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RESEARCH ARTICLE

The Activity Test of Ethanol Extract in Hibiscus Leaves (*Hibiscus Tiliaceus* L.) as the α-Glucosidase Enzyme Inhibitor by Using ELISA (*Enzyme Linked Immunosorbent Assay*) Reader

Sukmawati*, **Masdiana Tahir**, **Nining Sulfiani**

Faculty of Pharmacy, Universitas Muslim Indonesia.

***Corresponding Author:** Sukmawati

Abstract

Hibiscus leaves (*Hibiscus tiliaceus* L.) was a plant from the family of *Malvaceae*, which contained triterpenoid, saponins, flavonoids, and polyphenols compounds that functioned as antidiabetic mellitus. The purpose of this study was to determine the activity of α-glucosidase enzyme inhibitor from the ethanol extract of hibiscus leaves (*Hibiscus tiliaceus* L.). The test was carried out through in vitro by using α-glucosidase enzyme and p-nitrofenil-α-D-glukopiranosida (PNPG) substrate, and measured by ELISA reader. The simplicia was macerated with alcohol 96%. The reaction of α-glucosidase enzyme and p-nitrofenil-α-D-glukopiranosida substrate produced yellow p-nitrofenol. The reaction product was measured at a wavelength of 405 nm. The results showed that ethanol extract of hibiscus leaves (*Hibiscus tiliaceus L.*) had activity as an inhibitor of α-glucosidase enzyme with *Inhibitor Consentration* IC50 37.98 µg / mL and could be categorized as active.

Keywords: *Hibiscus tiliaceus L., Hibiscus leaves, A-glucosidase enzyme inhibitor.*

Introduction

Indonesia is rich in biodiversity which can be used in every aspect of human's life. The use of plants as medicine is a form of the utilization of biodiversity in Indonesia [1]. One of the plants that has been known and commonly used in Indonesia is hibiscus leaves (*Hibiscus tiliaceus* L.) [2].Hibiscus leaves (*Hibiscus tiliaceus* L.) is a plant from a family of *Malvaceae*. In traditional medication, hibiscus leaves are used to treat fever, ulcer, tonsils, laxative sputum, menstrual laxatives, anti-inflammatory, antitoxic, anti-inflammatory, and hair fertilizers. Besides, it is also used for diabetes mellitus treatment [3, 2].

Hibiscus leaves contains triterpenoid, saponin, flavonoid, and polifenol compounds [2].According to [4, 5], most of plants which contain bioactive compounds such as glucoside, alkaloid, terpenoid, flavonoid and saponin function as antioxidant and anti-diabetes. In addition, many researches prove that phytochemicals compounds have ability as α-glucosidase enzyme inhibitor such as triterpenoid dan flavonoid [6, 7].

Stated that polyphenol can act as natural inhibitor in hydrolyzing carbohydrate from the enzyme so it can help to inhibit the increase of blood glucose. The α-glucosidase enzyme is an enzyme located in the wall of small intestine. This enzyme works in food's carbohydrate hydrolysis to become glucose and other monosaccharides. This enzyme inhibition causes obstruction in glucose absorption.

The α-glucosidase enzyme inhibition is one of the therapies available for the diabetes mellitus patient type II [8]. The acarbose is the inhibitor of α-glucosidase synthetically enzyme which has been widely used for the treatment of diabetes mellitus type II patients, yet this medicine is reported to cause various side effects.

Therefore, the natural inhibitor is needed to treat diabetes mellitus because it has a low side effect with the use of natural ingredients through the utilization of medical plants which can be isolated and marketed [6].

In the research that has been done by [9], the ethanol extract of hibiscus plants (*Hibiscus tiliaceus* L.) can decrease the blood glucose in

white male laboratory rat with the dose of 5.625 g/kg BW which is proportional to 63 mg/kg BW dose of metformin. However, the research does not reveal any specific mechanism to decrease blood glucose level. It encouraged the researcher to further test the sample of hibiscus leaves (*Hibiscus tiliaceus* L.) in decreasing the blood glucose level through in vitro by the mechanism of inhibiting the α-glucosidase enzyme.

