Testing the Inhibition Activity of the Alpha-Glucosidase Enzyme of Melinjo Leaf (*Gnetum gnemon* L.) Extract and Fractions In Vitro

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Abstract. High blood sugar levels are one of the metabolic disorders associated with diabetes mellitus. Oral antidiabetic therapy using α-glucosidase inhibitor agents. By blocking the α-glucosidase enzyme, the research aims to ascertain the antidiabetic potential of melinjo leaf (Gnetum gnemon L.) extracts and fractions. The inhibition test was carried out by using a microplate reader to measure the absorbance of p-nitrophenol produced as a product of the enzymatic reaction at a wavelength of 405 nm. Ethanol extract, ethanol fraction, ethyl acetate fraction, and n-hexane fraction had IC50 values of 589.39, 494.51, 99.61, and 56.82, respectively. The α-glucosidase enzyme is more likely to be inhibited by the ethyl acetate and n-hexane fractions than by the ethanol extract and ethanol fraction. According to phytochemical analyses, the n-hexane fraction contained steroids while the ethyl acetate fraction contained flavonoids, polyphenols, tannins, and saponins. The α-glucosidase enzyme may be inhibited by the n-hexane and ethyl acetate fractions.

Keywords: melinjo leaf (*Gnetum gnemon* L.), α-glucosidase enzyme, IC_{50}, n-hexane fraction

1 Introduction

Diabetes mellitus is a chronic illness marked by elevated blood glucose levels brought on by the body's inability to either create insulin or efficiently utilize the insulin that is produced [1]. Diabetes mellitus is also known as the silent killer because sufferers often do not realize it and sufferers often find out when complications have occurred [2]. According to the International Diabetes Federation (IDF), 537 million persons worldwide, aged 20 to 79, are estimated to have diabetes. It is anticipated that this figure will rise even further, reaching 643 million in 2030 and 784 million in 2045. Indonesia has the seventh-highest diabetes prevalence worldwide. 2018 saw a rise in Indonesia's diabetes mellitus prevalence from 6.9% to 8.5% [3]. The increasing

SICBAS 2023, November 03, Palembang, Indonesia

DOI 10.4108/eai.3-11-2023.2347958

prevalence of diabetes mellitus from year to year shows the need for serious attention in treating this disease.

One treatment strategy for diabetes mellitus involves blocking the body's ability to absorb glucose through the action of enzymes like α-glucosidase [4]. After eating, the body's blood glucose levels rise due to the α-glucosidase enzyme's ability to catalyze the hydrolytic cleavage of oligosaccharides into monosaccharides, which speeds up the small intestine's absorption of glucose. An α-glucosidase enzyme inhibitor is required to reduce or delay the intestinal absorption of glucose, hence preventing an increase in postprandial blood glucose levels [5].

Acarbose is one medication that can be used to treat type II diabetes mellitus. Alpha-glucosidase is inhibited by acarbose to work. The use of natural medicines is currently an alternative therapy because long-term usage of synthetic antidiabetic pharmaceuticals, like acarbose, can produce various side effects, including abnormalities of the digestive tract system, such as nausea, vomiting, abdominal discomfort, and bloating [6,7]. Given its potential and low side effects, it is being considered more.

For a very long time, studies on α-glucosidase inhibition have been conducted.Therefore, the search for new, more effective α-glucosidase inhibitors with fewer side effects and lower costs remains an interesting area of research. What has the potential to be developed as an α -glucosidase inhibitor is melinjo leaves.

The melinjo (*Gnetum gnemon* L.) plant is traditionally used by the community as an antihypertensive, lowering blood sugar levels, and as a diuretic [8]. People use parts of the melinjo plant such as seeds as food in the form of chips. Melinjo leaves are composed of several chemicals, including tannins, flavonoids, and saponins [9]. Melinjo leaf extract has been found to include secondary metabolites such as alkaloids, flavonoids, steroids, saponins, and tannins, according to qualitative tests[10]. Quantitative analysis shows that melinjo leaf extract contains large amounts of secondary metabolites. The highest levels of tannin were reported (7.32-8.40%), saponin (5.32-7.49%), and flavonoids (0.96%) which shows that melinjo leaves are very active and are thought to have high antioxidant activity so that they can fight various diseases [10].

