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Effects of dihydrocubebin, a lignan isolated from Indonesian plant *Piper cubeba*, on the histamine release from rat mast cells

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SUMMARY

The fruits of *Piper cubeba* L. are used traditionally to treat respiratory disorders in Indonesia. In order to determine the compounds responsible for this activity, the fruits were extracted with n-hexane followed by ethanol to give n-hexane and ethanol extracts. Based on tracheospasmolytic assay on these two extracts, the n-hexane extract was more active to inhibit trachea contraction than that of ethanol extract. Upon bioassay guided isolation of the n-hexane extract, a tracheospasmolytic active compound was isolated and identified as dihydrocubebin [(3,4),(3′,4′)-bis-methylenedioxy-9,9′dihydroxylignan] (1). Compound 1 was tested further for its ability to inhibit histamine released from mast cells, using rat basophilic leukemia (RBL-2H3) cell line and rat peritoneal mast cells RPMCs) as models; and DNP_{24}-BSA, thapsigargin, ionomycin, compound 48/80 and PMA were used as inducers for histamine released from mast cell. The test result showed that 1 inhibited histamine release from RBL-2H3 cells induced by DNP_{24}-BSA, thapsigargin and ionomycin. In addition, 1 suppressed histamine release from RPMC induced by either thapsigargin or ionomycin. However, 1 did not inhibit histamine release from RPMC induced by either compound 48/80 or combination PMA-sub optimum dose of ionomycin. Therefore, it was concluded that the inhibitory effects of 1 on the histamine released from mast cells may involve mechanisms related to intracellular Ca^{2+} signaling events or downstream processes of intracellular Ca^{2+} signaling in mast cells.

Key words: *Piper cubeba* L.; Dihydrocubebin; Histamine; Mast cells

INTRODUCTION

Indonesia has been reported for its first biodiversity of the world. *Piper cubeba* L.f commonly present in a traditional preparation (*jamu*) intended to cure respiratory disorder. Initial study indicated that n-hexane extract of *Piper cubeba* inhibited contraction of isolated guinea pig trachea induced by methacholine (Wahyuono *et al.*, 1999). Many plants of genus Piper have been studied for their anti-allergy effects such as of *Piper longum* using rat-lung perfusion as a model (Nayampali and Satoskar, 1980). Ethanolic extract of *Piper betle* leaves decreased histamine and GM-CSF produced by an IgE-mediated hypersensitive reaction, and inhibited eotoxin and IL-8 secretion as well in a TNF-α and IL-4-induced allergic reaction

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(Wirotesangthong *et al.*, 2007). While methanolic extract of *Piper nigrum* leaves inhibited histamine release from rat peritoneal mast cell, a powerful inhibition on DNFB-induced cutaneous reaction at 1 h and 24 h after DNFB challenge in mice, which passively sensitized with anti-DNP IgE antibody upon oral administration of this extract (Hirata *et al.*, 2008).

Asthma is a lung disease characterized by airways obstruction, airways inflammation, and increased airway responsiveness. Allergic reaction has a main role on development of airway obstruction and pathogenesis of asthma (Busse and Reed, 1993; Holt *et al.*, 1999). Mast cell is a crucial effector cell in the pathogenesis of asthma, especially asthma with an allergic basic (Brown *et al.*, 2008). Mast cells are strategically located in pulmonary system to participate in lung allergic reactions by release rapidly mediators such as histamine and other lipid mediators (Busse and Reed, 1993). A therapeutic strategy for treating allergic airway diseases is to inhibit mast cells degranulation and to inhibit the release of mast cells mediators such as histamine.

At present we report the effect of dihydrocubebin [(3,4),(3′,4′)-bis-methylenedyoxy-9,9′dihydroxylignan] (1), a lignan isolated from *Piper cubeba* fructus (Prabhu and Mulchandani, 1985; Wahyono, 2005) to inhibit histamine released from mast cells. In the study, rat basophilic leukemia (RBL-2H3) cells and rat peritoneal mast cells (RPMCs) were used; and DNP_{24}-BSA, thapsigargin, ionomycin, compound 48/80 and PMA were used as inducers for histamine release from mast cells.

MATERIALS AND METHODS

Materials

Dihydrocubebin (1) was isolated from *Piper cubeba* L.f fruits by Dr. Wahyono from Gadjah Mada University (GMU), Indonesia. The histamine release inducers used in the study were ionomycin, thapsigargin, compound 48/80, and phorbol myristate acetate (PMA) from Sigma Chemical Co. (St.Louis, MO, USA). Dinitro-phenylated 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, NY, USA). Fetal calf serum was obtained from JRH Biosciences (Kansas, USA). Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) was purchased from Dojindo (Kumamoto, Japan), and o-phthalaldehyde was from Wako Pure Chemical Industires (Osaka, Japan).

