Inhibitory activity of *Sargassum hystrix* extract and its chloroform fractions on inhibiting the α-glucosidase activity

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Abstract. Seaweed is one of the marine biota that has several benefits for health and cosmetics. The objective of this study was to isolate and test the activity of chloroform fraction of *Sargassum hystrix* seaweed extract in inhibiting the α-glucosidase and identification of the active fraction compounds. *S. hystrix* was extracted by maceration, then partitioned using chloroform and methanol. The chloroform fraction was further separated by column chromatography to obtain purer compounds. The extract of *S. hystrix*, chloroform fraction resulting from partitioning and chloroform fractions from column chromatography were tested for inhibitory activity against the α-glucosidase. The active chloroform fraction inhibits the α-glucosidase then identified its compounds using Gas Chromatography-Mass Spectrophotometry (GC-MS). The result showed that chloroform fraction (IC\textsubscript{50} = 0.726 mg/mL) had a higher inhibitory activity of the α-glucosidase than acarbose (IC\textsubscript{50} = 1.890 mg/mL). Column chromatography separation produced seven chloroform fractions. The highest inhibition activity was found in the second chloroform fraction (41.62%), third (51.39%), and fourth (30.49%). Based on GC-MS analysis, several compounds were thought to be able to inhibit the α-glucosidase. The identified compounds were 2-Hexadecene-1-ol, 3,7,11,15-tetramethyl-; Hexadecanoic acid, methyl ester; 9-Octadecenoic acid, methyl ester; Phytol; Stigmasta-5,24(28)-dien-3-ol, (3.beta,24Z)- and 6-Hydroxy-4,4,7a-trimethyl-5,6,7a-tetrahydro benzofuran-2 (4H )-one.

1. Introduction

Diabetes mellitus (DM) is a metabolic disease with characteristic hyperglycemia that occurs due to insufficiency of insulin function [1]. WHO [2] reported that in 2014 around 422 million adults suffered from diabetes mellitus, when compared to 1980, which was 108 million people, diabetes mellitus increased from 4.7% to 8.5% of the world's population. DM caused 1.5 million deaths in 2012.

One therapy for treating diabetes is to reduce postprandial blood glucose levels by inhibiting α-glucosidase [3]. Acarbose is a drug that works competitively to inhibit α-glucosidase [4]. The use of chemical drugs is considered less safe and has side effects, so there are many efforts to search for natural ingredients that have the potential to be antidiabetic, including natural ingredients from marine organisms [5]. *Sargassum* sp. is one type of brown seaweed that has the potential as an antidiabetic...
2. Bioactive compounds in *Sargassum sp*. Antidiabetic potentials, such as alkaloids, phenols, flavonoids, glycosides, lipids, proteins, and carbohydrates, and terpenoids [7,8]. One type of brown seaweed which has the potential as an antidiabetic was *Sargassum hystrix* [1]. Research that examines the content of secondary metabolites in *S. hystrix* has not been widely used, especially regarding active compounds as inhibitors of α-glucosidase enzymes. Fitramadan [9] reported that loliolida, halogenated furanone, and 4,4'-dihydroxy biphenyls in ethyl acetate fractions of *S. hystrix* were able to inhibit the activity of these enzymes.

Seaweed has a lot of secondary metabolites contents, to obtain and identify these compounds it is necessary to do the separation with the right solvent. According to Widodo [10], the chloroform fraction can be used to isolate secondary metabolites, which are soluble in semi-polar solvents. Chloroform fraction in *Ficus deltoidea* plants with a concentration of 5000 µg/mL (0.5%) was able to inhibit the action of the α-glucosidase by 6.49% [11]. Based on this, the chloroform fraction of *S. hystrix* extract is thought to have acted as an inhibitor of the α-glucosidase enzyme. Therefore, this research is expected to be used as a reference for the development of antidiabetic drugs through further research.

2. Materials and methods

2.1. Materials

The raw materials used were *S. hystrix*, ethanol, methanol, n-hexane, chloroform, ethyl acetate (KGaA, Germany), silica gel 60 (KGaA, Germany), acarbose, α-glucosidase Type I from *Saccharomyces cerevisiae* (Sigma-Aldrich, USA), p-nitrophenyl-α-D-glucopyranoside (p-NPG) (Sigma-Aldrich, USA), and TLC Silica gel 60 F254 (KGaA, Germany).

