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Research Paper

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Inhibitory Activity of *Aegle marmelos* L. (Bael) Leaf Ethanolic Extract Against α-Glucosidase

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Abstract

Background: Diabetes Mellitus (DM) is a significant global health problem characterized by hyperglycemia. A key therapeutic strategy for managing postprandial hyperglycemia is the inhibition of the α-glucosidase enzyme, which slows glucose absorption. **Objectives:** This study was designed to evaluate the *in vitro* α-glucosidase inhibitory activity of an ethanolic extract derived from Bael (*Aegle marmelos* L.) leaves. **Methods:** Bael leaves were dried, processed into simplicia, and subsequently extracted via maceration using 96% ethanol to yield an ethanolic extract. The α-glucosidase inhibitory capacity of the extract was assessed spectrophotometrically using an ELISA reader. The assay quantified the absorbance of *p*-nitrophenol, the product of the enzymatic reaction with the *p*-nitrophenyl-α-D-glucopyranoside (PNPG) substrate, at a wavelength of 405 nm. Acarbose was utilized as the positive control. **Key findings:** Acarbose, at concentrations of 0.2, 0.4, 0.6, 0.8, and 1 ppm, exhibited potent, concentration-dependent inhibition of 39.07%, 44.90%, 51.24%, 56.42%, and 62.81%, respectively. In comparison, the *A. marmelos* leaf extract, at concentrations of 300, 400, 500, 600, and 700 ppm, yielded inhibition values of 11.67%, 20.70%, 29.73%, 44.65%, and 53.37%. The calculated IC_{50} value for acarbose was 0.569 µg/mL, whereas the IC_{50} for the *A. marmelos* extract was determined to be 667.17 µg/mL **Conclusions:** These findings indicate that while acarbose possesses exceptionally potent α-glucosidase inhibitory activity, the ethanolic extract of *A. marmelos* leaves exhibits very weak inhibitory capacity under the tested conditions.

Keywords: Maja leaves, Extract, Alpha-Glucosidase enzyme, IC50

Introduction

Diabetes mellitus (DM) is a group of chronic diseases characterized by elevated blood glucose levels (hyperglycemia) as well as disturbances in carbohydrate, protein, and fat metabolism. This condition occurs because the pancreatic beta cells are unable to produce an adequate amount of insulin, or because the body cannot effectively utilize insulin, leading to increased blood sugar levels [1].

Diabetes mellitus is one of the most urgent global health problems and has received great attention in recent decades. This disease ranks seventh as a cause of death in the United States and worldwide. The mortality rate due to diabetes reaches 82.4 per 100,000 population, with a total of about 5.2 million deaths globally [2]. Based on data from the International Diabetes Federation, in 2021, 537 million people in the world were living with diabetes. This number is predicted to increase to 643 million by 2030, and reach 783 million adults aged 20–79 years by 2045. This increase is influenced by factors such as population growth, the aging process, and the rising rates of obesity [3].

One therapeutic strategy that can be applied in the treatment of Diabetes Mellitus is by inhibiting the action of enzymes that play a role in the glucose absorption process in the body, such as the alpha-glucosidase enzyme. This enzyme functions to accelerate glucose absorption in the small intestine by catalyzing the breakdown of oligosaccharides into monosaccharides through hydrolysis, thereby increasing blood glucose levels after eating. Therefore, the use of alpha-glucosidase enzyme inhibitors is necessary to slow or delay glucose absorption in the intestine, thus preventing an increase in postprandial blood glucose levels [4].

Alpha-glucosidase inhibitors (AGIs) are one of the antidiabetic agents used in the management of diabetes mellitus that work by inhibiting the action of the alpha-glucosidase enzyme on the wall of the small intestine. Several alpha-glucosidase inhibitors used clinically, such as acarbose, voglibose, and miglitol, have been widely use [5]. However, there are disadvantages in the use of synthetic drugs for managing diabetes mellitus, which often cause side effects on the digestive system such as abdominal pain, diarrhea, and bloating [6]. The use of natural materials can be an alternative therapy to manage postprandial hyperglycemia in people with diabetes mellitus, with advantages such as low side effects and more affordable prices compared to synthetic diabetes drugs sold on the market [1].

