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Anti-allergic effects of Marmin, a coumarine isolated from Aegle marmelos Correa: In vitro study

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Abstract

Marmin or (7-(6',7'-dihydroxygeranyl-oxy)coumarin is an active compound isolated from Aegle marmelos Correa. The study aimed to investigate the effects of marmin on the histamine release from rat mast cell. The study was performed by using rat basophilic leukemia (RBL-2H3) cell line and rat peritoneal mast cells (RPMCs). The histamine release from these cells was determined by using HPLC fluorometric method. In this study, marmin succeeded to inhibit the histamine release from RBL-2H3 cell line induced by DNP_{24}-BSA, thapsigargin or ionomycin. In addition, marmin suppressed $^{45}Ca^{2+}$ influx on RBL-2H3 cell line induced by thapsigargin. Marmin also succeeded to inhibit the histamine release from RPMCs induced by thapsigargin. However, marmin showed weak inhibitory effects on the histamine release from RPMCs induced by compound 48/80, PMA or ionomycin. Based on the results, the inhibitory effect of marmin on the histamine release from mast cells high depends on the type of mast cell and also involves mechanisms related to intracellular Ca^{2+} signaling events by blocking Ca^{2+} influx into mast cells.

Keywords: Aegle marmelos Correa; marmin; histamine release; mast cell.

Introduction

Mast cell is a mediator involved in many different acute and chronic inflammatory processes. The cells act in both delayed and immediate hypersensitivity reactions [1,2]. Allergy (type I hypersensitivity reaction) can be triggered in present of allergen such as grass pollen, product from dust mites, foodstuffs or some drugs. First step of allergy stimulation is production of antibodies of the IgE type, and then bind to FcεRI receptors on membrane surface of mast cell and eosinophils. Subsequently, a series of intracellular signaling events is generated, and then trigger granule exocytosis, which releases allergy mediators from mast cells such as histamine, eicosanoids and cytokines. Histamine has most important role in the inflammatory reactions and immune responses. Therefore, these mediators induce location-dependent effects such as hay fever in the nose, the initial phase of asthma in the bronchial tree, urticaria in the skin or gastrointestinal tractus irritations [3,4].

Many antiallergy drugs have been developed from several sources including from natural products. Natural products have served as a major source of drugs for centuries, and about half of the pharmaceuticals in use today are derived from natural products [5]. Over 50% of the best-selling pharmaceuticals in use are derived from natural products [6]. Natural products contain diverse chemical compounds as sources of therapeutics. Recent progress to discover drugs from natural product sources has resulted in compounds that are being developed to treat some diseases [7]. Therefore it is urgent and promising to discover new potential antiallergy drug isolated from natural products.

Aegle marmelos Correa is a species belongs to Rutaceae family. They originate from and grow widely in some areas of the Southeast and South Asia countries. This plant has been widely used as an ancient and modern traditional medicine for treatment of various disorders in Indonesia, Malaysia and its surrounding areas. Aegle marmelos Correa has been reported having several pharmacological activities such as antiproliferative, anti-inflammatory, antipyretic, analgesic, antioxidant, antifungal, antiviral, hypoglycemic, and antidiabetes [8-14]. Several compounds of this plant have been isolated and evaluated for their pharmacological effects [15-17]. It is necessary to focus and develop the compounds to be effective drugs.

In the present study, we investigated the in vitro antiallergic effect of marmin, a coumarine compound isolated from Aegle marmelos Correa. Marmin (7-(6',7'-dihydroxygeranyl-oxy) coumarin (Fig. 1) was isolated from the bark and fresh root extracts of Aegle marmelos Correa [15,16,18]. In the study, we used two kinds of mast cell, rat basophilic leukemia (RBL-2H3) cells and rat peritoneal mast cells (RPMCs). DNP_{24}-BSA, thapsigargin, ionomycin, compound 48/80, and PMA were used as inducers for histamine release from mast cell. The result of these studies may provide useful information for further discovering pharmacologically traditional plants isolated-active compounds for treatment some disease related to histamine or mast cells.

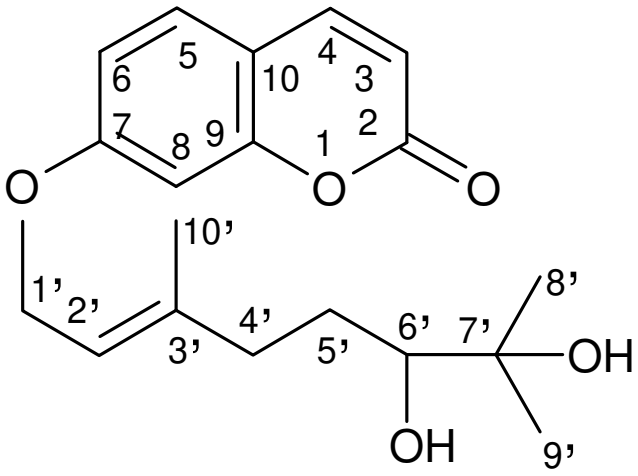


Figure 1. Chemical structure of marmin.

Materials and Methods

Preparation of marmin

Marmin or (7-(6',7'-dihydroxygeranyl-oxy) coumarin was isolated from Aegle marmelos Correa. The chemical structures of the compounds are shown in Fig.1. The plant was collected from area around Yogyakarta, Indonesia [18]. Aegle marmelos was identified by a botanist at Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. The voucher specimen was deposited in herbarium of the department.