2.2. Collection, identification, and preparation of seaweed

Fresh seaweed collected from Minajaya beach, West Java were identified in the Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada. The sample was cleaned from impurities such as sand and gravel attached, and then the wet sample is measured for its water content. The wet sample is dried in the room for 4 to 5 days [12]. The dried sample was then cut into small pieces and blended to form a powder.

2.3. Seaweed extraction and partition

Extraction of secondary metabolite *S. hystrix* based on the method of Yang *et al*. [13]. The sample powder was extracted using 60% methanol at room temperature for 24 hours with a ratio of raw material and solvent 1:8 (b/v). The solution was filtered and then evaporated using a rotary evaporator (60 rpm, 40 °C) obtaining *S. hystrix* extract. Extracts were partitioned using a liquid-liquid partition method with solvents whose polarity increased, from non-polar to polar. Comparison of extracts with solvents used was 1:15 (b/v). *S. hystrix* extract was dissolved with methanol and stirred until homogeneous. Before partitioning, the extract was added with water first with a ratio of methanol: water (3:1) (v/v). Comparison of partitions with chloroform was 1:1 (v/v). The chloroform fraction was then concentrated to obtain the chloroform fraction of *S. hystrix* extract.

2.4. Chloroform fraction separation by columns chromatography

Column chromatography separation using the silica gel 60 stationary phases and the mobile phase eluent the results of thin-layer chromatography (TLC). The column used was 3 cm in diameter and 40 cm in length. Chloroform was included in determining the presence or absence of faults, then chloroform fraction of *S. hystrix* extract. As much as 0.5 grams were dissolved in a chloroform solvent, then carefully put into the column. The samples were eluted using five solvents with multilevel polarity (step gradient polarity). The solvent volume was two times the column volume.
used, which is 424 mL, accommodates in vial bottles measuring 15 mL, and TLC tests were carried out. Eluate with the same Rf value was combined and dried.

2.5. \( \alpha \)-glucosidase inhibitory activity

The \textit{in vitro} inhibitory activity of the \( \alpha \)-glucosidase by acarbose, \textit{S. hystrix} extract, chloroform fraction, and chloroform fractions had used the method of Mayur \textit{et al.} [14]. The test solution (K) consisted of 75 \( \mu \)L phosphate buffer pH 7 25 \( \mu \)L \( \alpha \)-glucosidase enzyme 0.2 unit mL\(^{-1}\). Sample solution (S1) consisted of 25 \( \mu \)L of various concentration samples, 50 \( \mu \)L phosphate buffer pH 7, and \( \alpha \)-glucosidase 25 \( \mu \)L 0.2 mL\(^{-1}\) unit. The sample solution (S0) consisted of 25 \( \mu \)L of various concentration samples and 75 \( \mu \)L of phosphate buffer pH 7. Then all solutions were added 25 \( \mu \)L of 0.5 mM p-NPG and incubated for 30 minutes at 37 \( ^\circ \)C. The reaction was stopped by adding 100 \( \mu \)L Na\(_2\)CO\(_3\) 0.2 M. The amount of p-nitrophenol released will be measured on the microplate reader with a wavelength of 405 nm. The following formula calculates the percentage of inhibition:

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

Note:
K: Control absorbance - blank
S1: Absorbance of the sample with the addition of enzymes
S0: Absorbance of the sample without the addition of enzymes

The data of inhibition activity was converted into a linear regression equation. Namely, the relationship of the sample concentration log (x-axis) to the percentage of inhibition (y-axis) and the results were used to calculate the value of x at a value of 50. Furthermore, the value of the sample activity expressed by IC\(_{50}\) was obtained from antilog x. Values a and b were obtained from the linear regression equation \( y = a + bx \). IC\(_{50}\) values were calculated using the formula: IC\(_{50}\) = (50-a)/b.

2.6. Identification of active compounds of chloroform fraction of \textit{S. hystrix} extract

The active fraction of the highest enzyme inhibition activity results was analyzed by Gas Chromatography-Mass Spectrophotometry (GC-MS) to determine the compounds contained therein. GC-MS was conducted at Puslabfor Bareskrim Polri Jakarta. GC analysis was carried out with 6890 GC Method, and the column was equipped with Agilent 19091S-433. Helium was used as a carrier gas at a flow rate of 1 mL/minute. Capillary columns with a length of 30 meters, diameters of 0.25 mm, and thickness of 0.25 \( \mu \)m. The temperature program on the column is the initial temperature of the 80\( ^\circ \)C column until it reaches the final temperature of 290\( ^\circ \)C.