One of the plants utilized in traditional Indonesian medicine is the bael leaf (*Aegle marmelos* L.), which has also long been used as a herbal remedy to treat diabetes mellitus in the traditional Indian medicine system. Bael leaves contain various active compounds such as alkaloids, flavonoids, terpenoids, saponins, tannins, and steroids that are thought to have antidiabetic activity. Bael leaf extract has been shown to have a significant effect in preventing the increase in blood glucose levels in alloxan-induced

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hyperglycemic rats at a dose of 200 mg/kgBW. Research showed that administration of bael leaf extract at doses of 600 mg/kgBW and 1200 mg/kgBW could reduce blood glucose levels in alloxan-induced diabetic mice [7]. Based on these results, the researcher is interested in studying the potential of bael leaf extract (*Aegle marmelos* L.) as an antidiabetic agent through the inhibitory activity of the alpha-glucosidase enzyme.

Materials and Methods

Materials and Reagents

All chemicals and reagents were of analytical grade. Acarbose (positive control), α-glucosidase (from *Saccharomyces cerevisiae*, EC 3.2.1.20), and *p*-nitrophenyl-α-D-glucopyranoside (PNPG) were procured for the assay. Solvents and buffers included 70% (v/v) ethanol, phosphate buffer solution (pH 7.0), sodium carbonate (Na_2CO_3), and deionized water (Aquadest). Key laboratory equipment included an analytical balance, glassware (volumetric flasks, graduated cylinders), a mortar and pestle, a rotary evaporator, a Laminar Air Flow (LAF) cabinet, an incubator, calibrated micropipettes, Eppendorf tubes, and a 96-well microplate reader (Spectrostar Nano, BMG Labtech).

Plant Collection and Extract Preparation

Fresh leaves of *Aegle marmelos* L. (Bael) were collected from the Pangkajene and Islands Regency, Indonesia. The plant material was taxonomically authenticated. In accordance with the protocol, leaves were collected between 6:00 and 8:00 AM, selecting the fifth leaf from the shoot apex to ensure chemical stability, and were picked directly by hand. The leaves were subjected to wet sorting, washed thoroughly with running water to remove contaminants, and subsequently air-dried in the shade until constant weight was achieved.

The dried leaves were mechanically pulverized into a fine powder (simplicia). An aliquot of 300 g of the simplicia was extracted by maceration with 70% ethanol at ambient temperature for 3 x 24 hours, with intermittent agitation. The resulting filtrate was pooled and filtered. The solvent was removed *in vacuo* using a rotary evaporator at a controlled temperature of 50°C to yield a viscous, crude ethanolic extract. The final extract was weighed, and the percentage yield was calculated using the formula: *Yield (%) = (Weight of crude extract (g) / Initial weight of simplicia (g)) x 100%*

Preparation of Assay Solutions

All assay solutions were prepared according to the methods described by Maryam et al. (2020), with minor modifications, using phosphate buffer (pH 7.0) as the diluent unless otherwise noted [8]. A stock solution (1000 µg/mL) of the *A. marmelos* extract was prepared by dissolving 10 mg of the crude extract in 10 mL of buffer, which was then serially diluted to obtain working concentrations of 700, 600, 500, 400, and 300 µg/mL. For the positive control, a stock solution (100 µg/mL) of acarbose was prepared by dissolving 10 mg in 100 mL of buffer and subsequently diluted to working concentrations of 1.0, 0.8, 0.6, 0.4, and 0.2 µg/mL. The substrate solution (5 mM PNPG) was prepared by dissolving 15 mg of PNPG in 10 mL of deionized water. The enzyme solution was prepared from a stock (1 mg in 100 mL buffer, 28 U/mL) and diluted with cold buffer to a final working concentration of 0.25 U/mL. Finally, a 200 mM Na_2CO_3 stop solution was prepared in CO_2-free deionized water.

