

ANTI-ALLERGY POTENTIAL OF *AVERRHOA BILIMBI* LINN. FRUIT WATER EXTRACT SHOWN BY ITS SUPPRESSIVE EFFECT ON THE DEGRANULATION OF RBL-2H3 CELLS

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ABSTRACT

Allergy rhinitis (AR), as reported by the World Allergy Organization (WAO), is one of the highest prevalence allergies affecting 10-30% of all adults and up to 40% of children. In Indonesia, current evidence showed that the prevalence of AR is increasing. *Averrhoa bilimbi* Linn. fruit (AF), or locally known as *belimbing wuluh* has potentials to treat many diseases due to the abundant of polyphenol content, including to the treatment of allergies. Therefore, this study was aimed to investigate the anti-allergy potential of AF *in vitro*. The anti-allergy effect of *Averrhoa bilimbi* Linn. fruit water extract (AFWE) was examined using RBL-2H3 cells. At first, the cytotoxicity effect of AFWE was determined by WST-8 assay. The release of β -hexosaminidase by RBL-2H3 cells was also measured to evaluate degranulation suppression activity of AFWE. Lastly, calcium assay was employed to investigate the intracellular calcium concentration ($[Ca^{2+}]_i$). Results demonstrated that AFWE does not show any cytotoxicity at any given concentration. In addition, AFWE at 1.25 mg/mL showed sufficient inhibitory effect towards degranulation by RBL-2H3 cells. Moreover, the degranulation-suppressing activity of AFWE was resulted from the inhibition of calcium-dependent signaling pathways. Unfortunately, the properties of active substances from AFWE have not been investigated. To conclude, this study indicated that AFWE has potential as an alternative treatment for allergic diseases.

Keywords: Allergic rhinitis, anti-allergy, *averrhoa bilimbi*, beta-hexosaminidase, RBL-2H3 cells

ABSTRAK

Rinitis alergi (AR), sebagaimana dilaporkan oleh *World Allergy Organization* (WAO), merupakan salah satu alergi dengan prevalensi tertinggi yang mempengaruhi 10-30% orang dewasa dan 40% anak-anak. Di Indonesia, bukti terkini menunjukkan terjadinya peningkatan prevalensi AR. Buah *Averrhoa bilimbi* Linn. (AF), atau yang dikenal sebagai belimbing wuluh, berpotensi untuk mencegah atau mereduksi risiko beberapa penyakit, termasuk pada penyakit alergi, karena melimpahnya kandungan polifenol. Oleh sebab itu, studi ini bertujuan untuk menginvestigasi potensi anti-alergi AF secara *in vitro*. Pengujian efek anti-alergi dari ekstrak air buah *Averrhoa bilimbi* Linn. (AFWE) dilakukan pada sel RBL-2H3. Efek sitotoksitas dari AFWE diuji menggunakan metode WST-8. Lalu, pelepasan β -hexosaminidase dari sel RBL-2H3 diukur untuk mengevaluasi aktivitas penekanan degranulasi dari AFWE. Terakhir, uji kalsium dilakukan untuk menginvestigasi konsentrasi kalsium intraselular ($[Ca^{2+}]_i$). Hasil studi menunjukkan bahwa AFWE tidak memiliki efek sitotoksik di setiap konsentrasi yang diujikan. Selain itu, AFWE pada konsentrasi 1,25 mg/mL menunjukkan efek penghambatan yang cukup terhadap degranulasi sel RBL-2H3. Aktivitas penekanan degranulasi oleh AFWE dihasilkan dari adanya penghambatan pada *calcium-dependent signaling pathway*. Akan tetapi, properti kandungan aktif dari AFWE belum dapat terinvestigasi. Sebagai kesimpulan, studi ini mengindikasikan bahwa AFWE memiliki potensi sebagai sumber pangan alternatif untuk memperbaiki kondisi pasien dengan penyakit alergi.

Kata kunci: Anti-alergi, *averrhoa bilimbi*, beta-hexosaminidase, rinitis alergi, sel RBL-2H3

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INTRODUCTION

Allergic rhinitis (AR) is defined as an inflammation of the membrane lining in the nasal cavity which is indicated by one or more symptoms such as sneezing, nasal itching, nasal congestion, and nasal discharge (Bousquet *et al.*, 2008). As reported by World Allergy Organization (WAO), AR affected 10-30% of the total population and up to 40% of children worldwide (Pawankar *et al.*, 2011). In Indonesia, the latest national report by International Study of Asthma and Allergies in Childhood (ISAAC) found that the prevalence of AR was less than 5% (Mallol *et al.*, 2013). However, current evidence showed that the prevalence is increasing, particularly in the big cities, including Bandung and Surabaya to 38% and 23%, respectively (Fauzi *et al.*, 2015; Soegiarto *et al.*, 2019).