Flavonoids have antidiabetic activity through their function as antioxidants which can bind free radicals so that they can reduce oxidative stress and result in reduced insulin retention. Additionally, flavonoids stop pancreatic β cells from becoming damaged and dysfunctional, which has antihyperglycemic properties. Another mechanism aspect of flavonoids as antidiabetics is their ability to inhibit GLUT 2 (Glucose Transporter type 2) which is the major transporter of glucose in the intestine. By inhibiting GLUT 2, glucose levels increase blood decreases [11].

Thus, the purpose of this study is to determine which fraction has the most ability to prevent the alpha-glucosidase enzyme from acting and identify the chemical compound groups contained in this fraction. In this study, extraction using the universal solvent ethanol 96% and to demonstrate antidiabetic potential, fractionation utilizing the liquid-liquid partition method with multiple solvents with varying degrees of polarity can be performed. Using the substrate p-nitrophenyl-A-D-glucopyranoside (PNPG), the α-glucosidase inhibitory activity of the three fractions was assessed using the spectrophotometric technique [12]. The IC_{50} values of the three fractions were compared with the IC_{50} value of the reference (acarbose).

2 Methodology

2.1 Material

The material for this research is melinjo leaves, ethanol 96%, n-hexane, ethyl acetate, chloroform (PT. Bratachem, Palembang), ammonia, sulfuric acid, Mayer's reagent, p hydrochloric acid, $FeCl_3$ reagent, anhydrous acetic acid (Merck & Co®), Bovine serum albumin (Sigma Aldrich), acarbose (PT. Dexa Medica), alpha-glucosidase enzyme (Sigma Aldrich G5003-100UN-PW), and para nitrophenyl substrate α-D-glucopyranoside (PNPG) (Sigma Aldrich N1377).

2.2 Sample collection and identification

Sample of melinjo leaves obtained from the Indralaya area, South Sumatra, which have been determined at the UPT Plant Conservation Center Purwodadi Botanical Gardens-LIPI, Pasuruan, East Java.

2.3 Extraction of melinjo leaves

The extract was made by extracting 2 kg of simplicia melinjo leaves and macerating them with 96% ethanol solvent for 3 days at room temperature, shielded from light, and with frequent stirring. After that, an ethanol extract is obtained by filtering it. To get a concentrated ethanol extract, the solvent was evaporated at 50°C using a rotary evaporator.

2.4 Liquid-liquid partition

Next, the thick ethanol extract was fractionated with n-hexane, ethyl acetate, and ethanol in order of polarity using the liquid-liquid method, then the solvent was evaporated to obtain n-hexane fraction, ethyl acetate fraction, and ethanol fraction.

Before testing for antidiabetic activity, the extracts and fractions obtained were subjected to phytochemical tests to confirm secondary metabolites. Parameters tested include the identification of flavonoids, steroids, triterpenoids, saponins, alkaloids, and tannins.

Three drops of ammonia, ten milliliters of chloroform, and 0.5 gram of extracts or fraction melinjo leaves were added for the alkaloid test. After adding ten drops of sulfuric acid to the ammonia fraction to make it more acidic, the acid fraction is divided into three sections. Reagents from Dragendorf, Mayer, and Wagner were added to each section. Red precipitate upon adding Dragendorf reagent, white precipitate upon adding Mayer reagent, and brown precipitate upon adding Wagner reagent indicate successful outcomes [13].

In a flavonoid test, up to 0.1 g of extracts or fraction melinjo leaves were cooked for five minutes in 5 mL of ethanol. After the findings are filtered, 10 drops of strong hydrochloric acid are added to the filtrate. Up to 0.1 g of magnesium powder is added. Whereas the hue orange-yellow denotes the presence of chalcones, flavones, and aurons, orange-red to purple-red shows the presence of flavonoids [14].

Five minutes were spent boiling 0.5 grams of the sample in 5 milliliters of distilled water in a test tube. After that, the tube was given a vortex shake and left for ten minutes. The production of steady, non-vanishing foam is an indication of saponin [13].

Ten milliliters of distilled water were used to dissolve five grams of extract ethanol melinjo leaves, which were then boiled for five minutes and filtered. Four to five drops of FeCl3 are

applied to the resulting filtrate. Dark blue or blackish-green hues are indicative of positive results for phenol compounds [13]. The presence of a violet-green tint indicates that tannins are having a good effect.

Testing of steroid and triterpenoid compounds was carried out using a lower layer that had been obtained from the identification of alkaloids, dropped on a drop plate, and allowed to dry. The dry bottom layer was added with 1 drop of acetic anhydride (CH_3CO), then stirred and added 3 drops of concentrated sulfuric acid (H_2SO_4). Positive results for steroids if they change color to blue or green. Triterpenoid positive results if the color changes to red or purple [13].

