Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2015; 7(6): 420-425

ISSN- 0975 1556

Research Article

Effects of Zeorin and Dustanin Isolated from *Aegle marmelos* Correa on Histamine Released from RBL-2H3 Cells

Agung Endro Nugroho[1,]i, Navista Sri Octa Ujiantari[2], Sugeng Riyanto[2], Mohd. Aspollah Hj. Sukari [3], Kazutaka Maeyama[4]

[1]*Department of Pharmacology and Clinical Pharmacy,*
[2]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia;*
[3]*Faculty of Science and Environmental Studies, Universiti Putra Malaysia;*
[4] *Department of Pharmacology, Informational Biomedicine, Ehime University Graduate School of Medicine, Shitsugawa, Toon-shi, Ehime 791-0295, Japan*

Available Online: 31st October, 2015

ABSTRACT
Two compounds have been isolated from the leaves and stem barks of *Aegle marmelos* Correa. They were zeorin (6α,22-dihydroxyhopane) and dustanin (15α,22-dihydroxyhopane). *A. marmelos* has an indication as anti-inflammatory and anti-allergy. In the study, these compounds were evaluated for their inhibitory effect on histamine release from mast cell culture, rat basophilic leukemia (RBL-2H3) cell line, a tumor analog of mast cells. Histamine release from mast cell was induced by DNP_{24}-BSA and thapsigargin, an immunologic and non-immunologic inducers respectively. The histamine release was determined by using HPLC with fluorometric detector. In the study, only zeorin markedly exhibited inhibitory activity on histamine release from mast cell induced by DNP_{24}-BSA. Zeorin showed potent inhibitory effect by 40% decrease of histamine release. However, dustanin did not alter the histamine release from mast cells induced by thapsigargin. Based on the results, the inhibitory effect of zeorin is contributed by some mechanisms related to alteration on intracellular Ca^{2+} concentration. Molecular docking was conducted to examine possible mechanisms of zeorin and dustanin on Sarcoplasmic Reticulum Ca^2+-ATPase. Our results suggest that zeorin does not blockade the sarcoplasmic reticulum Ca^{2+}-ATPase. In conclusion, zeorin isolated from A. marmelos inhibited the histamine release from mast cell by some mechanisms related to alteration on intracellular Ca^{2+} concentration however might be not related to blokage on sarcoplasmic reticulum Ca^{2+}-ATPase.

Keywords : *Aegle marmelos* Correa, RBL-2H3 cell, histamine, thapsigargin, DNP_{24}-BSA

INTRODUCTION

Aegle marmelos Correa is a plants widely used for ancient and modern traditional medicines for treatment of various disorders. The plant originates from India, and also grows widely in some areas of the southeast countries such as Vietnam, Laos, Cambodia, Thailand and Indonesia. The plant is known as Maja (Java's name) or Bila (Bali's name) in Indonesia, and Bael in its native country India[14,5]. The name of Maja is related to the Indonesian famous ancient empire, Majapahit based on the island of Java in 13th until 16th century. Reportedly, the plant have been studied for its several pharmacological activities such as antiallergy[15]; antihistamine[16,17]; antiasthma[18]; antiinflammatory[2]; antimuscarinic[11]; antimicrobial[10]; wound healing[5]; gastric ulcer protection[3]; immunomodulator[7]; hypoglycemic[22]; antidiabetes[21]. The content of many compounds in a plant can cause various pharmacological activities. In case of *A. marmelos*, several compounds have been successfully isolated and investigated for their pharmacological activities. Previously, *A. marmelos* collected from the areas of Yogyakarta (Indonesia) was studied for its active compounds including zeorin (6α,22-dihydroxyhopane) and dustanin (15α,22-dihydroxyhopane) (Fig. 1). Both of them, can be obtained from petroleum ether extract of the leaves of *A. marmelos* Correa[20]. Inflammatory reactions involve some mediators including mast cells that act in delayed and immediate hypersensitivity reactions[4,6,23]. Mast cells have important role in allergy reaction. Mast cells can be induced by the present of allergen such as grass pollen, product from dead house dust mites, and foodstuffs. These allergens can evoke the production of IgE, and the antibody bind to the mast cells. Subsequently, the cells release allergy mediators such as histamine, cytokines and eicosanoids that can trigger allergy and inflammatory reactions[19]. In this study, zeorin and dustanin were investigated for their effect on histamine release from mast cells.

