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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation　Anti-allergic activity of bioactive compounds in coffee beans (*Coffea arabica* L.)
（コーヒー (*Coffea arabica* L.)豆に含まれる生理活性成分の抗アレルギー効果）

学位論文要約：
Dissertation Summary

Coffee is one of the most consumed drinks in the world. It is one of the best-selling commodities in the world market. Coffee is included in the *Coffea* genus that have around 100 species. However, only two of them are often cultivated and provide economic value, namely *Coffea canephora* (produces robusta coffee) and *Coffea arabica* L. (produces arabica coffee). Among of them, arabica coffee is the most widely commercialized type and has a high selling value. Coffee is preferred by consumers as a refreshing drink because it has a distinctive taste and aroma. This taste is due to the presence of complex chemical compounds, including caffeine, chlorogenic acid, and trigonelline. Besides being consumed daily of its flavor, coffee is also consumed because of its health benefits. These health benefits are influenced by the bioactive compounds contained in coffee.

Among the several compounds contained in coffee, caffeine and trigonelline are bioactive compounds that are widely used for its health benefits. Caffeine is one of the compounds in coffee that is also contained in other agricultural commodities such as tea and cocoa. Coffee has become one of the plants with caffeine content which is very often consumed by humans besides tea. Caffeine itself is classified into drugs and proven safe for consumption in certain doses. People consume caffeine every day for concentration and memory enhancement, and also physical performance improvement. Caffeine can also provide health benefits, such as a source of antioxidants, anti-cancer, anti-bacterial, and anti-inflammatory activities. Meanwhile, trigonelline is an alkaloid contained in coffee beans which indirectly contributes to the formation of flavor-forming compounds in coffee. 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.

In this study, caffeine and trigonelline contents in green coffee beans were detected by using High Performance Liquid Chromatography (HPLC). The reason for choosing green coffee beans as a sample is because these beans have not been roasted which will affect the concentration of caffeine and trigonelline. HPLC is a sample analysis method that uses the principle of chromatography. In chromatography, analysis is done by separating molecules based on differences in structure or composition. The separation occurs when the sample moves through the stationary phase because it is carried by the mobile phase. The various components in the sample will be separated based on differences in their affinity for the stationary phase. Compounds that can interact actively with the stationary phase will move more slowly so that they can separate from other compounds with weak interactions. Detection of caffeine and trigonelline content in coffee is carried out to prove the presence of both compounds in coffee. Thus, the results of studies on anti-allergic activity of caffeine and trigonelline can be used as a basis for the development of coffee commodities as functional food.

Allergies are known as hypersensitivity reactions and can be interpreted as immunological reactions to antigens that are unnatural in someone who has previously been exposed to certain allergens. Based on the mechanism of the reaction, allergies consist of four types namely type I (IgE-mediated hypersensitivity), type II

(antibody-mediated cytotoxic hypersensitivity), type III (immune complex-mediated hypersensitivity) and type IV (delayed-type hypersensitivity). Types I to III include immediate type allergic reactions, whereas type IV includes delayed type allergic reactions. These types of allergy can be seen in Figure 1.