Culture of RBL-2H3 cells

The RBL-2H3 cells were cultured in MEM containing 15% fetal calf serum and antibiotics (penicillin and streptomycin) in a flask in a humidified atmosphere (5% CO_2) at 37 °C as described by Barsumian *et al.* (1981). For the assay of histamine release, cells were seeded into 24-well culture plates at a density of 5×10^5 cells/0.4 ml per each well. The cells were incubated overnight at 37 °C. For 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 (119 mM NaCl, 5 mM KCl, 25 mM PIPES, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 40 mM NaOH, and 0.1% bovine serum albumin, pH 7.2), and pre-incubated 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 pre-incubation, 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 weighing between 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. RPMCs were isolated by injection of 25 ml phosphate buffered saline (PBS) pH 7.4 containing 5 IU/ml heparin and 0.1% 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 re-suspended in 2 ml PBS buffer containing 0.1% 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 min 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 re-suspended in 1 ml of PIPES buffer. Mast cell preparations were about 95% pure as assessed by toluidine blue staining.

For the assay of histamine release, 120 μl of RPMCs suspension (2×10^4 cells/ml) was pre-incubated 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 pre-incubation, 20 μl of stimulant (100 μg/ml 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

Histamine released in the medium was measured by HPLC-fluorometry as described previously (Yamatodani *et al,* 1985). 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 5 mM 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.

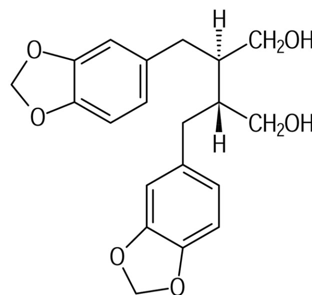

Fig. 1. Dihydrocubebin (1).

Statistical analysis

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

RESULTS

Effects on histamine release from RBL-2H3 cells

DNP_{24}-BSA was used as an antigen to stimulate histamine release from IgE-sensitized RBL-2H3 cells. DNP_{24}-BSA (20 ng/ml) stimulated histamine release from RBL-2H3 cells by 34.56 ± 1.05% (n = 3) (Fig. 2). Pre-incubation of RBL-2H3 cells with 1 for 30 minutes significantly inhibited the histamine and β-hexoaminidase enzyme release induced by DNP_{24}-BSA in a dose-dependent manner. At the highest dose (100 μM), this compound inhibited the release of histamine from RBL-2H3 cells by 52.05 ± 3.85% (n = 3) (Table 1). IC_{50} value of its

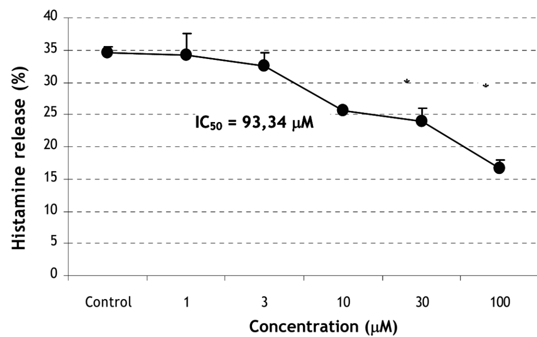

Fig. 2. Effect of dihydrocubebin on histamine released from RBL-2H3 cells in the presence of DNP-BSA 20 ng/ml. Data represent mean ± SEM, and are three independent experiments. *$P < 0.05$ compared to the negative control value.

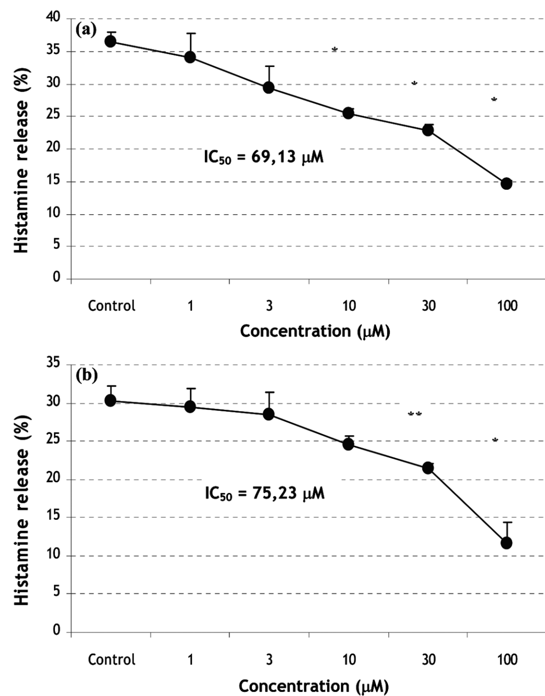

Fig. 3. Effect of dihydrocubebin on histamine released from RBL-2H3 cells in the presence of either thapsigargin 0.5 μM (**a**); or ionomycin 1 μM (**b**). Data represent mean ± SEM, and are three independent experiments. *$P < 0.05$, **$P < 0.1$ compared to the negative control value.