Materials and Methods

The Collection and Processing of the Sample

The research sample of the hibiscus leaves (*Hibiscus tiliaceus* L.) was taken from District of Bontocani, Bone Regency, North Sulawesi Province.The hibiscus leaves (*Hibiscus tiliaceus* L.) was then washed from the stuck dirt by running water, then the leaves were cut into smaller pieces. Then, they were dried through aeration process, and triturated.

The Preparation of Ethanol Extract Sample [10]

The sample of hibiscus powder was weighed 100 g (*Hibiscus tiliaceus* L.) and placed into a maceration container and added by ethanol solvent 96% until soaked, and left out until three days in a closed container and protected from the direct sunlight while being stirred periodically. After 3 x 24 hours, it was filtered and the residue was macerated again with ethanol 96%. The re-maceration was done twice for approximately 2 x 24 hours. The extract from the maceration or filtrate which was produced was collected in one place and evaporated until it became thick ethanol extract of hibiscus leaves.

The Activity Test of α-Glucosidase Enzyme Inhibitor

The activity test of α-glucosidase enzyme inhibitor was completed through: [6, 11] The Making of Acarbose Solvent. The acarbose was measured 5 mg, and put into a 5 ml volumetric flask with the concentration 1000 ppm, and added by 5 µL DMSO, then added pH 7 phosphate buffer until reaching limit mark. After that, it was diluted from mother liquor, pipetted from each solvent 250 µl (50 ppm), 500 µl (100 ppm), 750 µl (150 ppm), 1000 µl (200 ppm), 1250 µl (250 ppm) into the 5 mL volumetric flask, and added by pH 7 phosphate buffer until reaching limit mark.

- ***The Making of Sample Solvent.***

The making of 1000 ppm mother liquor. The 25 mg ethanol extract was added into 25 mL volumetric flask. To make 1000ppm solvent, it was added by 3 drops DMSO and added by phosphate buffer pH 7 until reaching limit mark, so it would reach 1000 ppm concentration. Then, it was diluted from mother liquor, pipetted from each solvent 250 µl (50 ppm), 500 µl (100 ppm), 750 µl (150 ppm), 1000 µl (200 ppm), 1250 µl (250 ppm) into the 5 mL volumetric flask, and added by pH 7 phosphate buffer until reaching limit mark.

- ***The Preparation of α-glucosidase Enzyme Inhibitor (0,25 U/mL)***

The 1 mg α-glucosidase enzyme was diluted with 1 mL of phosphate buffer pH 7 (28 U/mL). Then the α-glucosidase enzyme solvent was made with the concentration of 0, 25 U/mL, by pipetting 89, 28 µL and the volume was supplemented with 10 mL pH 7 phosphate buffer.

- ***The Preparation of 5 mM p-nitrophenyl-α-D-glucopyranoside (PNPG) substrate***

The 15,062 mg p-nitrophenyl-α-D-glucopyranoside (PNPG) substrate was measured, and diluted in 10 mL pH 7 phosphate buffer so there would be 5 mM PNPG substrate concentration.

- ***Acarbose Test***

Each mixture of reagents used in this test contained 50 µL pH 7 phosphate buffer, and added by 25 µL PNPG (5 mM), then added 10 µL of acarbose solvent from each concentration series that have been composed which are 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm. Then added by 25 µL α-glucosidase enzyme solvent (0, 25 U/mL) which was later incubated for 30 minutes in 37°C temperature. The reaction was stopped by adding 0, 2 M of 100 µL natrium carbonate solvent. The sample was taken from the uptake by ELISA *reader* at 405 nm wavelength.