2.5 Inhibitory assay against alpha-glucosidase

Three fractions were used for the α-glucosidase inhibition test: n-hexane fraction, ethyl acetate fraction, and ethanol fraction. The test was carried out using the spectrometric method with slight modifications [15, 16]. A total of 10 µL of extract/compound with various concentrations (in DMSO) was added with pH 6.8 phosphate buffer and 250 µL of 10 mM PNPG substrate. Following five minutes of incubation at 37°C, 250 µL of an enzyme solution containing 0.07 U/mL was added, and the combination was once more incubated for thirty minutes at 37°C. Next, the enzymatic process was stopped by adding 2000 µL of 200 mM Na_2CO_3. Using a microplate reader, the samples' absorbance was determined at λ 405 nm. Testing was carried out in triplicate. The same test was carried out on blank (DMSO without sample) and acarbose (positive control).

Measurement of the absorbance of control samples and blank controls was carried out in the same way, but after the first incubation, Na_2CO_3 was added first, then the second incubation, the glucosidase enzyme was then introduced, and the absorbance was determined after that. Percent inhibition is calculated according to the following formula.

$$\% \text{ inhibition} = [(Ao - A1) / Ao] \times 100\%$$

Note: Ao = blank absorbance – blank control absorbance
A1 = sample absorbance – control sample absorbance

The IC_{50} value is determined by making a linear regression between concentration and percent inhibition. Applying the linear regression formula, Y = a + b X, where y represents the percentage of inhibition and the X axis represents the sample concentration. The Excel application was used to analyze data to ascertain how fractions affected the alpha-glucosidase enzyme's ability to function. To test the best inhibitory power of the fraction against the enzyme α -glucosidase, it can be seen from the IC_{50} value.

3 Result and Discussion

3.1 Phytochemical test

Fractionation and the maceration method were used to extract Melinjo leaves. Using a separating funnel, the liquid-liquid partition method is used to carry out the fractionation procedure. Fractionation aims to separate active compound components from the resulting extract based on their polarity [17]. As a result, the non-polar solvent n-hexane, the semi-polar solvent ethyl acetate, and the polar solvent ethanol are used in the fractionation process. Semi-polar substances found in cell walls can be dissolved by the semi-polar solvent ethyl acetate.

Alkaloids, glycosides, phenolic compounds, terpenoids, and aglycones can all be extracted using semi-polar solvents [13].

Ethanol 96% solvent was used in a maceration process to extract the powder from Melinjo leaf simplicia. The thick extract was 428.326 g with a yield percentage of 21.416%. The percent yield states the amount of active compound content extracted in the solvent used. The greater the yield value, the greater the content of active compounds that are attracted. Using a liquid-liquid partition technique, fractionation is done in phases according to the polarity of the solvent, starting with non-polar, semi-polar, and polar solvents. The yield percentage of the n-hexane fraction extracted from melinjo leaves was 8.124%. 38.560% was the yield percentage of the ethyl acetate fraction produced from melinjo leaves. In the meantime, 54.117% of the ethanol fraction of melinjo leaves was yielded.

Phytochemical screening aims to identify chemical compounds contained in plants which is carried out using test reagents. Table 1 displays the findings of the phytochemical screening of melinjo leaf fractions and extracts.

Table 1. The phytochemical test result of ethanol extract and fraction melinjo leaf

Secondary metabolites	Ethanol Extract	Ethanol Fraction	Ethyl Acetate Fraction	N-hexane Fraction
Alkaloids				
Mayer	+	+	-	-
Wagner	+	+	-	-
Dragendorff	+	+	-	-
Flavonoids	+	+	+	-
Tannins	+	+	+	-
Phenolics	+	+	+	-
Steroids	+	+	-	-
Triterpenoids	-	-	-	+
Saponins	+	+	+	-

Note: + = detected; - = not detected

In the ethanol extract and ethanol fraction of melinjo leaves, there are alkaloids, flavonoids, tannins, phenolics, steroids, and saponins. Steroids are present in the n-hexane fraction and flavonoids, tannins, phenolics, and saponins in the ethyl acetate fraction. Semi-polar solvent ethyl acetate is capable of extracting glycosides, aglycones, alkaloids, phenolic compound, and terpenoids [13]. Alpha-glucosidase enzyme inhibition may be caused by the chemical components of plants, such as alkaloids, flavonoids, tannins, saponins, quinones, and steroids/triterpenoids [18, 19, 20].