inhibitory effect was used as the parameter of potency. The IC_{50} of the inhibitory effects of this compound on histamine induced by DNP_{24}-BSA were 93.34 mM.

Table 1. The inhibitory effects of dihydrocubebin on the histamine released from RBL-2H3 cells and its IC_{50} values

No.	Conc. (μM)	histamine release inhibition (%)		
		DNP_{24}-BSA	thapsigargin	ionomycin
1.	1	0.78 ± 9.71	6.84 ± 10.67	2.51 ± 8.19
2.	3	6.05 ± 6.38	19.38 ± 8.91	5.88 ± 9.68
3.	10	26.00 ± 0.49	30.25 ± 1.86	18.69 ± 3.51
4.	30	30.51 ± 5.93	37.64 ± 2.58	29.27 ± 2.22
5.	100	52.05 ± 3.85	59.75 ± 1.48	61.35 ± 8.88
IC_{50} value (μM)		93.34	69.13	75.23

Concentration of DNP_{24}-BSA, thapsigargin and ionomycin were 20 ng/ml, 0.5 μM and 1 μM, respectively. Data represent mean ± SEM of three independent experiments.

Thapsigargin (0.5 μM) and ionomycin (1 μM), which act on calcium ion influx and intracellular calcium pathways, stimulated histamine release by 36.44 ± 1.54% (n = 3); and 30.22 ± 1.99% (n = 3), respectively (Fig. 3). Dihydrocubebin (1) succeeded to inhibit the histamine release induced by both of which in a dose-dependent manner. At the dose of 100 μM, the compound strongly inhibited the release of histamine from RBL-2H3 cells by 59.75 ± 1.48% (n = 3); and 61.35 ± 8.88% (n = 3), respectively (Table 1). The IC_{50} of the inhibitory effects of 1 on histamine release induced by both inducers were 69.13 and 75.23 μM respectively.

In the absence of histamine secretogogue, possibility of histamine release from RBL-2H3 cells by 1 was observed. The effect was considered significant if the compound caused spontaneous histamine release of more than 10% (Ikawati *et al.*, 2001). Dihydrocubebin at 100 μM induced the spontaneous histamine release on RBL-2H3 cells by 1.08 ± 0.97% (n = 3).

Effects on histamine release from RPMCs

Compound 48/80, PMA, thapsigargin, and ionomycin were used for stimulating the histamine release rat peritoneal mast cells (RPMCs). Compounds 48/80, which acts on G proteins in mast cells, stimulated histamine release from RPMCs by 73.26 ± 1.41% (n = 3). Dihydrocucebin at 0.1-100 μM did not significantly

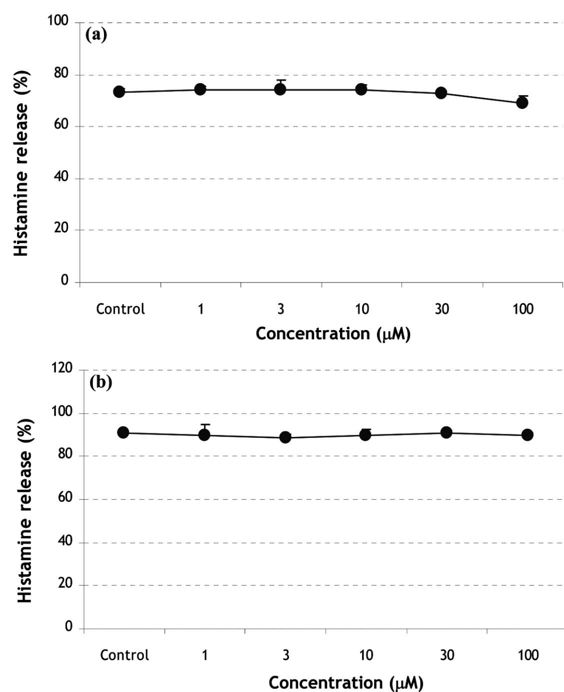

Fig. 4. Effect of dihydrocubebin on histamine released from rat peritoneal mast cells in the presence of either compound 48/80 10 μg/ml (**a**); or phorbol myristate acetate 10 nM-ionomycin 0.1 μM (**b**). Data represent mean ± SEM, and are three independent experiments.