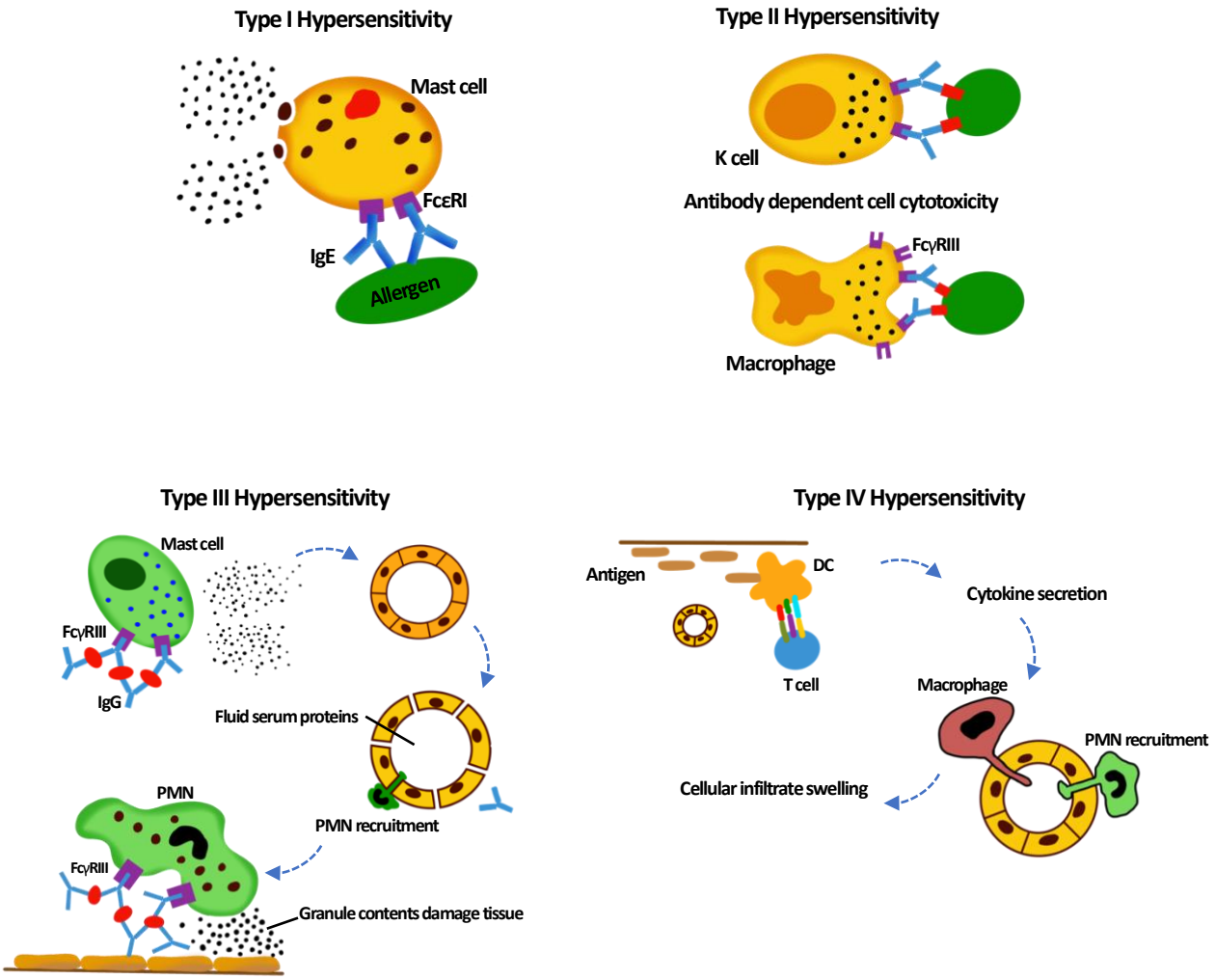

Figure 1. Types of allergy

Type I allergic reaction is initiated with the binding between allergens and IgE molecules attached to the high-affinity IgE receptor (FcεRI) on the surface of mast cells and basophils. Mast cells and basophils are granular immune cells that play an important role in allergic reactions. Rat basophilic cell line RBL-2H3 cells, like mast cells and basophils, respond by releasing of a series of mediators that evoke a potent allergic response, which is started by a cross-linking between allergens and IgE that binds to FcεRI. In the subsequent exposure, allergen will be bound to the IgE that primed on the mast cell, and it will activate the mast cell. Activated mast cell will release allergy mediators (β-hexosaminidase), and this step is called degranulation processes (Figure 2). β-Hexosaminidase is a common degranulation marker. It is suitable as a biomarker for degranulation of RBL-2H3 cells due to the easy, rapid detection. This β-hexosaminidase will be distributed in the bloodstream, and it will cause allergy symptoms, such as red and itchy eyes, hives, and runny nose. Inhibition of the degranulation process is expected to be able to inhibit allergic reactions. In this study, evaluation of anti-allergic activity especially those related to anti-degranulation of caffeine and trigonelline were performed by *in vitro* and *in vivo* models.

