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Research Article

Effects of Lupene-ol and Lupene-on from *Aegle marmelos* Correa on Histamine Release: *In Vitro* and *In Silico* Studies

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ABSTRACT

Aegle marmelos is a tree native to India, and also present in Southeast Asia including Indonesia. Traditionally, *A. marmelos* is used as anti-inflammatory and anti-allergy. Exploration of its active compounds have been isolated and investigated for their pharmacological activities. Previous study reported that 20(29)-lupene-3α-ol and 20(29)-lupene-3-on can be isolated from the leaves and stem barks. In the study, these lupane-type triterpenes were evaluated for their inhibitory effect on histamine release from basophilic leukemia (RBL-2H3) cell line, a tumor analog of mast cells. The release of histamine from this mast cell was immunologically and non-immunologically stimulated by DNP_{24}-BSA and thapsigargin, respectively. The histamine release was determined by using HPLC with fluorometric detector. In the study, these lupane-type triterpenes obviously exhibited inhibitory activity on histamine release from mast cell induced by DNP_{24}-BSA. However, these compounds did not alter the histamine release from mast cells induced by thapsigargin. *In silico* study was carried out to examine the interaction between lupene-ol and lupene-on on Sarcoplasmic Reticulum Ca^{2+}-ATPase. Both of the compounds blockade the sarcoplasmic reticulum Ca^{2+}-ATPase and inhibite the Ca^{2+} uptake from intracellular cytosolic. Based on the results, the inhibitory effect of lupene-ol and lupene-on might be unrelated to intracellular Ca^{2+} concentration.

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

INTRODUCTION

Inflammation is a response of tissue damage that involves immune cells, blood vessels, some mediators including mast cells. This cell acts in delayed and immediate hypersensitivity reactions (Galli, 1993; Stone *et al*., 2010; Gilfillan *et al*., 2011). Mast cells have important contribution in inflammatory and allergy processes. Mast cells can be induced by tissue injury or present of allergen such as grass pollen, product from dead house dust mites, foodstuffs. The induction can activate the production of IgE. The antibody cross-links to a high affinity receptor (FcεRI) on the surface of mast cells. Rapidly, the mast cells release the allergy mediators such as histamine, cytokines and eicosanoids. The mediators are responsible to trigger allergy and inflammatory reactions (Rang *et al*., 2003; Gilfillan *et al*., 2011).

Aegle marmelos Correa originates from India, and is widely present as naturalised species in some areas of the Southeast Asia countries such as Vietnam, Laos, Cambodia, Thailand and Indonesia. In Indonesia, *A. marmelos* is known as Maja (Java's name) or Bila (Bali's name). Whilst in its native country India, the plant is popularly known as Bael (Nugroho *et al*., 2011a; Gautam *et al*., 2014). The plant is widely used for ancient and modern traditional medicines for treatment of various disorders. *A. marmelos* have been studied for its several biological activities. Its activities include hypoglycemic (Sachdewa *et al*., 2001); antidiabetes (Sabu and Kuttan, 2004); antiallergy (Nugroho *et al*., 2011b); antiinflammatory (Benni *et al*., 2011); antihistamine (Nugroho *et al*., 2011c); antiasthma (Nugroho *et al*., 2012); antimuscarinic (Noviarsito *et al*., 2014); antimicrobial (Mujeeb *et al*., 2014); wound healing (Gautam *et al*., 2014); immunomodulator (Govinda and Asdaq, 2011); gastric ulcer protection (Das and Roy; 2012).

The pharmacological activities of a plant is contributed by its active compounds. Several compounds have been successfully isolated from *A. marmelos*. Some of them have been investigated for their pharmacological activities such as 20(29)-lupene-3α-ol and 20(29)-lupene-3-on (Fig. 1). Lupene-ol and lupene-on are pentacyclic triterpenes that can be isolated from petroleum ether extract of the stem bark of *A. marmelos* Correa (Riyanto, 2003). However, these compounds can be obtained from other plants such as *Bruguiera parviflora*, *Anadenanthera colubrina*, *Diospyros maritima*, *Gardena saxatilis*