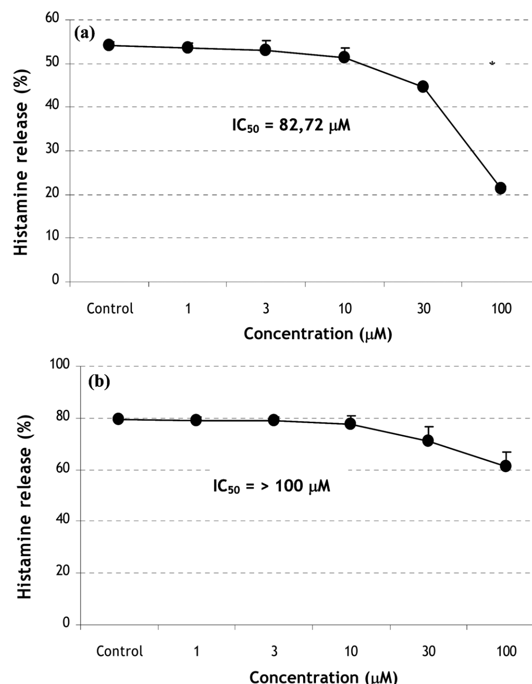

Fig. 5. Effect of dihydrocubebin on histamine released from rat peritoneal mast cells in the presence of either thapsigargin 0.5 μM (**a**); or ionomycin 1 μM (**b**). Data represent mean ± SEM, and are three independent experiments. *$P < 0.05$ compared to the negative control value.

inhibit compound 48/80-induced histamine release.

To assess the effect of 1 on protein kinase C-mediated histamine release, combination of PMA, a modulator of PKC, and low-dose ionomycin were used. RPMCs release 90.93 ± 0.66% (n = 3) of their total histamine content in response to combination 10 nM PMA and 0.1 μM ionomycin (Fig. 4). In this experiment, 1 (0.1 - 100 μM) did not significantly influence the histamine release.

In RMPCs, thapsigargin (0.5 μM) and ionomycin (1 μM) induced histamine release by 54.02 ± 0.97% (n = 3); and 79.52 ± 0.60% (n = 3), respectively (Fig. 5). Dihydrocubebin dose-dependently inhibited both thapsigargin- and ionomycin-induced histamine released from RPMC. At the dose of 100 μM, the compound potently inhibited the release of histamine release from RBL-2H3 cells by 60.65 ± 1.21% (n = 3); and 23.23±7.19 % (n = 3), respectively (Table 2). The IC_{50} of the inhibitory effects of 1 on histamine release induced by both of which were 82.72 μM and > 100 μM, respectively.

Table 2. The inhibitory effects of dihydrocubebin on the histamine release from RBL-2H3 cells

No.	Conc. (mM)	histamine release inhibition (%)	
		thapsigargin	ionomycin
1.	1	0.85 ± 2.24	0.47 ± 1.61
2.	3	1.89 ± 4.26	0.40 ± 1.22
3.	10	5.13 ± 4.34	2.22 ± 4.12
4.	30	17.52 ± 0.61	10.96 ± 7.61
5.	100	60.65 ± 1.21	23.23 ± 7.19
IC_{50} value (μM)		82.72	> 100

Concentration of thapsigargin and ionomycin were 0.5 μM and 1 μM, respectively. Data represent mean ± SEM of three independent experiments.

In RPMCs experiments, 1 at 100 μM induced the spontaneous histamine release by 0.23 ± 0.27% (n = 3), and this value was lower than this in RBL-2H3 cells.

DISCUSSION

Upon tracheospasmolytic guided isolation method, dihydrocubebin (1) was isolated from n-hexane extract of *Piper cubeba* L.f fruits traditionally used to treat respiratory disorders. It was suggested that 1 contributed anti-asthmatic activity present in the *Piper cubeba* L.f fruits. In present study, we studied the effect of dihydrocubebin on the histamine release from rat mast cell i.e. rat basophilic leukemia (RBL-2H3) cells and rat peritoneal mast cells (RPMCs).