In brief, dried ground powder of bark and fresh root were extracted successively with petroleum ether, chloroform and methanol. Chloroform extract of both were chromatographed over silica gel, and selected fractions were further chromatographed using mini column to yield several fractions. Selected fractions were combined and concentrated, and the solid obtained was recrystallized to yield marmin. Methanol extract of dried ground powder of bark or petroleum ether extract of dried ground powder of fresh root were separated using vacuum column chromatography with gradient

elution to yield several fractions. Selected fractions were combined and evaporated, then separated by using gravity column chomatography to provide marmin.

Materials

The histamine release inducers used in the study were ionomycin (Calbio-Chem), thapsigargin, compound 48/80, and phorbol myristate acetate (Sigma, Chemical). Dinitrophenylated bovine serum albumin (DNP_{24}-BSA) as an antigen and monoclonal IgE against DNP_{24}-BSA purified from supernatant in IgE producing hybridoma, were produced in our laboratory. Eagle's minimum essential medium (MEM) and antibiotics (combination of penicillin G sodium and streptomycin sulfate) were purchased from Gibco (Grand Island, New York). Fetal calf serum was obtained from JRH Biosciences (Kansas, USA), piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) and bovine serum albumin (BSA) were purchased from Dojindo (Kumamoto, Japan), and o-phthalaldehyde was purchased from Wako Pure Chemical Co. (Osaka, Japan). PIPES buffer consisted of NaCl (119 mM), KCl (5mM), PIPES (25 mM), glucose (5.6 mM), $MgCl_2$ (0.4 mM), $CaCl_2$ (1 mM), NaOH (40 mM), and BSA (0.1 %) and was adjusted to pH 7.20.

Culture of RBL-2H3 cells

RBL-2H3 cells were cultured in MEM containing 15% fetal calf serum (FCS) and antibiotics (penicillin and streptomycin) in a flask in a humidified atmosphere (5% CO_2) at 37°C [19]. The cells were seeded into 24-well culture plates at a density of 5 x 10^5 cells/0.4 ml per each well, and then incubated overnight at 37°C. In DNP_{24}-BSA experiments, the cells were sensitized with 0.5 μg/ml of monoclonal IgE. On the second day, the medium was removed, and the cells were washed twice with 500 μl of PIPES buffer, and preincubated for 10 min at 37°C after addition of 180 μl PIPES buffer either without (as a negative control) or with the drug. After 10 min preincubation, 20 μl of stimulant (200 ng/mL DNP_{24}-BSA, 5 μM thapsigargin, or 10 μM ionomycin) were added to each well and the plate was incubated at 37°C for 30 min.

Isolation of RPMCs

Male Wistar rats (250-300 g, 3-4 months) were used. The animal experiments were conducted according to the guidelines of the Animal Care Committee of the Ehime University, and all experimental protocols had been approved by this Committee. Rats were killed by decapitation and exsanguination. RPMCs were isolated by injection of 25 mL phosphate buffered saline (PBS) pH 7.4 containing 5 IU/ml heparin and 10% BSA into the peritoneal cavity and the abdomen was massaged for about 120 s. Afterwards, the peritoneal cavity was opened carefully, and the fluid containing mast cells were collected. The collected mast cells were centrifuged at 1,000 rpm for 5 min at room temperature and then resuspended in 2 ml PBS buffer containing 10% BSA. Peritoneal mast cells were separated from the other components (macrophages and lymphocytes) by layering on 4 ml of 38% BSA, and centrifuging at 2,000 rpm for 20 minutes at 4°C. After the upper layer containing other components was aspirated and discarded, the remaining cell pellet was washed with 6 ml PBS buffer and resuspended in 1 ml of PIPES buffer. Mast cell preparations were about 95% pure as assessed by toluidine blue staining.

One hundred twenty μl of RPMC suspension ($2x10^4$ cells/ml) was preincubated for 10 min at 37°C after addition of 60 μl PIPES buffer either without (as a negative control) or with drugs at a range of concentrations (0.1 – 100 μM). After 10 min preincubation, 20 μl of stimulant (100 μM compound 48/80, 5 μM thapsigargin, 10 μM ionomycin, or a combination of 100 nM PMA and 1 μM ionomycin) was added to each well and the plates were incubated at 37°C for 30 min.

Assay of histamine release

Level of histamine release was measured by HPLC-fluorometry as described previously study [20]. After 30 min incubation, the plates were

centrifuged at 3,000 rpm for 5 min and 50 µl of the supernatant was mixed with 250 µl of 3% perchloric acid containing 5mM Na_2-EDTA. After addition of 30 µl of 2 M KOH/1 M KH_2PO_4 and centrifugation at 10,000 x g for 15 min at 4°C , 50 µl of the supernatant was injected directly onto a column packed with TSKgel SP-2SW cation exchanger (Tosoh, Tokyo). For measuring the total histamine content in cells, 350 µl of PIPES buffer was added to 6 wells and the cells were then sonicated. Fifty microlitres cell homogenate was used for the histamine assay described above. Histamine was eluted with 0.25 M potassium phosphate at a flow rate of 0.6 ml/min, and post-labeled with o-phthalaldehyde under alkaline conditions and detected using a F1080 Fluorometer (Hitachi, Tokyo) at excitation and emission wavelengths of 360 and 450 nm, respectively. The values were expressed as a percentage of net histamine release.