3.2 Inhibitory assay against α-glucosidase

The melinjo leaf fraction's α-glucosidase enzyme inhibitory activity test seeks to determine the potential of the fraction used as an antidiabetic based on the IC_{50} value. In this study, acarbose was used as a comparison. The α-glucosidase enzyme test's inhibitory activity is represented by the IC50 value that is derived from the linear regression analysis of the percent inhibition and concentration data. Table 2 displays information on the fractions' IC50 values and % inhibition.

Table 2. The inhibition of α-glucosidase enzyme activity from melinjo leaf extracts and fractions

Test	Concentration (ppm)	% Inhibition	Regresi linear	IC_{50}
Acarbose (control)	3.125	23.08463	Y = 0.2128X + 22.314	130.10340
	12.5	23.14884		
	25	30.17080		
	50	32.13304		
Extract of ethanol	0.78125	2.00077	Y = 0.0796X + 3.0849	589.3857
	1.5625	2.76872		
	6.25	4.92873		
	25	5.82766		
	50	6.54938		
Fraction of ethanol	0.78125	11.04662	Y = 0.7242X + 11.415	494.5087
	1.5625	13.21947		
	3.125	14.30333		
	25	15.57724		
	50	16.73045		
Fraction of ethyl acetate	0.390625	14.77334	Y = 0.3523X + 14.908	99.6082
	0.78125	13.88211		
	6.25	18.88275		
	50	32.32567		
Fraction of n-hexane	0.390625	11.89161	Y = 0.6705X + 11.901	56.8218
	0.78125	16.44793		
	12.5	17.7501		
	25	25.08026		
	50	47.79247		

From the IC_{50} values in Table 2, the IC_{50} values for the extract of ethanol and the fraction of ethanol are 589.3857 and 494.5087 respectively, while the fraction of ethyl acetate and fraction of n-hexane exhibit IC50 values of 99.6082 and 56.8218 µg/mL. The extract of ethanol and ethanol fraction of melinjo leaves have weak antidiabetic activity because the IC_{50} value is greater than 100 µg/mL and also has a greater IC_{50} than acarbose. The greater the activity in suppressing the α-glucosidase enzyme's functioning, the smaller the IC_{50} value [21]. The fraction of ethyl acetate and n-hexane fraction of melinjo leaves are included in the active antidiabetic category, where the ethyl acetate fraction and n-hexane fraction have the lowest IC_{50}. Comparatively, the IC50 of acarbose is 8.68141 µg/mL. If a fraction's IC50 value is less than 10 µg/mL, it is considered highly active as an α-glucosidase enzyme inhibitor; if it is between 10 and 100 µg/mL, it is considered active; and if it is greater than 100 µg/mL, it is considered weak [25].

Table 3. IC_{50} values and activity of melinjo leaf extracts and fractions

Test	IC_{50}	Activity
Acarbose	130.1304	medium
Ethanol extract	589.3857	weak
Ethanol fraction	494.5087	weak
Ethyl acetate fraction	99.60820	active
n-hexane fraction	56.82177	active

Tests on ethanol extract, ethanol fraction, ethyl acetate fraction, and n-hexane fraction were carried out after obtaining the IC_{50} value for acarbose. The IC_{50} value obtained from each extract and fraction was compared with the IC_{50} value of acarbose to see whether the fraction could inhibit alpha-glucosidase activity. The test results show that acarbose, which IC_{50} value of 130.1304 ppm can inhibit alpha-glucosidase activity. When compared to the IC50 value of the four extracts and fractions that were examined, this value is relatively high. With a value of 56.82177 ppm, the n-hexane fraction has the lowest IC50 value. Meanwhile, the fraction of ethyl acetate, fraction of ethanol, and extract of ethanol have IC_{50} values of 99.6082 ppm, 494.5087 ppm, and 589.3857 ppm (data can be seen in Table 3).

