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Inhibitory Activity of *Sargassum hystrix* Extract and Its Methanolic Fractions on Inhibiting α-Glucosidase Activity

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ABSTRACT

Seaweed has a great potential in the pharmaceutical field, one of them as antidiabetic. The purposed of this study was to know the inhibitory activity of *Sargassum hystrix* extract and its methanol fraction in inhibiting α-glucosidase activity. *S. hystrix* was extracted using methanol, then partitioned using chloroform, ethyl acetate, and methanol. Methanol fraction then separated by column chromatography to obtain the compound. The crude extract, the partitioned methanol fraction, and the column chromatography fraction were tested for its activity on inhibiting the α-glucosidase. The compounds of active fraction were analyzed using *gas chromatography-mass spectrometry* (GC-MS). The inhibitory activity (IC_{50}) of the crude extracts and the partitioned methanol fraction were 0.35±0.05 and 0.02±0.00 (mg/mL), respectively. The column chromatography fractions that had an inhibitory activity to α-glucosidase were M2 (23.46±1.63%), M3 (30.88±4.53%), M4 (73.64±3.47%), and M7 (53.48±1.56%). GC-MS showed that the suspected compound which had inhibiting α-glucosidase in methanol fraction were 9-Octadecenoic acid, 1-Heptadecanecarboxylic acid,9,12-Octadecadienoic acid (Z, Z), and Octadecanoic acid methyl ester.

Keywords: α-Glucosidase, Fraction, Methanol, *Sargassum hystrix*

INTRODUCTION

Diabetes mellitus is one of the chronic diseases that occur when the pancreas was no longer able to produce insulin or the insulin produced cannot be utilized by the body (IDF, 2015). The inability of the pancreas to produce insulin or use it effectively causes increased glucose levels in the blood (hyperglycemia) if it lasts in the long term can caused body damage, failure of various organs, and tissues. Among the degenerative diseases, diabetes was one of the non-communicable diseases that its prevalence continues to increase and was expected to increase up to 642 million people by 2040 (IDF, 2015). Indonesia ranked sixth after China, India, the United States, Brazil, and Mexico with a population of 10.3 million people and was expected to increase to 16.7 million by 2045 (IDF, 2017).

Various efforts have been done to overcome diabetes, among others with nonpharmacologic therapy such as weight control, diet, and exercise and pharmacological therapy such as hormone insulin and oral hypoglycemic drugs. Patients generally feel difficult when undergoing nonpharmacologic therapy so that therapy was mostly done pharmacological therapy (Hanafeld, 2007). However, the use of chemical drugs was considered less safe and had side effects such as flatulence, weight gain, and an increase in digestive problems (Stein *et al*., 2014). Nowadays continuous drug searches of natural products to deal with diabetes, especially those come from plants (Lee and Jeon, 2013). The use of natural inhibitors can be an effective therapy in the postprandial management of glycemia with minimal side effects compared to drugs such as acarbose (Souza *et al*., 2012). Acarbose is an oligosaccharide that can delay the breakdown of carbohydrates (Narkedhe *et al*., 2011).

Seaweed is one of the potential natural ingredients to be utilized both in the medical, cosmetic, and food fields, among which is brown seaweed. Brown seaweed is a potential source of metabolites such as carotenoids, laminarin,

alginate, fucoidan, mannitol, and phlorotannin (Demirel *et al*., 2012; Jaswir *et al*., 2011). One type of brown seaweed that has the potential to be developed in the medical field was *Sargassum hystrix*. *S. hystrix* had various activities, among others, can inhibit α-amylase and α-glucosidase (Samudra *et al*., 2015), capable of lowering blood glucose levels of diabetic rats (Nurfahmi *et al*., 2018), and has the highest antioxidant activity compared to *S. polyceratium, S. Angustifolium, S. filipendula, S. cinereum, S. siliquosum, S. mcclurei* (Budhiyanti *et al*., 2012). Thus *S. hystrix* had potential as an antidiabetic, but its compound activity was not widely known. This study aim was to know the inhibitory activity of *S. hystrix* extract and its methanol fraction in inhibiting α-glucosidase activity.

