

MARMIN, A COMPOUND FROM *AEGLE MARMELLOS* CORR., RELAXES THE OVALBUMIN-INDUCED CONTRACTION OF TRACHEA

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ABSTRACT

In the previous study, marmin isolated from *Aegle marmelos* Correa has been evaluated for its effects on the contraction of guinea pig tracheal chain (GPT) induced by histamine and compound 48/80. Marmin inhibited the histamine-induced contraction in competitive manner. Marmin also successfully inhibited compound 48/80-induced contraction. In the study, we investigated the effect of marmin on immunologically induced GPT contraction. In addition, we also investigated the effect of marmin to GPT relaxation contracted by a single concentration of histamine. In present study, marmin succeeded to inhibit the contraction of GPT induced immunologically by 3 µg/mL ovalbumin. In addition, marmin also relaxed the precontraction of GPT induced by 3 x 10⁻⁵ M histamine. The value of pD₂ of marmin was 4.42±0.02.

Keywords: Marmin, *Aegle marmelos* Corr., Guinea pig tracheal chain (GPT), Ovalbumin.

INTRODUCTION

A lot of medicinal plants have been used empirically to treat various diseases. One of them is *Aegle marmelos* Corr. It has been used in traditional Indian medicine. This plant originates from and grows widely in some areas of the Southeast and South Asian countries such as India, Sri Lanka, Indonesia, Malaysia and Vietnam. *Aegle marmelos* Correa has been reported possessing several biological activities such as antifungal, anti-inflammatory, analgesic, antipyretic, antioxidant, antidiabetes, antiproliferative¹⁻⁵.

Marmin is a coumarin derivative isolated from stem bark and root of *Aegle marmelos* Corr⁶. Chemical structure of marmin is shown in figure 1. Previously, marmin succeeded to inhibit the guinea pig tracheal chain (GPT) contraction induced by series concentration of histamine in competitive manner. In addition, marmin also potently inhibited GPT contraction induced by compound 48/80, an intracellular histamine stimulant⁷. Based on the results, marmin is potential to be developed into a new drug for treating allergy.

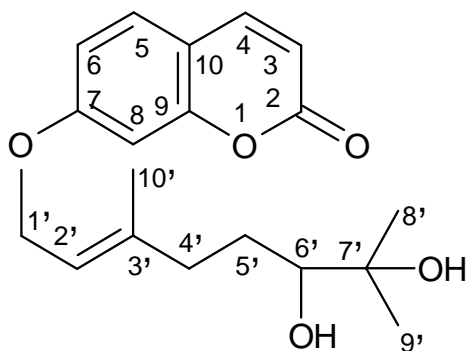


Fig. 1: Chemical structure of marmin

Allergy is a condition in which the immune system over reacts to allergens such as proteins, dusts, feathers, and pollens. The results of these reactions are hay fever, asthma, rhinitis allergy, or shock anaphylactic⁸. Allergy, which is also called type I hypersensitive, invokes immune system to produce IgE that would bind to FcεRI on mast cells surface. In the next challenge of allergen, it would be recognized by IgE and bind to it. The IgE and allergen binding can stimulate degranulation of mast cells⁹.

Degranulation of mast cells has an important role in the occurrence of allergies¹⁰. Mediators released from mast cells are histamine, heparin, and neutral proteases (trypsin, chymase, and kininogenase).

The mediators would cause allergy symptoms in the skin, gastrointestinal tract, respiratory tract, and blood circulation⁸. Allergic symptoms appear as pruritus, redness, temporary loss of consciousness, digestive disorders, nausea, vomiting, diarrhea, bone pain and mental disorders resulting from impaired brain function¹¹. Mast cells may be induced immunologically or non-immunologically by external stimuli¹².

Histamine plays an important role in the pathophysiology of diseases associated with allergies such as asthma, allergic rhinitis, conjunctivitis, anaphylactic shock, and urticaria. In the respiratory tract, histamine quickly causes itching, sneezing, mucus and also spasmus onset. Bradykinin, histamine, leukotrienes, and PAF (platelet activating factor) work together synergistically in the capillaries so they would induce vasodilatation, vascular permeability change, and cause cellular adhesion¹³.

Based on these facts, a study of marmin, isolated from *Aegle marmelos* Corr., on contraction of GPT induced by endogenous histamine is very interesting to do. Our previous study showed that marmin was able to inhibit GPT contraction induced non-immunologically by compound 48/80⁷. In this study, endogenous histamine is produced from mast cell degranulation stimulated immunologically by ovalbumin. In addition, this study also investigated the relaxation effect of marmin in tracheal smooth muscle.