Uptake of $^{45}Ca^{2+}$

RBL-2H3 cells in a 24 well-plate were incubated for overnight at at 37°C. Then, the cells were washed twice with 500 µl of PIPES buffer and then preincubated for 10 min at 37°C in 180 µl PIPES buffer either without (as a negative control) or with the drug. After preincubation, PIPES buffer containing $^{45}Ca^{2+}$ (5µCi/mL) and Ca^{2+} uptake stimulant (thapsigargin) was added into each well, and the plate was incubated at 37°C for 15 min. After this time, the reaction was stopped by washing with ice-cold Ca^{2+}-free buffer containing 100 µM La^{3+}. The cells were lysed with 0.3 ml of 0.1% Triton X, and 100 µL of the solution was combined with 10 ml of scintillation cocktail for radioactivity counting. The values were expressed as the percentage of maximum uptake in the absence of inhibitor compounds.

Analysis of data

The percentage of net histamine release was calculated according to the following equation: [Histamine concentration (in supernatant of cells stimulated) – histamine concentration (in the supernatant of unstimulated cells)] / [Total histamine content – histamine concentration (in the supernatant of unstimulated cells)] X 100.

Whereas, the percentage of inhibition of histamine release or Ca^{2+} influx was calculated according to the following equation: [histamine release without drug (negative control) – histamine release with drug treatment] / histamine release without drug (negative control) X 100.

Statistical analysis

All data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test were used for statistical analyses. P-values less than 0.05 were considered significant.

Results

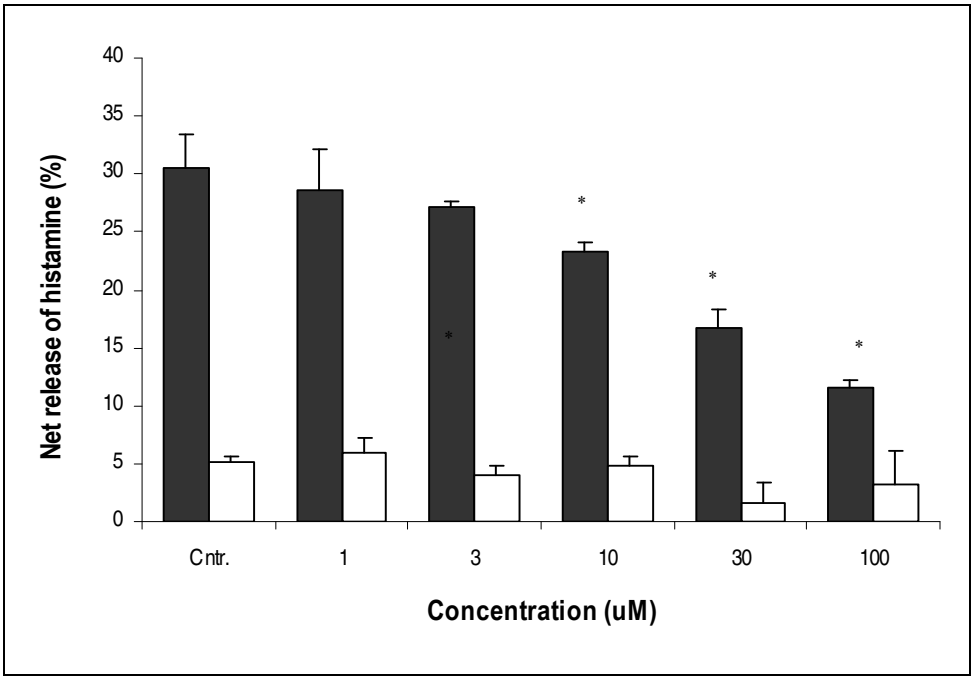
Effects on Histamine Release from RBL-2H3 Cells

In present study, DNP_{24}-BSA (20 ng/mL) stimulated histamine release from RBL-2H3 cells by 30% (Fig. 1a). The histamine release was markedly inhibited by marmin in a concentration-dependent manner. At maximum dose (100 µM) marmin inhibited the histamine release from RBL-2H3 by 62.06 ± 1.95%.

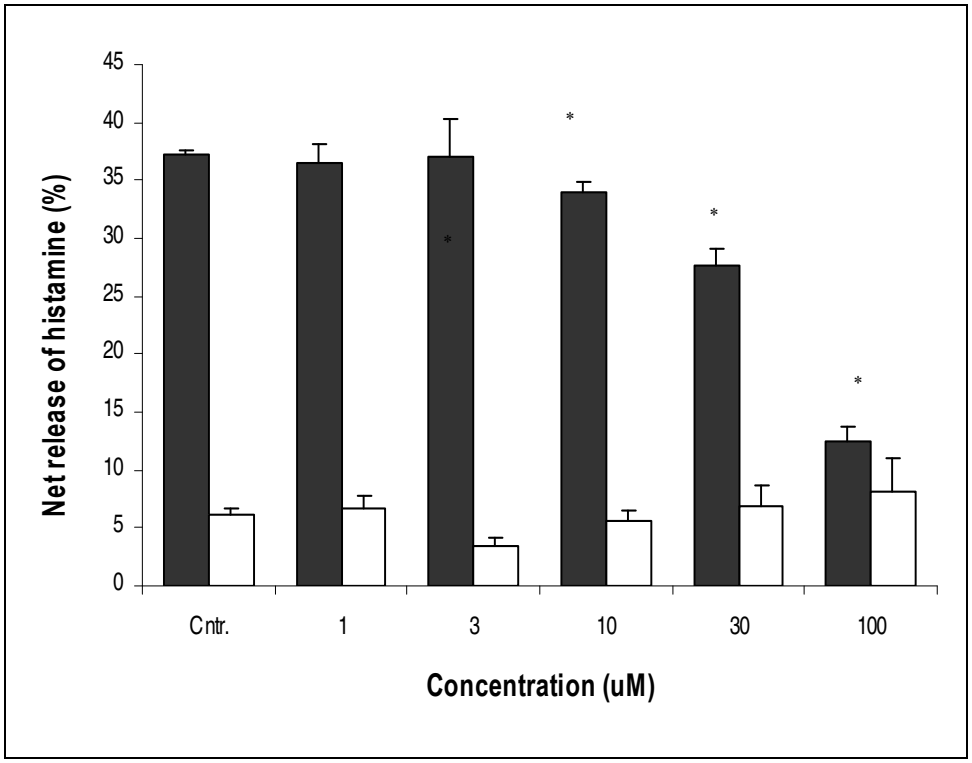
Thapsigargin and ionomycin, which act on Ca^{2+} influx and intracellular calcium pathways [21-22] stimulated histamine release by 37.2±0.4%; and 33.2±1.9%, respectively (Fig. 1b and 1c). Marmin suppressed the histamine release successfully when induced by 0.5 µM thapsigargin or 1 µM ionomycin. Marmin showed a significant effect at the dose of 10 µM in thapsigargin experiment, and 30 µM in ionomycin experiment. At the maximum dose (100 µM), marmin suppressed the histamine release about 60% in both experiments. Marmin effect was presumed to be related to intracellular Ca^{2+} signaling events in mast cells. Table 4