MATERIAL AND METHODS

Materials

The raw material used was *S. hystrix* obtained from Pantai Minajaya, Sukabumi, West Java. Other materials used were phosphate buffer, chloroform, ethyl acetate, and methanol (E-Merck), thin layer chromatography (TLC Silica Gel 60 F254 E-Merck), acarbose (Glucobay), α-glucosidase (Type I Saccharomyces cerevisiae G5003-100UN Sigma-Aldrich), silica gel 60 size 70-230 mesh (E-Merck), p-Nitrophenyl-α-D-glucopyranoside (p-NPG) (Sigma-Aldrich), and Na_2CO_3 (E-Merck). Tools used include blender (Philips), analytical scales (Denver Instrument Company AA-200), chromatography column (Iwaki Pyrex), rotary evaporator (Heidolph Instrument Laborota 4000), GC-MS instrument (Agilent), oven (Eyela Natural Oven NDO-451SD), Incubator (Memmert IN 110), UV-Vis (Pacific Image Electronics), microplate (IWAKI Pyrex), and ELISA microplate reader (Heales MB-580).

Seaweed extraction and partition

S. hystrix extraction was performed according to the method of Yang *et al*. (2011), i.e., 500g samples were extracted by maceration using 4L methanol at room temperature (1:8). Maceration was done for three days with solvent replacement every day. The filtrate obtained was further filtered and evaporated with a rotary evaporator (40°C, 60rpm). The evaporated *S. hystrix* extract was stored at -20°C.

The methanol extract was partitioned using an increasingly polar solvent starting from chloroform, ethyl acetate, and methanol. The dried extract was completely dissolved with a mixture of methanol:water (3:1). The ratio of extract and solvent was 1:15 (w/v). The solution was then partitioned with chloroform solvent (1:1) to obtain chloroform fraction and methanol fraction. The methanol fraction was repartitioned with ethyl acetate solvent to obtain ethyl acetate fraction and methanol fraction. The methanol fraction was concentrated and stored at -2°C.

Methanol fraction separation by chromatography columns

The silica gel was dissolved in 100mL of ethanol and inserted into a 3cm diameter column with a length of 40cm, then eluted using chloroform to ensure no bubbles were formed. The methanol fractions of the partitions of 1-1.5g were fed into the columns and eluted using five graded solvents with step grafting (step gradient polarity). The solvent used was based on the best TLC results. The volume of each solvent used was two times the volume of the column. The eluant was accommodated on a 15mL vial bottle and monitored by TLC. A sample having the same Rf value were combined as one fraction and evaporated.

α-glucosidase inhibitory activity

Inhibitory activity of α-glucosidase was performed according to Mayur *et al*. (2010) with modifications. Samples used were *S. hystrix* extract, partitioned methanol fraction, methanol fraction of column chromatography, and acarbose as a comparison. The preparation of the S1 solution was prepared by preparing as much as 50μL of the pH 7 phosphate buffer inserted into the microplate and then adding 25μL of 0.5mM p-NPG. After that, 25μL samples were added and 25μL of α-glucosidase (0.2Unit/mL) was added. The mixture was incubated for 30min at 37°C. The reaction was discontinued by the addition of 100μL of Na_2CO_3 0.2M. Preparation of the S0 solution was carried out in the same manner, but a phosphate buffer replaced the addition of the α-glucosidase. While the preparation of K and B solutions was carried out in the same manner as S1 and S0, the sample was replaced by a phosphate buffer. Each test system performed three replications. The inhibitory activity was measured by the amount of *p*-nitrophenol produced by measuring its absorbance using an ELISA microplate reader at a wave length of 405nm.

Table I. Effect of acarbose and *S.hystrix* extract concentration on α-glucosidase inhibitory activity

Inhibitor	Concentration (mg/mL)				
	10	5	2.5	1.25	0.625
Acarbose	82.29±3.04	77.52±6.08	62.24±1.53	46.37±2.66	15.22±4.26
S. hystrix extract	97.31±1.45	95.88±1.87	89.34±5.00	69.84±4.00	55.20±5.08

The following formula calculates the percentage of inhibition:

$$\text{Inhibition activity (\%)} = \frac{(K - B) - (S1 - S0)}{(K - B)} \times 100$$

Note: K = control with enzyme addition; B = control without enzyme addition; S1 = sample with addition of enzyme; S0 = sample without addition of enzyme.