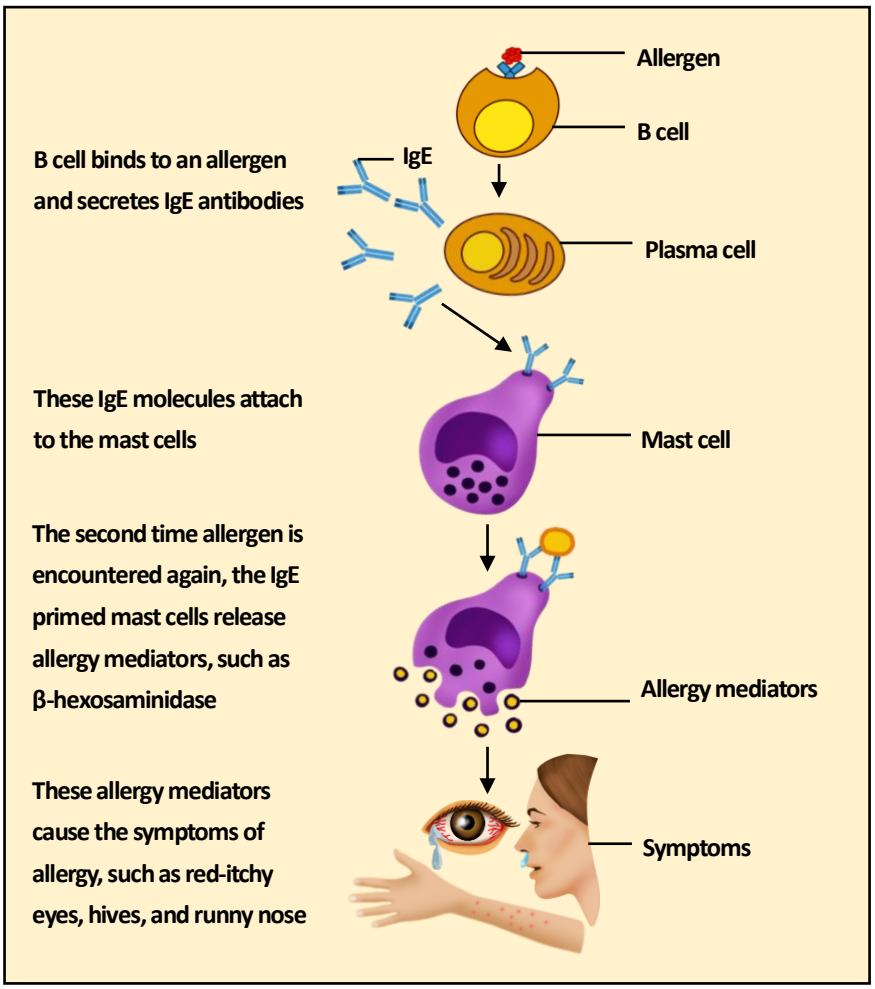

Figure 2. The role of mast cells in allergic mechanisms

In the first study, the anti-degranulation activity of caffeine through *in vitro* and *in vivo* experiments were examined. Through *in vitro* experiment, it has been proven that the β-hexosaminidase activity released was suppressed by the addition of caffeine in a dose-dependent manner without cytotoxic effect. Significant inhibitory activity of β-hexosaminidase release was obtained at caffeine concentrations on 0.62, 2.50, and 10 mM. RBL-2H3 cells sensitized with anti-DNP IgE were treated with various concentrations of caffeine at 37°C for 10 min, and degranulation was induced with DNP-HSA antigen at 37°C for 30 min. Cell viability was measured using the WST-8 solution after various concentrations of caffeine or distilled water (DW) as control was added to anti-DNP IgE-sensitized RBL-2H3 cells followed by stimulation by DNP-HAS antigen. Caffeine was also able to suppress the release of Ca^{2+} in cells. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in mast cells increases by the activation of signaling pathways due to the interaction between antigen and FcεRI receptor via IgE bonds. $[Ca^{2+}]_i$ was measured using the calcium kit Fluo 3-AM. Anti-DNP IgE-sensitized cells were incubated with Fluo 3-AM for 1 h and subsequently incubated with caffeine (2.5 mM and 10 mM) or DW for 10 min. The treated cells were stimulated by DNP-HSA antigen, and the fluorescence intensity was measured. As result, $[Ca^{2+}]_i$ was more suppressed in the addition of caffeine with a higher concentration (10 mM). It indicates that the inhibitory effect of caffeine on antigen-stimulated degranulation is due to suppression of intracellular Ca^{2+} elevation. Furthermore, the anti-degranulation effect stimulated by thapsigargin was measured. Various concentrations of caffeine or DW as control was added to RBL-2H3 cells, and the treated cells were stimulated by thapsigargin for 30 min to induce degranulation. Released β-hexosaminidase was used as a marker of degranulation. As result, it was found that

