

IN VITRO ANTIDIABETIC ACTIVITY OF SARGASSUM HYSTRIX EXTRACT AND ITS ETHYL ACETATE FRACTIONS

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ABSTRACT

Seaweed has great potential in the pharmaceutical field, including as an antidiabetic agent. The purpose of this study was to isolate and identify the active fraction of *Sargassum hystrix* that inhibits α -amylase and α -glucosidase. *S. hystrix* was subjected to extraction using methanol, followed by partitioning using chloroform, ethyl acetate, and methanol. The ethyl acetate fraction was then separated by column chromatography to obtain more pure fractions. The crude extract, ethyl acetate fraction, and column chromatography fraction were tested for their ability to inhibit α -glucosidase. The active fractions of ethyl acetate that inhibited α -amylase and α -glucosidase were further identified using gas chromatography-mass spectrometry (GC-MS). The results showed that the seaweed *S. hystrix* has potential as an antidiabetic substance.

The ethyl acetate fraction of the *S. hystrix* extract had $IC_{50}=0.014$ mg/mL for α -amylase inhibition and $IC_{50}=0.009$ mg/mL for α -glucosidase inhibition. The compounds presumed to have inhibitory activity against α -amylase ($58.52\pm 0.40\%$ inhibition at 50 μ g/mL) and α -glucosidase ($39.76\pm 0.03\%$ inhibition at 150 μ g/mL) were 1,2-benzenedicarboxylic acid, 1,3,5-benzenetriol, flamenol, and eicosanoic acid. The pure active compound of the ethyl acetate fraction was pentadecanoic acid, which was suspected to be an inhibitor, with $67.38\pm 6.84\%$ inhibition of α -amylase activity at 50 μ g/mL and $18.90 \pm 2.82\%$ inhibition of α -glucosidase activity at 150 μ g/mL.

Keywords: *Sargassum hystrix*, antidiabetic activity, α -glucosidase, ethyl acetate fraction

1. INTRODUCTION:

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (ADA, 2012). Chronic hyperglycemia in diabetes is related to long-term damage, dysfunction or failure of several organs of the body, especially the eyes, nerves, heart and blood vessels (Elekofehinti et al., 2013). The WHO (2012) reported that approximately 346 million people in the world suffer from DM. Among degenerative diseases, diabetes is a noncommunicable disease whose prevalence continues to increase and is expected to increase to 642 million people by 2040 (IDF, 2016). In terms of diabetes, Indonesia ranks sixth after China, India, the United States, Brazil, and Mexico, with 10.3 million people affected, and this number is expected to increase to 16.7 million by 2045 (IDF, 2017).

Various efforts have been made to overcome diabetes, including with nonpharmacologic therapy such as weight control, diet, and exercise and pharmacological therapy such as hormone insulin and oral hypoglycemic drugs. Patients generally experience difficulty when undergoing nonpharmacologic therapy, so therapy is mostly performed with pharmacological therapy (Hanafeld, 2007). However, the use of chemical drugs has been considered less safe and has led to side effects such as flatulence, weight gain, and an increase in digestive problems (Stein et al., 2014). Currently, there is an ongoing search for natural product-based drugs to deal with diabetes, especially those derived 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).

Marine algae are a potential source of bioactive secondary metabolites with high development potential as new pharmaceutical agents. Bioactive compounds from brown marine algae have potential as antitumor, antifungal, antiviral, antioxidant, antihypertensive and antidiabetic agents (Gamal, 2010; Husni et al., 2014; Gotama et al., 2018). One type of brown seaweed that has the potential to be developed in the medical field is *Sargassum hystrix*. *S. hystrix* has various activities, including the ability to lower the blood glucose levels of diabetic rats (Nurfahmi et al., 2018), and has the highest antioxidant activity compared to *S. polyacratium*, *S. angustifolium*, *S. filipendula*, *S. cinereum*, *S. siliquosum*, and *S. mcclurei* (Budhiyanti et al., 2012). Thus, *S. hystrix* has potential as an antidiabetic agent, but its compound activity is not widely known. This study aimed to determine the inhibitory activity of *S. hystrix* extract and its ethyl acetate fractions in inhibiting α -amylase and α -glucosidase activity.

