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Antidiabetic and Anti-Inflammatory Activity of Sea Cucumber (*Holothuria* sp.) Captured from Madura Island, Indonesia

Hafiludin[1*], Ita Ainur Rohmah[1], Apon Zaenal Mustopa[2], Mojiono[3], Iffan Maflahah[3]

[1]Department of Aquatic Resource Management, University of Trunojoyo Madura, Bangkalan, East Java, 69162, Indonesia
[2]Research Center for Genetic Engineering, National Research and Innovation Agency (BRIN), Indonesia
[3]Deparment of Agroindustrial Technology, University of Trunojoyo Madura, Bangkalan, East Java, 69162, Indonesia

***Corresponding author: hafiludin@trunojoyo.ac.id**

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ABSTRACT

Holothuria sp. is a fishery commodity that has a high economic value and is very easy to find in Madura waters but has not been widely used. The high nutrition and bioactive compounds contained can be potential as drug and functional food candidates. This study aimed to determine the morphological and morphometric characteristics, analyze the proximate content, protein profiling, and its potential as an anti-inflammatory and anti-diabetic agent *in vitro*. The stages of this research consisted of raw material preparation, morphometric and morphological analysis, chemical characterization, protein extraction, and screening for anti-inflammatory activity using the protein denaturation method and anti-diabetic by inhibiting the α-glucosidase enzyme. The study used a completely randomized design and was analyzed using ANOVA with the Dunken further test. Sea cucumbers *Holothuria* sp. has a physical appearance of a black cylindrical shape with a length of around 12.94cm. *Holothuria* sp. has a high nutritional content with a protein of 3.62% and the molecular weight of meat protein ranges from 172.05-38.22 kDa and viscera of 187.96-56.92 kDa. The highest potential anti-inflammatory activity in 10,000ppm hydrolysate was 98.83% and antidiabetic activity in 10,000ppm hydrolysate was 85.34%. Sea cucumbers originating from Madura waters have the potential to be developed in the fungsional food and medicine sectors.

INTRODUCTION

Diabetes melitus (DM) is a disorder of metabolic activity mainly characterized by hyperglycemia and problematic bioprocessing of carbohydrates, lipids, and proteins as a result of declined insulin function. Diabetes mellitus is defined by very high glucose levels due to abnormalities in insulin production, or can also be caused by insulin experiencing resistance **(Rahman *et al*., 2022)**. Globally, the disease, reported in 2011, reached 366 million people, and without proper preventive actions, the number was expected to increase to 552 million in 2030 **(IDF, 2011)**.

Inflammation is a protective mechanism against agents that attack healthy cells locally. Such local cells underwent some reactions to remove the threats. The damaged cells are substituted by new tissues, named as inflammation **(Laksmitawati & Tiffani, 2020)**. When a tissue is inflamed, several chemical mediators such as histamine, 5-hydroxytryptamine or serotonin, prostaglandins, and leukotrienes, are released by cells. If not treated immediately, the inflammatory process causes vasomotor diseases, rhinorrhea, rheumatoid arthritis, and atherosclerosis **(Choudhary & Ahlawat, 2008; Mavridis *et al*., 2008; Novika *et al.*, 2021**). Inflammation is recognized as a pivotal biological mechanism contributing to the development of various consequences of diabetes mellitus, including neuropathy, cardiomyopathy, and nephropathy **(Hyrin, 2009; Rivero-González *et al*., 2017)**. In 2018, the Indonesian Ministry of Health released cases of joint disease in Indonesia recorded at 7.3% and the most common joint disease suffered is osteoarthritis or arthritis **(Novika *et al.*, 2021)**.

Sea cucumbers are one of the marine organisms that have the potential to produce bioactive compounds for inflammation treatments **(Bawole *et al.*, 2021)**. They are rich in collagen, polysaccharides, saponins, and other substances that are beneficial to the human body **(Zhang *et al*., 2021)**. Regarding their nutritional compositions, the creature contains high levels of protein (40-60%) but low levels of lipids, primarily polyunsaturated fatty acids (PUFAs), and minerals such as calcium, zinc, iron, and magnesium. It also contains vitamins A, B1, B2, and B3 **(Gajdosechova *et al*., 2020)**.