caffeine is able to inhibit extracellular influx of $[Ca^{2+}]_i$ into RBL-2H3 cells, so β-hexosaminidase release was suppressed compared to control in thapsigargin-induced RBL-2H3 cells. These data suggest that caffeine inhibits the degranulation caused by Ca^{2+} influx from the endoplasmic reticulum (ER). Next, the investigation of the signaling molecules was conducted by Western blotting. Allergy reaction initiates with the cross-linkage of antigen to FcεRI via IgE bonds that is followed by the phosphorylation of Lyn, Syk, and Fyn kinases as members of Src family of kinases. In this study, the results of immunoblot analysis showed the suppression of phosphorylation of Syk, Btk, PLCγ1, PI3K, and Akt kinase in antigen-stimulated RBL-2H3 cells. The phosphorylation levels of Syk, Btk, and PLCγ1 are located downstream of the tyrosine kinase Lyn. They were suppressed by caffeine, although the caffeine did not affect the phosphorylation of Lyn. Accordingly, the inhibition of Syk activation by caffeine may be induced by suppression of the phosphorylation of γ-ITAMs. Syk is recruited and activated, and then it activates downstream signaling which includes phosphorylation of Btk and PLCγ1/2. PLCγ1, which is a downstream target of Syk, was significantly inactivated by caffeine treatment. Reactive oxygen species (ROS) generated by antigen stimulation regulate PLCγ1/2 activation. The ROS production stimulate PLCγ1 to evoke $[Ca^{2+}]_i$ oscillations by stimulating Ca^{2+} release from the IP_3 pool and $STIM_1$-regulated Ca^{2+} influx. Besides, the phosphorylation level of PI3K was decreased by treatment with caffeine. These results revealed that caffeine suppresses antigen-induced degranulation by RBL-2H3 cells through down-regulation of both the calcium-dependent signaling pathway and the calcium-independent pathway involved in degranulation (Figure 3). Thus, the suppression of degranulation might be important to alleviate the allergic symptoms.

Furthermore, the microtubule formation assay was conducted to prove that the suppression of degranulation might be caused by the inhibitory effect of caffeine on the microtubule formation. Microtubule formation is crucial for degranulation process and it has been reported to occur after elevation in intracellular calcium concentration. Caffeine was found to suppress degranulation by inhibiting microtubule formation. These results indicate that caffeine tends to inhibit the RBL-2H3 cells degranulation through prevention of microtubule formation. Next, the effect of caffeine on passive cutaneous anaphylaxis (PCA) in an *in vivo* mice model was tested. *In vitro* studies often conveniently eliminate whole organism physiological influences allowing for a detailed analysis of da compound's impact. In this case, the *in vitro* studies were conducted to understand the detailed mechanism of inhibition of caffeine on the degranulation reaction, while *in vivo* studies were used to observe dermal response to an allergen-IgE interaction. Therefore, the doses used in *in vivo* studies need to be adjusted based on their effects at physiological levels. Caffeine has side effects if taken as much as 15 mg/kg BW and fatal dose at 150-200 mg/kg BW; progressing side effects from mild effects including restlessness, nervousness, and irritability, to severer effects such as delirium, emesis, neuromuscular tremors, and convulsions. There were 3 groups of mice in this experiment, namely control group (orally administered by DW only), low caffeine group (1 mg/kg BW/day), and high caffeine group (5 mg/kg BW/day). Quantification of the allergic response by PCA reaction could be achieved by using Evans blue dye to measure the changes in vascular permeability after intravenous injection of antigen. An hour later after oral administration of caffeine, the antigen was injected intravenously along with Evans blue dye to measure the blue dye color that occurs due to mast cell IgE cross-linking and chemical mediators release. The intensity of the blue dye was decreased for the mice that were orally administered with caffeine, with the inhibition by higher caffeine dose being slightly less potent. Caffeine at a dose of 1 mg/kg BW/day inhibited the PCA reaction 64.1% compared to the control group. While at a dose of 5 mg/kg BW/day, the inhibition was 49.7% compared to the control group. In the first study, it was found that caffeine inhibits IgE-mediated type I allergic reaction through multiple mechanisms (Figure 3).