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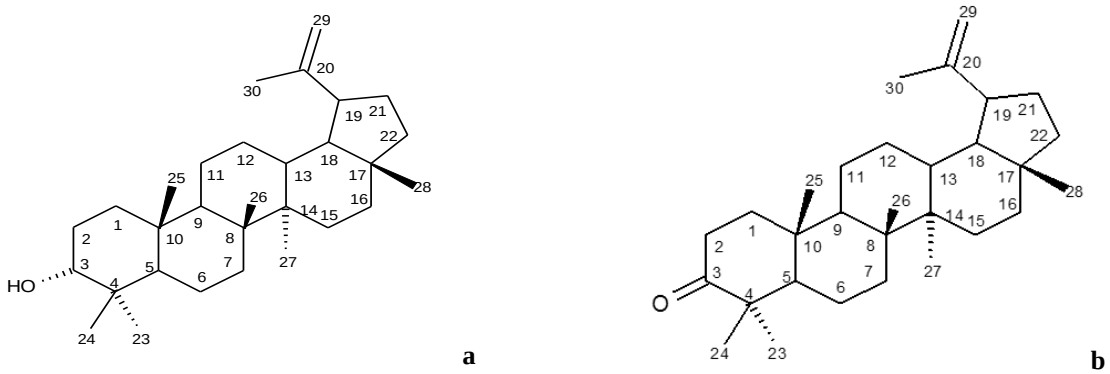

Figure 1: Chemical structure of lupene-ol **(a)** and lupene-on **(b)**

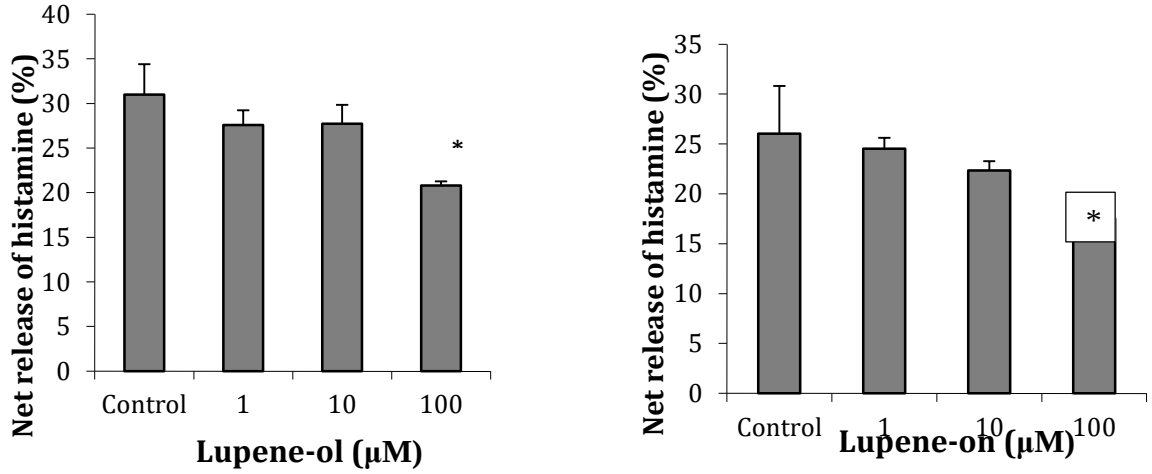

Figure 2: Effect of lupene-ol and lupene-on on the histamine release from RBL-2H3 cells in the presence of 20 ng/mL DNP-BSA. Data represent mean±SEM, and are three independent experiments. * Significant difference P<0.05 compared to the negative control value.