2. MATERIALS AND METHODS:

1.1. Materials

The main material used in this study was *S. hystrix* seaweed obtained from the coast of Gunungkidul Yogyakarta, Indonesia. The materials used for the extraction were ethanol, methanol, ethyl acetate, and sodium carbonate (E. Merck, Germany). The materials used to analyze the α -glucosidase inhibition activity were α -glucosidase from

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Saccharomyces cerevisiae type I (Sigma-Aldrich, USA), p-nitrophenyl- α -D-glucopyranoside (Sigma-Aldrich, USA), and acarbose (Bayer Pharmaceuticals, Indonesia).

1.2. Seaweed Extraction and Partitioning

S. hystrix extraction was performed as stated by Yang et al. (2011); i.e., 500 g samples were extracted by maceration using 4 L of methanol at room temperature (1:8). Maceration was conducted for three days with solvent replacement every day. The filtrate obtained was further filtered and evaporated using a rotary evaporator (40°C, 60 rpm). The evaporated *S. hystrix* extract was stored at -20°C. The methanol extract was subdivided using increasingly polar solvents from chloroform to ethyl acetate to methanol. The dried extract was completely dissolved by a mixture of methanol:water (3:1). The ratio of extract and solvent was 1:15 (w/v). The solution was then partitioned with ethyl acetate (1:1) to obtain the ethyl acetate fraction and methanol fraction. The methanol fraction was repartitioned with ethyl acetate to obtain the ethyl acetate fraction and methanol fraction. The ethyl acetate fraction was concentrated and stored at -20°C.

1.3. Ethyl Acetate Fraction Separation by Chromatography Columns

Silica gel was dissolved in 100 mL of ethanol, inserted into a column (3 cm diameter, 40 cm length) and then equilibrated using chloroform to ensure that no bubbles were formed. The ethyl acetate fractions of the partitions of 0.5-1.0 g were fed into the columns and eluted using five graded solvents with a 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 in a 15 mL vial bottle and monitored by TLC. A sample having the same R_f value was combined as one fraction and dried by evaporation.

1.4. Inhibition of α -Amylase Activity

The inhibitory activity against α -amylase was determined according to Husni et al. (2018) with modifications. A volume of test solution was made from 25 mL of sample extract at different concentrations and 25 mL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing 13 U/mL α -amylase. The test solution was mixed using a vortex mixer and incubated at 37°C for 10 min. Then, 25 mL of 1% soluble starch in 0.02 M sodium phosphate buffer was added to the test solution, and it was incubated at 37°C for 10 min. Next, the solution was further treated by the addition of 50 mL of 96 mM 3,5-dinitrosalicylic acid (DNS) and incubated for 5 min in a water bath. The solution was cooled at room temperature, and its absorbance was recorded at a wavelength of 550 nm. Absorbance values of samples were obtained and used to calculate the percentage (%) inhibition of the enzyme.

$$\text{Percentage Inhibition} = \frac{K-(S1-S0)}{K} \times 100\% \quad (\text{Eq. 1})$$

where:

K= Absorbance of control-blank

S1= Absorbance of sample with enzyme

S0= Absorbance of sample without enzyme

1.5. Inhibition of α -Glucosidase Activity

An inhibition test of α -glucosidase was performed as stated by Mayur et al. (2010). A test solution consisting of 50 mL of 0.1 M phosphate buffer (KH₂PO₄), pH 7, 25 mL of 0.5 mM p-nitrophenyl- α -D-glucopyranoside (PNP-G, as the substrate), 10 mL of the simple extract at various concentrations, and 25 mL of 0.2 U/mL α -glucosidase. The solution was mixed and incubated at 37°C for 30 min. The reaction was stopped by the addition of 100 mL of 0.2 M Na₂CO₃. Inhibition of enzyme activity was measured by the amount of p-nitrophenol formed by measuring the absorbance using a microplate reader at a wavelength of 405 nm. Absorbance values were obtained and then used to calculate the percentage inhibition of the enzyme.