showed the IC_{50} values of the inhibitory effects of marmin on the histamine release from RBL-2H3 cells induced by DNP-BSA, thapsigargin and ionomycin. These values represent potency of marmin effect in this study.

(a)



(b)



(c)

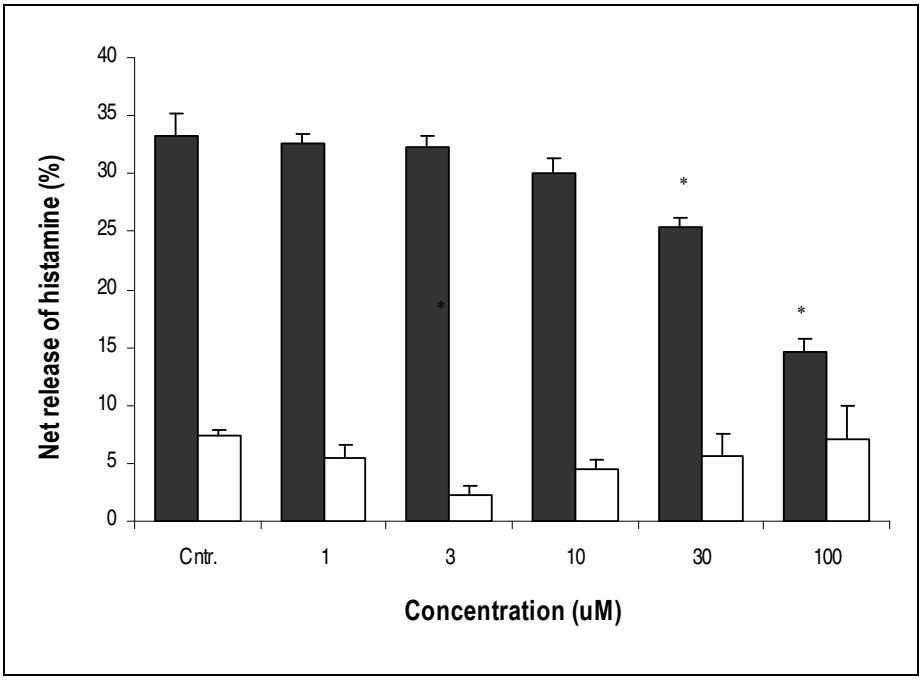


Figure 2. Effect of marmin on histamine release from RBL-2H3 cells in the presence (solid bar) or absence (open bar) of histamine stimulants i.e. DNP-BSA 20 ng/mL (a), thapsigargin 0.5 μM (b), or ionomycin 1 μM (c). Data represent mean±SEM, and are three independent experiments. *Significant difference ($P<0.05$) compared to the negative control value.

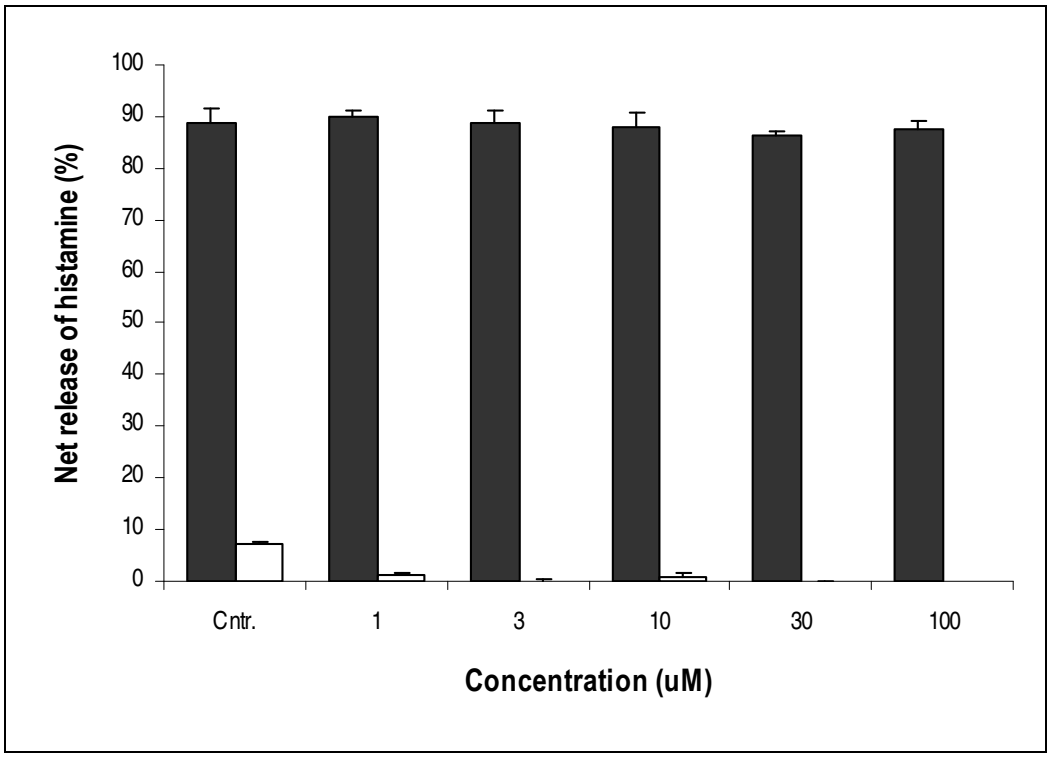
In the study, we also observed the possibility of induction of histamine release from RBL-2H3 cells by marmin with a series of concentration (1-100 μM), even though no histamine stimulant was added. The effect was considered significant if marmin caused spontaneous histamine release of more than 10 %. All concentrations of marmin showed low spontaneous histamine release, less than 10 % of the total histamine contained in RBL-2H3 Cells (Fig. 2).