MATERIALS AND METHODS

Materials

The main material used in the study was marmin obtained from Prof. Dr. Sugeng Riyanto, M.S., Apt. (Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta Indonesia). Male guinea pigs weighing 300-450 g were supplied by Experimental Animal Center of Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy Gadjah Mada University, Indonesia. Other materials were histamine, ovalbumin, Al(OH)₃, cromolyn sodium, compound 48/80 purchased from Sigma Chemical Co. (St. Louis, MO, USA), carbogen (Aneka Gas Industri), aquadest, Krebs buffer solution, DMSO (Merck, Germany), and saline (Otsuka Pharmaceutical, Indonesia).

Guinea pig sensitization procedure

Guinea pigs were adapted for 7 days. Afterwards, they were injected intraperitoneally with solution containing 10 µg ovalbumin and 1 mg Al(OH)₃ in 0.5 ml of saline solution. Injectable solution dosage was 0.5 ml/200 g BW. The solution was injected again after 2 weeks (on day 14) of the first injection. Guinea pigs were sacrificed at day 21 or three weeks after the first injection¹⁴.

Study on GPT contraction that induced immunologically

GPT was equilibrated for 60 minutes in an organ bath containing 20 mL Krebs buffer at 37°C. Subsequently, GPT was induced by 3 x 10⁻⁵ M histamine. GPT contractions were observed on the recorder. Then, the organ was washed for 45 minutes with replacing the Krebs buffer every 15 minutes. Subsequently, marmin (10 or 100 µM) was added to the organ bath at 10 minutes prior to the contraction induced immunologically with 3 µg/mL ovalbumin. GPT contractions that occurred were recorded up to 30 minutes after induction. Contractions that occurred were then transformed to the value of % contraction in response to histamine (3 x 10⁻⁵ M). Cromolyn sodium (100 µM) was used as a reference drug¹⁵.

Relaxation effects on the isolated guinea pig trachea

GPT was equilibrated for 60 minutes in an organ bath that contains 20 mL Krebs buffer at 37°C. Subsequently, the tracheal smooth muscle was induced by 3 x 10⁻⁵ M histamine. Tracheal contractions were then recorded on the recorder. After the maximum contraction was reached, then series concentration of marmin (5; 10; 20; 40; 80; 100 µM) were added to the organ bath. Responses that occurred were then recorded on the recorder¹⁶.

Data analysis

EC₅₀ (concentration of agonist which can produce a response by 50% of maximum response) was calculated based equation 1. The values are then converted into pD₂ (-Log. EC₅₀) as in equation 2. This value represents potency of the effect of agonist on the tracheal smooth muscle. Subsequently, the data was presented as an average of pD₂ agonist ±SEM (standard error of mean).

$$\text{Log EC}_{50} = \left(\frac{50 - Y_1}{Y_2 - Y_1} \right) \times (X_2 - X_1) + X_1 \quad (1)$$

Explanation:

Log EC₅₀ : the logarithm of agonist concentrations that can produce a response by 50%

of the maximum response

X₁: Log. Concentration with the appropriate response below 50%

X₂: Log. Concentration with the appropriate response above 50%

Y₁: % response appropriate below 50%

Y₂: % response appropriate above 50%

$$\text{pD}_2 = -\text{Log. EC}_{50} \quad (2)$$

AUC (area under curve) of immunologically induced tracheal contraction was calculated using equation 3. AUC₀₋₃₀ value was used to calculate the percentage of inhibition of contraction (equation 4).

$$\text{AUC}_{0-30} = \sum_{x=0}^{30} \frac{(y_x + y_{x-1})(z_x - z_{x-1})}{2} \quad (3)$$

Explanation:

AUC₀₋₃₀ : area under the curve from minute 0 to minute 30 (minute.% contraction response)

y_{x-1} : large of area % contraction at minute (n-1) (%contraction response)

y_x : large of area % contraction at minute (n) (%contraction response)

z_x : minute (n)

z_{x-1} : minute (n-1)

$$\text{Percentage of contraction inhibitory (\%)} = \frac{(\text{AUC}_{0-30})_0 - (\text{AUC}_{0-30})_n}{(\text{AUC}_{0-30})_0} \times 100\% \quad (4)$$

Explanation:

(AUC₀₋₃₀)₀ : Mean AUC₀₋₃₀ control (minute.% contraction response)

(AUC₀₋₃₀)_n: AUC₀₋₃₀ treatment (minute.% contraction response)

All data were presented as mean ± the standard error of the mean (SEM). Unpaired t test and one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test were used for statistical analyses. P-values less than 0.05 were considered significant.