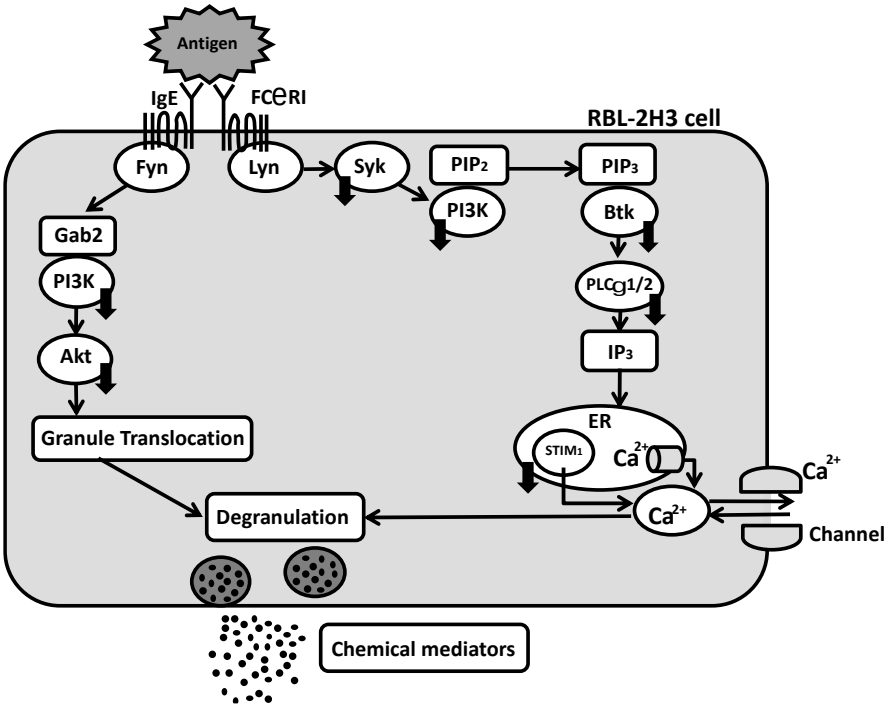

Figure 3. Mechanism of action for degranulation inhibition signaling pathways of caffeine

In the second study, the anti-allergic activity of trigonelline related to anti-degranulation was carried out by *in vitro* and *in vivo* models. Through an *in vitro* experiment, it was shown that trigonelline can inhibit antigen-stimulated degranulation of RBL-2H3 cells without cytotoxic effect. 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 mM 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 enzyme-linked immunosorbent assay (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, 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 Ca^{2+} into the mast cells. This influx of Ca^{2+} results in an unstable mast cell membrane that is easily penetrated by chemical mediators. $[Ca^{2+}]_i$ 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. The level of $[Ca^{2+}]_i$ 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 Ca^{2+} concentration. Measuring $[Ca^{2+}]_i$ reveals that trigonelline suppresses degranulation of mast cells through inhibiting the calcium-dependent pathway. To further investigate whether trigonelline inhibits degranulation through regulating the signaling pathway, the phosphorylation of some signaling molecules was examined. In this study, the results of immunoblot analysis showed the suppression of phosphorylation of PLCγ1, PI3K, and Akt kinase in antigen-stimulated RBL-2H3 cells. The phosphorylation of PLCγ1, PI3K, and Akt kinase was suppressed by adding trigonelline, which means that trigonelline inhibits degranulation of the cells. PLCγ1, a downstream target of Syk, was significantly inactivated by trigonelline.

Suppressing the phosphorylation of PLCγ1 inhibited the release of Ca^{2+}, which affected the suppression of mast cell degranulation. However, suppressing degranulation through the independent calcium pathway also needs to be investigated. Microtubule formation assay was conducted to examine trigonelline ability to inhibit the degranulation of mast cell through independent calcium pathway. 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. The results showed that microtubules spread out to the plasma membrane after 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 calcium-dependent and independent pathways (Figure 4). Furthermore, the anti-allergic effect of trigonelline by *in vivo* experiment was examined using PCA model mice. 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 inhibited the PCA reaction 45.7% compared with the control group, while the dose of 30 mg/kg BW inhibited the reaction 59.5% compared with the control group.