Effects on Histamine Release from RPMCs

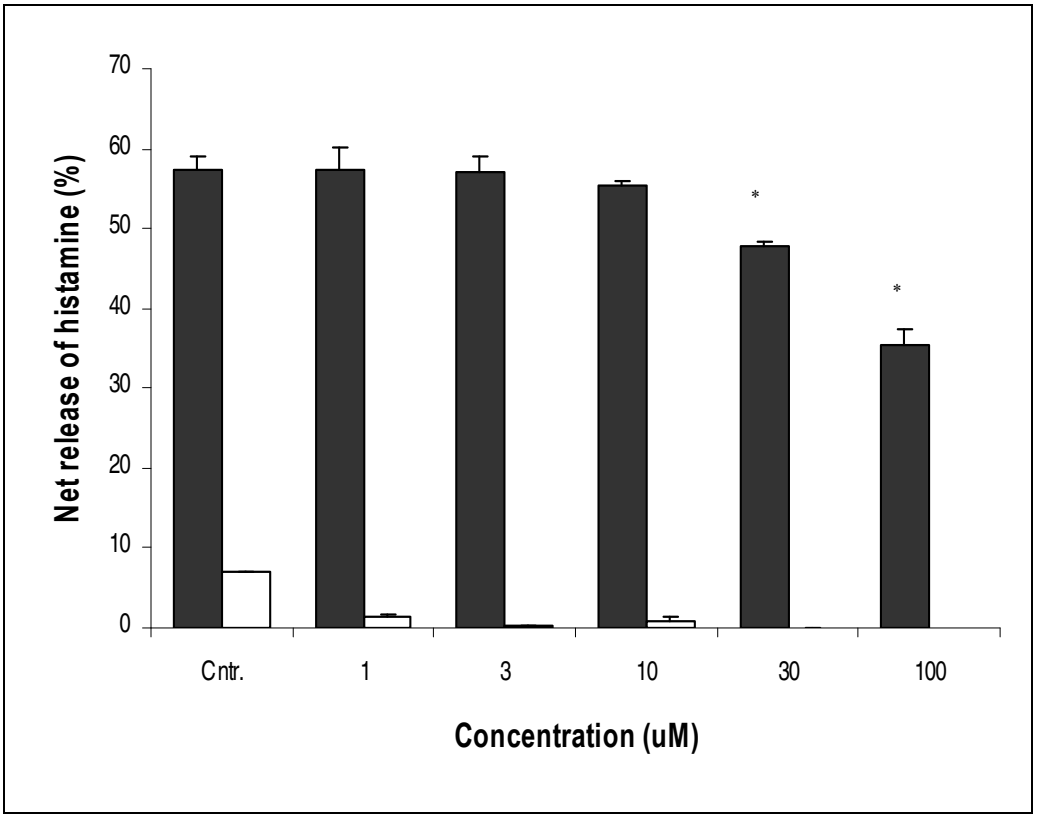
Compound 48/80, thapsigargin, ionomycin, and PMA were used for stimulating the histamine release rat peritoneal mast cells (RPMCs). The net histamine release from RPMCs affected by marmin in the presence or absence of histamine stimulants are shown in Fig. 3. Compound 48/80, acting directly on G proteins in mast cells [25-26] increased histamine release from RMPCs by more than 88.88±2.82 % of the total cellular content of histamine. In the study, marmin could not suppress the histamine release induced by 10 μM compound 48/80. Marmin effect was presumed to be not related to signaling events in G proteins activation pathways.

Thapsigargin (0.5 μM) and ionomycin (1 μM), which stimulate the Ca^{2+} signaling in mast cell, increased histamine release from RMPCs by 57.38±1.66%; and 77.84±2.08%, respectively (Fig. 3b and 3c). Marmin showed weak inhibitory effects on the histamine release induced by ionomycine, and at high dose (100 μM) could suppress the histamine release weakly only 13% of the total histamine content. However, marmin still succeeded to deplete the histamine when thapsigargin used as a histamine stimulant. Marmin showed a gradual inhibitory effect, and then finally suppressed the histamine release until 40 % at the highest dose. Nevertheless, this effect is still less potent than this on RBL-2H3 cells.