RESULTS

Effect on guinea pig tracheal contraction that induced immunologically

The effect of marmin on contraction profiles of GPT induced immunologically by ovalbumin are presented in figure 2. Measurement of contraction of the GPT induced immunologically by ovalbumin was performed for 30 minutes. Contraction response data was then converted to the values of AUC (area under curve) at minute 0-5, 5-10, 10-15, 15-20, 20-25, and 25-30 (Fig. 3).

In figure 2, the contraction response began to appear at 2 minutes after induction by using 3 µg/mL ovalbumin. Maximal contraction response was achieved at 3 minute after induction. After that, the response gradually decreased to near base line. Concentration-response curves due to pretreatment of 10 µM marmin, 100 µM marmin and 100 µM cromolyn sodium were lower than this of control group (fig. 2). It indicates that 10 µM marmin, 100 µM marmin, and 100 µM cromolyn sodium could inhibit the GPT contraction induced immunologically by ovalbumin.

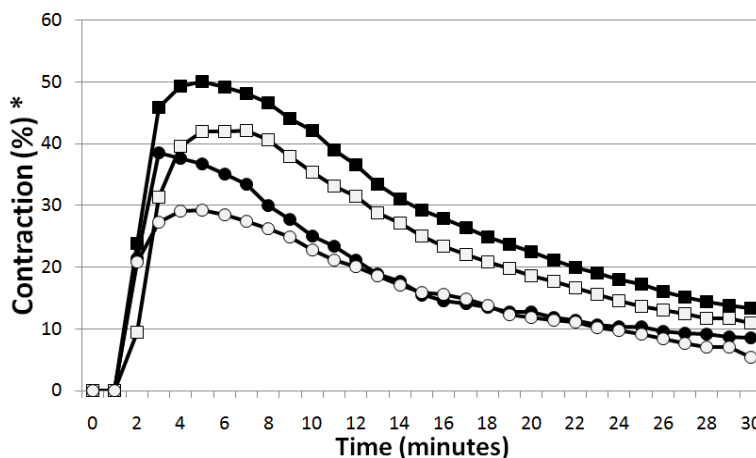


Fig. 2: Time-response curves to ovalbumin in the absence (■) or presence of marmin at concentration of 10 µM (□), 100 µM (●), 100 µM sodium cromolyn (○) in guinea-pig tracheal smooth muscle (Data represent mean±SEM, n=5-10).

*compare to the maximum contraction of tracheal smooth muscle induced by histamine.

In figure 3, marmin seem to be able to inhibit contractions of immunologically induced GPT. It appears from the area under curve (AUC) (minute.% contraction) of marmin groups in comparison to this of control group. However, the AUC values of marmin at concentration of 10 µM did not show a significant difference in comparison to this of

control treatment ($p > 0.05$). Whereas, 100 µM marmin showed a significant difference in comparison to control treatment at AUC_{5-10} , AUC_{10-15} , AUC_{15-20} , and AUC_{20-25} ($p < 0.05$). While the treatment of 100 µM cromolyn sodium also showed a significant difference with the control at AUC_{5-10} , AUC_{10-15} , AUC_{15-20} , and AUC_{20-25} ($p < 0.05$).