- ***Acarbose Blank Test***

Each reagent mixture in this test contained 50 µL pH 7 phosphate buffer, added by 25 µL PNPG (5 mM), and then added by 10 µL

acarbose solvent from each concentration series that was made which were 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm. After that, 25 µL pH 7 phosphate buffers was added and incubated for 30 minutes in 37°C temperature. The reaction was stopped by adding 0, 2 M of 100 µL natrium carbonate solvent. The sample was taken from the uptake by ELISA *reader* at 405 nm wavelength.

- ***Control Test***

Each reagent mixture in this test contained 50 µL pH 7 phosphate buffer and 5 mM of 25 µL p-nitrophenyl α-D-glucopyranoside (PNPG), then added by 10 µL pH 7 phosphate buffer and 25 µL α-glucosidase solvent (0.25 U/mL), then incubated for 30 minutes in 37°C temperature. The reaction was stopped by adding 0, 2 M of 100 µL natrium carbonate solvent. The sample was taken from the uptake by ELISA *reader* at 405 nm wavelength.

- ***Control Blank Test***

Each mixture of reagents used in this test contained 50 µL ph 7 phosphate buffer and 5 mM of 25 µL p-nitrophenyl α-D-glucopyranoside (PNPG), then added 35 µL ph 7 phosphate buffer then incubated for 30 minutes in 37°C temperature. The reaction was stopped by adding 0, 2 M of 100 µL natrium carbonate solvent. The sample was taken from the uptake by ELISA *reader* at 405 nm wavelength.

- ***Test of Hibiscus Leaves' (Hibiscus Tiliaceus L.) Ethanol Extract Sample***

To test the ethanol extract sample of hibiscus leaves (*Hibiscus tiliaceus* L.), it was needed 50 µL pH 7 phosphate buffer, added by 25 µL PNPG (5 mM), and added by 10 µL sample solvent from each concentration series had been made which were 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm. Then 25 µL α-glucosidase enzyme solvent (0, 25 U/mL) was added into the sample. The reaction was stopped by adding 0, 2 M of 100 µL natrium carbonate solvent. The sample was taken from the uptake by ELISA *reader* at 405 nm wavelength.

- ***Test of Hibiscus leaves' (Hibiscus tiliaceus L.) Ethanol Extract Sample Blank***

To test the ethanol extract sample of hibiscus leaves (*Hibiscus tiliaceus* L.), it was needed 50 µL pH 7 phosphate buffer, added by 25 µL PNPG (5 mM), and added by 10 µL sample solvent from each concentration series had been made which were 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm. Then 25 µL pH 7 phosphate buffer was added into the sample and incubated for 30 minutes in the temperature of 37°C. The reaction was stopped by adding 0, 2 M of 100 µL natrium carbonate solvent. The sample was taken from the uptake by ELISA *reader* at 405 nm wavelength.

Result

Inhibition percentage from α-glucosidase can be calculated by the following formula [11]:

$$\textbf{\% inhibition} = \frac{B-S}{B} \text{ x } 100\%$$

Note:

B= difference in control absorbance with blank control absorbance.
S= difference in sample absorbance with blank sample absorbance.

The value of IC_{50} was determined by making a curve between percent inhibitions versus the concentration or the curve between the probit versus log concentration until the regression equation is obtained. From the regression equation, it could be measured that the amount of extract concentration which had the inhibitory ability of α-glucosidase enzyme activity was 50%. Through the equation of linear regression, y = a + bx, where x axis was the sample concentration and y axis was % of the inhibition, so the value of IC_{50} could be calculated by the formula [6, 12]:

$$IC_{50} = \frac{50-a}{b}$$

Note:
- Intercept from x and y axis plot
- slope of x and y axis plot
- stated at 50
- the value of IC_{50}

Table 1: The of inhibition energy level toward α-glucosidase enzyme [13]:

Intensity	Value of IC_{50}
Very active	$IC_{50} \leq 25$ µg/mL
Active	25 µg/mL < $IC_{50} \leq 50$ µg/mL
Less active	50 µg/mL < $IC_{50} \leq 100$ µg/mL
Not active	$IC_{50} > 100$ µg/mL

Discussion

Determination result showed that the plant used in this research was Hibiscus leaves (*Hibiscus tiliaceus* L.) from the family of *Malvaceae*. The Hibiscus leaves produce secondary metabolite which have a broad biological activity. The early stage in this research was composing the extract. The extract was obtained from the extraction process by maceration method by using ethanol 96% in room temperature. The result from the Hibiscus leaves (*Hibiscus tiliaceus* L.) extraction can be seen in Table 2.