AR is stimulated by the cross-link of antigen to immunoglobulin E (IgE) bound on high-affinity IgE receptors, known as FcεRI (Ishida *et al.*, 2013). This action stimulates the calcium dependent signaling pathway (Lyn-Syk-LAT-PLCγ) in FcεRI receptor expressing-cells, such as mast cells and basophils, which leads to Ca²⁺ liberation from endoplasmic reticulum (ER) (Sun *et al.*, 2014). As the results, the cells are degranulated and chemical mediators are released such as β-hexosaminidase, histamine, and inflammatory cytokines, inducing acute allergic responses (Metcalf *et al.*, 2009).

Averrhoa bilimbi Linn. is a Southeast Asian endemic plant species, which is underutilized. It is used as one of food ingredients in Indonesian traditional dishes, such as *garang asam*, *sayur asem*, and *asem-asem*. *Averrhoa bilimbi* Linn. fruit (AF) is also used as ethnomedicine for skin care (Ahmed and Alhassan, 2016), to treat syphilis (Samuel *et al.*, 2010), whooping cough, obesity, hypertension, and diabetes (Ahmed and Alhassan, 2016). Besides, scientific studies revealed the capability of AF as antihypertensive (Lestari *et al.*, 2018), antihyperlipidemic (John and Pta, 2019), antidiabetic (Kurup and Mini, 2014), antimicrobial (Mokhtar and Aziz, 2016), anti-inflammatory (Suluvoy *et al.*, 2017), and anticancer (Nair *et al.*, 2016). However, assessment of AF for its anti-allergic potentials has not been done to date.

In phytochemical studies conducted by Hasanuzzaman *et al.* (2013) and Yan *et al.* (2013), AF was reported to be a promising source of polyphenols, which include phenolic acids, flavonoids, and tannins. Hasanuzzaman and colleagues also found that the polyphenols are present in aqueous extract of AF. Polyphenols, particularly flavonoids, have been strongly associated with the alleviation and prevention of IgE-mediated allergic diseases. Polyphenol-induced alleviation of allergic reactions is done through the reduction of expression of MHC-II on dendritic cells, causing the reduction the antigen presentation from dendritic cells to T_H2 cells. This action leads to the suppression of inflammatory cytokines (IL-4 and IL-13) released by T_H2 cells, resulting in the reduction of B cells recruitment and the reduction of antigen specific IgE production (Singh *et al.*, 2011; Tanaka and Takahashi, 2013).

To analyze anti-allergy potential of AF water extract (AFWE), Rat Basophilic Leukemia (RBL)-2H3 cells were used in this *in vitro* study. RBL-2H3 cells mimic the properties of mast cells and express high levels of FcεRI on the surface of the cells when activated by IgE-allergen complex (Fu *et al.*, 2019).

MATERIALS AND METHOD

This study was performed at Animal Cell Technology Laboratory, Ehime University, Japan and at Indonesia International Institute for Life Sciences (i3L), Jakarta, Indonesia. The cytotoxicity effect, suppression of β-Hexosaminidase release, and suppression of intracellular Ca²⁺ concentration ([Ca²⁺]_i) were investigated. In this study, unnoted materials were purchased from either Nacalai Tesque (Kyoto, Japan) or Fujifilm Wako Pure Chemical (Osaka, Japan).

Sample preparation

Averrhoa bilimbi Linn. fruits were from Tangerang, Indonesia, and randomly sampled. The fruits were washed and freeze-dried at Pilot Plant, i3L. The freeze-dried fruits (the whole fruit, including flesh, peel and seeds) were made into powder and soaked in distilled water (DW) at concentration of 2 g/mL overnight at room

temperature. The extract was centrifuged twice at 4,000 rpm for 3 min and 15,000 rpm for 20 min, sequentially. pH of the supernatant was adjusted to 7.4 using 0.1 M of NaOH and the supernatant was filtered. The solution was freeze-dried again to obtain the water-soluble substances in the extract. The dried form of the water-soluble extract was rehydrated with DW at 20 mg/mL. The extract solution was sterilized using a 0.22 μm filter syringe in sterile condition and stored at -35°C until further used. Prior to use, the extract was further diluted into 10.00, 5.00, 2.50, and 1.25 mg/mL. The processed extract was then brought to Japan for further analysis.