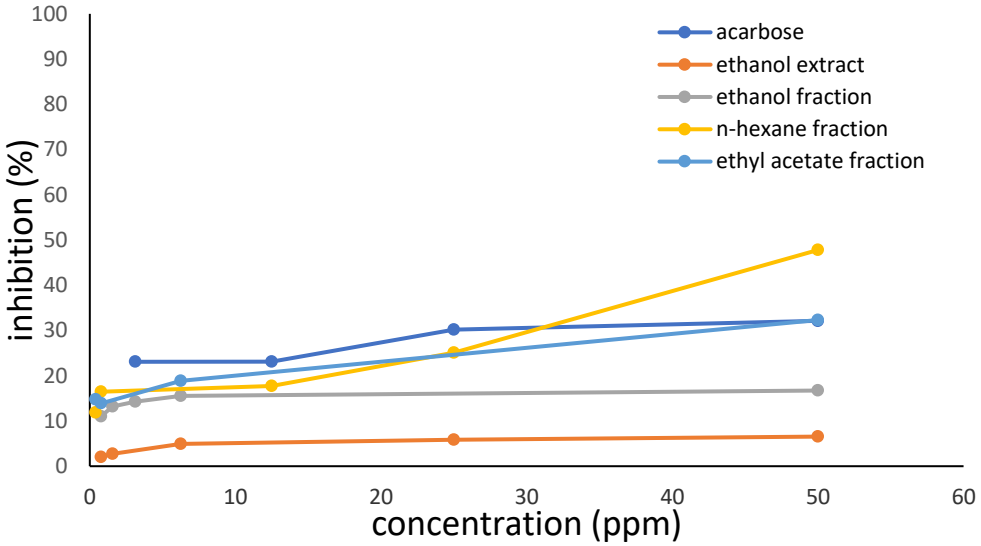

Figure 1. Graph of α -glucosidase enzyme inhibition from melinjo leaf extracts and fractions and acarbose.

The IC_{50} value of acarbose that is too far different from the test fraction may be because acarbose is an α-glucosidase inhibitor which has a strong inhibitory impact on alpha-glucosidase in mammals but not on alpha-glucosidase from *Saccharomyces cerevisiae* and *Bacillus stearothermophilus*. Meanwhile, while catechin does not inhibit human α-glucosidase, it does have an inhibitory impact on Saccharomyces cerevisiae and Bacillus stearothermophilus [23]. Quercetin, in addition to catechin, inhibits α-glucosidase against Saccharomyces cerevisiae, with an IC50 value of less than 15μM [18].

The concentration needed to inhibit 50% of an enzyme's activity is known as the IC50 value, and it is calculated using the percent inhibition value obtained. This number is then compared to the sample and used to measure the extract's inhibitory strength against the enzyme. The alpha-glucosidase activity's inhibitory capacity increases with decreasing IC50 value and vice versa. Based on the IC_{50} value of each melinjo leaf extract and fraction, it shows the ability to have antidiabetic activity through inhibition of the alpha-glucosidase enzyme. The fraction of n-hexane and fraction of ethyl acetate had the smallest IC_{50} values among the ethanol fraction and ethanol extract so they were classified as actively inhibiting the α-glucosidase enzyme. The n-hexane fraction has the best ability to inhibit the α-glucosidase enzyme among the four fractions and extracts. The n-hexane fraction contains steroid compounds, where steroids have the effect of inhibiting the work of the α-glucosidase enzyme so that It can interfere with and

lessen the small intestine's ability to absorb glucose [24]. Meanwhile, the fraction of ethyl acetate fraction contains phenolics, flavonoids, saponins, and tannins. The fraction of ethanol and extract of ethanol were classified as weak in inhibiting the α-glucosidase enzyme.

Alkaloids, flavonoids, and terpenoids' secondary metabolites have the innate ability to block A-glucosidase. The ability of these secondary metabolites to lower blood glucose levels has been studied both in vitro and in vivo [25]. Alkaloids, flavonoids, steroids, and glycosides are examples of bioactive substances with hypoglycemic properties [26].

This activity probably occurs due to the presence of steroids and flavonoids which are active as antidiabetics with a working mechanism of inhibiting the α-glucosidase enzyme in melinjo leaf samples dissolved in n-hexane and ethyl acetate solvents. Acarbose was chosen as a comparison because acarbose is an antidiabetic drug that works by inhibiting the α-glucosidase enzyme circulating in Indonesia and also acarbose has become an internationally recognized comparison. Moreover, in terms of structure, acarbose has almost the same structure as the substrate p nitrophenyl-α-D-glucopyranoside. Based on the test results, acarbose as a comparison has an IC_{50} value of 130.1304 μg/mL which has the potential to be very active as an inhibitor of the α-glucosidase enzyme.