Identification of active compound by GC-MS

The active fraction inhibiting the α-glucosidase enzyme was identified by the Gas Chromatography-Mass Spectrometry (GC-MS) method, which was tested at the Center for Forensic Laboratory, Kepolisian Republik Indonesia Jakarta. The steps taken were five µg sample dissolved in methanol. The sample was inserted in the injection port at 290°C. The steam-shaped sample was carried by Helium with a flow rate of 1mL/min through a GC column with an oven temperature starting at 80 to 290°C. Detection of compounds takes place in MS by the mechanism of firing of compounds by electrons into ionized molecules and recording fragmentation patterns. The fragmented mass components were compared with the reference data standard WILEY and (NIST) libraries indicated by a similarity index percentage (SI).

Data analysis

The percentage data of inhibition was then converted to a linear regression equation calculating the IC_{50} value. The IC_{50} values of the linear regression results of each sample were statistically tested using SOVS (one-way ANOVA) and Tukey HSD test with 95% confidence level.

RESULT AND DISCUSSION

Inhibitory activity of α-glucosidase by *S. hystrix* extract

The effect of *S. hystrix* extract concentration on α-glucosidase activity is showed in Table I. The highest concentration was 10mg/mL and the lowest was 0.625mg/mL with mean of inhibitory inhibition *S. hystrix* extract of 97.31±1.46 and 55.21±5.07%, respectively, while acarbose was 82.30±3.047 and 15.23±4.26%. Based on the data (Table I), the higher sample concentration had higher inhibitory activity against α-glucosidase. The inhibitory activity of *S. hystrix* extract was much higher than that acarbose as commercial drugs at all concentrations, so the *S. hystrix* extract was more effective in inhibiting the α-glucosidase than acarbose. This can happen because in the extract of *S. hystrix* there was a content of secondary metabolite compounds such as alkaloids, terpenoids, phenols, and tannins (Nur'aini, 2017) which had ability to inhibit the activity of α-glucosidase, as reported by Kumar *et al*. (2011). Various activities that have been reported include polyphenolic compounds and phlorotannin from *S. hystrix* and *Eucheuma denticulatum* showed the ability to inhibit the α-glucosidase (Pratiwi, 2013). According to Kumar *et al*. (2011) and Matanjun *et al*. (2008), suggest that polyphenol was more prevalent in brown algae than red algae, so this may result in *S. hystrix* extract having higher inhibitory activity against α-glucosidase.

Inhibitory activity of α-glucosidase by *S. hystrix* methanol fraction

Concentrations of 100µg/mL fraction can inhibit enzyme activity at 73.22±0.72% and at the lowest test concentration at 35.26±3.52% (Figure 1). The higher concentration used, the value of the inhibitory activity was also greater. Tests with similar concentrations of acarbose were also performed, but the acarbose did not show any inhibitory and negative values. This may occur due to lack of acarbose inhibitory activity against α-glucosidase at those concentration.

The value of inhibition of each sample then was used to determine the value of IC_{50}. The IC_{50} of *S. hystrix* extract and methanol fraction were 0.35±0.05 and 0.02±0.00 (mg/mL), respectively (Table II). This value was much lower than acarbose which was 1.89±0.12 (mg/mL). Thus, *S. hystrix* extract and methanol fraction have better activity than acarbose, while the methanol fraction has much better activity than acarbose or *S. hystrix* extract.

The IC_{50} of acarbose was almost same as the IC_{50} acarbose obtained by Fitramadan (2013) of 2.1mg/mL, while the value of IC_{50} obtained by Samudra (2013) was much larger at 6.66mg/mL. It was also revealed by Pratiwi (2013) that inhibiting enzyme research was difficult to compare with other studies because the components present in each algae were different. In addition, the method of extraction, purification method, and the level of purity of the active components were also different.

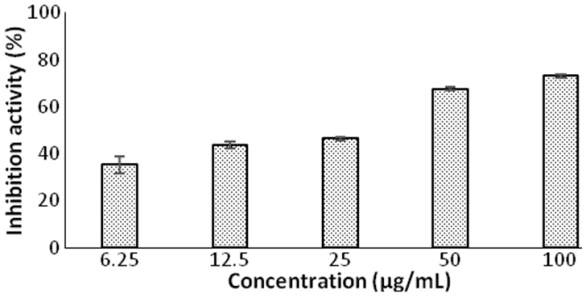

Figure 1. Effect of methanol fraction concentration on α-glucosidase inhibitory activity (Azizi, 2018).