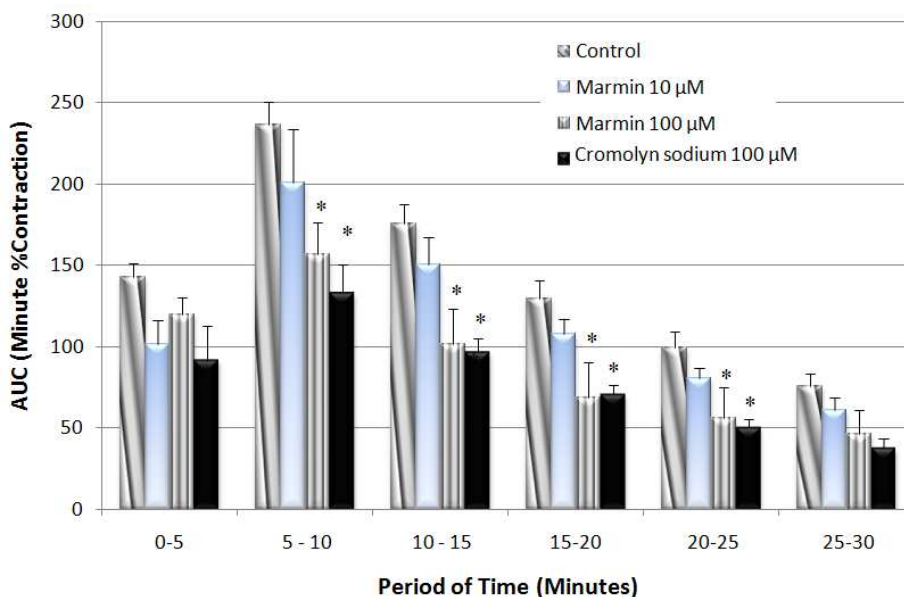


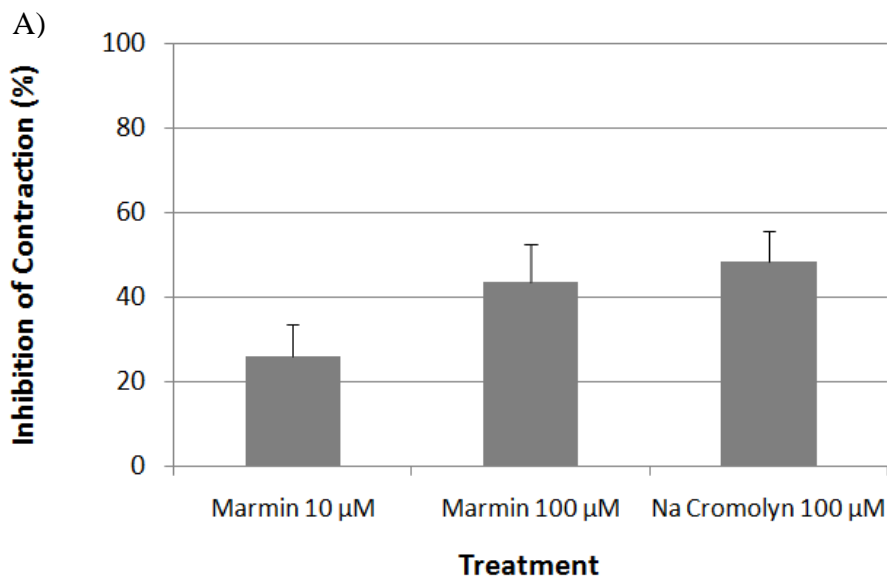
Fig. 3: The percentage of tracheal contraction (AUC) vs. period of time after ovalbumin administration in the absence or presence of marmin at concentration of 10 µM, 100 µM, or 100 µM cromolyn sodium (Data represent mean±SEM, n=5-10).

*Significant difference $P < 0.05$ compared to the control value.

The value of AUC_{0-30} was used to calculate the percentage of inhibition of GPT contraction (%) that was induced immunologically. The percentage of inhibitory effect of marmin (10 or 100 µM) in comparison to the value of 100 µM cromolyn sodium (positive control) were shown in fig. 4A. In the study, increasing concentration of marmin caused an increase in the percentage of inhibitory effect on the contraction.

The average of percentage of inhibitory effect of marmin (10 and 100 µM) was $25.86 \pm 7.57\%$ and $43.49 \pm 9.24\%$, respectively. Whereas, 100 µM cromolyn sodium showed inhibitory effect of $48.44 \pm 7.13\%$

Maximum contraction response in the guinea pig tracheal smooth muscle induced immunologically by ovalbumin was also observed (Fig. 4B). The contraction response reached a maximum during 2-8 minutes after administration of 3 µg/mL ovalbumin. Treatment of 100 µM cromolyn sodium could significantly decrease the percentage of the maximum contraction ($P < 0.05$). Whereas, there were no significant difference in percentage of maximum contraction after treatment of marmin 10 or 100 µM ($p > 0.05$). It indicates that marmin (10 and 100 µM) did not inhibit the maximum contraction of the guinea pig trachea induced immunologically by ovalbumin, while 100 µM cromolyn sodium succeeded to inhibit the maximum contraction.