Table 2: Percent yield of Hibiscus Leaves' (*Hibiscus tiliaceus* L.) ethanol extract

Solvent Type	Weight of Dry Sample (g)	Weight of the Extract (g)	Percent Yield (%)
Ethanol 96%	100	4,487	4,847

Maceration method was chosen because it has many advantages compared to other methods. The main advantages of maceration method are it uses simple procedure and tools, also reduces the damage to the chemical component contained in the sample due to the heating process [14].

Ethanol was used as the solvent because it has two groups with different polarity which are hydroxyl group that is polar and alkyl group that is non-polar, so by the existence of these groups it is expected that the compounds with different polarity levels will be extracted into the ethanol [15].

The extraction process was done twice to optimally draw the bioactive compound in simplicial powder. The solvent from filtrate of the extraction result was evaporated until producing 4,847 gram thick extract with 4,847% percent yield. This activity test of α-glucosidase enzyme inhibitor used ELISA reader in 405 nm wavelength.

The use of his method has been commonly used by previous enzyme inhibitor researchers and this method has numerous advantages such as the simple and economical working techniques, and has a fairly high sensitivity [16].

In the activity test of α-glucosidase enzyme inhibitor, there were six solvents tested which were acarbose solvent, blank acarbose solvent, control solvent, blank control solvent, sample extract of hibiscus leaves (*Hibiscus tiliaceus* L.) and blank sample

solvent of hibiscus leaves (*Hibiscus tiliaceus* L.). The solvent of acarbose and sample extract of Hibiscus leaves (*Hibiscus tiliaceus* L.) was made in five variations which were 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm.

These variations of concentration were made so that they could be used to make regression equation to calculate IC_{50}. Besides, there was a control test to substitute the sample solvent by using buffer to observe the enzyme activity without adding the extract. On the other hand, blank solvent in both sample and control was used as correction.

This correction was to ensure that the natrium carbonate had actually inhibited the enzyme's action and to know if there was an absorbance that was detected from other compounds than p-nitrophenol with ELISA reader (microplate reader) at 405 nm wavelength.

The components in this activity test were 10 µL extract solvent, pH 7 phosphate buffer and 0.5 mM of 25 µL p-nitrophenyl α-D-glucopyranoside (PNPG), 25 µL α-glucosidase solvent (0.2 unit/mL) and 0.2 M of 100 µL natrium carbonate solvent.

The phosphate buffer solvent was used in pH 7 because α-glucosidase enzyme could work optimally in pH 7. During the activity test,

the test solvent was incubated for 30 minutes the temperature of 37°C. The incubation functioned as to give time for the enzyme to work.

The temperature of 37°C was chosen because it was the optimum work temperature for α-glucosidase enzyme. After the incubation, the natrium carbonate was added, which worked as the stopper of hydrolysis reaction. The principal of α-glucosidase enzyme inhibition process through in vitro was known based on the hydrolysis reaction in p-nitrophenyl-α-D-glucopyranoside substrate that produces p-nitrophenol and α-D-glucose with yellow color. (Figure: 1).