MATERIALS AND METHODS

Materials

Zeorin and dustanin were isolated from *A. marmelos* Correa by Prof. Dr. Sugeng Riyanto (Faculty of Pharmacy, Universitas Gadjah Mada Indonesia). The chemical structure of these compounds are provided in Fig. 1.

**Author for Correspondence*

Thapsigargin (Sigma, Chemical) and dinitrophenylated bovine serum albumin (DNP_{24}-BSA) were used for *histamine*-releasing agent. Monoclonal IgE against DNP_{24}-BSA was obtained from the supernatant of IgE producing hybridoma. Eagle's minimum essential medium (MEM) and antibiotics (combination of penicillin G sodium and streptomycin sulfate) were obtained from Gibco, Grand Island New York. Other materials were fetal calf serum (JRH Biosciences Kansas, USA), piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) and bovine serum albumin (BSA) were purchased (Dojindo Kumamoto, Japan), and *o*-phthalaldehyde (Wako Pure Chemical Co. Osaka, Japan). The composition of the pH 7.2 adjusted-PIPES buffer was 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 %).

Cell Culture

RBL-2H3 cell (rat basophilic leukimia cell line) was cultured in MEM medium containing 15% fetal calf serum (FCS) and antibiotics (combination of penicillin and streptomycin). The cell was incubated in a humidified atmosphere (5% CO_2) at 37°C. In the assay of histamine release, the cells were cultured in 24-well plate at a density of 5 x 10^5 cells/0.4 ml per each well, and maintained overnight at 37°C. In the DNP_{24}-BSA experiment, the cells were then sensitized with monoclonal IgE (0.5 µg/ml). On the next day, the MEM was removed, and the cells were washed twice with 500 µl of PIPES buffer. The cells were then preincubated with 180 µl of PIPES buffer either in absence (control) or presence of the drug for 10 min at 37°C. Afterward, 20 µl of histamine-releasing agents (200 ng/mL DNP_{24}-BSA or 0.5 µM thapsigargin) were added into each well, and incubated at 37°C for 30 min.

Assay of histamine release

Histamine release assay was carried out using HPLC-fluorometry regarding to our previous studies[12,13]. After previous incubation, the plates were centrifuged at 3,000 rpm for 5 min, and the supernatant was collected in a 1.0 *mL* microcentrifuge *tube*. After sequent addition of 250 µl of 3% perchloric acid containing 5mM Na_2-EDTA and 30 µl of 2 M KOH/1 M KH_2PO_4, the tubes were centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was injected directly onto the HPLC column packed with TSKgel SP-2SW cation exchanger (Tosoh, Tokyo). In total histamine content assay, the cells, six wells were added with 350 µl of PIPES buffer and the cells were sonicated. Fifty microlitres of these cell homogenates were subjected for the histamine assay. The histamine of these homogenates or supernatants 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.

Molecular Docking Study

The X-ray Crystallographic structure of Sarcoplasmic Reticulum Ca^{2+}-ATPase (ID:2C8K) was retrieved from Protein Data Bank (PDB) (www.rcsb.org) with a resolution of 2.80 Å and loaded into MOE working environment (underlicensed Faculty of Pharmacy UGM) ignoring water molecules and heteroatoms. Preparation receptor was done using AMBER method. The structure was protonated at temperature of 310 K, pH of 7.4 and a salt concentration of 0.1. The two test compunds, Zeorin and Dustanin was constructed through Marvin Bean Software and energy minimized using PM3 method with MOE. Docking was performed using MOE and pocket was used as the binding site using forcefield method (MMFF). Protocol docking was validated first where thapsigargin as native ligand and RMSD was calculated. Triangle matcher docking placement methodology and poses were generated. Thirty docking conformations were generated for each ligand and these conformations were ranked based on the free binding energies that were generated by London dG scoring function. Results showed list of docked conformation with least docking score and give the best overlay was chosen for each compounds for the analysis.