$$\text{Percentage Inhibition} = \frac{K-(S1-S0)}{K} \times 100\% \quad (\text{Eq. 2}) \quad \text{where:}$$

K= Absorbance of control-blank

S1= Absorbance of sample with enzyme

S0= Absorbance of sample without enzyme

1.6. Identification of Active Compounds by GC-MS

The active fraction inhibiting α -amylase and α -glucosidase was identified by the gas chromatography-mass spectrometry (GC-MS) method, which was tested at the Center for Forensic Laboratory, Kopolisian Republik Indonesia Jakarta. Five micrograms of each sample dissolved in methanol was used. The sample was injected in the injection port at 290°C. The volatilized sample was carried by helium with a flow rate of 1 mL/min through a GC column with an oven temperature ramping from 80 to 290°C. Detection of compounds takes place in the MS system by the mechanism of bombarding compounds with electrons to form ionized molecules and recording fragmentation patterns. The fragmented mass components were compared with the reference data standard WILEY and NIST libraries, as indicated by a similarity index percentage (SI).

1.7. Statistical analysis

The data in this study were extract concentration versus percent inhibition of the enzyme, and the data were then plotted to obtain the regression equation. The IC₅₀ activity value of the *S. hystrix* extract and its fraction against α -glucosidase were obtained from the regression equation. The IC₅₀ values were tested statistically with one-way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS) at 95%.

3. RESULTS AND DISCUSSION:

3.1. Inhibition of α -Amylase Activity

The α -amylase inhibitory activity of the *S. hystrix* extract and standard (acarbose) can be seen in Figure 1, which indicates that the higher concentrations used increased the α -amylase inhibitory activity. Acarbose at the highest concentration (10 mg/mL) had an inhibitory activity of 91.97±6.67%, and at the lowest concentration (0.625 mg/mL), it had an inhibitory activity of 25.83±3.65%; by comparison, the *S. hystrix* extract at the highest concentration (10 mg/mL) had an inhibitory activity of

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96.51±2.053% and at the lowest concentration (0.625 mg/mL) had an inhibitory activity of 67.31±4.49%. Thus, the inhibitory activity of the *S. hystrix* extract was higher than that of acarbose. Samudra et al. (2015) reported that *S. hystrix* extract exhibited inhibitory activity against α -amylase due to the presence of polyphenol compounds in *S. hystrix* seaweed that could act as α -amylase inhibitors. Other studies also reported that the α -amylase inhibitory activity by *S. polycystum* and *S. wightii* extracts as well as *S. yezoense* extract was higher than that of acarbose (Unnikrishnan et al. 2015; Park et al., 2017).

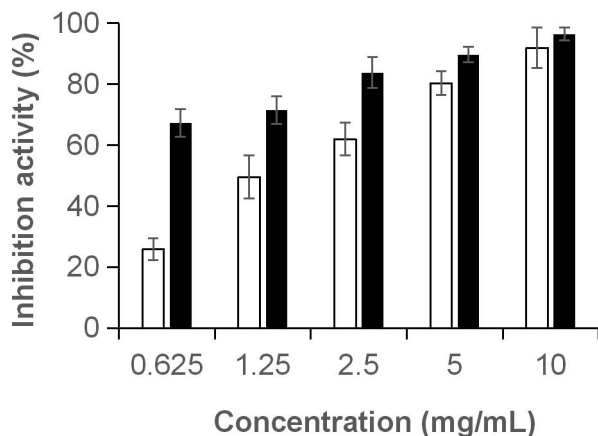


Figure 1. Effect of *Sargassum hystrix* extract () and acarbose () concentration on the inhibition activity of α -amylase.