RBL-2H3 cells seeding and sample treatment

RBL-2H3 cells seeding was performed according to Ishida *et al.* (2013) with minor modification. Cells were seeded into 96-well plate in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 100 $\mu\text{g}/\text{mL}$ of streptomycin, 100 U/mL of penicillin, and 5% of fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA) at 4×10^5 cells/well. The cells were sensitized with DNP-IgE (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 37°C in a humidified 5% of CO_2 incubator. The cells were washed using modified Tyrode's buffer (1.8 mM CaCl_2 , 5.6 mM glucose, 20 mM HEPES, 5mM KCl, 1 mM MgCl_2 , 135 mM NaCl, and 0.05% BSA, pH 7.4) twice and treated with 120 μL of AFWE at different concentration for 10 min. Cells degranulation was induced by adding 10 μL of DNP-HSA diluted in modified Tyrode's Buffer, and the cells were incubated for 30 min.

Cytotoxicity assay

Cytotoxicity of AFWE on RBL-2H3 cells were measured using WST-8 assay kit (Kishida Chemical, Osaka, Japan) according to the manufacturer's instruction. In brief, after RBL-2H3 cells were seeded in a 96 well plate, they were treated with AFWE and were degranulated (as induced by DNP-HSA). The cells were then washed once with 200 μL of phosphate buffered saline (PBS). Then, 110 μL of 5% FBS-DMEM containing 5% of WST-8 solution was added to each well. After that, the cells were incubated for

25 min at 37°C . Cells viability was measured through absorbance measurement using microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm.

β -Hexosaminidase assay

β -Hexosaminidase assay was performed according to Ishida *et al.* (2013) with minor modification. In brief, after 30-min of incubation following the degranulation of the cells, the degranulation process was terminated by incubating the cells on ice for 10 min. Supernatant was collected, and the cells were lysed by using a hand sonicator in a 0.1% Triton X-100 Tyrode's buffer for 5 s. The cell lysate and supernatant were transferred into a new 96-well plate, and 100 μL of 0.1 mM citrate buffer (pH 4.5) containing 3.3 mM p-nitrophenyl-2-acetoamid-2-deoxy- β -d-glucopyranoside (Wako Pure Chemical Industries) was added to each well. The microplate was incubated at 37°C for 25 min, and the reaction was terminated by adding 100 μL of 2 M glycine buffer (pH 10.4). β -Hexosaminidase release was measured using microplate reader (Model 550; Bio-Rad Laboratories) at 405 nm and the rate of release was calculated according to the formula: β -Hexosaminidase Release (%) = $\frac{\{A_{\text{supernatant}} - A_{\text{blank of supernatant}}\}}{\{(A_{\text{cell lysate}} - A_{\text{blank of cell lysate}}) + (A_{\text{supernatant}} - A_{\text{blank of supernatant}})\}}$.

Calcium assay

Intracellular Ca^{2+} concentration was measured using Calcium Kit-Fluo 3 AM (Dojindo Laboratories, Kumamoto, Japan). Following RBL-2H3 cells sensitization with DNP-IgE, the cells were washed twice with PBS and were labeled using 100 μL of Fluo-3 AM fluorescence dye. The cells were incubated for 1 h. Labeled cells were rinsed with PBS and treated with AFWE at different concentrations. Then, the cells were degranulated by adding DNP-HSA and the $[\text{Ca}^{2+}]_i$ was measured by using fluorescence microplate reader (SH-8000 Lab; Corona Electric, Ibaraki, Japan) at $\lambda_{\text{ex}} = 480 \text{ nm}$, $\lambda_{\text{em}} = 530 \text{ nm}$ for 2 min, in which fluorescent intensity was monitored every 10 s.

Statistical analysis

All experiments in this study were conducted in two batches with triplication in each batch. The data were presented as mean \pm standard error mean (SEM). One-way analysis of variance (ANOVA) and Tukey's test were conducted to obtain the statistical significance between AFWE and control.

RESULTS

In this study, the effect of AFWE on viability of RBL-2H3 cells was first to be examined by treating the cells with different concentrations AFWE ranging from 1.25 to 10.00 mg/mL. As shown in **Figure 1**, AFWE did not show any cytotoxicity at any given concentration on RBL-2H3 cells. These results indicated that all concentrations of AFWE can be further analyzed for its anti-allergy effect.

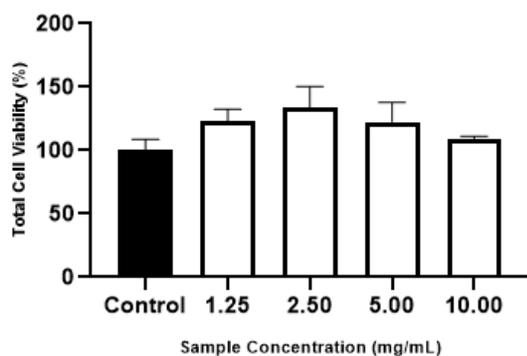


Figure 1. RBL-2H3 cells viability. Results were presented as the mean \pm SEM (n = 6). Data were collected in two batches (independent measurement) with three replications in each batch.