Analysis of data

Parameters calculated in the study were percentage of net histamine release and spontaneous histamine release. The percentage of net histamine release was calculated according to the following equation :

$$\text{Net release of histamine (\%)} = \frac{A-B}{C-B} \times 100 \quad (1)$$

Whilst, the spontaneous histamine release, the release of histamine in absence of histamine stimulant, was calculated with the following equation:

$$\text{Spontaneous histamine release (\%)} = \frac{B}{C} \times 100 \quad (2)$$

A = Histamine concentration (in supernatant of cells stimulated)

B = Histamine concentration (in the supernatant of unstimulated cells)

C = Total histamine content

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

In the study, induction of RBL-2H3 cells by DNP_{24}-BSA and thapsigargin resulted in histamine release by 30.98±3.44% and 40.56±1.61% of the total histamine content in the cells, respectively. DNP_{24}-BSA stimulates the histamine release through IgE sensitization on mast cells. Whereas, thapsigargin acts to stimulate the histamine release related to Ca^{2+} influx and intracellular calcium pathways in mast cells. In the study, we observed the inhibitory effect of zeorin and dustanin ranging 1-100 µM on the histamine release from RBL-2H3 cells. Zeorin could decrease the histamine release from mast cells induced by DNP_{24}-BSA in a concentration-dependent manner. However, dustanin did not alter this histamine release. At the concentration of 100 µM, zeorin reduced

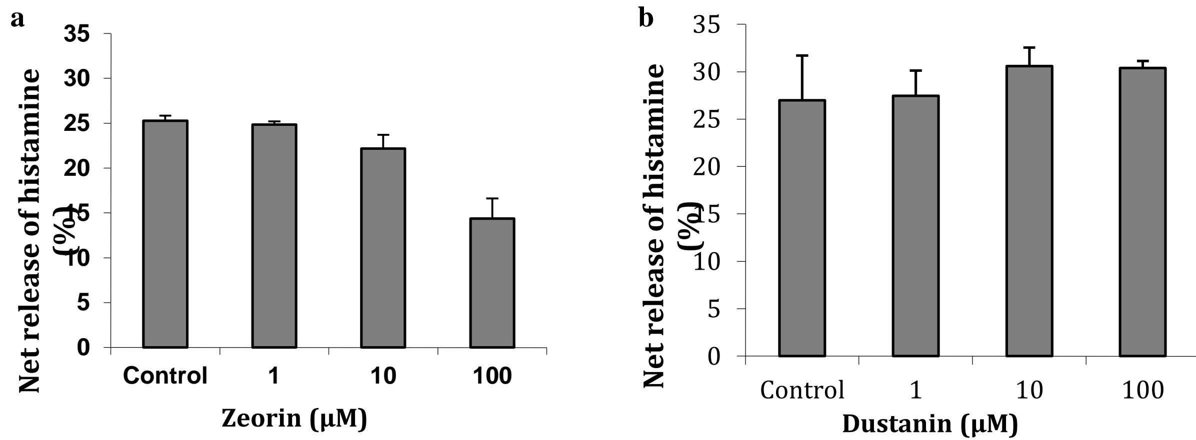

Figure 1. Effect of (a) zeorin and (b) dustanin on the histamine release from RBL-2H3 cells in the presence of 20 ng/mL DNP-BSA. Data represent mean±SEM, and are three independent experiments. * Significant difference P<0.05 compared to the negative control value.