Sea cucumbers from Madura Sea waters have quite a lot of potential, but their utilization is still limited to food consumption, namely being made into traditional food called crackers "kerupuk terung". Its utilization with high economic value still needs to be done. Sea cucumbers are marine invertebrates known for their high nutritional value and diverse bioactive compounds. They are rich in proteins, omega-3 fatty acids, vitamins, and minerals, making them a valuable food source **(Xu *et al.*, 2018; Ramalho *et al.*, 2020; Maskur *et al.*, 2024**). Peptide and protein compounds as well as bioactive compounds in sea cucumbers can function as antioxidants, anti-inflammatories and antidiabetics **(Xu *et al.*, 2018; Hossain *et al.*, 2022; Li *et al*., 2023; Sari *et al*., 2023)**.

Numerous studies discovering the nutrition and bioactivity of sea cucumbers have been reported. Sea cucumbers contain a number of important nutrients **(Rasyid *et al.*, 2020; Nguyen *et al.*, 2022; Widianingsih & Hartati, 2023)**. Sea cucumbers also contain a number of bioactive compounds **(Xu *et al.*, 2018; Man *et al*., 2023)**. **Ringo *et al*. (2017)** stated that the active ingredients of sea cucumber (*Holothuria*) can be useful as antibacterial agents. In addition, **Putram *et al.* (2017)** reported its effects on anticancer. Another work revealed the potential of sea cucumber for antibacterial agents due to content of steroid, saponin, and triterpenoid **(Nimah *et al*., 2012)**. Research on the use of sea cucumbers from Madura waters as anti-inflammatory and antidiabetic has never been conducted, but this is important as a basis for information on the development of sea cucumbers as pharmaceutical ingredients in the future. The study aimed to analyze the antidiabetic and anti-inflammatory activities of isolates and hydrolysates of sea cucumber protein (*Holothuria* sp.) from Madura Sea waters.

MATERIALS AND METHODS

1. Sample collection

Sea cucumbers (*Holothuria* sp.) were captured in Talango Island, a part of Madura Island, Indonesia. The samples were taken in living form in their habitat, namely at the coordinate points 7° 6'46.08"S and 114° 3'5.43"T. The samples used were a type of black sea cucumber with a size of around 13cm. They were immediately cleaned and frozen in a cool box. The samples were then cut from the meat and innards using scissors, by cutting from the posterior to the anterior.

2. Protein extraction procedures

Protein extraction was carried out based on the method of **Wen *et al.* (2024)**, with slight modifications. Sea cucumber protein was extracted by soaking the samples in acetone for 24h (1:2 w/v). Furthermore, the pellets were collected by centrifugation at 5000rpm at 4°C for 30min and then freeze-dried. The sample was dissolved in 5% Sodium Deodesyl Sulfate and incubated at 85°C then centrifuged at 12,000rpm for 15min.

3. SDS PAGE experiment

Sodium Deodesyl Sulfate-Polyacrilamide Gel Electrophoresis (SDS PAGE) procedures followed a previous method **(Budiarto *et al*., 2016)**. The gels were prepared as follows: 10% for lysate samples, 12% for hydrolysate, and 4% stacking gel for both samples. Staining was performed by CBB while washing was carried out using a solution 1 (metanol 30% and acetic acid 5%) and solution 2 (methanol 5% and acetic acid 7%) for 2-3 h until protein bands appeared clearly.

4. Enzymatic hydrolysis (Budiarto *et al.* 2016)

The sample was adjusted to pH 7. Protease enzyme was added to the sample at a ratio of 1:100 v/v. Hydrolysis was carried out with a waterbath shaker at 37℃ for 18 hours. Enzyme inactivation in hydrolysis was carried out with a heat lock at 95℃ for 15 minutes and then centrifuged at 12,000 rpm at 4℃ for 15 minutes. The supernatant in the form of hydrolysate was collected for further testing.

