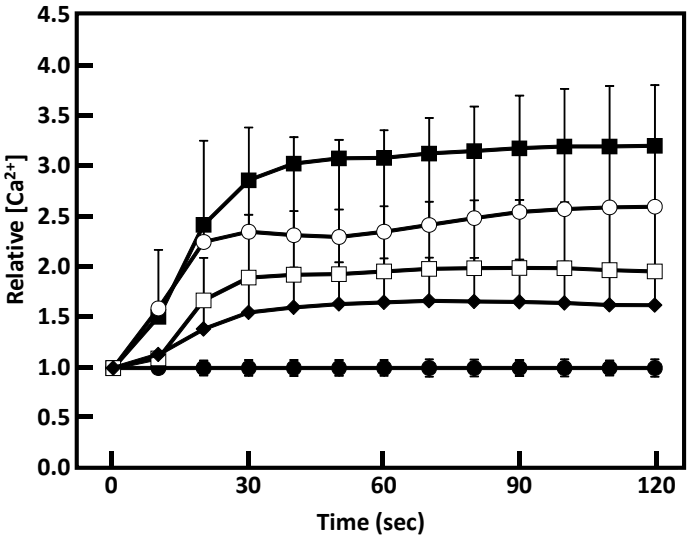


**Figure 3.** Effect of EEC on [$Ca^{2+}$]i in RBL-2H3 cells stimulated with antigen. After anti-DNP IgE-sensitized RBL-2H3 cells were incubated with Fluo-3 AM, the cells were treated with various concentrations of EEC or ethanol as control. Fluorescence intensity was measured immediately after inducing degranulation by treating with DNP-HSA. Closed circle, ethanol-treated cells not stimulated with DNP-HSA; closed square, ethanol-treated cells stimulated with DNP-HSA; open circle, 18 μg/ml of EEC-treated cells stimulated with DNP-HSA; open square, 35 μg/ml of EEC-treated cells stimulated with DNP-HSA; closed diamond, 70 μg/ml of EEC-treated cells stimulated with DNP-HSA. Experiments were performed in triplicate, and error bars indicate standard deviation.

## 4. Discussion

Screening in search of candidates for functional food materials revealed that EEC could have a significant effect in alleviating allergic symptoms. The release of the enzyme β-hexosaminidase which together with histamine is considered a major mediator of the acute inflammation and early hypersensitivity responses in allergic rhinitis was dose-dependently suppressed by EEC. Moreover, the relative viability of RBL-2H3 cells was not affected by all concentrations of EEC tested, indicating that the suppressive effect of EEC on degranulation of RBL-2H3 cells was not caused by cytotoxicity. Amit *et a*l. [3], found similar results with the spice cinnamon. Our results also showed that there was a decrease in intracellular calcium concentration after treatment with EEC. It is known that intracellular calcium mobilization precedes mast cell degranulation. Also, the measurement of intracellular calcium serves as an important marker for mast cell activation and it has been shown to be inhibited in many other anti-allergic foodstuffs [1,5].

## 5. Conclusions

This study reveals that EEC inhibits mast cell allergic reactions by suppressing degranulation. However, the effect of EEC on degranulation *in vivo* and its effect on intracellular signaling pathways involved in degranulation process have not yet been clarified. Such experiments will be performed in the future.