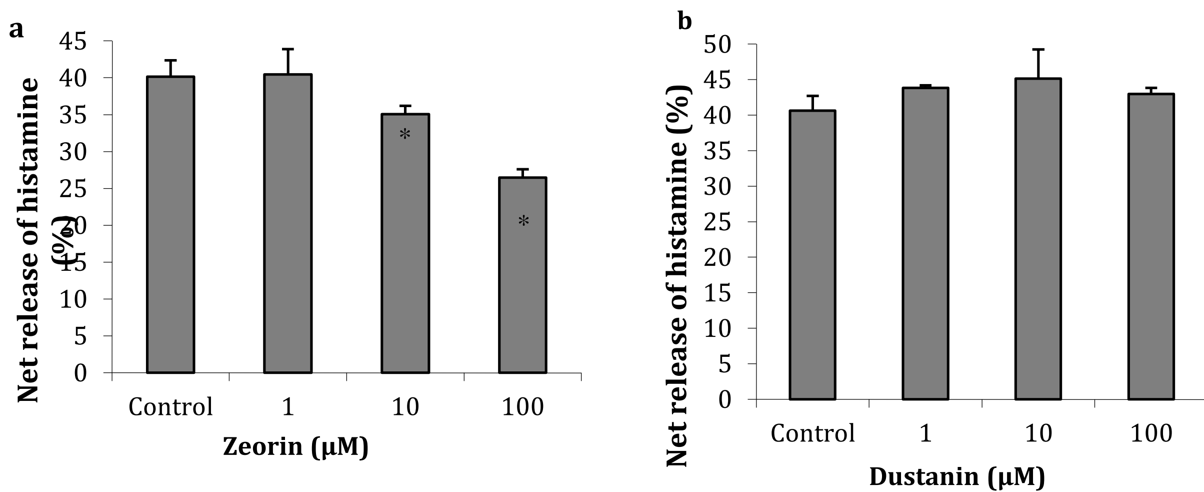

Figure 2. Effect of (a) zeorin and (b) dustanin on the histamine release from RBL-2H3 cells in the presence of thapsigargin 0.5 µM. Data represent mean±SEM, and are three independent experiments. * Significant difference P<0.05 compared to the negative control value.

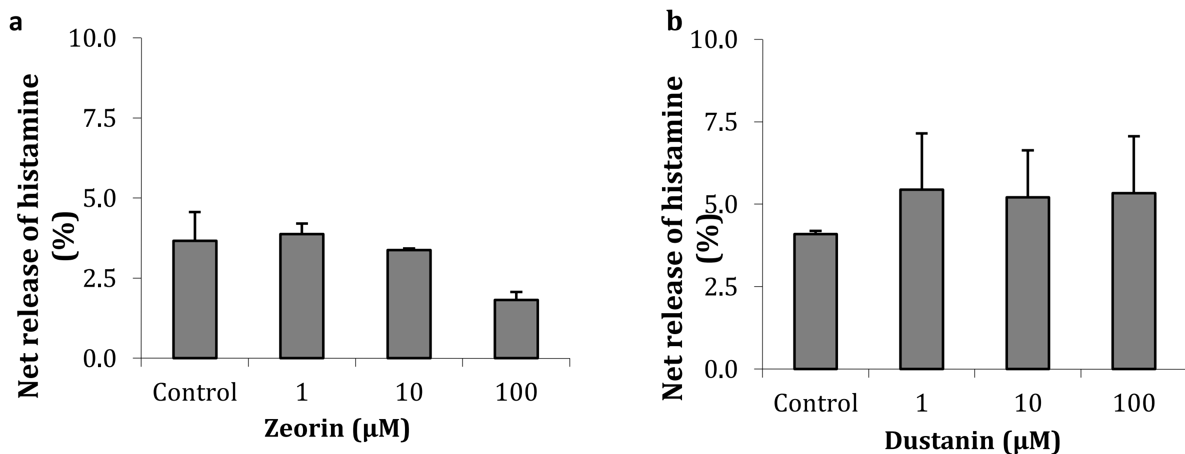

Figure 3. Effect of (a) zeorin and (b) dustanin on the histamine release from RBL-2H3 cells in the absence of histamine inducers (spontaneous histamine release). Data represent mean±SEM, and are three independent experiments.

the histamine release from RBL-2H3 cells induced by DNP24-BSA by 43.17±8.86% (Fig 2). Somewhat different

to previous results, only zeorin successfully inhibited the histamine release induced by thapsigargin. The compound exhibited significant inhibitory effects at 10 and 100 μM by reducing the histamine release by 12.12±3.12% and 34.12±3.12%, respectively (Fig. 3). Dustanin did not influence the histamine release induced by thapsigargin. This inhibitory effect of zeorin on the histamine release was presumed to related to Ca2+ signaling pathway in the mast cell. In the study, we also observed the effect of the drug on the histamine release from RBL-2H3 cells in absence of histamine-releasing agents. This effect is considered significant if the drug exhibits a spontaneous histamine release of more than 10%. Zeorin and dustanin at all concentrations exhibited low spontaneous histamine