(a)


Net release of histamine (%)
100
90
80
70
60
50
40
30
20
10
0
Cntr.
1
3
10
30
100
Concentration (uM)

(b)


Net release of histamine (%)
70
60
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Cntr.
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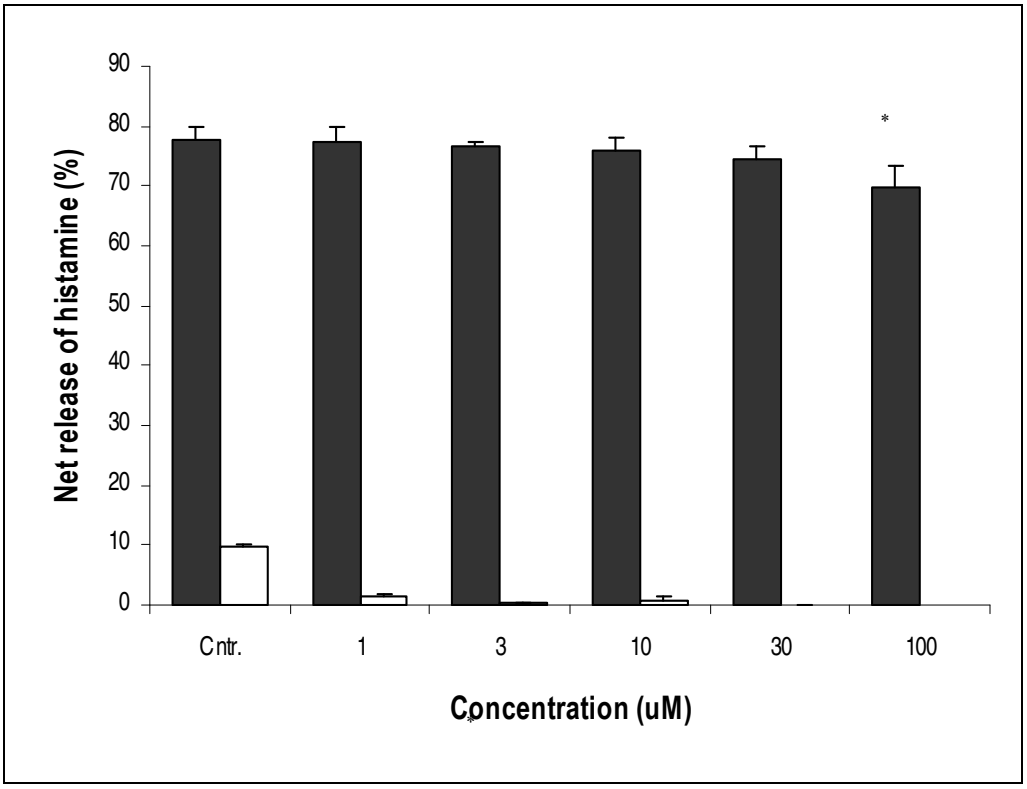
Concentration (uM)

(C)



(D)

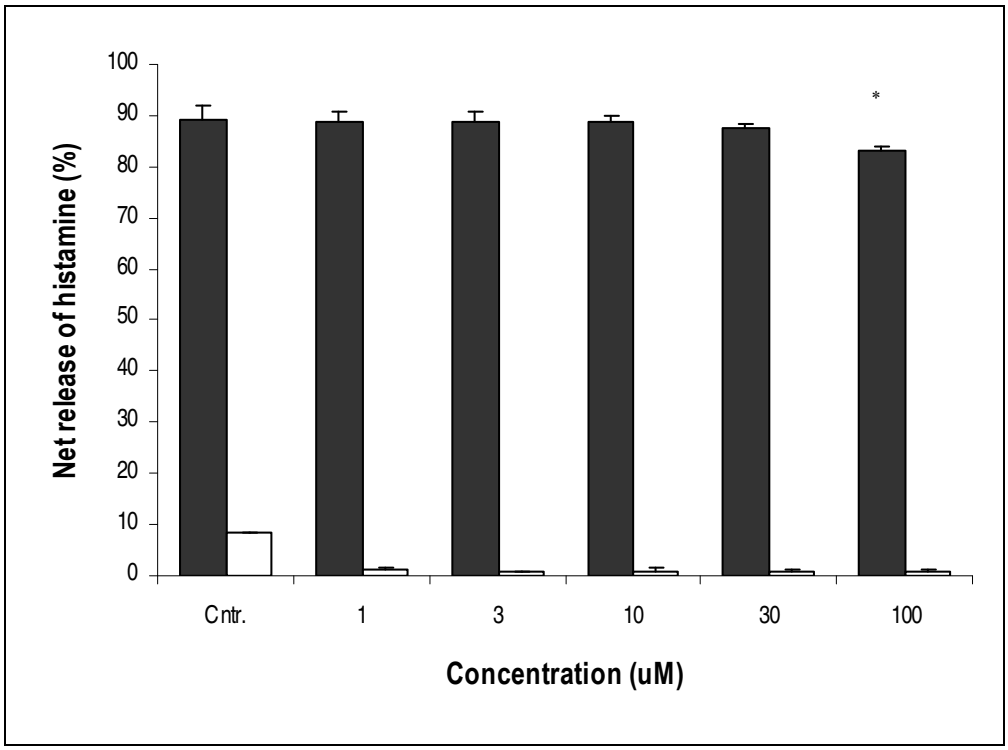


Figure 3. Effect of marmin on histamine release from rat peritoneal mast cells (RPMCs) in the presence (solid bar) or absence (open bar) of histamine stimulants i.e. compound 48/80 10 μM (a), thapsigargin 0.5 μM (b), ionomycin 1 μM (c); or phorbol myristate acetate 10 nM-ionomycin 0.1 μM (d).Data represent mean±SEM, and are three independent experiments. *Significant difference ($P<0.05$) compared to the negative control value.