The way flavonoids work as antidiabetics can be by reducing aldose reductase and regenerating pancreatic cells, as well as increasing insulin release and calcium ion absorption [27]. By preventing damage to pancreatic beta cells, antioxidant activity reduces the absorption of glucose and intra-pancreatic processes. Flavonoids can also increase the intestinal mucosal layer so that glucose intake into the intestine will be hampered [28]. Another mechanism is inhibiting GLUT-2, the major glucose transporter in the intestine. Flavonoids can also inhibit phosphodiesterase thereby increasing cAMP in pancreatic beta cells which will then stimulate the release of protein kinase which stimulates insulin secretion [29].

Alkaloids have the ability to lower blood glucose levels through a variety of mechanisms, including blocking the intestinal absorption of glucose, boosting blood glucose transport, inducing the synthesis of glycogen, and blocking the synthesis of glucose by blocking the enzymes fructose 1,6 bisphosphatase, glucose 6-phosphatase, and glucose 6-phosphate dehydrogenase. Enzymes involved in gluconeogenesis include fructose 1,6-bisphosphatase and glucose 6-phosphatase. The production of glucose from substrates other than carbs can be decreased by inhibiting the enzymes fructose 1,6-bisphosphatase and glucose 6-phosphatase [30]. Increased insulin secretion can be caused by the sympathetic nerve stimulating effect (sympathomimetic) of alkaloids which has the effect of increasing insulin secretion [30, 31].

Tannins are free radical scavengers and increase glucose uptake in the blood through insulin mediator activity thereby reducing blood glucose levels [32]. The mechanism of action of tannins in reducing blood glucose levels is through the mechanism that tannins reduce nutrient absorption by inhibiting glucose absorption in the intestine, besides inducing cell regeneration. Pancreatic β which affects adipose cells thereby strengthening insulin activity. The mechanism of proanthocyanidins in reducing blood glucose levels is to suppress oxidative stress associated with the inflammatory process due to diabetogenic induction. Suppression of oxidative stress through inhibition of lipid peroxidation and generation of ROS (Reactive Oxygen Species) [33].

Saponin also reduces glucose absorption in the intestine, inhibiting the GLUT-1 glucose transporter, boosting the sensitivity of insulin receptors in tissues, as well as glucose consumption and glycogen storage in peripheral tissues. The effect of saponins on the structure of cell membranes can prevent the body from absorbing tiny nutrition molecules that need to be

taken fast, like glucose [30]. Saponin can prevent the rise in blood sugar levels by preventing the small intestine from absorbing glucose and by preventing the stomach from emptying, which results in food absorption taking longer and blood glucose levels improving [34].

Thus, the activity of inhibiting the alpha-glucosidase enzyme inhibitor possessed by the extract of ethanol, fraction of ethanol, fraction of ethyl acetate, and fraction of n-hexane of water melinjo leaves and ethanol extract is inseparable from the content of secondary metabolite compounds contained therein. Phytochemicals from the melinjo plant (Gnetum gnemon L.) which have antidiabetic biological activity by effectively inhibiting the work of the enzymes alpha-amylase and alpha-glucosidase and are epi-gnetin C, gnetin C, gnetin L, gnetin E, trans-resveratrol, gnemonoside A, gnemonoside C, and gnemonoside D, [35]. Most melinjo structures are in the form of stilbene [9, 36]. The isolation results from the hexane fraction of melinjo leaves are 2,3-dihydroxypropyl icosanoate, oleic acid, and ursonic acid [37]. Melinjo leaves include dehydrovomifoliol, β-sitosterol, β-sitosterol glucoside, cinnamic acid, and vanillic acid [38].

4 Conclusion

Phytochemical tests found that the ethanol extract and ethanol fraction contained alkaloids, flavonoids, tannins, phenolic, saponins, and steroids,; the fraction of ethyl acetate contained flavonoids, phenolic, tannins, and saponins while the n-hexane fraction contained steroids. The IC_{50} value for extract of ethanol, fraction of ethanol, fraction of ethyl acetate, and n-hexane fraction was 589.39; 494.51; 99.61, and 56.82. Compared to the extract of ethanol and the fraction of ethanol, the ethyl acetate and n-hexane fractions are more likely to block the alpha-glucosidase enzyme.

Acknowledgments. This article's study was funded by the DIPA of Universitas Sriwijaya 2023. Nomor SP DIPA-023.17.2.677515/2023 on the 10th of November 2022. By the Rector's Decree Number: 0189/UN9.3.1/SK/2023 on the 18th of April 2023

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