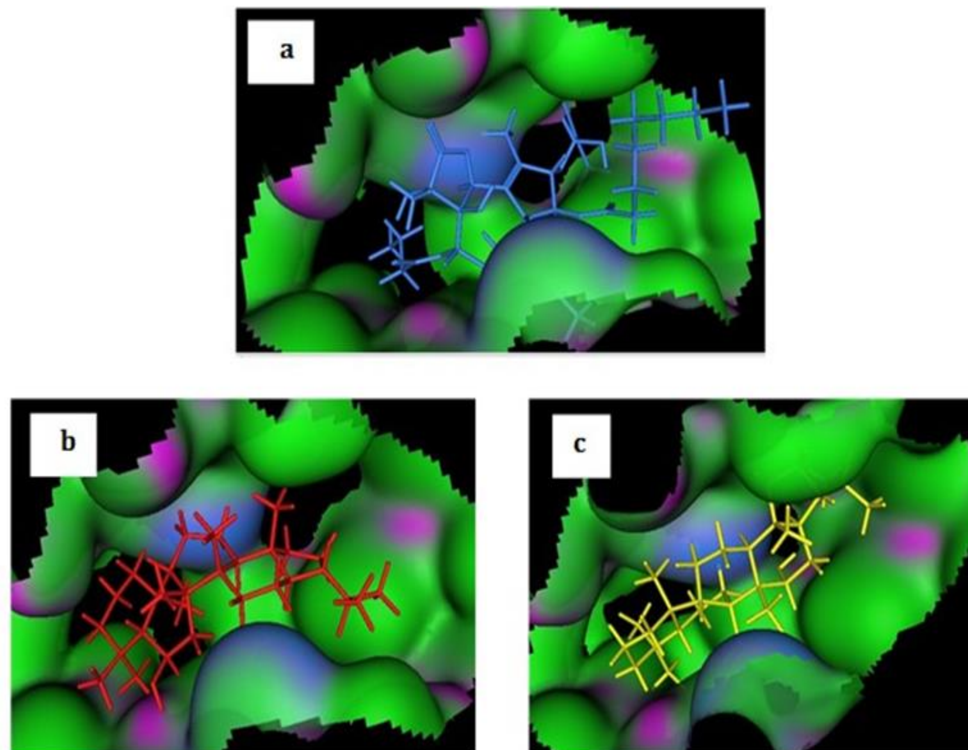

Figure 4. Interaction (a) thapsigargin, (b) zeorin, and (c) dustanin on the pocket. All three compounds have non-polar moiety and give hydrophobic interaction (green colour) toward amino acid residues around biding site.