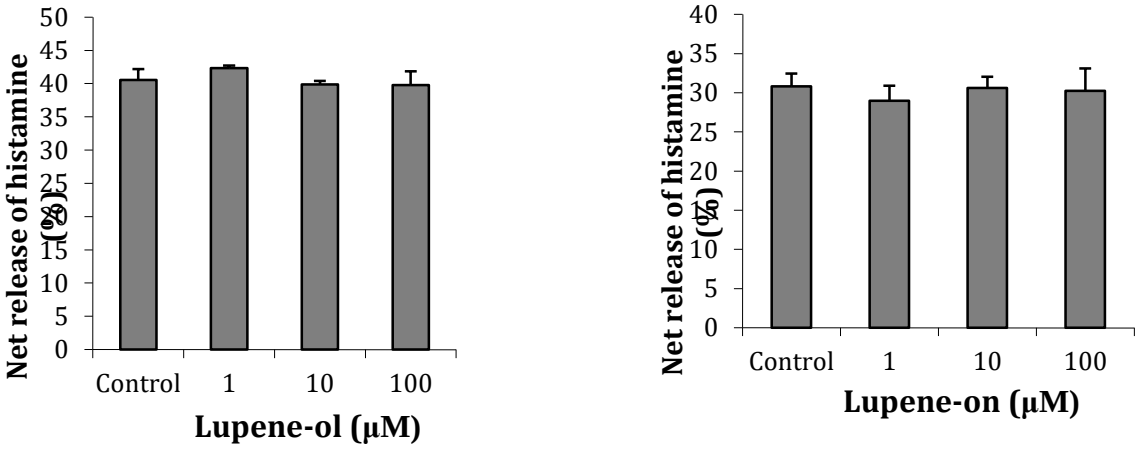

Figure 3: Effect of lupene-ol and lupene-on on the histamine release from RBL-2H3 cells in the presence of thapsigargin 0.5 µM. Data represent mean±SEM, and are three independent experiments. *Significant difference P<0.05 compared to the negative control value.

(Chumkaew *et al*., 2005; Gutierrez-Lugo *et al*., 2004, Kuo *et al*., 1997; Suksamrarn *et al*., 2003). Reportedly, these compound exhibited inhibitory effect on inflammation. Lupene-ol inhibited cytokine production (IL-4, IL-5 and IL-13) on asthmatic mice (Vasconcelos *et al*., 2008). In the study, lupene-ol and lupene-on were investigated for their inhibitory effect on histamine release from mast cells.

MATERIALS AND METHODS

Materials

These lupane-type triterpenes (lupene-ol and lupene-on) were isolated from *A. marmelos* Correa by Prof. Dr. Sugeng Riyanto (Faculty of Pharmacy, Universitas Gadjah Mada Indonesia). Fig. 1 shows the chemical structure of these compounds. Thapsigargin (Sigma, Chemical) and dinitrophenylated bovine serum albumin (DNP_{24}-BSA) were used as histamine-inducing agents. 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

MEM medium containing 15% fetal calf serum (FCS) and antibiotics (combination of penicillin and streptomycin) was used to culture the RBL-2H3 cell (rat basophilic leukimia cell line). Incubation was done in a humidified atmosphere (5% CO_2) at 37°C. 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 to assay the histamine release. The cells were then sensitized with monoclonal IgE (0.5 μg/ml) to do the DNP_{24}-BSA experiment. On the next day, the MEM was removed, and the cells were washed twice with 500 μl of PIPES buffer. Preincubation of the cells was done by 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

HPLC-fluorometry was used to do histamine release assay according to our previous researches (Nugroho *et al*., 2009; Nugroho *et al*., 2010). After previous incubation, the plates were centrifuged at 3,000 rpm for 5 min. This supernatant was then 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.

In Silico Study

In silico study was done to give more explanation about the negative effect of lupene-ol and lupene-on on histamine release induced by thapsigargin. The Sarcoplasmic Reticulum Ca^{2+}-ATPase, its X-ray structure (ID:2C8K; 2.80 Å) was downloaded from Protein Data Bank (PDB) and loaded into MOE working environment (underlicensed Faculty of Pharmacy UGM). Protein target was prepared with AMBER method with deleted its water groups and heteroatom. The structure was protonated at temperature of 310 K, pH of 7.4 and a salt concentration of 0.1. The two test compunds, Lupene-ol and Lupene-on 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.

RESULT

The mechanism of histamine release by DNP_{24}-BSA is sensitization of IgE on mast cells, while the histamine release of thapsigargin by Ca^{2+} influx and intracellular calcium pathways in mast cells. From the study, the result of histamine release from induction of RBL-2H3 cells by DNP_{24}-BSA and thapsigargin is 30.98±3.44% and 40.56±1.61% from the content total of histamine.

Lupene-ol and lupene-on showed inhibitory effect in 1-100 μM on the histamine release from RBL-2H3 cells.