The combination of PMA (10 nM), a modulator of protein kinase C (PKC) [27-28], and low-dose ionomycin (0.1 μM) increased histamine release by 89.23±2.62% in RPMCs (Fig. 3d). Marmin also did not success to deplete the histamine release from RPMCs. At highest dose only, marmin showed a significant inhibitory effect on the histamine release by 6.77±0.98%. The potency of marmin effect on the histamine release from RPMCs is shown in table 1. This value is expressed as IC_{50}.

In absence of histamine stimulator, marmin did not show significant spontaneous histamine release in RMPCs. All concentrations of marmin showed low spontaneous histamine release, less than 10 % of the total histamine contained in RMPCs. (Fig. 3).

Inhibition of $^{45}Ca^{2+}$ influx

Previously, marmin showed promising effects in RBL-2H3 when induced by histamine stimulant acting on Ca^{2+} signaling pathway such as thapsigargin and ionomycin. This study investigated the effect of marmin on intracellular Ca^{2+} signaling. The study was conducted by direct measurement of radiolabelled Ca^{2+} uptake in RBL-2H3 cells after stimulation with thapsigargin (Fig. 4). In parallel with previous results, marmin inhibited intracellular $^{45}Ca^{2+}$ incorporation concentration-dependently. Moreover, at highest dose (100 μM) marmin successfully suppress the Intracellular accumulation of $^{45}Ca^{2+}$ by 75.94±4.15%.

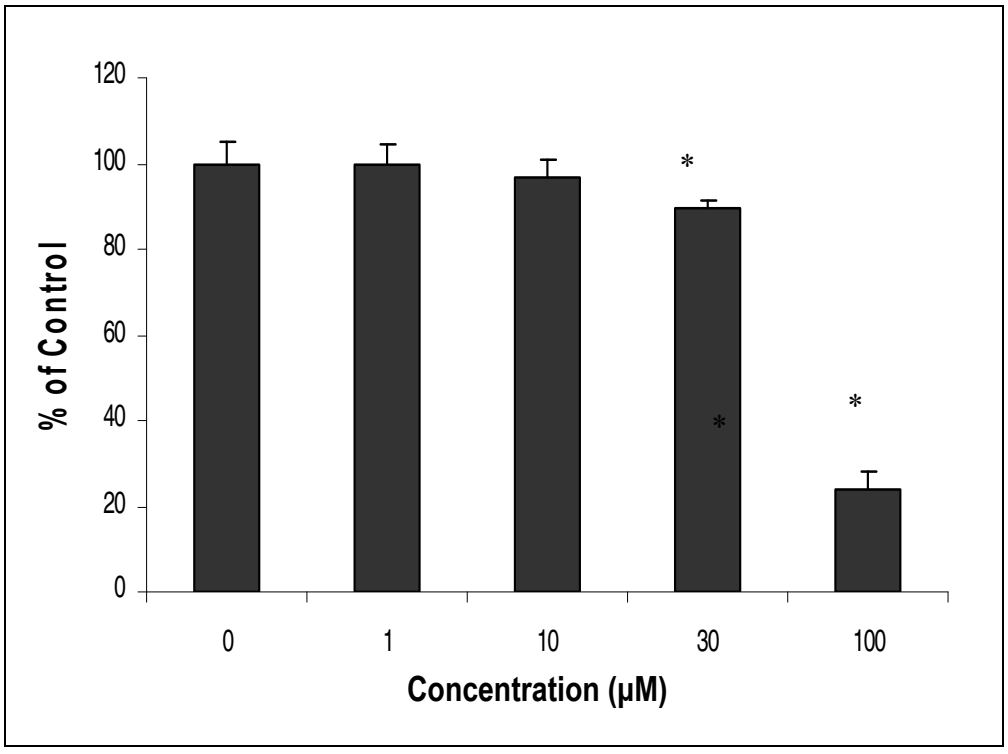


Figure 4. Inhibition of $^{45}Ca^{2+}$ uptake by marmin isolated from Aegle marmelos Correa in thapsigargin-stimulated RBL-2H3 cells. The data were representative of 3 independent experiments. *Significant difference ($P<0.05$) compared to the negative control value.

Table 1. The mean of IC_{50} of marmin effects on the histamine release from RBL-2H3 cells and RPMCs with several histamine release inducers.

Histamine inducer	IC_{50} (μM)	% inhibition of histamine release at 100 μM
1. RBL-2H3 cell lines		
DNP-BSA	49.38	62.06 ± 1.95
Thapsigargin	69.12	68.70 ± 3.54
Ionomycin	87.18	55.96 ± 3.53
2. RPMCs		
Compound 48/80	-	-
Thapsigargin	> 100	39.35 ± 3.60
Ionomycin	>> 100	10.32 ± 4.47
PMA-low dose of ionomycin	>> 100	6.77 ± 0.98

Discussion

Coumarin derivatives isolated from plants are considered potential to be developed as anti-allergic agents. Compound 3,4- dimethyl-7 -[4 -(p-chlorobenzyl) -pyperazine-1 -yl] -propoxicoumarin. dihydrochloride was reported to possess an activity as a histamine receptor antagonist. The compound also inhibited antigen-induced histamine release from sensitized human leucocytes, and inhibited the re-uptake of histamine by isolated human leukocytes [23]. Cnidicin and its related coumarins isolated from the root extract of Angelica koreana inhibited the degranulation process by inhibiting the β-hexoaminidase release from RBL-2H3 cells [24]. Other coumarins, 7-methoxycoumarin and 7-hydroxycoumarin also showed inhibitory effects on mediator release from RBL-2H3 cells. The study of structure-activity relationship among coumarine compounds indicated that oxigen-containing moiety at the 7-position of coumarin structure has a main role for the inhibitory effect [25].