5. Inhibition of α-glucosidase (Xu *et al.*, 2018)

A total of 30μL of metabolite samples, 36μL of phosphate buffer pH 6.8, and 17μL of p-nitrophenyl-α-D-glucopyranoside (PNPG) 5 mM were incubated at 39℃ for 5 minutes, 17μL of α-Glucosidase was added and incubated at 39^{0}C for 15 minutes. This reaction was stopped by adding 100µL of Na_2CO_3 with a concentration of 200 mM. Inhibition of α-Glucosidase was measured using an Elisa Reader with an absorbance of 405nm. Acarbose was used as a positive control for α-Glucosidase inhibitors. The inhibitory ability of the α-Glucosidase enzyme can be calculated by the formula:

$$Inhibition\ (\%) = \frac{Blank\ Absorbance - Sample\ Absorbance}{Blank\ Absorbance} x\ 100\% \qquad (1)$$

6. Anti-inflammatory activity (Nguemnang *et al.*, 2019)

100µL of sample metabolites and 500µL of 1% BSA were mixed, then incubated for 15 minutes at 37^0C and denatured at 70^0C for 5 minutes. The blank test solution used a metabolite solvent solution, and a positive control used diclofenac with a specific concentration. A mixture of metabolites + 1% BSA, blank solution, and positive control diclofenac were injected into a microplate and then measured using an Elisa Reader with an absorbance of 620nm. The inhibition can be calculated using the formula:

$$Inhibition\ (\%) = \frac{Blank\ Absorbance - Sample\ Absorbance}{Blank\ Absorbance} x\ 100\% \qquad (2)$$

7. Statistical analysis

Proximate parameter analysis consists of moisture, ash, lipid, fibre, protein, and carbohydrate, while protein profile analysis with SDS-PAGE uses quantitative analysis of sea cucumber protein weight. Analysis of sea cucumber (*Holothuria* sp.) activity test using a completely randomized design was followed with the one-way ANOVA test carried out with Duncan's further test.

RESULTS AND DISCUSSION

1. Characteristics of sea cucumber

Sea cucumber aged 1-2 years was captured from Talango waters, an area of Sumenep. It measured up to 97-238.156g, with average length of 12.94cm (Fig. 1). It appeared reddish black in color, with round-cylinder shapes. Its flesh was black outside, but light-white inside. While the flesh texture was slick, the visceral appeared orange-grey in color. In terms of yield, majority of the sea cucumber parts went toward flesh with 82%, whilst its visceral was only 6%. The difference is the dirt and water contained in the digestive tract.

(a) (b) (c)

Fig. 1. a) Physical appearance of sea cucumber; b) Sea cucumber after being separated, c) Oval

2. Chemical composition

The nutritional content of aquatic organisms, such as fish and shellfish, is a result of their internal metabolism and is stored in their meat and organs. This content includes proximate components like protein, fat, water, ash, and carbohydrates. The results of measuring the proximate content of sea cucumbers originating from Madura Sea waters can be seen in Table (1).

Table 1. Nutritional content (proximate) of fresh sea cucumber *Holothuria* sp.

Nutritional Content	***Holothuria* sp. (Madura)[1]**	***Holothuria scabra* (Bungin)[2]**	***Holothuria scabra* (Belitung)[2]**	***Holothuria scabra* (Makasar)[2]**	***Holothuria scabra* (Lampung)[2]**
Moisture (%)	91,59 ± 0,43	84,55	87,84	87,95	83,40
Ash (%)	1,50 ± 0,16	7,38	5,24	6,57	7,90
Lipid (%)	0,57 ± 0,10	0,78	1,22	0,48	0,90
Fiber (%)	2,1 ± 0,52	-	-	-	-
Protein (%)	3,62 ± 0,07	6,95	5,22	4,78	5,73
Carbohydrate	0,61 ± 0,30	0,34	0,48	0,22	2,07

Noted: [1]) current study; [2]) **(Ardiansyah *et al.*, 2020)**.