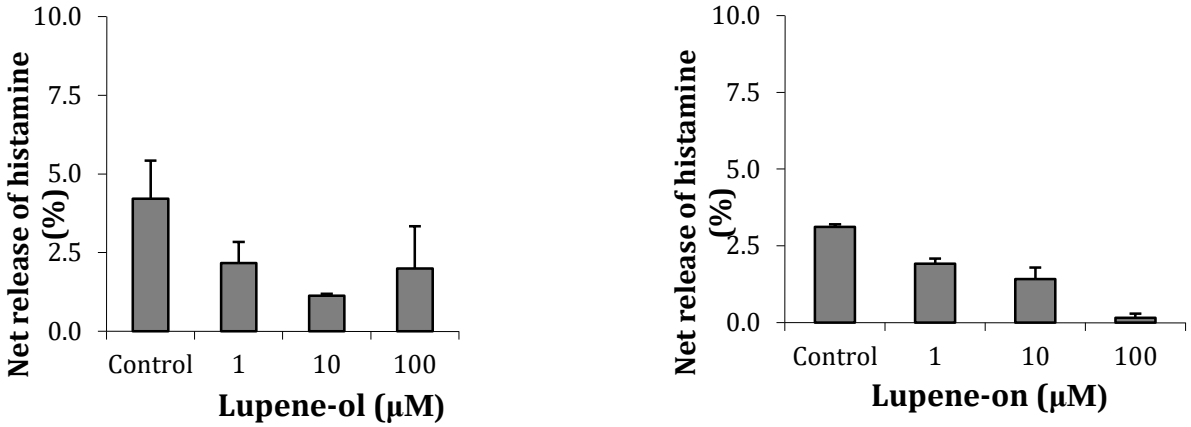

Figure 4: Effect of lupene-ol and lupene-on on the histamine release from RBL-2H3 cells in the absence of histamine inducers (spontaneous histamine release). Data represent mean±SEM, and are three independent experiments.

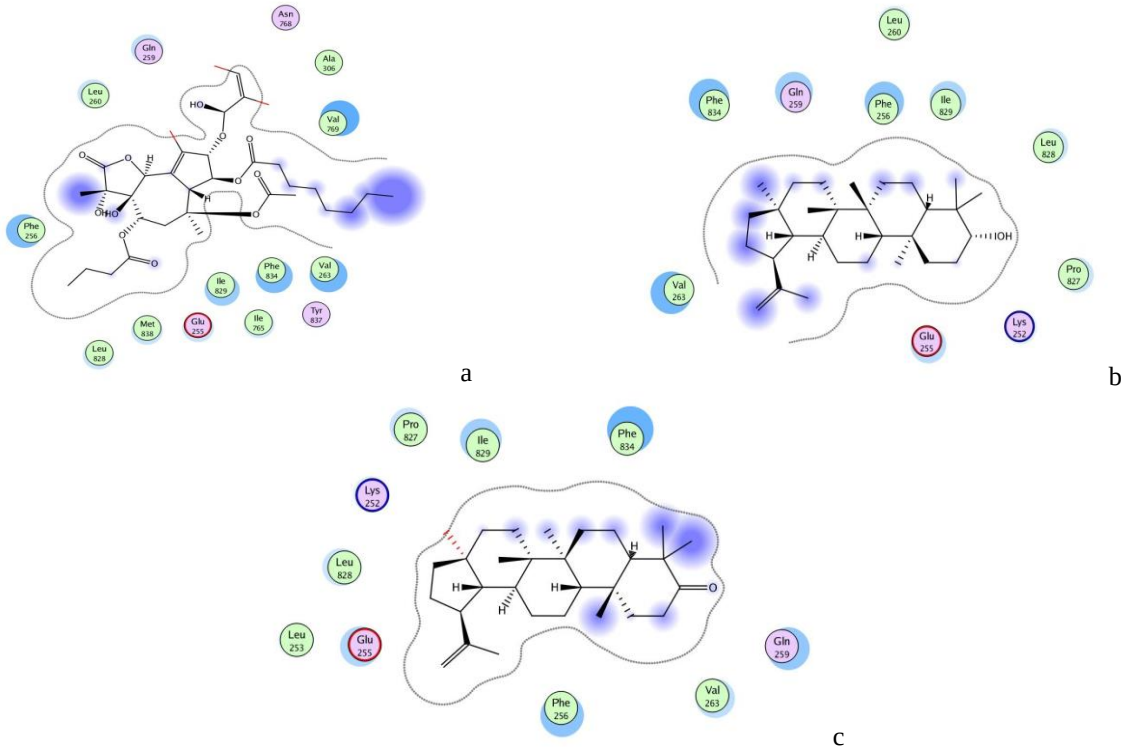

Figure 5: 2D interaction between (a) thapsigargin, (b) lupene-ol, and (c) lupene-on on acid amino residues around the pocket binding site. All of the compounds have similar interaction in binding amino acid residues.

Lupene-ol and lupene-on could decrease the histamine release from mast cells induced by DNP_{24}-BSA in a concentration-dependent manner. At the concentration of 100 µM, these compounds reduced the histamine release from RBL-2H3 cells induced by DNP_{24}-BSA by 32.82±1.43% and 32.64±4.46%, respectively (Fig 2). However, Lupene-ol and lupene-on did not influence the histamine release induced by thapsigargin. This inhibitory effect of these compounds on the histamine release was presumed to not related to Ca^{2+} signaling pathway in the mast cell (Fig 3).