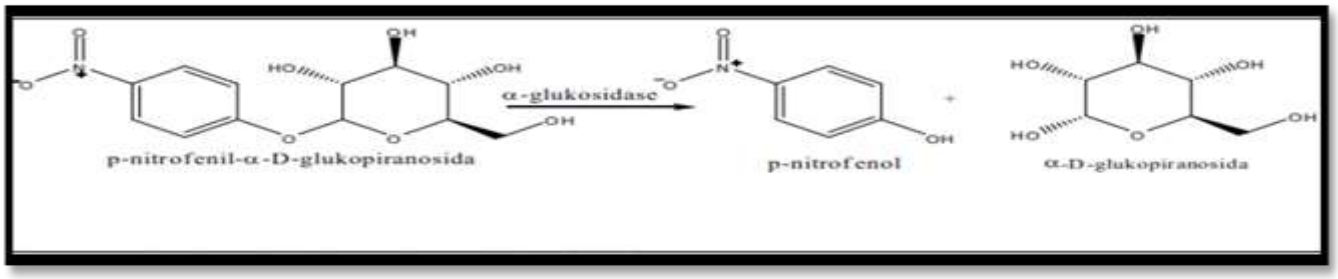

Fig.1: The enzymatic reaction equation of α-glucosidase dan p-nitrophenyl- α-D-glucopyranoside [17]

The weaker the yellow color from p-nitrophenol was an indication of the higher α-glucosidase inhibition activity. The number of glucoses made was equal to the number of p-nitrophenol. The greater the sample activity in suppressing enzyme activity, indicated by the lower absorbance value [18]. Acarbose was used as the comparison standard in the test of α-glucosidase enzyme inhibitor activity. The use of the standard as the parameter was useful to determine if the test solvent has either same, lower, or higher activity compared to the standard use. The test result of acarbose inhibition activity which was the α-glucosidase active inhibitor compounds that was used as the comparison in this research can be seen in Table 3.

Table 3: The absorbance measurement results of acarbose α-glucosidase enzyme inhibition

Cpncentration (µg/mL)	Absorbance				% inhibition	IC_{50} (µg/mL)	Energy of enzyme inhibition
	Control	Blank Control	Acarbose	Blank Acarbose			
50	1,586	0,022	1,203	0,012	23,84	683,12	Not active
100			0,966	0,966	33,75		
150			1,023	0,012	35,35		
200			1,110	0,015	36,63		
250			1,054	0,018	39,13		

The test result of Hibiscus leaves' (*Hibiscus tiliaceus* L.) ethanol extract as α-glucosidase enzyme inhibitor can be seen in Table 4.

Table 4: The test result of Hibiscus leaves' (*Hibiscus tiliaceus* L.) ethanol extract as α-glucosidase enzyme inhibitor

Concentration (µg/mL)	Absorbance				% inhibition	IC_{50} (µg/mL)	Energy of enzyme inhibition
	Control	Blank Control	Sample	Blank sample			
50	1,802	0,002	0,709	0,006	60,94	37,98	Aktif
100			0,427	0,035	78,22		
150			0,302	0,032	85		
200			0,207	0,043	90,88		
250			0,128	0,044	95,33		

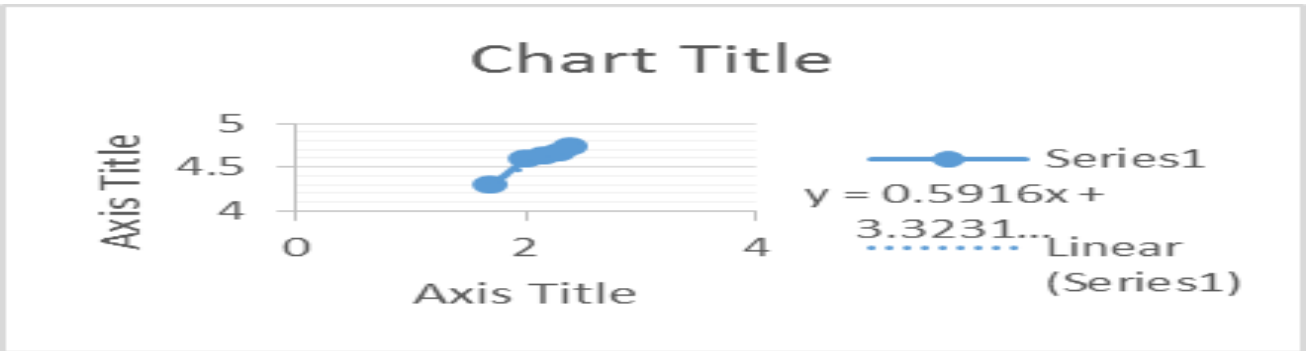

Fig.2: The regression equation to calculate the value of IC_{50} α-glucosidase enzyme inhibitor activity by acarbose.