Table II. IC_{50} value of *S. hystrix* extract, methanol fraction and acarbose in inhibiting α-glucosidase.

Sample	IC_{50} (mg/mL)
Acarbose	1.89±0.12[a]
S. hystrix extract	0.35±0.05[b]
Methanol fraction	0.02±0.00[c]

Note: The letters a, b, c show the relationships between treatments. The same letter shows no real difference between treatments and vice versa (Azizi, 2018).

Results showed that *S. hystrix* extract, methanol fraction, and acarbose had significantly different ($P<0.05$). According to Fitramadan (2013), it also showed that the activity of fraction in inhibiting α-glucosidase was higher than that of acarbose.

Inhibitory activity of α-glucosidase by methanol fraction of *S. hystrix*

Separation was obtained by 7 fractions and each was tested for inhibiting activity at concentration 150µg/mL (Figure 2). Fraction M1 has no inhibitory activity indicated by a negative inhibitory value, this was because fraction 1 was a composite of the first 10 vials. The initial shelter was assumed to be a chloroform solvent before the first solvent descends, so that at the initial running the compound contained in the sample has not yet descended. The M2, M3, M4 fractions indicate inhibition with a substantial inhibition of 23.46±1.63, 30.88±4.53, and 73.64±3.47%. The M4 fraction has the greatest inhibitory activity. It was possible that the compound in the M4 sample was the most important compound in inhibiting the activity of the α-glucosidase in the methanol fraction. The M5 fraction does not indicate inhibitory activity. This may be due to the fraction of M5 being the result of a solvent containing no content of the compound. This was supported by the clear color of the container and when the separation of TLC there was no spot or stain formed. The fractions of M6 and M7 indicate the presence of inhibitory activity with respective inhibitory values of 9.03±0.73 and 53.48±1.56%. This may be due to the fraction being a polar solvent. The TLC results also show that the compounds detected in the methanol fraction were compounds that have a relatively high degree of polarity.

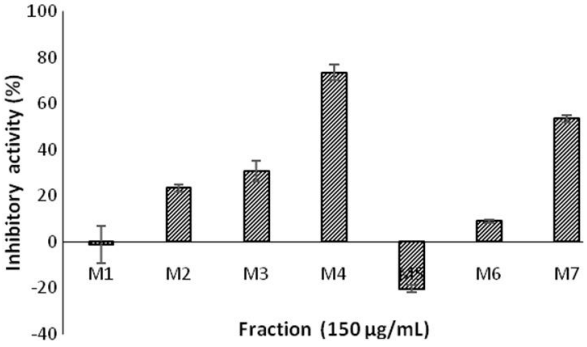

Figure 2. Inhibitory activity of *S. hystrix* fractions in inhibiting α-glucosidase activity (Azizi, 2018).

Identification of active compound using GC-MS

GC-MS analysis was performed against fraction which able to inhibit α-glucosidase activity that fraction of M2, M3, M4, and M7 (Table III). The 9-Octadecenoic acid compounds were present in the fractions of M2, M3, and M4 with the largest percentage of areas 70.63, 66.80, 79.41 (%), respectively. Based on the fraction inhibition values and percentage of area formed, more dominant the 9-Octadecenoic acid compounds in the sample, the greater inhibitory activity of α-glucosidase. According to Yang, *et al*. (2017), 9-Octadecenoic acid has an inhibitory activity against α-glucosidase with an IC_{50} value of 4.8±0.9µg/mL. Compound 9-Octadecenoic acid was a long-chain unsaturated fatty acid and was a fatty acid with a wide and abundant presence in nature (NCBI, 2017a).