The α -amylase inhibitory activity of the ethyl acetate fraction of the *S. hystrix* extract can be seen in Figure 2, which shows that the lowest concentration (0.024 μ g/mL) has an inhibitory activity of 54.44±2.69% and that the highest concentration (0.390 μ g/mL) has an inhibitory activity of 92.45±2.36%. The inhibitory activity of the *S. hystrix* ethyl acetate fraction in this study was much higher than that of the *Sargassum polycystum* ethyl acetate fraction, which was 77.00% at a concentration of 1000 μ g/mL, and the ethyl acetate fraction of *Myagropsis myagroides*, which was 58.43% at a concentration of 5 mg/mL (Unnikrishnan et al., 2015; Pak et al., 2015).

Table 1 shows the IC_{50} values of α -amylase inhibition by the *S. hystrix* extract, ethyl acetate fraction, and acarbose. There were significant differences between the IC_{50} values of the *S. hystrix* extract, ethyl acetate fraction, and acarbose. These values indicated higher inhibitory activity than that of other marine algae, for example, the chloroform extract of *Chaetomorpha aerea* (IC_{50} -408.90 μ g/mL) and methanol extract of *Chlorodesmis* (IC_{50} -147.60 μ g/mL) (Unnikrishnan et al., 2015). Senthilkumar & Sudha (2012) reported that the IC_{50} values of water extracts of marine algae (*S. polycystum*, *R. corticata* and *G. lactuca*) on α -amylase inhibition were 60 μ g/mL, 67 μ g/mL, and 82 μ g/mL, respectively. Nwosu et al. (2011) reported that the methanol extract of *A. nodosum* has a relatively smaller IC_{50} value (0.1 μ g/mL).

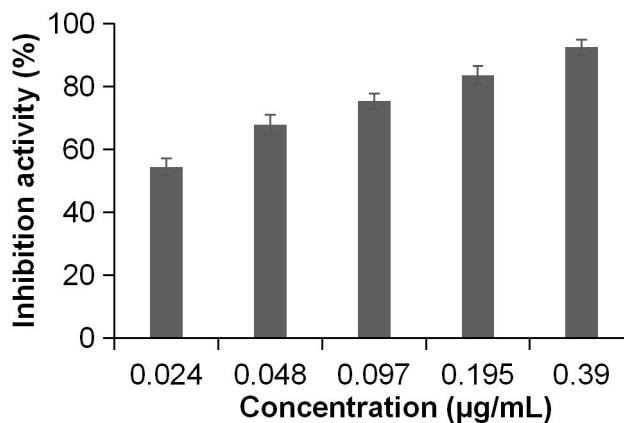


Figure 2. Effect of ethyl acetate fraction concentration on the inhibition of α -amylase activity.

3.2. Inhibition of α -Glucosidase Activity

The ability of each sample to inhibit the activity of α -glucosidase is presented in Figure 3. The results obtained showed that 10 mg/mL acarbose inhibited α -glucosidase by 82.39±3.03%, while 10 mg/mL *S. hystrix* extract inhibited the activity by 97.31±1.46%. The results are supported by research from Unnikrishnan et al. (2015), in which the *S. polycystum* methanol extract had 96.00% α -glucosidase inhibition at 1 mg/mL (better than in our study) compared with a value of 88% with 1 mg/mL acarbose. This result confirms that the *S. hystrix* extract has a better ability to inhibit the activity of α -glucosidase than the commercial drug acarbose.

The results obtained for the ethyl acetate fraction of the *S. hystrix* extract in terms of inhibiting α -glucosidase activity were 84.47±4.01% at 100 μ g/mL and 44.38±7.50% at 6.25 μ g/mL (Figure 4). According to Firdaus and Prihanto (2014), the use of ethyl acetate with *Sargassum* sp. led to the highest value of α -glucosidase activity inhibition compared with the use of n-hexane and ethanol; in addition, the ethyl acetate fraction inhibited α -glucosidase better than acarbose (15.26±4.26%) at the lowest concentration of 625 μ g/mL. This result is supported by research by Unnikrishnan et al. (2015), who noted that the use of ethyl acetate with *Sargassum wightii* led to an α -glucosidase inhibitory value of 91.00% at a concentration of 1000 μ g/mL, higher than the value of 88% with the equivalent concentration of acarbose.