The ash content in food ingredients indicates the presence of minerals contained in the ingredients. Minerals are essential in maintaining overall body function in cells, tissues, and organs. Table (1) shows that the ash content obtained in this study was 1.50%. Meanwhile, the ash content in the study of **Ardiansyah *et al.* (2020)** of *Holothuria scabra* sea cucumbers obtained from the waters of Bungin, Belitung, Makassar, and Lampung ranged from 5.24- 7.90%. The high and low ash content obtained in the test can be caused by differences in the types of sea cucumbers and their water environments. The habitat of sea cucumbers also affects the nutritional content in them **(Sroyraya *et al.*, 2017; Ardiansyah *et al*., 2020; Widianingsih & Hartati, 2023)**. Habitat and water conditions affect food intake in sea cucumbers, and impact their metabolic processes and nutritional content as a result of the metabolic process. Each marine organism has different abilities in regulating and accumulating heavy metals in the area where it lives, dramatically affecting the ash content in the ingredients **(Pangulimang *et al.*, 2023)**.

Aquatic organisms contain fat and oil at varying levels in one biota and another**.** Fat content analysis determines the fat content in the sea cucumber meat studied. Table (1) shows that the average fat content of *Holothuria* sp. sea cucumbers is 0.57%. However, in a study by **Song *et al.* (2023)**, *Apostichopus japonicus* sea cucumbers contain an average fat content of 4%. Differences in fat content contained in sea cucumbers can be caused by several factors, namely species, food availability in their habitat, age of the biota, and metabolic rate of the biota. The fat content will increase with age because the biota is entering its reproductive phase.

Crude fibre is a collection of fibre content in food ingredients that cannot be digested. The components of this crude fibre consist of cellulose, lignin, pentose, and many others. Fiber content analysis is used to determine the total fibre content in the ingredients. The average crude fibre content of *Holothuria* sp. sea cucumbers is 2.1%, as seen in Table (1). The high fibre content contained in the ingredients can be used as a source of functional food and is suitable for consumption by people with obesity **(Kumayanjati, 2020)**.

High protein content in food ingredients can provide good nutritional value. The protein functions as a building block, regulator, and catalyst. Sea cucumber meat is rich in various types of protein, namely glycine, glutamic amino acids, and arginine because most of the sea cucumber body is composed of collagen found in muscle tissue at 70%**.** The results measuring the average protein content of sea cucumber *Holothuria* sp. obtained a value of 3.62% (Table 1). The results of this study differ from those conducted by **Feng *et al.* (2021)** on sea cucumber *Stichopus japonicas* with differences in several seasons; the protein content obtained ranged between 52.4 and 38.8%. The protein value difference is thought to be due to age, food consumed, aquatic habitat, and metabolic rate. The unstable nature of protein also plays a role in the protein content results obtained. In addition, high protein levels can be seen from the high ash content and low-fat content obtained **(Oedjoe, 2017)**.

Carbohydrates are one type of macronutrient that functions as the body's primary energy source. They are composed of carbon, hydrogen, and oxygen molecules. The average carbohydrate content analysis value of *Holothuria* sp. sea cucumbers in Table (1) is 0.61%; in other studies, it shows that the carbohydrates contained in *Holothuria scabra* sea cucumbers are around 2.07- 0.22% **(Ardiansyah *et al.*, 2020)**. Carbohydrates play an important role because they are the primary source of energy for living things. Fishery products' carbohydrates do not contain fibre and are generally glycogen **(Pangulimang *et al.*, 2023)**. The carbohydrate content of raw materials plays a vital role in the characteristics of food ingredients in the form of taste, color, and texture.

Variations likely influence the differences in proximate composition obtained in sea cucumber living conditions, such as habitat, climate, season, location, and sampling time. In general, the influence of season on the nutritional composition of sea cucumbers is the most significant factor compared to other factors **(Liu *et al*., 2021)**. Differences caused by season are closely related to the breeding phase, and temperature impacts metabolic activity **(Feng *et al*., 2021)**. Previous studies have shown that physiological performance is an essential factor that reflects the growth of sea cucumbers at fluctuating temperatures; on the other hand, temperature also affects food sources and metabolic activity **(Yu *et al*., 2016)**.