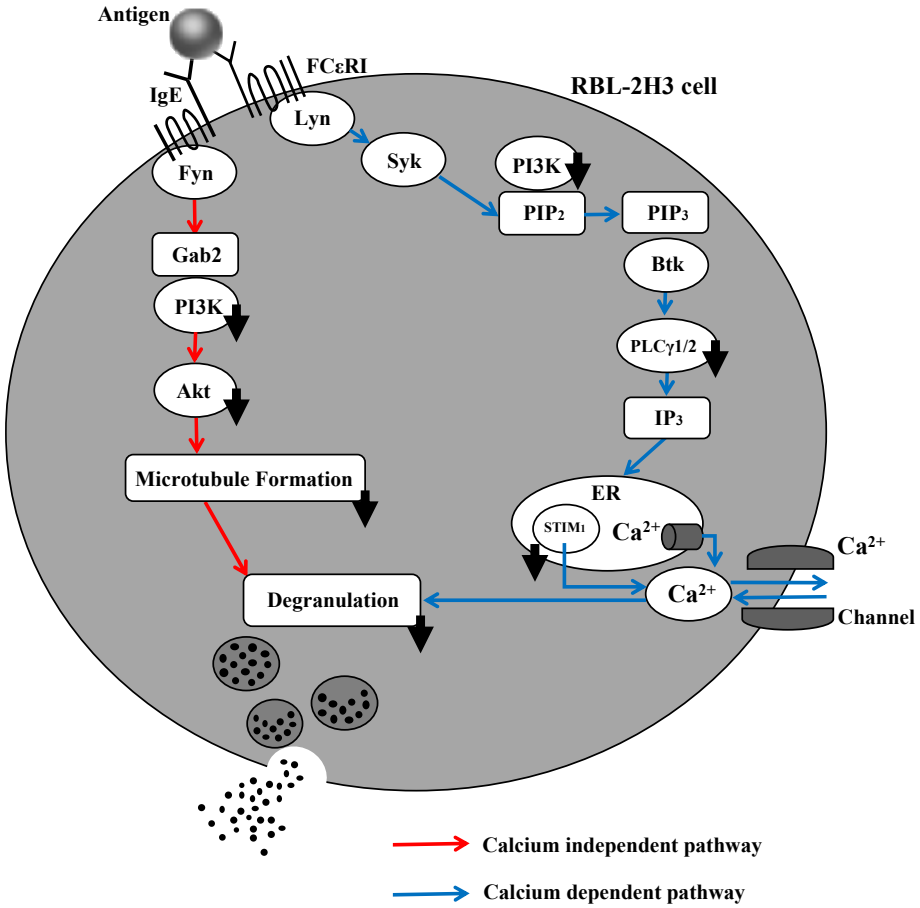

Figure 4. The mechanism of action of the signaling pathways of trigonelline in inhibiting degranulation

In summary, caffeine and trigonelline, as bioactive compounds contained in coffee, shown a potency as a functional food by its anti-allergic activity. This activity is mainly demonstrated by its ability to suppress degranulation in the mast cells. There is a slight difference between the mechanism of action of caffeine and trigonelline samples against the degranulation inhibition signaling pathway. The results of immunoblot analysis showed that caffeine suppressed the antigen-stimulated degranulation by inhibited phosphorylation of Syk, Btk, PLCγ1, PI3K, and Akt in antigen-stimulated RBL-2H3 cells. While trigonelline only showed phosphorylation inhibition of PLCγ1, PI3K, and Akt. However, both caffeine and trigonelline could inhibit phosphorylation of PLCγ1 as an essential step in the signaling pathway regulating FcεRI-dependent degranulation. Stimulation of PLCγ evokes $[Ca^{2+}]_i$ oscillations by stimulating Ca^{2+} release from the IP_3 pool and $STIM_1$-regulated Ca^{2+} influx. In other words, the inhibition of phosphorylation of PLCγ1 is a key to the suppression of $[Ca2^{+}]_i$ that affects the inhibition of degranulation through the calcium-dependent pathway. Meanwhile, the calcium-independent pathway shows that both caffeine and trigonelline could inhibit the microtubule formation in RBL-2H3 cells. In general, caffeine and trigonelline have relatively similar degranulation inhibition mechanisms. The similarity in the mechanism of inhibition is likely because both are alkaloids that have almost identical molecular structures.