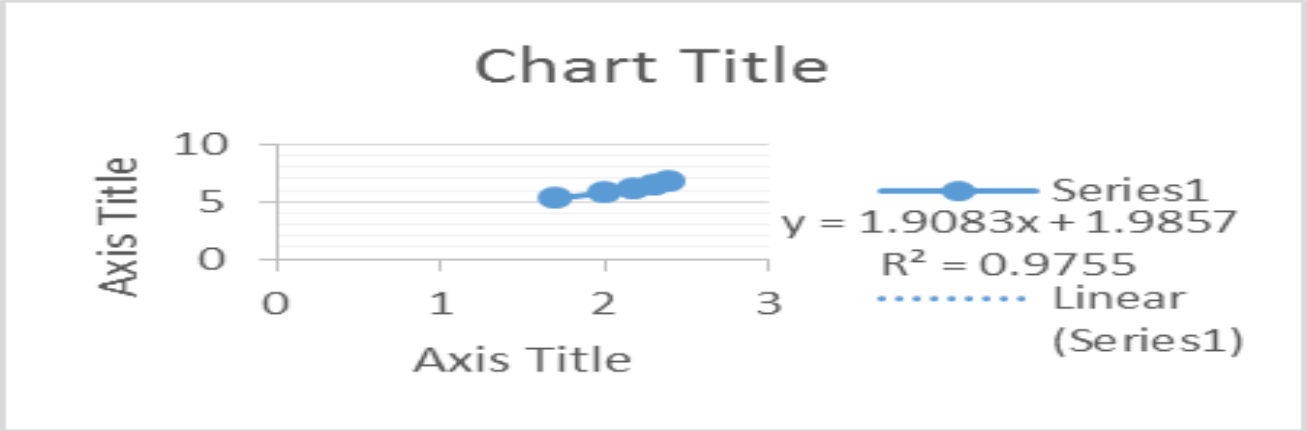

Fig.3: The regression equation to calculate the value of IC_{50} α-glucosidase enzyme inhibitor activity by the sample of Hibiscus leaves (*Hibiscus tiliaceus* L).

The test result on the α-glucosidase enzyme inhibitor activity showed the value of IC_{50} reached 683, 12 µg/mL. The energy of enzyme inhibition was categorized as non-active. While the ethanol extract in Hibiscus leaves (*Hibiscus tiliaceus* L.), the value of IC_{50} reached 37, 98 µg/mL. This energy of enzyme inhibition was in active category. The high value of IC_{50} in acarbose was caused by the less sensitivity of acarbose to α-glucosidase that came from the bacteria and yeast.

The acarbose was more effective in inhibiting the α-glucosidase that came from mammals, such as sucrose and maltase [19]. Another thing that might be the cause of the high IC_{50} in acarbose was the pure compound, while the tested sample was in a form of rough extract so that in the solvent extract still consisted of more than one inhibitor compounds that could cause higher inhibition energy. The activity of α-glucosidase enzyme inhibitor in Hibiscus leaves (*Hibiscus tiliaceus* L.) Extract could not be separated from bioactive compound works in it. This fact is supported by some researches that have been proved that phytochemicals compound has the ability to inhibit the work of α-glucosidase enzyme, such as the compounds from the group of triterpenoids dan flavonoid [6].

Conclusion

From this research, it was concluded that the ethanol extract in Hibiscus leaves (*Hibiscus tiliaceus* L.) had the activity as α-glucosidase enzyme inhibitor with the value of IC_{50} reached 37,98 µg/mL and it could be categorized as active.

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