In Vitro α-Glucosidase Inhibition Assay

The inhibitory assay was performed in a 96-well microplate according to the modified standard procedure described by Maryam et al. (2020) [8]. For the test wells (S1), 30 µL of the sample extract (or acarbose solution) was added to 36 µL of phosphate buffer (pH 7.0). Subsequently, 17 µL of the α-glucosidase solution (0.25 U/mL) was added, and the mixture was pre-incubated at 37°C for 5 minutes. Following pre-incubation, the enzymatic reaction was initiated by the addition of 17 µL of the 5 mM PNPG substrate. The plate was incubated for a further 15 minutes at 37°C, and the reaction was terminated by adding 100 µL of the 200 mM Na_2CO_3 stop solution. The absorbance of the liberated *p*-nitrophenol, corresponding to enzyme activity, was quantified spectrophotometrically at 405 nm using the microplate reader. Concurrently, a series of control wells were prepared and processed alongside the test wells: a blank (B1) representing 100% enzyme activity (containing 66 µL buffer, 17 µL enzyme, and 17 µL substrate); a sample blank (S0) to correct for intrinsic sample color (containing 30 µL sample, 36 µL buffer, and 17 µL substrate, with the enzyme replaced by buffer); and a buffer blank (B0) (containing 83 µL buffer and 17 µL substrate, with both enzyme and sample replaced by buffer). All wells, including controls, received 100 µL of the stop solution before the final absorbance reading.

Data Analysis

All experiments were conducted in triplicate. The percentage of α-glucosidase inhibition was calculated by correcting for both sample and buffer absorbance using the formula: *Inhibition (%) = [(A_B1 - A_B0) - (A_S1 - A_S0)] / (A_B1 - A_B0) x 100%*

Where *A_B0* is the absorbance of the sample, *A_B1* is the sample blank, *A_S0* is the blank (control), and *A_S1* is the buffer blank. The IC_{50} value (the concentration of inhibitor required to achieve 50% enzyme inhibition) was determined by plotting the percentage of inhibition versus the log of the inhibitor concentration. A linear regression equation (y = ax + b) was derived, and the IC_{50} was calculated by solving for x when y = 50 [9].

Results and Discussion

The ethanolic maceration of 300 g of dried *Aegle marmelos* L. simplicia yielded 14.7587 g of a thick, dark brownish-black extract (Table 1), corresponding to a final extraction yield of 4.919% (Table 2). The *in vitro* α-glucosidase inhibitory activity was subsequently determined, with the raw absorbance data for the acarbose standard and *A. marmelos* extract detailed in Table 3 and Table 4, respectively. From these measurements, the percent inhibition was calculated, revealing a clear dose-dependent relationship for both substances. The acarbose positive

control exhibited potent inhibition, increasing from 39.0665% at 0.2 µg/mL to 62.8108% at 1.0 µg/mL (Table 5). Similarly, the *A. marmelos* extract showed progressively increasing inhibition with concentration, ranging from 11.6692% at 300 µg/mL to 53.3739% at 700 µg/mL (Table 6). Finally, the half-maximal inhibitory concentrations (IC_{50}) were calculated to quantify potency, as summarized in Table 7. A significant difference in activity was observed: acarbose demonstrated exceptionally strong inhibition (IC_{50} = 0.569 µg/mL), while the *A. marmelos* extract exhibited considerably weaker activity (IC_{50} = 667.169 µg/mL).

This study utilized bael leaf (*Aegle marmelos* L.) as the sample. The objective of this research was to investigate the potential of bael leaf as an antihyperglycemic agent by evaluating its inhibitory mechanism against the *α*-glucosidase enzyme and to determine its IC_{50} value.The harvested bael leaves were processed into a dried simplicia to reduce or eliminate water content. This drying process is essential as moisture can serve as a medium for fungal and bacterial growth, thereby preventing sample degradation due to microbial contamination.