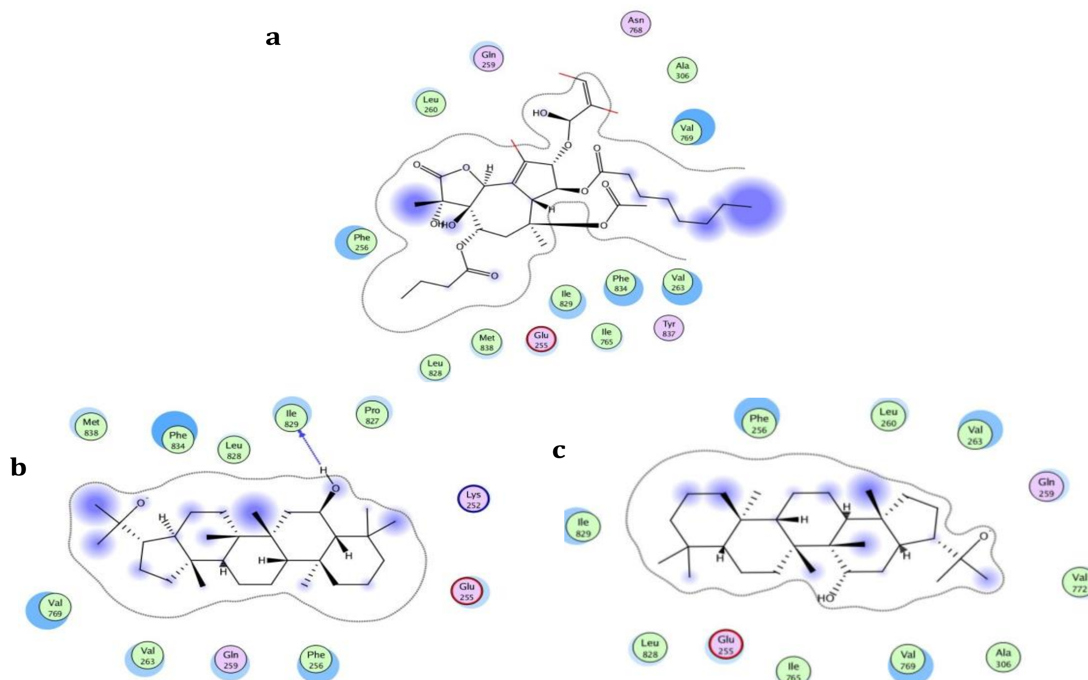

Figure 5. 2D interaction between (a) thapsigargin, (b) zeorin, and (c) dustanin on acid amino residues around the pocket binding site. Both of (a) thapsigargin and (c) dustanin have similar interaction in binding amino acid residues such as Leu260, Ala306, Ile765.

release (less than 10 % of the total histamine content) (Fig 4). Molecular docking was performed in order to confirm the effect of zeorin on intracellular Ca^{2+} signaling pathway in mast cells. Firstly, docking validation was performed to calculate RMSD value. In the study, the calculated RMSD value was 1.8977 (lower than 2,0). Based on docking result, the scores of interaction of thapsigargin, zeorin, and dustanin on the protein target were -8.9803; -8.7725; and -9.002, respectively. The interaction represents an energy interaction. Lower score shows stronger affinity for binding protein.