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%. Lupene-ol and lupene-on at all concentrations exhibited low spontaneous histamine release (less than 10 % of the total histamine content) (Fig 4).

In order to give more explanation the inhibitory effect of lupene-ol and lupene-on histamine release not related to Ca^{2+} signaling pathway, molecular docking was caried out. Docking validation was done firstly 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, lupene-ol, and lupene-on on protein target were -8.9803; -9.5191; and-9.2311, respectively. The interaction represents an energy

interaction. Lower score shows stronger affinity for binding protein.

DISCUSSION

An unfavorable response of the immune system, allergy, can be happened by allergens, such as grass pollen, dust mite, certain foodstuffs or some drugs. The pathological process of allergic responses such as anaphylaxis, allergic rhinitis, allergic asthma, involved mast activation through the activation of IgE type attaching to the cell surface via the high-affinity FcεRI receptors. Cross linkage mechanism of certain allergen from the activation of the receptor into IgE antibody molecules can produce a series of intracellular celluler signaling including the initiation of protein tyrosine kinases and an increase of intracellular Ca^{2+} levels. These processes can raise the granule exocytosis and then put out the mediators including preformed mediators (serine proteases, histamine, serotonin, proteoglycans), lipid mediators (thromboxane, prostaglandin D2, leukotrine C4, platelet-activating factor) and cytokines/chemokines. Erythema, edema, ithcing, sneezing, cough, mucus secretion etc are the result from the present of preformed mediators and lipid mediators in immediate reaction, meanwhile cytokines/chemokines provide late phase reactions (inflammation, persistent asthma) that happen 6-14 hours after the immediate reaction (Metcalfe *et al*., 1997; Rang *et al*., 2003; Stone *et al*., 2010).

As an important mediator to produce the response of allergy, histamine only needs one minute to reduce half of its concentration in blood and has a powerful response on smooth muscle, endothelial cells, nerve endings and mucous release. The pathway of histamine metabolism in human body is through methylation process by histamine N-methyltransferase to tele-ethyl histamine and oxidation by diamine oxidase to imidazole acetaldehyde. The stimulation of histamine release also can be happened by some signaling pathways, one of the pathways is the alternation of IgE antibody with antigen that would activate 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). Interaction between IP_3 and its receptor that happened on endoplasmic reticulum (ER) would release calcium ion and increase the concentration of cytosolic calcium. These processes make an emptiness of calcium on the ER stores and activates calcium ion influx that will increase the concentration of cytosolic calcium. 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 (Metcalfe *et al.*, 1997; Beaven *et al.*, 1987; Stone *et al*., 2010; Gilfillan *et al*., 2011).

Lupene-ol and lupene-on, lupane-type triterpenes are active compounds can be isolated from *Aegle marmelos* Correa. These lipane triterpenes provided from petroleum ether extraction of plant's stem bark and separated using vacuum column chromatography over silica gel (Riyanto, 2003). In the study, lupene-ol and lupene-on 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 (Metcalfe *et al.* 1997; Liu *et al.*, 1980; Stone *et al*., 2010; Gilfillan *et al*., 2011)

Based on the results, the lupane tripterpenes did not influence this histamine release. 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 (Rang *et al*., 2003; Metcalfe *et al.*, 1997; Stone *et al*., 2010). In this case, the effect of lupene triterpenes might be not related to this pathway.

In silico study with molecular docking was done to give more explanation about lupene-ol and lupene-on did not influence histamine release induced by thapsigargin. Its presumed that both of compounds did not decrease intracellular Ca^{2+}. Docking results showed both of compounds had lower score and indicated its affinity stronger than thapsigargin which give possibility to blockades this transporter. Both of compounds 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 5), it can be seen that there were some amino acid residues which bind the molecule. However, lupene-ol and lupene-on inhibitory effect on histamine release induced by DNP_{24}-BSA might through another signaling pathway. Further investigation should be conducted to give another action mechanism lupene-ol and lupene-on in decreasing histamine release in mast cells.

CONCLUSION

Lupene-ol and lupene-on (lupene triterpenes) isolated from *Aegle marmelos* Correa inhihited the histamine release from mast. The inhibitory effect of lupene-ol and lupene-on might be unrelated to intracellular calcium signaling.

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