3. Protein profiles

The yield of sea cucumber meat and offal protein isolates produced was around 0.04 and 1.39%. This result was obtained from 269g of fresh sea cucumber meat and 38.92g of offal. The low yield value was caused by the highwater content in sea cucumbers, which affected the concentration of the solvent used. Other factors that influenced it were the type of solvent, extraction method, temperature, and particle size of the sample. The characteristics of the sea cucumber meat isolate produced were black, and the offal isolate was yellowish white.

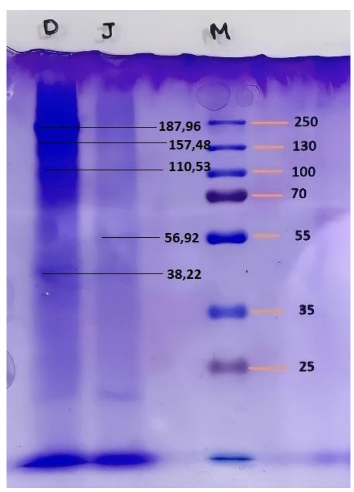

Fig. 2. Profile of SDS-PAGE sea cucumber isolates (D. meet, J. viscera, M. Marker)

The results of SDS-PAGE of sea cucumber meat isolates obtained protein profiles with molecular weights of 172.05, 157.48, 110.53 kDa, and 38.2 kDa. At the same time, the molecular weight of the viscera samples was 187.96 and 56.92 kDa. In their study, **Khirzin *et al*. (2015)** showed that the molecular weight of collagen peptides from sea cucumbers was 130.33 kDa. The results of the molecular weight of the protein band from *Holothuria* sp. sea cucumbers in this study are lower than those of **Syamsudin *et al.***

(2017) that *Holothuria leucospilota* collagen protein has 3 protein bands with molecular weights of 247.29, 166.43 and 138.35 kDa. Differences in species can cause thickness in the protein bands formed. Differences in sample concentration can cause other factors, the type of enzyme used during hydrolysis. These will affect the formed band's weight, peptide-cutting pattern, and thickness **(Prasetyo, 2016)**.

4. Anti-inflammatory activity of sea cucumber (*Holothuri* sp.)

An anti-inflammatory refers to a substance or treatment that reduces inflammation, which is the body's immune response to harmful stimuli such as pathogens, damaged cells, or irritants. Inflammation is characterized by redness, swelling, pain, heat, and loss of function **(Ivanescu & Corciova, 2017; Nwosu *et al*., 2019)**. Anti-inflammatory agents work by inhibiting various components of the inflammatory process, thereby alleviating these symptoms. The results of measuring the anti-inflammatory activity of sea cucumber extract from Madura Sea waters can be seen in the Fig. (3).

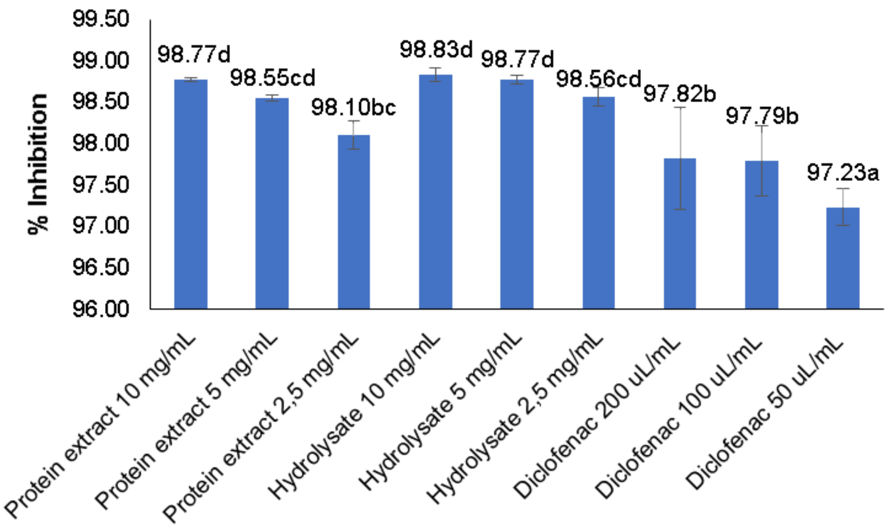

Fig. 3. Anti-inflammatory activity of sea cucumber (*Holothuria* sp.) isolate and protein hdrolyzate