DISCUSSION

Allergy is a hypersensitivity response of the immune system that can be triggered by allergens, such as grass pollen, dust mite, certain foodstuffs or some drugs. The pathogenesis of allergic diseases such as anaphylaxis, allergic rhinitis, allergic asthma, involved mast activation through production of IgE type attaching to the cell surface via the high-affinity FcεRI receptors. Activation of the receptor by cross-linkage of certain allergen into IgE antibody molecules can generate a series of intracellular celluler signaling including the activation of protein tyrosine kinases and an increase of intracellular Ca^{2+} levels. These events can evoke the granule exocytosis and then release the mediators including preformed mediators (serine proteases, histamine, serotonin, proteoglycans), lipid mediators (thromboxane, prostaglandin D2, leukotrine C4, platelet-activating factor) and cytokines/chemokines. Preformed mediators and lipid mediators contribute in immediate reaction including erythema, edema, ithcing, sneezing, cough, mucus secretion etc. Cytokines/chemokines mediate late phase reactions (inflammation, persistent asthma) that occur 6-14 hours after the immediate reaction[9,19,23]. Histamine is a substantial mediator in the generation of allergy reaction. Histamine has short half-time (1 minute), and metabolized by histamine N-methyltransferase to tele-,ethyl histamine and by diamine oxidase to imidazole acetaldehyde. Histamine has significant effects on smooth muscle, endothelial cells, nerve endings and mucous release. Some signaling pathways are involved in the release of histamine from mast cells. Interaction of an antigen with its specific IgE antibody on mast cell surface stimulates tyrosines phosphophorylation of phospholipase C-γ1 (PLC-γ1) and then hydrolysis of phosphatidyl inositol 4,5-biphosphate (PIP_2) to form inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 interacts with its receptor on endoplasmic reticulum (ER) to liberate calcium ion. The increase of cytosolic calcium concentration due to emptying of the ER stores of calcium causes calcium ion influx through store operated calcium entry. This increase of intraceluller calcium ion by IP_3 and protein kinase C (PKC) activation by DAG synergistically induce granules exocytosis and in turn release the histamine from mast cells[1,6,9,23]. Zeorin and dustanin were isolated from petroleum ether extraction of fresh leaves of *A. marmelos* Correa. This was fractionated using vacuum column chromatography and developed by gradient elution to provide this hopane[20]. In the study, zeorin succeeded to decrease the histamine release from RBL-2H3 cells, a type of *mucosal* mast cells, induced by DNP_{24}-BSA, a specific antigen for monoclonal IgE antibody. This antigen cross-link into IgE antibody molecules on FcεRI receptors to evoke subsequent intracellular signal transductions involved in mast cell. In turn, the signal can trigger granules exocytosis and release the histamine from mast cells[6,8,9,23]. Based on the results, zeorin markedly decreased the histamine release from mast cells induced by thapsigargin. Thapsigargin is categorized as a sesquiterpene lactone that is isolated from a plant, *Thapsia garginica*. Thapsigargin is histamine-releasing agents that acts on the intracelluler calcium signaling pathways in the mast cells. The compound acts on ATP-dependent Ca^{2+} pump (Ca^{2+}-ATPase) in the sarco/endoplasmic reticulum. Thapsigargin blockades this transporter non-competitively, and the cytosolic calcium can not be stored in endoplasmic reticulum. It causes the increase of cytosolic calcium concentration, and in turn plays a major role in the opening of cell membrane calcium channels store and calcium influx (store operated calcium entry). This increase of intracelluler calcium triggers histamine release from mast cells[9,19,23]. In this case, zeorin might inhibited the histamine release from mast cells related to intracelluler calsium signaling pathways. Further explanation was done to investigate interaction zeorin and dustanin toward sarcoplasmic reticulum Ca^{2+}-ATPase (Fig 5). Docking results showed that dustanin had lowest score and indicated its affinity as well as thapsigargin which give possibility to blockades this transporter. Dustanin might inhibite the Ca^{2+} uptake from intracellular cytosolic to sarcoplasmic reticulum and could not affect the histamine release because intracellular Ca^{2+} remain the same. From 2D interaction (Fig 6), it can be seen that there were some amino acid residues which bind the molecule. Thapsigargin and dustanin bind similar some amino acid resiudues such as Leu260, Ala306, Ile765. Zeorin's score implied its affinity toward Ca^{2+}-ATPase weaker than thapsigargin. It means that zeorin could not compete with thapsigargin to bind this transporter. We suggest that zeorin does not blockade Ca^{2+} uptake to sarcoplasmic reticulum because its lower affinity. However, zeorin inhibitory effect in histamine release might through another signaling pathway. Further investigation should be conducted to give more explanation action mechanism zeorin in decreasing histamine release in mast cells.

CONCLUSION

Zeorin (hopane triterpene) isolated from *Aegle marmelos* Correa inhibited the histamine release from mast cells. The inhibitory effect of zeorin is closely related to alteration on intracellular calcium however might be not related to blokage on sarcoplasmic reticulum Ca^{2+}-ATPase.

ACKNOWLEDGMENT

Authors thank to the Department of Pharmacology, School of Medicine Ehime Universiyt Japan and The Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada for facilitating and supporting the study.