In this study, Bael leaves were extracted using the maceration method. This technique was selected because Bael leaves are known to contain heat-sensitive compounds, such as flavonoids. Maceration is an extraction process that does not rely on heat, making it a relatively safe method for preserving thermolabile compounds. Moreover, maceration is a simple, effective, and cost-efficient method [9]. The maceration was performed using 70% ethanol as the solvent. Ethanol was selected due to its numerous advantages: it is non-toxic, more effective at extracting a broad range of compounds compared to methanol or water, miscible with water, and evaporates readily [10]. Furthermore, ethanol concentrations above 20% inhibit microbial growth. The maceration process was conducted for 5×24 hours at room temperature in a sealed container with occasional agitation. After five days, the filtrate was concentrated using a rotary evaporator at approximately 50°C. From an initial 300 grams of sample, 14.758 grams of thick extract was obtained, corresponding to a final yield of 4.919%.

The α-glucosidase inhibitory activity of the bael leaf extract was assayed using a microplate reader, also known as an ELISA (Enzyme-Linked Immunosorbent Assay) reader. This instrument is a spectrophotometer equipped with a diffraction grating or filters to define the wavelength, capable of reading between 400–750 nm. The working principle of the ELISA reader involves a fiber-optic-like illumination system that directs light into the sample wells. The light is transmitted through the sample (1-3 mm diameter), and the transmitted light is captured by a detector. The signal is then amplified, converted into an electrical signal, and read as absorbance. The instrument is also connected to a computer for data recording, analysis, and interpretation. The ELISA method was selected for its simplicity, cost-effectiveness, and high sensitivity, even when using minimal sample volumes.

In this study's absorbance measurements, both sample and blank absorbances were recorded. DMSO (dimethyl sulfoxide) was used as the control for the blank solution, as it was the solvent used to dissolve the sample. Furthermore, "sample blank" controls (lacking the enzyme) were used to correct for any intrinsic absorbance from the colored extract that could interfere with the measurement at the operating wavelength. The absorbance was quantified at a wavelength of 405 nm.

This research employed an α-glucosidase enzyme derived from recombinant *Saccharomyces cerevisiae*, with *p*-nitrophenyl-α-glucopyranoside (PNPG) as the substrate. The α-glucosidase enzyme reacts with the PNPG substrate to produce glucose and *p*-nitrophenol. The latter product is a chromogen, indic ated by a variable yellow color change corresponding to its absorbance [11]. Fainter yellow color signifies lower *p*-nitrophenol production, indicating that the sample effectively inhibited the enzymatic reaction; consequently, a higher level of inhibition is recorded.

The positive control (comparator standard) used was acarbose, tested at concentrations of 0.2, 0.4, 0.6, 0.8, and 1 ppm. Acarbose is an FDA-approved α-glucosidase inhibitor [12]. It functions as a competitive inhibitor because its structure mimics the glycosidic bond of the substrate. Its mechanism involves occupying the active site of the α-glucosidase enzyme, thereby preventing the enzyme from cleaving the substrate [13].

To evaluate the extract's inhibitory potential, five concentrations of the bael leaf sample were tested: 300, 400, 500, 600, and 700 ppm. This concentration range was used to establish a dose-response curve and determine the percent inhibition at each level. Percent inhibition was calculated by comparing the absorbance of the sample wells against the blank (100% activity) wells. The results indicated a dose-dependent response for both substances. For acarbose, the percent inhibition ranged from 39.0665% (0.2 ppm) to 62.8108% (1 ppm). For the bael leaf extract, inhibition ranged from 11.6692% (300 ppm) to 53.3739% (700 ppm). These inhibition percentages were used to calculate the IC_{50} value, which represents the concentration required to inhibit 50% of the α-glucosidase activity. A lower IC_{50} value indicates greater inhibitory activity. The calculated IC_{50} value for acarbose was 0.569 µg/mL, whereas the Bael Leaf extract yielded an IC_{50} of 666.33 µg/mL.

The IC_{50} value of the sample was then compared to that of acarbose to evaluate its relative potency. According to the classification system described by Najma et al. (2021), α-glucosidase inhibitory activity can be categorized as: very strong ($IC_{50} \leq 25$ µg/mL), strong (25 µg/mL < $IC_{50} \leq$ 50 µg/mL), weak (50 µg/mL < $IC_{50} \leq 100$ µg/mL), or very weak ($IC_{50} > 100$ µg/mL). Based on this classification, the acarbose IC_{50} value (0.569 µg/mL) falls into the 'very strong' category. In contrast, the bael leaf sample (666.33 µg/mL), while demonstrating measurable activity, is classified as a 'very weak' inhibitor.