Dinitrophenylated bovine serum albumin (DNP_{24}-BSA) is a specific antigen for monoclonal IgE antibody (Bottcher *et al.,* 1980; Liu *et al.,* 1980). The cross-linkage of antigen into IgE antibody molecules on FceRI receptors, and in turn can generate a series of intracellular signaling such as the activation of protein tyrosine kinases and an increase of intracellular Ca^{2+} levels. Finally, these subsequent signaling events trigger the granule exocytosis releasing histamine from mast cells (Metcalfe *et al.,* 1997). In our study, 1 markedly inhibited the histamine release from RBL-2H3 cells induced by DNP_{24}-BSA. This finding indicates that 1 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 transduction involved in mast cell degranulation.

Futhermore, we investigated whether the inhibitory effect of 1,5-bis(4′-hydroxy-3′-methoxyphenyl)-1,4-pentadiene-3-on is related to intracellular Ca^{2+} signal. For this purpose, we used thapsigargin and ionomycin. Thapsigargin, a sesquiterpene lactone isolated from plant *Thapsia garginica*, is able to release mediators from isolated rat mast cells (Patkar *et al.,* 1979), its target is sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) to prevent the Ca^{2+} store refilling. The RE-store depletion because of intracelluler Ca^{2+} released from RE has a major role in the opening of cell membrane Ca^{2+} channels to cause Ca^{2+} influx in mast cells (Metcalfe *et al.,* 1997; Thastrup *et al.,* 1990). Ionomycin is a polyether antibiotic with a high affinity for calcium ions. Ionomycin is obtained in pure form from fermentation broths of *Streptomyces conglobatus* sp. nov. Trejo by solvent extraction (Liu *et al.,* 1978; Westley *et al.,* 1979). Ionomycin can release histamine from mast cells by increasing Ca^{2+} intracellular from both intracellular Ca^{2+} pools and Ca^{2+} influx (Huang and Putney, 1998). In the study, 1 successfully inhibited the histamine release from rat mast cell induced by either thapsigargin or ionomycin. This finding indicates that the effect of this compound on the release of mast cells mediators involve mechanisms related to intracellular Ca^{2+} signaling events or downstream processes of intracellular Ca^{2+} signaling in mast cells.

Compound 48/80 is a histamine secretagogue which able to increase the rate of GTP S binding to G-proteins (Mousli *et al.,* 1990; Palomaki *et al.,* 2006). The activation of G-proteins can trigger intracellular signaling events such as activation of phospolipase C, protein kinase C, and Ca^{2+} signaling, and finally results in the release of histamine from these cells. By this compound, only restricted types of mast cells are activated, and these are of the connective tissue type. Mucosal mast cell such as RBL-2H3 cells, do not respond to this secretagogue (Metcalfe *et al.,* 1997). In the study, 1 did not show significant effect on compound 48/80-induced histamine release. This fact indicates that the inhibitory effect of 1 is not related to signaling events in G protein activation pathway.

Phorbol myristate acetate (PMA) stimulates histamine release by activating protein kinase C (PKC) signaling event in mast cells (Okano *et al.,* 1986; Bergstrand *et al.,* 1992). PMA does not stimulate histamine release to the same extent as other histamine secretagogue (Okano *et al.,* 1985). In order to promote granule exocytosis and mediator release from mast cells, activation of PKC is markedly enhanced in present of a large Ca^{2+} influx which causes translocation of PKC to the membrane (Katakami *et al.,* 1984; Chakravarty, 1990). In RPMCs, activation of PKC alone also caused a few amount of histamine

release but the combination with Ca ionophore induced the high amount of histamine release (Heiman and Crews, 1985). A sub-effective dose of Ca^{2+} ionophore is often used concomitantly with PMA to induce high amount of histamine release (Yen *et al.*, 1992; Shin *et al.*, 2004). In the study, 1 did not affect the histamine release from RMPCs induced by PMA and ionomycin in combination. It suggests that this compound did not alter the interaction between PKC and intracellular Ca^{2+} during granule exocytotic processes.

In previous study, the n-hexane and ethanol extract of *Piper cubeba* fruit induced the histamine release in absence of antigen (Ikawati *et al.,* 2001). In contrast, 1 that was isolated from n-hexane extract of *Piper cubeba* fruit did not induce any obvious spontaneous histamine release from either RBL-2H3 cells or RPMCs. This evidence suggests that there are other compounds in this n-hexane extract and not 1, responsible for the spontaneous histamine release.

In conclusion, 1 inhibited histamine release and may involve in the mechanisms related to intracellular Ca^{2+} signalings or downstream processes of intracellular Ca^{2+} signalings in mast cells. Nevertheless, further study is required to investigate the detail mechanism of 1 in mast cells.

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