2.7. Data analysis

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

3. Results and discussion

3.1. Inhibitory activity of \( \alpha \)-glucosidase by \textit{S. hystrix} extract

The effect of \textit{S. hystrix} extract on \( \alpha \)-glucosidase activity was shown in Table 1. The highest concentration was 10 mg/mL and the lowest was 0.625 mg/mL with a mean of inhibitory inhibition \textit{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 1), the higher sample concentration had higher inhibitory activity against \( \alpha \)-glucosidase. The inhibitory activity of \textit{S. hystrix} extract was much higher than that of acarbose as commercial drugs, so the \textit{S. hystrix} extract was more effective in inhibiting the \( \alpha \)-glucosidase than acarbose. Nur’aini \textit{et al.} [15] reported that the extract of \textit{S. hystrix} there was a content
of secondary metabolite compounds such as alkaloids, terpenoids, phenols, and tannins which could inhibit the activity of α-glucosidase. According to Kumar et al. [16] and Matanjun et al. [18], suggested 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.

**Table 1.** Effect of acarbose and *S. hystrix* extract concentration on α-glucosidase inhibitory activity [18].

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

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

Figure 1 showed that the highest inhibitory activity in the chloroform fraction of *S. hystrix* extract was 54.58 ± 7.07% at a concentration of 1000 µg/mL and the lowest inhibition activity was at 62.5 µg/mL with a value of 23.93±3.52%. The result of the inhibition activity of the α-glucosidase in the chloroform fraction of *S. hystrix* extract showed that the higher the concentration used, the inhibitory activity also increased.

![Figure 1. Effect of chloroform fraction concentration on α-glucosidase inhibitory activity [18].](image)

Table 2 showed the IC₅₀ value of the sample testing for inhibition α-glucosidase. The IC₅₀ value obtained from the chloroform fraction was 0.726±0.164 mg/mL had higher inhibitory activity than acarbose, which was 1.890±0.146 mg/mL. Table 2 also showed that the chloroform fraction in inhibiting the α-glucosidase activity was higher than that of the acarbose comparison solution. The high activity of chloroform fraction was possible because the presence of compounds contained in the fraction consists of several compounds that may play a role in inhibiting the α-glucosidase action. According to Milovic et al. [19], chloroform solvents were able to impede α-glucosidase in *Cystoseira barbata*, *Cymodocea nodosa*, *Halimeda tuna*, and *Codium exchanges* with IC₅₀ of 9.98±3.34, 11.48±3.57, 19.16±0.65, and 13.85±1.41 µg/mL, respectively, compared to the IC₅₀ acarbose (59.8 ± 12.3 µg/mL).
Table 2. The IC\textsubscript{50} value of \textit{S. hystrix} extract, chloroform fraction, and acarbose in inhibiting α-glucosidase.

| Sample                   | IC\textsubscript{50} (mg/mL) |
|--------------------------|------------------------------|
| Acarbose                 | 1.890 ± 0.146\textsuperscript{a} |
| \textit{S. hystrix} extract | 0.343 ± 0.052\textsuperscript{c} |
| Chloroform fraction      | 0.726 ± 0.164\textsuperscript{b} |

Note: The letters a, b, c show the relationships between treatments. The same letter shows no real difference between treatments and vice versa [18].

The IC\textsubscript{50} value of the chloroform fraction was higher when compared with IC\textsubscript{50} from \textit{S. hystrix} extract. Because more compounds that have the potential to inhibit α-glucosidase activity was polar in the extract, whereas the chloroform fraction only attracts semi-polar compounds. The semi-polar compounds that were interested in having an inhibitory activity of the α-glucosidase were no higher than the polar compounds in the extract. Fitriana [20] also showed that IC\textsubscript{50} in Ashitaba leaf extract was 28.63 µg/mL, while IC\textsubscript{50} in the chloroform fraction was 76.16 µg/mL.

3.3. Inhibitory activity of α-glucosidase by methanol fraction of \textit{S. hystrix}

Based on Figure 2, the inhibition activity in each fraction was different. The highest inhibition activity was found in chloroform fraction two, three, and four with a value of 41.62±10.54, 51.39±8.53, and 30.49±8.66\%, respectively. The three fractions were identified as compounds using GC-MS. If the inhibitory activity was compared between the partitioned chloroform fraction (500 µg/mL) and the column chloroform fraction (400 µg/mL), it was seen that the inhibition activity of column chloroform fraction was higher. The difference in inhibitory activity was thought to be caused by a purer compound in the chloroform fraction as a result of column chromatography compared to the results of the partition.