Table III. Compounds were suspected as α-glucosidase inhibitors in the active fraction

Compounds	Area (%)				α-glucosidase inhibitors	
	M2	M3	M4	M7	Inhibiting ability	Reference
9-Octadecenoic acid	70.63	66.80	79.41	-	IC_{50} 4.8±0.9µg/mL	Yang, *et al.*, 2017
1-Heptadecanecarboxylic acid/ Octadecanoic acid	3.69	4.64	6.46	-	IC_{50} 46.2±2.5µg/mL	Yang, *et al*., 2017
9,12-Octadecadienoic acid (Z,Z)- / Linoelaidic acid	-	0.32 & 0.96	2.10 & 0.84	-	IC_{50} 3.4±1.5µg/mL	Yang, *et al*., 2017
Octadecanoic acid, methyl ester	-	-	-	8.10	78.1±5.2 %	Artanti, *et al*., 2012

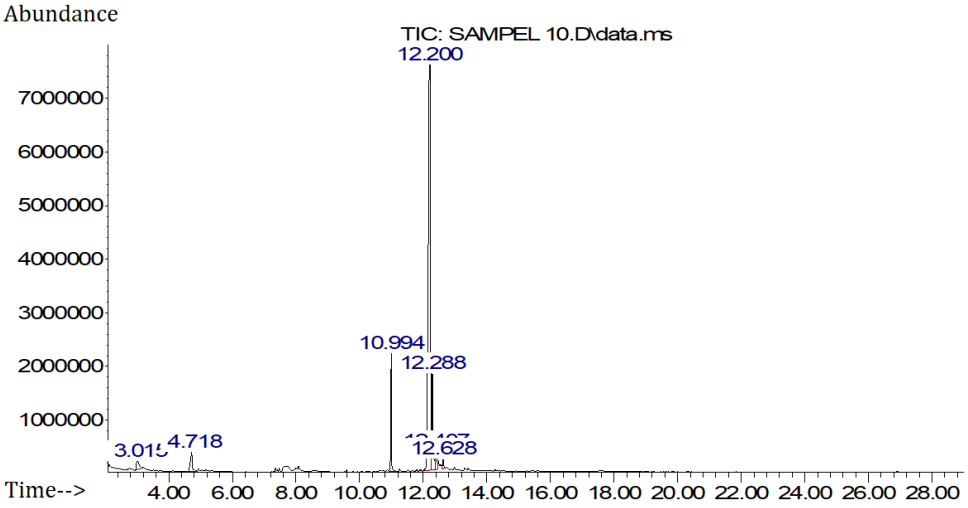

Figure 3. Chromatogram of *S. hystrix* M4 fraction by GC-MS analysis (Azizi, 2018).

These fatty acids were soluble in organic solvents such as ether, acetone, benzene, chloroform, carbon tetrachloride, ethanol, methanol (Lide and Milne, 1994), and water with 0.01 mg/L solubility at temperature 25°C (NCBI, 2017a). The 9-Octadecenoic acid fatty acid has a molecular weight of 282g/mol with a boiling point of 286°C at 100 mm Hg and a melting point of 16.3°C (NCBI, 2017a).

The 1-Heptadecanecarboxylic acid compound was present in the fraction of M2, M3, M4 with a small percentage of area of 3.69, 4.64, 6.46 (%), respectively. According to Yang *et al*. (2017), the 1-Heptadecanecarboxylic acid compound has inhibitory activity against α-glucosidase with IC_{50} value of 46.2±2.5µg/mL. The 1-Heptadecanecarboxylic acid compound also called Octadecanoic acid or stearic acid was a long-chain saturated fatty acid with 18 carbon atoms. Stearic acid was found in various fats of animals and plants. Stearic acid was white solid as crystalline or powder and it smells light. Stearic acid has a molecular weight of 284g/mol with a boiling point of 232°C at 15mm Hg and a melting point of 68.8°C. Stearic acid dissolves in acetone, chloroform, carbon disulphide (NCBI, 2017b), slightly soluble in ethanol, benzene and water soluble solvents with 0.597mg/L solubility at 25°C (Yalkowsky and Yan, 2003).