Fig. (3) explains that the anti-inflammatory activity of protein extracts and sea cucumber hydrolysis results using diclofenac drug control obtained results ranging from 98.10-98.83%; these results are higher than the diclofenac control, which is 97.23-97.82%. The anti-inflammatory activity of protein hydrolysate is higher than that of sea cucumber protein extract; the higher the concentration used, the higher the anti-inflammatory activity produced. The treatment of extract concentration and protein hydrolysate from sea cucumbers had a significant effect on the value of inhibiting inflammation caused by BSA (α <0.05). These results are higher than those of a previous study **(Moelyono *et al*., 2018)**, in which the highest anti-inflammatory in sea cucumbers (78.90%) was obtained at a dose of 1000ppm.

The high anti-inflammatory activity in sea cucumber protein hydrolysate from Madura waters is due to the protein components being broken down into proteins with low molecular weight sizes so that they are possible in the form of peptide compounds. This is in accordance with the statements **(Ghanbari *et al.*, 2016; Song *et al.*, 2016; Jiang *et al.*, 2024)** that protein in the form of peptides from sea cucumbers can function as an anti-inflammatory. Sea cucumber peptides have been found to exhibit anti-inflammatory properties through various mechanisms, making them potential candidates for pharmaceutical and nutraceutical applications. Sea cucumber peptides have been discovered to possess anti-inflammatory activities, regulating physiological functions through multiple pathways **(Jiang *et al.*, 2024)**, inhibit the production of pro-inflammatory cytokines and nitric oxide in macrophages, indicating their anti-inflammatory potential **(Man *et al*., 2023)**.

5. Antidiabetic activity of sea cucumber *(Holothuri* sp.*)*

The antidiabetic potential of the results of the isolation and hydrolysis of *Holothuria* sp. sea cucumbers was measured by calculating the inhibitory activity of the lysate and hydrolysate of *Holothuria* sp. sea cucumbers against the α-Glucosidase enzyme. **Mariyanti (2017)** stated that the α-Glucosidase enzyme functions in the breakdown of carbohydrates into glucose and other monosaccharide derivatives; therefore, compounds that inhibit enzyme activity show potential as antidiabetic agents. The α-Glucosidase enzyme inhibition test used Acarbose and p-nitrophenyl- α-D-glucopyranoside (PNPG) as substrates. The inhibitory reaction of the α-Glucosidase enzyme will hydrolyze p-nitrophenyl-α-D-glucopyranoside, which will become p-nitrophenol, is marked by yellow and glucose. Enzyme activity was measured based on the results of p-nitrophenol absorbance of the lysate and hydrolysate of *Holothuria* sp. sea cucumbers. It can inhibit the activity of the enzyme α-Glucosidase so that p-nitrophenyl will be reduced.

The inhibition of the α-glucosidase enzyme in Fig. (4) shows that the sea cucumber protein hydrolysate has the highest inhibitory activity against the α-glucosidase enzyme, namely 85.34% at a concentration of 10,000 ppm, 74.24% at a concentration of 5,000 ppm and 48.62% at a concentration of 2,500 ppm. The inhibitory activity of the α-glucosidase enzyme in the sea cucumber isolate *Holothuria* sp. at a concentration of 10,000 ppm was 62.99%, a concentration of 5,000 ppm was 47.65%, and at a concentration of 2,500 ppm, the inhibitory activity was 54.48%. Antidiabetic activity in sea cucumber protein hydrolysate is higher than its protein extract. The higher the concentration used in both, the higher the antidiabetic activity, but vice versa in the acarbose control. The treatment of hydrolysate concentration and sea cucumber protein extract from Madura waters significantly affected the value of antidiabetic activity ($\alpha<0.05$).