## References


1. Nishi, K.; Teranishi, M.; Yasunaga, S.; Iitsuka, M.; Matsumoto, S.; Sugahara, T. The major whey protein β-lactoglobulin inhibits IgE-mediated degranulation of RBL-2H3 cells and passive cutaneous anaphylaxis in mice. *Int Dairy J***2014**, *39*, 89-95, 10.1016/j.idairyj.2014.05.006.
2. Ren, M.; Tang, Q.; Chen, F.; Xing, X.; Huang, Y.; Tan, X. *MahuangFuziXixin* decoction attenuates Th1 and Th2 responses in the treatment of ovalbumin-induced allergic inflammation in a rat model of allergic rhinitis. *J Immunol Res* **2017**, *2017*, 1-12, 10.1155/2017/8254324.
3. Kandhare, A.D.; Aswar, U.M.; Mohan, V.; Thakurdesai, P.A. Ameliorative effects of type-A procyanidins polyphenols from cinnamon bark in compound 48/80-induced mast cell degranulation. *Anat Cell Biol***2017**, *50*, 275-283, 10.5115/acb.2017.50.4.275
4. Galli, S.J.; Tsai, M.; Piliponsky, A.M. The development of allergic inflammation. *Nature* **2008**, 454, 445-454, 10.1038/nature07204.
5. Prakash, A.; Ebenezer, A.J.; Vasanth, S.; Nagarajan, G.; Elden B.T. Effect of *Ocimumtenuiflorum* Linn extract on histamine mediated allergic inflammation in human mast cells. *JBAPN***2017**, *7*, 10-17, 10.1080/22311866.2016.1275983.
6. Bernstein, D.I.; Bernstein, C.K.; Deng, C.; Murphy, K.J.; Bernstein, I.L.; Bernstein, J.A.; Shukla, R. Evaluation of the clinical efficacy and safety of grapeseed extract in the treatment of fall seasonal allergic rhinitis; a pilot study. *Ann Allergy Asthma Immunol***2002**, *88*, 272-278, 10.1016/S1081-1206(10)62008-X.
7. Resnick, E.S.; Bielory, B.P.; Bielory, L. Complementary therapy in allergic rhinitis. *Curr Allergy Asthma Rep* **2008**, *8*, 118-125, 10.1007/s11882-008-0021-y.
8. Hwang, K.-A.; Hwang, Y.-J.; Song, J. Anti-allergic effect of *Aster yomena* on ovalbumin-sensitized mouse and RHL-2H3 cells via Th1/Th2 cytokine balance. *J Funct foods***2018**, *44*, 1-8, 10.1016/j.jff.2018.02.026
9. Naik, S.R.; Bhagat, S.; Shah, P.D.; Tare, A.A.; Ingawale, D.; Wadekar, R.R. Evaluation of anti-allergic and anti-anaphylactic activity of ethanolic extract of *Zizyphusjujuba*fruits in rodents. *Rev Bras Farmacogn***2013**, *23*, 811-818, 10.1590/S0102-695X2013000500014.
10. Onishi, S.; Nishi, K.; Yasunaga, S.; Muranaka, A.; Maeyama, K.; Kadota, A.; Sugahara, T. Nobiletin, a polymethoxy flavonoid, exerts anti-allergic effect by suppressing activation of phosphoinositide 3-kinase. *J Funct Foods***2014**, *6*, 606-614, 10.1016/j.jff.2013.12.005.
11. Mizusaki, A.; Nishi, K.; Nishiwaki, H.; Sugahara, T. Suppressive effect of ethanol extract from passion fruit seeds on IgE production. *J Funct Foods* **2017**, *32*, 176-184, 10.1016/j.jff.2017.02.030.
12. Ishida, M.; Sasaki, T.; Nishi, K.; Tamamoto, T.; Sugahara, T. Suppressive effect of ethanol extract from mango (*Mangiferaindica* L.) peel on IgE production *in vitro* and *in vivo*. *Biosci Biotech Bioch***2018**, *82*, 732-739, 10.1080/09168451.2017.1412250.
13. Osakabe, N.; Takano, H.; Sanbongi, C.; Yasuda, A.; Yanagisawa, R.; Inoue, K.; Yoshikawa, T. Anti-inflammatory and anti-allergic effect of rosmarinic acid (RA); inhibition of seasonal allergic rhinoconjunctivitis (SAR) and its mechanism. *Biofactors***2004**, *21*, 127-131, 10.1002/biof.552210125.
14. Sim, J.H.; Lee, H.S.; Lee, S.; Park, D.E.; Oh, K.; Hwang, K.-A.; Kang, H.-R.; Ye, S.-K.; Kim, H.-R. Anti-asthmatic activities of an ethanol extract of *Aster yomena* in an ovalbumin-induced murine asthma model. *J Med Food***2014**, *17*, 606-611, 10.1089/jmf.2013.2939.
15. Bachiega, T.F.; de Sousa, J.P.; Bastos, J.K.; Sforcin, J.M. Clove and eugenol in noncytotoxic concentrations exert immunomodulatory/anti-inflammatory action on cytokine production by murine macrophages. *J Pharm Pharmacol***2012**, *64*, 610-616, 10.1111/j.2042-7158.2011.01440.x.
16. Xu, J.-S.;Li, Y.; Cao, X.; Cui, Y.The effect of eugenol on the cariogenic properties of *Streptococcus mutans* and dental caries development in rats.*ExpTherMed***2013**, *5*,1667-1670,10.3892/etm.2013.1066.
17. Sulieman, A.M.E.; Boshra, I.M.O.; El Khalifa, E.A.A. Nutritive value of clove (*Syzygiumaromaticum*) and detection of antimicrobial effect of its bud oil. *Res J Microbiol***2007**, *2*, 266-271, ISSN 1816-4935.
18. Tanko, Y.; Mohammed, A.; Okasha, M.A.; Umar, A.H.; Magaji, R.A. Anti-nociceptive and anti-inflammatory activities of ethanol extract of *Syzygiumaromaticum* flower bud in wistar rats and mice. *Afr J Tradit Complement Altern Med* **2008**, *5*, 209-212, 10.4314/ajtcam.v5i2.31275.