The anti-allergy effect of AFWE was examined through β -Hexosaminidase assay to check its capability to suppress the release of β -Hexosaminidase by the degranulated cells (**Figure 2**). The suppression effect of AFWE towards β -Hexosaminidase release was reported as the ratio of β -Hexosaminidase that was released-to-supernatant to the total of β -Hexosaminidase presence in both supernatant and lysed cells. The results showed that AFWE significantly suppresses β -Hexosaminidase release in a dose dependent

manner, suggesting that AFWE has a degranulation-suppressing activity. Moreover, the results demonstrated that 1.25 mg/mL of AFWE was sufficient to inhibit β -Hexosaminidase release from RBL-2H3 cells. However, 2.50 mg/mL of AFWE showed more potent inhibition.

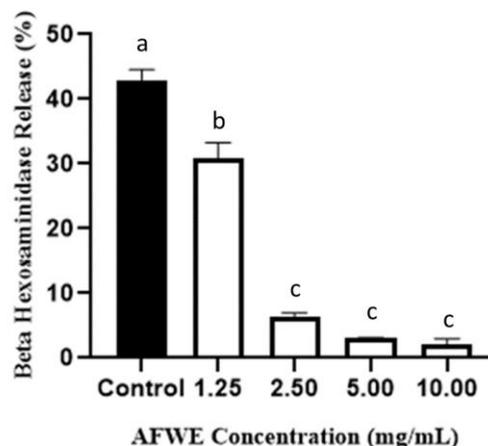


Figure 2. β -Hexosaminidase release. Results were presented as the mean \pm SEM (n = 6). Data were collected in two batches (independent measurement) with three replications in each batch. Statistically significant differences between samples are represented by different alphabets ($p < 0.05$).

In order to investigate the effect of AFWE to calcium dependent signaling pathway, $[Ca^{2+}]_i$ was examined. As shown in **Figure 3**, $[Ca^{2+}]_i$ is rapidly elevated in non-treated RBL-2H3 cells, while the elevation of $[Ca^{2+}]_i$ was suppressed by the addition of AFWE. These results suggested that the degranulation-suppressing activity of AFWE was resulted from the inhibition of calcium dependent signaling pathways.

DISCUSSION

Averrhoa bilimbi Linn. is an underutilized natural source that is commonly found in Indonesia and other tropical countries, such as Malaysia and Philippines. Its leaves and fruits are used as food ingredients and ethnomedicine (Ahmed and Alhassan, 2016), and recently, scientific studies

showed that *Averrhoa bilimbi* Linn. fruit (AF) has high potentials to be a functional food, because it was reported to exert several beneficial health effects, such as antihypertensive (Lestari *et al.*, 2018), antihyperlipidemic (John and Pta, 2019), antidiabetic (Kurup and Mini, 2014), antimicrobial (Mokhtar and Aziz, 2016), anti-inflammatory (Suluvoy *et al.*, 2017), and anticancer (Nair *et al.*, 2016). Phytochemical studies also revealed the abundance content of polyphenols in AF, especially flavonoids, tannins, and phenolic compounds. Previously, numerous studies revealed the association of polyphenols with anti-allergy (Juríková *et al.*, 2015; Magarone and Jirillo, 2012; Tanaka *et al.*, 2019). Therefore, the potential benefits of AFWE as an anti-allergy is important to be investigated.

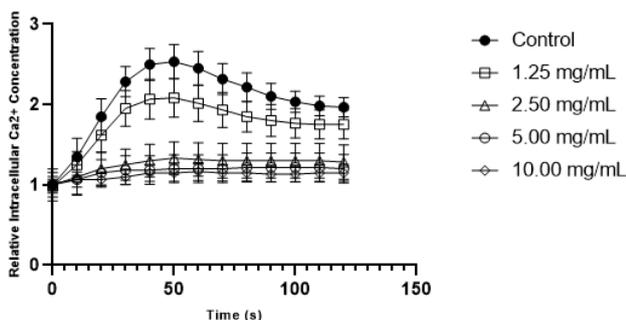


Figure 3. $[Ca^{2+}]_i$ level in RBL-2H3 cells following the treatment by control and AFWE. Results were presented as the mean \pm SEM (n = 6). Data were collected in two batches (independent measurement) with three replications in each batch

In this study, the cytotoxic effect of AFWE was first examined and results showed that AFWE does not possess cytotoxic effect toward RBL-2H3 cells. After the safety of AFWE towards RBL-2H3 cells was confirmed, the potential of AFWE to inhibit degranulation of RBL-2H3 cells was examined. The results demonstrated that AFWE successfully suppresses degranulation on RBL-2H3 cells in a dose dependent manner, denoted by the suppression of β -Hexosaminidase release (**Figure 2**). Moreover, as shown in **Figure 3**, the suppression of β -Hexosaminidase release by AFWE was caused by the suppression of

intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). These results suggested that AFWE has a good potential as an anti-allergy.