The 'very weak' inhibitory ability of the Bael Leaf sample, as indicated by its high IC_{50} value, may be attributed to several factors. First, the crude extract contains a complex mixture of various active compounds, and the specific inhibitory agent may be present at a low concentration. Second, potential compound instability or degradation may have occurred during the research process, particularly due to heat exposure (approx. 50°C) during the rotary evaporation step. Finally, other factors, such as improper or careless storage of the extract and sam ple, could also have influenced the stability of the active compounds.

Tabel 1 Yield Calculation

Weight of Sample	300 grams
Weight of Empty Beaker	82,6793 grams
Weight of Beaker + Extract	97,438 grams
Weight of Extract	14,758 grams
% Yield	**Yield =** $\frac{\text{Weight of Extract}}{\text{Weight of Simplicia}}$ **x 100 %** $\frac{14,7587}{300}$ **x 100 %** **= 4,919 %**

Tabel 2 Bael Leaf Extract Results

Type of Extract	Simplicia Weight (g)	Extract Weight (g)	Percent Yield (%)
Thick extract	300	14,7587	4,919 %

Tabel 3 Acaarbose Absorbance

No	Concentration (ppm)	Standard Control, S0	Standard, S1	Blank Control, B0	Blank, B1	Standard Data, S0-S1	Blank Data, B0-B1
1	0,2	0,093	1,294	0,088	2,059	1,201	1,971
2	0,4	0,098	1,184	0,088	2,059	1,086	1,971
3	0,6	0,114	1,075	0,088	2,059	0,961	1,971
4	0,8	0,123	0,982	0,088	2,059	0,859	1,971
5	1	0,142	0,875	0,088	2,059	0,733	1,971

Tabel 4 Sample Absorbance

No	Concentration (ppm)	Standard Control, S0	Standard, S1	Blank Control, B0	Blank, B1	Standard Data, S0-S1	Blank Data, B0-B1
1	300	0,126	1,867	0,088	2,059	1,741	1,971
2	400	0,151	1,714	0,088	2,059	1,563	1,971
3	500	0,163	1,548	0,088	2,059	1,385	1,971
4	600	0,18	1,271	0,088	2,059	1,091	1,971
5	700	0,225	1,144	0,088	2,059	0,919	1,971

Tabel 5 Percent Inhibition of Acarbose

Standard	Concentration (ppm)	Percent Inhibition (%)
Acarbose	0,2	39,0665
	0,4	44,9011

	0,6	51,2430
	0,8	56,4181
	1	62,8108

Tabel 6 Percent Inhibition of Sample

Sample	Concentration (ppm)	Percent Inhibition (%)
Bael Leaf Sample	300	11,6692
	400	20,7002
	500	29,7311
	600	44,6474
	700	53,3739

Tabel 7 IC_{50} Values

	IC50 Value (µg/mL)
Acarbose	0,569
Bael Leaf Extract	667,169

Conclusion

Based on the findings of this study, it can be concluded that the ethanolic extract of *Aegle marmelos* leaves demonstrated dose-dependent inhibitory activity against the α-glucosidase enzyme, with inhibition percentages ranging from 11.6692% at 300 ppm to 53.3739% at 700 ppm. The calculated IC_{50} value for the *A. marmelos* extract was determined to be 667.16 µg/mL, whereas the positive control, acarbose, exhibited an IC_{50} of 0.569 µg/mL. Therefore, while the bael leaf extract shows a measurable ability to inhibit the α-glucosidase enzyme, its activity is categorized as weak, in contrast to the very potent inhibition demonstrated by acarbose.

Supplementary Material

None

Author Contributions

SEK : Conceptualization, Methodology, Writing-Original Draft. **W** : Data Curation, Formal Analysis, Visualization. **IFA** : Supervision, Funding Acquisition, Writing- Review & Editing.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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