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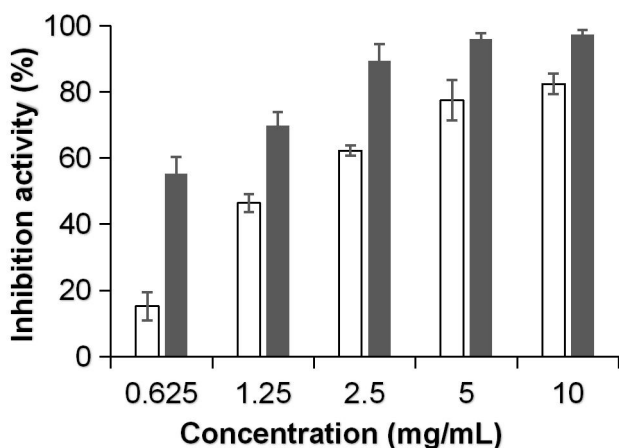


Figure 3. Effect of *Sargassum hystrix* extract () and acarbose () concentration on the inhibition activity against α -glucosidase.

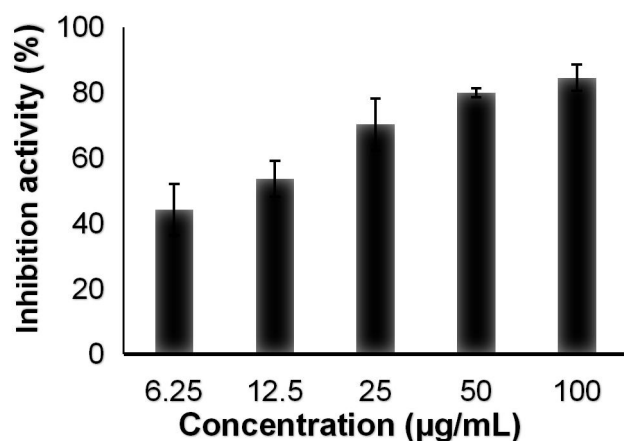


Figure 4 - Effect of ethyl acetate fraction concentration on the inhibition of α -glucosidase activity.

The inhibition activity against α -glucosidase showed that the extract of *S. hystrix* is a better inhibitor of α -glucosidase than acarbose. Based on the IC_{50} values of the three samples that were tested, the inhibition of α -glucosidase activity from the largest to smallest value was acarbose, *S. hystrix* extract and its ethyl acetate fraction (Table 1). The resulting inhibition values of the *S. hystrix* extract, ethyl acetate fraction, and acarbose were significantly different ($P < 0.05$). According to Fitriamadan (2013), the activity of the fraction in inhibiting α -glucosidase was higher than that with acarbose. Nur'aini et al. (2018) reported that the extract of *S. hystrix* contained secondary metabolite compounds such as alkaloids, terpenoids, phenols, and tannins, which could inhibit the activity of α -glucosidase. According to Kumar et al. (2012) and Matanjun et al. (2008), polyphenol is more prevalent in brown algae than red algae, so this may result in the *S. hystrix* extract having higher inhibitory activity against α -glucosidase.

Phenols are one of the bioactive components that can inhibit the activity of α -amylase and α -glucosidase. Polyphenols inhibit these enzymes in the breakdown of

carbohydrates into glucose. Phenol has an inhibitory effect on α -amylase through bond hydroxylation and ring substitution on the β bond. The principle is similar to that of inhibition by acarbose, i.e., by promoting delays, disaccharide carbohydrate hydrolysis and absorption of glucose and inhibiting the metabolism of sucrose into glucose and fructose (You et al., 2012). On the other hand, phlorotannin is another phenolic component that can inhibit the function of α -amylase and α -glucosidase. As a polyphenol, phlorotannin inhibits enzymes that work in the breakdown of carbohydrates into glucose. The principle is also similar to that of acarbose inhibition, which promotes a delay in hydrolysis and absorption of carbohydrates, and disaccharides inhibit the metabolism of glucose and sucrose into glucose and fructose (You et al., 2012).