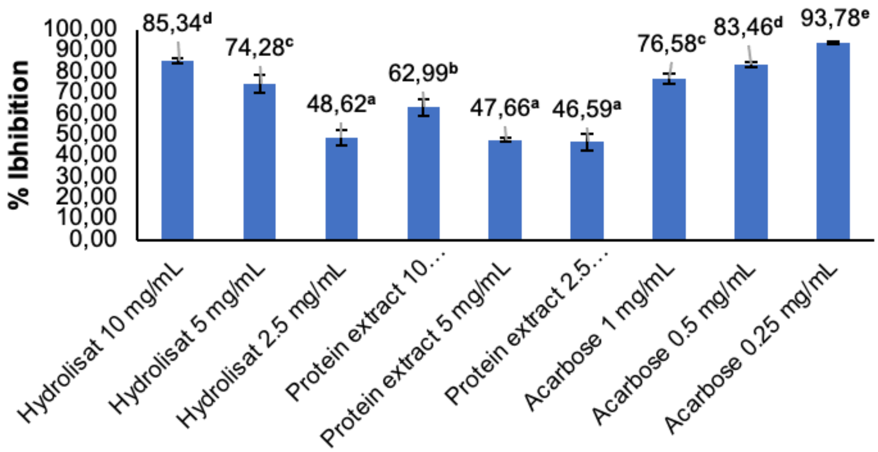

Fig. 4. Antidiabetic activity of sea cucumber (*Holothuria* sp.) isolate and protein *hdrolyzate*

The inhibitory reaction by the hydrolysate and extract protein of the sea cucumber *Holothuria* sp. occurred due to the slowing of postprandial carbohydrate absorption, thereby reducing blood sugar levels **(Karnila, 2012)**. The ability to inhibit the activity of the α-Glucosidase enzyme is influenced by the concentration of metabolite compounds **(Mariyanti, 2017)**; the higher the concentration of metabolite compounds used, the greater the chance of the inhibitor covering the active site of the enzyme, metabolite compounds are also able to bind to enzymes and enzyme-substrate complexes.

The results of this study are not much different from the study conducted by **Mogharabi *et al.* (2017)**, that the inhibitory activity of the α-glucosidase enzyme in the extracts of sea cucumbers *Holothuria leucospilota* and *Stichopus hermani* had maximum inhibitory activities of 34 and 40%, respectively. Sea cucumbers contain bioactive compounds that can reduce glucose levels. Saponin is one of the secondary metabolite compounds from holothuria. Saponin is a triterpene glycoside that has broad pharmacological activity. Saponin from sea cucumber isolation is known as holothurian or frondoside **(Rabsanjani & Nurhidayati, 2023)**. In addition to saponin compounds, fucoidan is another compound that is thought to play a role in reducing glucose levels. The body wall of sea cucumbers is composed of fucoidan. Holothuria fucosylated chondroitin sulfate (FuCs) is composed of sulfate and L-fucose groups in polysaccharides. This fucoidan compound is thought to influence the antioxidant activity of the sea cucumber hydrolysate sample in this study, which reached 8.4% at a concentration of 10 mg/mL because fucoidan can be obtained by enzymatically hydrolyzing sea cucumbers **(Gustini *et al.*, 2022)**.

CONCLUSION

Sea cucumbers (*Holothuria* sp.) obtained from Madura Sea waters have a physical appearance of reddish black, white meat, gray-orange innards, and chewy texture. The average body length is around 12.94cm, weighing 97-238.15g. It has a high nutrient content, containing 3.62% protein the molecular weight of meat protein ranges from 172.05-38.22 kDa and viscera of 187.96-56.92 kDa. Isolates and hydrolysates of sea cucumber proteins (*Holothuria* sp.) also have protein and enzyme inhibitory activity. A sample of 10,000ppm showed the highest anti-inflammatory activity isolates with a percentage of BSA denaturation inhibition of 98.83%. The highest antidiabetic activity was in a sample of 10,000ppm hydrolysate with a percentage of α Glucosidase inhibition of 85.34%.

CONFLICT OF INTEREST

The author declares that there are no conflicts of interest regarding the authorship or publication of this research.

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