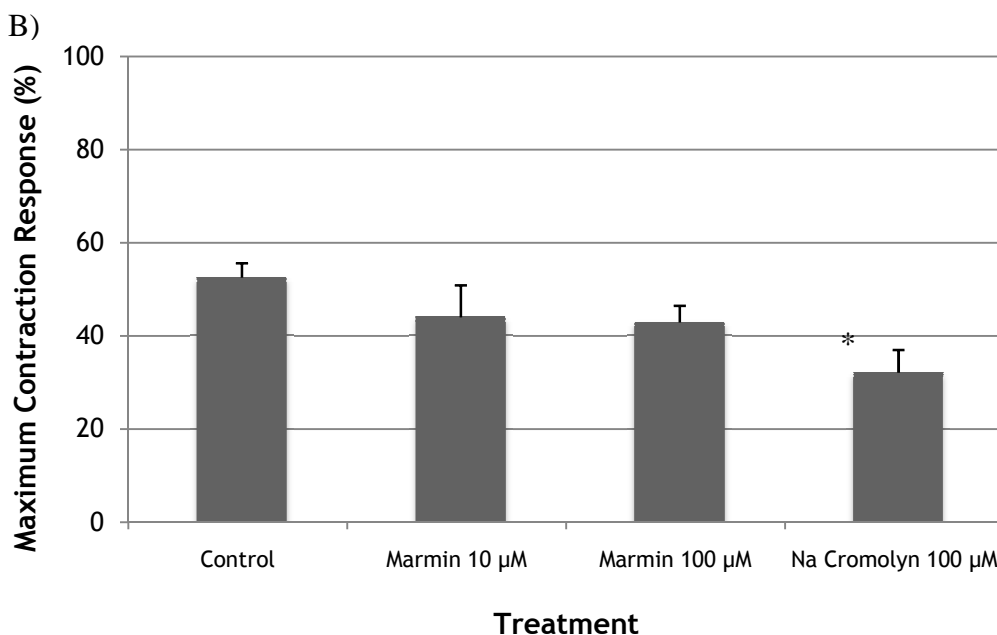


Fig. 4: (A) Histogram of the percentage of inhibitory effects of 10 µM marmin, 100 µM marmin and 100 µM cromolyn sodium (positive control) on ovalbumin-induced contraction. (B) Histogram of the percentage of maximal contraction of GPT induced by ovalbumin in the absence or presence of marmin at concentration of 10 µM, 100 µM, or 100 µM sodium cromolyn (Data represent mean±SEM, n=5-10).

*Significant difference P<0.05 compared to the control value.

Relaxation effect in isolated guinea pig trachea.

In the study, relaxation effect of marmin was also investigated. Marmin with a gradual concentration (5-100 µM) was administered on GPT that was previously contracted with 3×10^{-5} M histamine. It would activate the H₁ receptor then resulted in smooth muscle tracheal contraction. Subsequently marmin (5-100 µM) were added to relax the GPT contraction. The relaxation response induced by marmin was converted to percentage of the response.

Marmin relaxation effects began at a concentration of 10 µM (Fig. 5). Marmin (10 µM) relaxed the tracheal smooth muscle by $4.66 \pm$

2.04%. When marmin given at concentration of 20 µM, the mean of relaxation percentage was $19.38 \pm 4.61\%$. While the administration with a concentration of marmin 20; 40; 80; and 100 µM produced relaxation effect by $19.38 \pm 4.61\%$, 47.80 ± 3.68 ; 90.55 ± 1.15 , and $100.00 \pm 0.00 \%$. The pD₂ value of relaxation effect of marmin was 4.42 ± 0.02 .

These results confirm previous research conducted by Takase et al. that marmin has a relaxation effect on isolated guinea pig ileum precontracted by acetylcholine and histamine¹⁷. Relaxation thought come from the influence or antagonism on H₁ receptor by marmin.