In the study, we evaluated the antiallergic effects of marmin, a coumarine isolated from Aegle marmelos Correa. Marmin was investigated for its effects on histamine release from rat mast cells induced by several histamine stimulants. Marmin was isolated from dried ground powder of bark and fresh root of Aegle marmelos Correa, then using petroleum ether, chloroform and methanol for extraction solvents. Marmin was provided after subsequent purification using chromatography methods and recrystallization [18].

There are only few available literatures concerning the pharmacological activity of marmin. Takase et al. reported that marmin isolated from Aurantii fructus immaturus protect the gastric mucosal on the experimental acute gastric lesions by maintenance of the mucosal barrier integrity, inhibition on gastric motor activity, and prevent the effects of acetylcholine and histamine [26].

In the present study, marmin inhibited histamine release from RBL-2H3 cells induced by DNP_{24}-BSA, an specific antigen for monoclonal IgE antibody [27-28]. Its fact indicates that the compound might alter the effect of DNP_{24}-BSA on mast cells by affecting its interaction with IgE on the mast cell surface or by altering intracellular signal transductions involved in mast cell degranulation.

To evaluate the inhibitory effect of marmin involving intracelullar Ca^{2+} levels, thapsigargin and ionomycin were used for stimulate the release of histamine from mast cell. Thapsigargin, a sesquiterpene lactone isolated from the plant Thapsia garginica, is a Ca^{2+} signaling stimulant. Its target is the ATP-dependent Ca^{2+} pump in the endoplasmic reticulum, and it can increase the concentration of

cytosolic free calcium ion [21-22]. Ca^{2+} release from intracellular store plays a major role in the opening of cell membrane Ca^{2+} channels to cause Ca^{2+} influx in mast cells [4]. Ionomycin, a selective Ca^{2+} ionophore, also induces histamine release from mast cells by increasing in intracellular Ca^{2+} concentration, both through the release from intracellular Ca^{2+} pools (endoplasmic reticulum) and via Ca^{2+} influx [29]. In our study, marmin inhibit successfully the histamine release from RBL-2H3 cells induced by thapsigargin and ionomycin. These data were supported by the finding that marmin potently suppressed $^{45}Ca^{2+}$ uptake from extracelullar side. RBL-2H3 cell is totally dependent on the influx of external Ca^{2+}. Blockage of Ca^{2+} influx cause a rapid decline in intracellular Ca^{2+} concentration and then decrease the release of histamine from this cells [30]. Its fact indicates that the effect of marmin on the release of histamine involve alteration on the influx of external Ca^{2+}.

In RPMCs experiments, marmin inhibited the histamine release induced by thapsigargin, even though the effect was lower than this in RBL-2H3 cells. Moreover, marmin showed low inhibitory effect when histamine stimulated by ionomycin. RMPCs still release low histamine in the absence of influx of external Ca^{2+} [31-32]. In this case, the intracellular Ca^{2+} pool has an important role in histamine secretion [33]. Figure 4, marmin could suppress the influx of external Ca^{2+} the but not complete that still allow a few amount of Ca^{2+} from external side to trigger intracellular Ca^{2+} pool depletion. These findings indicate that marmin may inhibit intracellular Ca^{2+} from Ca^{2+} influx rather than from intracellular Ca^{2+} pool.

In the study, marmin did not influence the histamine release from RPMCs induced by compound 48/80. Compound 48/80 is known to activate mast cell secretory processes by increasing the rate of GTP S binding to G-proteins (Go/Gi mixture) [34,35]. Compound 48/80 also stimulates histamine release from RPMCs in both the presence and absence of extracellular calcium [4]. Its fact indicates that the inhibitory effect of marmin is not related to signaling events in G protein activation pathway.

PMA is a histamine secretagogue that activates PKC signaling event in mast cells [36,37]. PMA does not elicit histamine release to the same extent as other inducers of histamine release [38]. Since the activity of PKC in promoting granule exocytosis and inflammatory mediator release from mast cells is dependent on the intracellular Ca^{2+} concentration [39,40], a sub-effective dose of calcium ionophore is often used concomitantly with PMA to stimulate histamine release [41-43]. In RPMCs, marmin showed very low inhibitory effect on the histamine release induced by PMA and ionomycin in combination. It is suggested that this compound may did not alter the interaction between PKC and intracellular Ca^{2+} during granule exocytotic processes.

As mentioned above that the mechanism of histamine release from mast cells involve several intracellular signaling pathways. In the study, marmin successfully influence the histamine release from mast cells when using DNP_{24}-BSA, thapsigargin and ionomycin in RBL-2H3 cells since this cell is totally dependent on Ca^{2+} influx. It is supported that marmin also successfully inhibit the influx of external Ca^{2+}. In the other hand, marmin did not influence or showed low inhibitory effects in RPMCs, a type of mast cell still release low histamine in absence of Ca^{2+} influx. Based on these facts, marmin is suggested to inhibit the histamine release from mast cell by blocking by blocking Ca^{2+} uptake. Nevertheless, further study is required to investigate the detail mechanism of marmin in mast cells. Moreover, the further in vivo study is very useful to provide information to further explain drug action.

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