REFERENCES

1. Beaven, MA, Guthrie, DF, Moore, JP, Smith, GA, Hesketh, TR, Metcalfe, JC. Synergistic signals in the mechanism of antigen-induced exocytosis in 2H3 cells: evidence for an unidentified signal required for histamine rele. *J Cell Biol*. 1987;105:1129-1136.
2. Benni JM, Jayanthi MK, Suresha RN. Evaluation of the anti-inflammatory activity of Aegle marmelos (Bilwa) root. *Indian J Pharmacol*. 2011;43(4):393-397.
3. Das SK, Roy C. The protective role of Aegle marmelos on aspirin-induced gastro-duodenal ulceration in albino rat model: a possible involvement of antioxidants. *Saudi J Gastroenterol*. 2012; 18(3):188-194.
4. Galli SJ. New concepts about the mast cell. *N Engl J Med.* 1993; 328(4):257-265.
5. Gautam MK, Purohit V, Agarwal M, Singh A, Goel RK. In vivo healing potential of Aegle marmelos in excision, incision, and dead space wound models. *Scientific World Journal*. 2014:1-9.
6. Gilfillan AM, Austin SJ, Metcalfe DD. Mast cell biology: introduction and overview. *Adv Exp Med Biol*. 2011;716:2-12.
7. Govinda HV, Asdaq SM. Immunomodulatory Potential of Methanol Extract of Aegle marmelos in Animals. *Indian J Pharm Sci*. 2011;73(2):235-240.
8. Liu, FT, Bohn, JW, Ferry, EL, Yamamoto, H, Molinaro, CA, Sherman, LA, Klinman, NR, Katz, DH. Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation, and characterization. *J Immunol.* 1980;124(6): 2728-2737.
9. Metcalfe, DD, Baram, D, Mekori, YA. Mast Cells. *Physiol Rev*. 1997;77(4): 1033-1064.
10. Mujeeb F, Bajpai P, Pathak N. Phytochemical evaluation, antimicrobial activity, and determination of bioactive components from leaves of Aegle marmelos. *Biomed Res Int.* 2014.
11. Noviarsito P, Riyanto S, Nugroho AE. Marmin of Aegle marmelos Correa antagonizes $AchM_3$ receptor : in silico and in vitro studies on isolated-guinea pig illeum smooth muscle. *Journal of Mathematical and Fundamental Sciences*. 2014;46A (3):278-289.
12. Nugroho, AE, Ikawati, I, Sardjiman, Maeyama, K. Effects of benzylidenecyclopentanone analogues of curcumin on histamine release from mast cells. *Biological Pharmaceutical Bulletin.* 2009;2(5):842-849.
13. Nugroho, AE, Sardjiman, Maeyama, K. Inhibitory effect of 2,5-bis(4-hydroxy-3-methoxybenzylidene) cyclopentanone on mast cell histamine mediated-rat paw edema. *Thai J. Pharm. Sci.,* 2010;34:107-116.
14. Nugroho, AE, Riyanto, S, Sukari, MA, Maeyama, K. Effects of aegeline, a main alkaloid of Aegle marmelos Correa leaves, on the histamine release from mast cells. *Pak J Pharm Sci.* 2011a; 24(3):359-367.
15. Nugroho, AE, Riyanto, S, Sukari, MA, Maeyama, K. Anti-Allergic Effects of Marmin, a Coumarine isolated from Aegle marmelos Correa leaves: In Vitro Study. *International Journal of Phytomedicine.* 2011b ;3(1):84-97.
16. Nugroho, AE, Anas, Y, Arsito, PN, Wibowo, JT, Riyanto, S, Sukari, MA. Effects of marmin: a compound isolated from aegle marmelos correa, on contraction of the guinea pig-isolated trachea. *Pak J Pharm Sci.* 2011c; 24(4):427-433.
17. Nugroho, AE, Sahid, NA, Riyanto, S, Maeyama, K, Ikawati, Z. Effects of marmin isolated from Aegle marmelos Correa on L-histidine decarboxylase enzyme in RBL-2H3 cells. *Thai J. Pharm. Sci.,* 2011d;35:1-7.
18. Nugroho, AE, Wibowo, JT, Riyanto, S. Marmin, a compound from aegle marmelos corr., relaxed the ovalbumin-induced contraction of trachea. *International Journal of Pharmacy and Pharmaceutical Sciences.* 2012;4(Supp 1):479-484.
19. Rang, HP, Dale, MM, Ritter, JM, Moore, PK. *Pharmacology*. 5th Ed. Sydney:Churchill Livingstone;2003.
20. Riyanto, S. Phytochemical Studies and Bioactivity Tests of Murraya paniculata Jack, Aegle marmelos Correa, and Zingiber amaricans Blume. *Dissertation*:Universiti Putra Malaysia. 2003.
21. Sabu, MC, Kuttan, R. Antidiabetic activity of Aegle marmelos and its relationship with its antioxidant properties. *Indian J Physiol Pharmacol*. 2004;48(1):81-88.
22. Sachdewa A, Raina D, Srivastava AK, Khemani LD. Effect of Aegle marmelos and Hibiscus rosa sinensis leaf extract on glucose tolerance in glucose induced hyperglycemic rats (Charles foster). *J Environ Biol.* 2011;22(1):53-57.
23. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils, J Allergy Clin Immunol. 2010;125(2 Suppl 2):S73-80.