Table 1. Inhibition activity (IC_{50}) values against α -amylase and α -glucosidase of the *Sargassum hystrix* extract, its ethyl acetate fraction and acarbose

Inhibitors	IC_{50} against α -amylase (mg/mL)	IC_{50} against α -glucosidase (mg/mL)
Acarbose	1.506 \pm 0.040 ^a	1.891 \pm 0.147 ^a
<i>Sargassum hystrix</i> extract	0.143 \pm 0.040 ^b	0.344 \pm 0.052 ^b
Ethyl acetate fraction	0.014 \pm 0.002 ^c	0.009 \pm 0.002 ^c

Values with different letters (a-c) in the same column indicate a significant difference ($P < 0.05$).

3.3. Inhibition of the α -Glucosidase Activity of Fractions

Ethyl acetate fractions were obtained after the ethyl acetate fraction was separated by column chromatography. The resulting ethyl acetate fractions were then combined based on the Rf value and the appearance of the solution color. The inhibitory activity against α -amylase and α -glucosidase of the ethyl acetate fraction after separation using column chromatography can be seen in Figure 5. At an ethyl acetate fraction concentration of 50 μ g/mL, the inhibitory activity against α -amylase ranged from 44.44 \pm 3.78 to 90.13 \pm 2.46%, while that against α -glucosidase (with a concentration of 150 μ g/mL) ranged from 10.35 \pm 1.28 to 58.61 \pm 0.46%. This inhibitory activity was greater than that of the n-hexane fractions, which was 19.16% at a concentration of 1 mg/mL (Pak et al., 2015).

The resultant inhibitory activity of the ethyl acetate fractions (F1-F8) was better than that of acarbose (55.21 \pm 5.09% at a concentration of 625 μ g/mL) in inhibiting α -glucosidase activity. Husni et al. (2014) reported that acarbose at a concentration of 1.74 \pm 0.25 mg/mL can inhibit 50% of the α -glucosidase activity. This result was in accordance with Subramanian et al. (2008), suggesting that acarbose could inhibit 50% of the α -glucosidase activity at a concentration of 6.2 \pm 0.33 mg/mL.

3.4. Active Compounds of the Ethyl Acetate Fractions

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Based on the results of the inhibition test shown in Figure 5, F6 has the highest yield, followed by further separation using preparative thin-layer chromatography. Based on the results of preparative TLC, an analysis using GC-MS was conducted. The GC-MS results found many fatty acids (FAs) such as benzenepropanoic acid, hexadecanoic acid, eicosanoic acid, 1,2-benzenedicarboxylic acid or phthalic acid and pentadecanoic acid. Some FA compounds that appear in the GC-MS results are reported to have potential as antidiabetic agents. Fatty acid compounds are able to bind to the active side of the substrate so that the active site of the enzyme cannot react with the substrate (Su et al., 2013). FA has double bonds that play a binding role in enzymes; the greater the number of double bonds is, the greater the inhibitory activity is (Teng & Chen, 2016).

1,2-Benzenedicarboxylic acid or phthalic acid has α -amylase and α -glucosidase inhibitor activity both in vitro and in vivo in Wistar rats (Save et al., 2015; Al-Hajj et al., 2016). Hexadecanoic acid or palmitic acid is known to be a free fatty acid that has anti-inflammatory and antidiabetic activity (Channabasava et al., 2014). Eicosanoic acid or arachidic acid is also referred to as having antidiabetic potential. The administration of arachidic acid supplements to Goto-Kakizaki rats, which are a genetic diabetes model, can reduce blood sugar levels by increasing insulin sensitivity (Song et al., 2003).

The GC-MS results indicate the presence of pure or single compounds found at spot E8, namely, pentadecanoic acid compounds. There are no specific reports on the activity of pentadecanoic acid directly regarding antidiabetic activity, but according to Gomathi & Elango (2015), pentadecanoic acid in the medicinal plant *Evolvulus alsinoides* has potential as an antioxidant. *Malva parviflora* seeds containing nine free fatty acids with 30.05% pentadecanoic acid are known to reduce blood glucose, LDLc, and TBARS levels while increasing plasma insulin and HDLc levels in diabetic rats (El-Gizawy & Hussein, 2015), so pentadecanoic acid is also considered to have antidiabetic potential; however, further research is needed to determine the ability of pentadecanoic acid compounds to inhibit α -amylase and α -glucosidase.