![Figure 2. Inhibitory activity of \textit{S. hystrix} chloroform fractions in inhibiting the α-glucosidase activity [18].](image)

3.4. Identification of active compound using GC-MS

Some compounds that were thought to have biological activity as antidiabetic as a result of GC-MS identification were presented in Table 3. There were 13 secondary metabolites including alkaloids, flavonoids, and terpenoids, which naturally act as inhibitors of the α-glucosidase enzyme. These secondary metabolites have been tested both in vitro and in vivo to reduce blood glucose levels [8]. Bioactive compounds that provide hypoglycaemic effects include alkaloids, flavonoids, steroids, and glycosides [21].
Table 3. Compounds were suspected as α-glucosidase inhibitors in the chloroform active fraction [18]

| Fraction number | Compounds                                      | Class      | Biological activity                                      |
|-----------------|------------------------------------------------|------------|----------------------------------------------------------|
| 2               | 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-       | Terpenoid  | Antidiabetes (Elmazar et al., 2013) [22]                 |
|                 | 9-Octadecenoic acid, methyl ester,             | Fatty acid | Antidiabetes (Ahmad et al., 2012) [23]                  |
|                 | Phytol                                         | Terpenoid  | Antidiabetes (Elmazar et al., 2013) [22]                 |
|                 | Stigmasta-5,24(28)-dien-3-ol, (3.beta.,24Z)-  | Steroid    | Antidiabetes (Lee et al., 2004) [25]                    |
| 4               | 6-Hydroxy-4,4,7a trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one | Terpenoid  | Antidiabetes (Hunyadi et al., 2012) [26]                |

Table 3 showed that the compounds in the chloroform fraction of S. hystrix have biological activities that were thought to have the potential as antidiabetic. The results of the identification of the second potential antidiabetic chloroform fraction compound were 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl (2.92%), Hexadecanoic acid methyl ester (11.66%), 9-Octadecenoic acid methyl ester (2.08%), and Phytol (1.70%). According to Elmazar et al. (2013) [22], a compound that was thought to be able to inhibit the activity of α-glucosidase, namely 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl, and phytol. Phytol has a potential role in the management of insulin resistance and metabolic disorders that accompany diabetes and/or obesity. Fatty acid compounds such as Hexadecanoic acid methyl ester and 9-Octadecenoic acid methyl ester were thought to have potential as antidiabetic [23]. Balogun et al. [24] also reported that some fatty acid compounds were thought to be able to inhibit the α-glucosidase. Compound that was considered to have the potential as antidiabetic in the third chloroform fraction was Stigmasta-5,24 (28)-dien-3-ol, (3.beta.,24Z) (6.40%). According to Lee et al. (2004) [25] Stigmasta-5,24 (28) -dien-3-ol, (3.beta., 24Z) was thought to have the potential as an antidiabetic. The compound was a natural compound possessed by seaweed, which was used in the treatment of diabetes by inhibiting damage to glycogen in the liver; this can also delay carbohydrate digestion and glucose absorption. Compound that was thought to have the potential as antidiabetic in the fourth chloroform fraction was 6-Hydroxy-4,4,7a-trimethyl-5,6,7,7-tetrahydro benzofuran-2(4H)-one (4.06%). Hunyadi et al. (2012) [26], also reported that this compound has the potential as an antidiabetic type 2 in Morus alba plants.

4. Conclusion
The chloroform fraction of S. hystrix extract was able to inhibit α-glucosidase activity with an IC$_{50}$ value of 0.726 mg/mL. The results of column chromatography isolation showed that the three fractions that had the highest α-glucosidase inhibition activity were the second chloroform fraction (41.62%), third (51.39%), and fourth (30.49%). The identification results of the active compound in inhibition of α-glucosidase chloroform fraction S. hystrix extract was thought to be 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl, hexadecanoic acid methyl ester, 9-Octadecenoic acid methyl ester, phytol, Stigmasta 5,24 (28)-dien-3-ol, (3.beta., 24Z), and 6-Hydroxy-4,4,7a-trimethyl-5,6,7,7-tetrahydro benzofuran-2(4H)-one.
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