Compounds 9,12-Octadecadienoic acid were present in the fractions of M3 and M4 (Figure 3) with a percentage of each area of 0.32 and 2.10 (%). According to Yang, *et al*. (2017), 9.12-Octadecadienoic acid has an inhibitory activity against high α-glucosidase with an IC_{50} value of 3.4±1.5µg/mL. Compound 9.12-Octadecadienoic acid was a polyunsaturated fatty acid widely found in glycoside plants. These fatty acids were essential fatty acids for mammalian nutrients and were used in the biosynthesis of prostaglandins and cell membranes. This fatty acid has a chemical formula $C_{18}H_{32}O_2$ with a molecular weight of 280 g/mol, a boiling point at 230°C (16mm Hg) and a melting point at -6.9°C. These fatty acids were

soluble in acetone, benzene, ethyl ether, and ethanol (Haynes, 2013) as well as water (1.59mg/L at 25°C) (NCBI, 2017c).

Octadecanoic acid compound, methyl ester was present in M7 fraction with area percentage of 8.10%. According to Artanti *et al*. (2012), the inhibition activity of Octadecanoic acid compound, methyl ester on α-glucosidase was 78.1±5.2% at 10μg/mL. The presence of Octadecanoic acid, methyl ester in the M7 fraction was not a dominant compound. Based on TLC results, M7 fraction was a fraction that has a high polarity level so it was suspected that there were non volatile compounds that can not be detected using GC-MS and have inhibitory activity against α-glucosidase. Octadecanoic acid compound, methyl ester was a long chain fatty acid with 19 carbon atoms. The trivial name of this fatty acid was methyl ester stearic acid. Octadecanoic acid, a crystal-shaped white methyl ester, has a molecular weight of 298 g/mol with a boiling point of 443°C and a melting point of 39.1°C. Octadecanoic acid, methyl ester soluble in ether, chloroform, alcohol, and soluble solvents in water (NCBI, 2017d),

The compounds in the fractions analyzed using GC-MS in this study were fatty acid compounds and some alcohol groups. This was similar to a study by Egua *et al*. (2013), ie fatty acid compounds or volatile oils were successfully isolated in polar fractions such as n-butanol and water. This was supported by the opinion of Lide & Milne (1994) which states that fatty acids can be isolated by methanol solvent. The absence of other types of polar compounds based on the results of identification using GC-MS was possible because GC-MS was a tool instrument capable of identifying volatile compounds. Non-volatile compounds can not be detected, so it was suspected that some compounds in the sample are undetectable by GC-MS especially non-volatile compounds, new compounds, or high-polarity compounds.

The type of inhibition performed by each isolated compound from the methanol fraction of the specific α-glucosidase was not yet known. In general, based on research conducted by Nguyen & Kim (2015) and Su *et al*. (2013), fatty acids such as oleic acid have high inhibitory activity against the α-glucosidase with the type of inhibition performed was competitive inhibition. The same type of inhibition was also carried out by compounds isolated from the green algae *Enteromorpha prolifera* and red algae *Polyopes lancifolia* (Chen *et al*., 2016; Kim *et al*., 2010). Commercial drugs such as acarbose also have a competitive inhibitory type by blocking the enzymatic reaction due to the presence of nitrogen groups in the compound (Goldstein, 2008).

Methanol extract and fraction of *S. hystrix* has a better ability than acarbose in inhibiting α-glucosidase acitivity. It's possible because *S. hystrix* extract contained several secondary metabolites which have been reported to have inhibitory activity against α-glucosidase such as alkaloids, terpenoids, phenols, and tannins (Nur'aini, 2017) while acarbose consists of purer compounds namely an oligocasaccharide. The methanol fraction from the extract of *S. hystrix* also had better inhibitory activity compared to acarbose. Based on the results of analysis by GC-MS, the resulting compounds in the form of fatty acids and dominant fatty acids contained in the fraction of fatty acids that have been reported to have good inhibitory activity against the α-glucosidase (Table III).

CONCLUSION

Methanol fraction of *S. hsytrix* extract able to inhibit α-glucosidase with IC_{50} value of 19μg/mL. Methanol fraction of column chromatographic separation having inhibitory activity against α-glucosidase were M2 fraction (23.46±1.63%), M3 (30.88±4.53%), M4 (73.64±3.47%), and M7 (53.48±1.56%). Compounds in the methanol fraction of *S. hystrix* extract suspected to actively inhibit α-glucosidase include 9-Octadecenoic acid, 1-Heptadecanecarboxylic acid, 9.12-Octadeca-dienoic acid (Z, Z) -, and Octadecanoic acid methyl esters.

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