4. CONCLUSIONS:

The seaweed *S. hystrix* has potential as an antidiabetic substance. The ethyl acetate fraction of the *S. hystrix* extract had $IC_{50}=0.014$ mg/mL for α -amylase inhibition and $IC_{50}=0.009$ mg/mL for α -glucosidase inhibition. The compounds presumed to have inhibitory activity against α -amylase ($58.52\pm 0.40\%$ at 50 μ g/mL) and α -glucosidase ($39.76\pm 0.03\%$ at 150 μ g/mL) were 1,2-benzenedicarboxylic acid, 1,3,5-benzenetriol, flamenol, and eicosanoic acid. The pure active compound of the ethyl acetate fraction was pentadecanoic acid, which was suspected to be an inhibitor, with $67.38 \pm 6.84\%$ inhibition of α -amylase activity at 50 μ g/mL and $18.90 \pm 2.82\%$ inhibition of α -glucosidase activity at 150 μ g/mL.

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In Vitro Antidiabetic Activity Of Sargassum Hystrix Extract And Its Ethyl Acetate Fractions

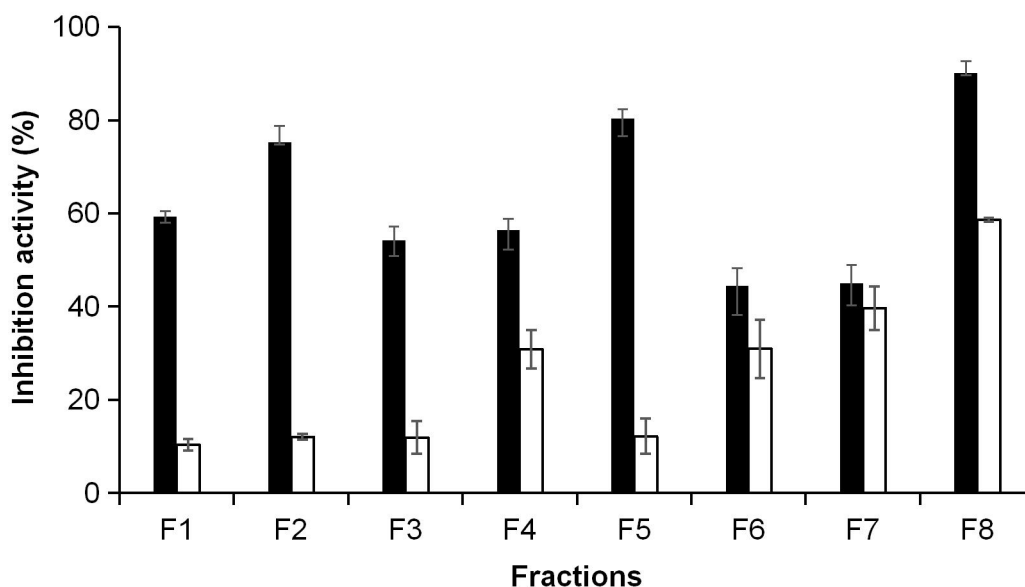


Figure 5. Inhibition activity against α -amylase () and α -glucosidase () by ethyl acetate fractions

Table 2. Compounds from *Sargassum hystrix* suspected to be inhibitors of α -amylase and α -glucosidase

Compound	Area (%) of Fraction			
	E2	E3	E6	E8
Eicosanoic acid			3.51	
1,3,5-Benzenetriol			67.00	
Flamenol			14.11	
1,2-Benzenedicarboxylic acid		5.33		
Hexadecanoic acid	27.47	13.60	6.75	
Pentadecanoic acid				100

Note: Fractions E2, E3, E6 and E8 of the ethyl acetate fractions after fractionation by preparative TLC.