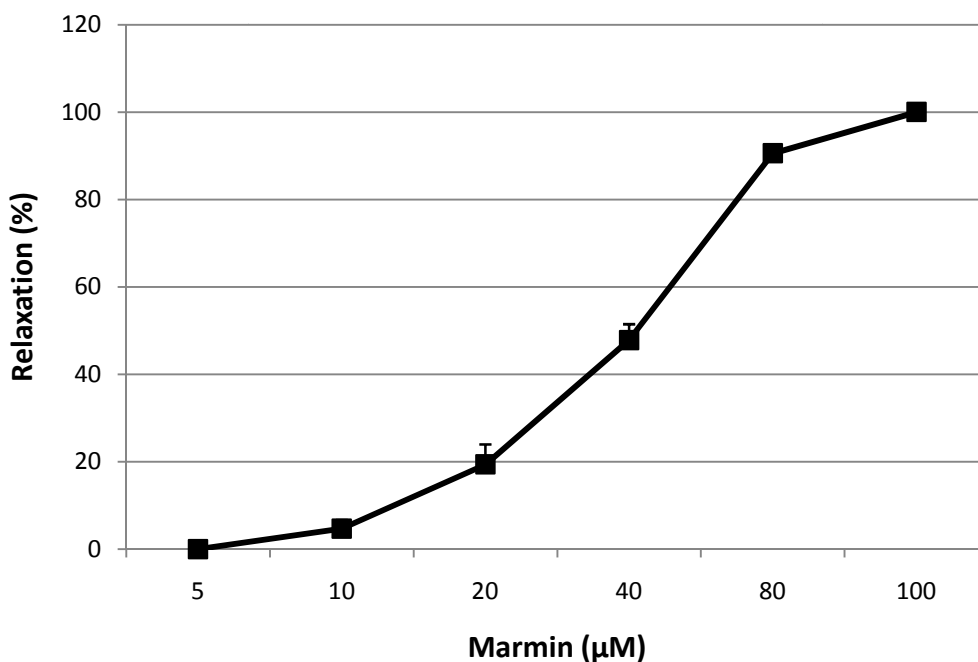


Fig. 5: The relaxant effect of marmin on isolated-tracheal smooth muscle of guinea pigs. Values are mean ± SEM of 5 experiments.

DISCUSSION

The study aimed to investigate the effect of marmin isolated from *Aegle marmelos* Corr. on GPT contraction induced immunologically by endogenous histamine and to determine the relaxation effect of marmin on GPT. In this study, endogenous histamine released from mast cells stimulated immunologically by ovalbumin.

In the study, marmin was isolated from petroleum ether, chloroform and methanol extracts of the roots and stem bark of the plant. The extracts were fractionated using chromatography (vacuum column, gravity column) and developed by gradient elution. The solid material obtained was recrystallized to yield the compound.

Allergens such as dust, pollen, and feathers can cause allergic responses such as itching, shortness of breath, to anaphylactic shock which can cause death in individuals with immune system hypersensitivity⁸. Allergic response that arises primarily related to activation of H₁ receptors because most of the compounds released from mast cell granules is histamine. Therefore, targeted treatment of allergies is the antagonism at histamine H₁ receptors or keep low the frequency of recurrence by using inhibitor of mast cell degranulation (mast cell stabilizer)⁹.

In the study, marmin succeeded to inhibit the contraction of guinea pig tracheal smooth muscle induced immunologically by ovalbumin. Ovalbumin contracted the tracheal smooth muscle by stimulating the histamine release from mast cell, and then the histamine stimulated H₁ receptors in tracheal smooth muscle resulting in a contraction. Inhibition of contraction is thought from the inhibition of histamine release from mast cells. In previous studies, marmin showed an inhibitory effect on the histamine release from RBL-2H3 cell through inhibition of Ca²⁺ influx¹⁸. Marmin could inhibit the activation of SOCC (store operated calcium channels) thereby inhibiting Ca²⁺ influx. Effect of calcium on the contraction of guinea pig trachea induced by immunological or non-immunological was not examined in this study. In addition, marmin competitively inhibited the contraction of the tracheal smooth muscle induced by histamine⁷. It indicates that marmin obviously suppressed the contraction induced immunologically by ovalbumin through two ways, (1) by inhibiting the histamine release from mast cells, and (2) by competitively disrupting the histamine receptor in tracheal smooth muscle.

Inhibitory effect of marmin on the guinea pig tracheal contraction induced immunologically and non-immunologically was thought come from the inhibition of histamine release from mast cells. However, these effects can also be caused by the influence marmin of H₁ receptors. Degranulation of mast cells produces the release of histamine that is able to activate the H₁ receptors and cause contraction response. Inhibition of the receptor activation will inhibit the response of smooth muscle contraction in guinea pig trachea. Based on the previous study, marmin was able to relax the guinea pig ileum previously contracted by a single concentration of histamine and acetylcholine¹⁷. Relaxation response is thought a result from inhibition of the H₁ receptor.

The finding contributes in the discovery of new potential anti-allergy and anti-asthma agent isolated from natural products. In the further study, it is necessary to investigate the mechanism of action of the relaxation effect of marmin. Other plants that are potential to be developed as anti-asthma or anti-allergy agents are *Delphinium denudatum* Wall¹⁹, and *Cynodon dactylon*²⁰.

CONCLUSION

Based on the results, marmin was able to inhibit the contraction of GPT induced immunologically by ovalbumin. Marmin was also able to relax the precontraction of GPT induced by 3×10^{-5} M histamine. The value of pD₂ of marmin was 4.42±0.02.

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