The anti-allergy effect of AFWE might be because of the presence of polyphenols, particularly flavonoids. Previous studies revealed the mode of action of polyphenols as anti-allergy is done through the reduction of expression of MHC-II on dendritic cells (Singh *et al.*, 2011). This action leads to the reduction of antigen presented from dendritic cells to T_H2 cells, causing suppression of inflammatory cytokines released by T_H2 cells. This results in the suppression of IgE production from B cells (Tanaka and Takahashi, 2013). In study by Yoo *et al.* (2014), aged black garlic extract fraction rich in polyphenols and flavonoids were reported to have potent inhibitory effect towards β -Hexosaminidase release. In addition, polyphenols from apple such as apple condensed tannins was also reported to have strong inhibitory effect toward β -Hexosaminidase release from RBL-2H3 cells (Kanda *et al.*, 1998). The polyphenols content in AF have not been fully identified yet. However, study by Muhamad *et al.* (2015) mentioned the presence of catechin in AF. Previously, it has been reported that the consumption of tea containing catechin has successfully alleviated the symptoms of mouse with Japanese cedar pollinosis (Maeda-Yamamoto *et al.*, 2007). The consumption of catechin and its derivatives was previously reported to have significant effects on inhibiting IgE-mediated allergy through the prevention of tyrosine phosphorylation (Maeda-Yamamoto *et al.*, 2004) and the production of IL-4 and IL-13 released by T_H2 cells (Singh *et al.*, 2011). Besides that, ethyl acetate fraction of AF also reported to contain quercetin (Kurup and Mini, 2017). Previously, quercetin has been elaborated for its potential to inhibit histamine release from $Fc\epsilon RI$ receptor expressing-cells (Scheller *et al.*, 2011), and to inhibit the activation of MHC-II in APCs (Gong and Chen, 2003). A study by Kempuraj *et al.* (2005) showed that quercetin and kaempferol suppress $[Ca^{2+}]_i$ in the mast cells. However, the presence of kaempferol and other flavonoids such as chrysin and apigenin in AF have not been identified yet, especially in aqueous extract.

Unfortunately, the molecular mechanism on how AFWE suppresses degranulation of RBL-2H3 cells is still unknown. Referring to the previous study, Lyn and Syk are frequently utilized as protein biomarkers in immunoblotting analysis as both of these proteins are responsible for $[Ca^{2+}]_i$ liberation, the activation of mitogen-activated protein kinases (MAPKs), and also the degranulation of the mast cells (Yoo *et al.*, 2014). Since the Lyn and Syk pathway induce the activation of MAPKs pathway, then the utilization of MAPKs, including p38 mitogen activated protein kinase, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated protein kinase (ERK), can also be alternative biomarkers (Lian *et al.*, 2015). In a study by Ishida *et al.* in 2013, PLC γ was also one of the biomarkers in the immunoblotting test. PLC γ has an important role in Ca^{2+} signaling (Putney and Tomita, 2012). In addition, Fyn and Gab2 are also candidates for protein biomarkers. Besides its responsibility for accumulation of PI3K, study by Nishida *et al.* (2005) showed that Fyn and Gab2 are also responsible for the formation of microtubules in mast cells. In this case, the microtubule leads to the translocation of granules to the plasma membrane and results in the degranulation of the cells. Therefore, further investigations are required to clarify the mechanism underlying the inhibitory activity of AFWE on degranulation of RBL-2H3 cells.

CONCLUSION

AFWE successfully demonstrated its anti-allergy potential through *in vitro* experiment using RBL-2H3 cells. Results showed that AFWE inhibit the release of β -Hexosaminidase from RBL-2H3 cells through the suppression of intracellular Ca^{2+} ion $[Ca^{2+}]_i$ concentration. These findings indicate the potential of *Averrhoa bilimbi* fruit as an alternative for patients with allergic rhinitis and/or other IgE-mediated allergies. Further investigation is needed to identify the active substance in AFWE and to elucidate its mode of actions.

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