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Antioxidant, Antibacterial, and Antifungal Characteristics on Crude Collagen Extracts of Sea Cucumber (*Stichopus hermanii*)

Rini Pramesti[1], Wilis Ari Setyati[1*], Nirwani Soenardjo[1], Dafit Ariyanto[2]

[1] Department of Marine Science, Faculty of Fisheries and Marine Sciences, Diponegoro University, Semarang 50275, Indonesia
[2] Research Center for Oceanography, National Research and Innovation Agency, Jakarta 14430, Indonesia

Corresponding Author Email: wilisarisetyati@yahoo.co.id

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ABSTRACT

Stichopus hermanii is a species of sea cucumber commonly found in the western Indo-Pacific region and has been extensively utilized by local communities for commercial purposes and health benefits. This study aims to evaluate the antioxidant, antibacterial, antifungal, and SPF properties of crude collagen extracted from *Stichopus hermanii*. The research methodology included extraction processes using an ethyl acetate solution, measurement of antioxidant activity through the DPPH method, antibacterial and antifungal testing using the paper disc diffusion method, and SPF value measurement through UV-Vis spectrophotometry. Results indicated that a crude collagen yield of 3.75% was successfully obtained from the golden sea cucumber. The crude collagen extract of the golden sea cucumber exhibited antioxidant activity categorized as strong, with an IC50 value of 56.82 µg/mL. The percentage of inhibition was directly proportional to the extract concentration. Antibacterial activity against *E. coli* and *S. aureus* was observed only in extracts at a concentration of 2000 µg/disc, with clear zones of 6.3±0.1 mm and 5.4±0.01 mm, respectively, at the end of the study. The collagen extract exhibited antifungal activity against *C. albicans* at a concentration of 500 µg/disc with a clear zone diameter of 17.2±1.6 mm at the end of the study, while no antifungal activity was observed against the test pathogen *Trichoderma* sp. The SPF value of the collagen extract was low, ranging from 0.2 to 1.46. The crude collagen extract from the golden sea cucumber *Stichopus hermanii* demonstrated potential as an antioxidant, antifungal, and antibacterial agent.

1. INTRODUCTION

Stichopus hermanii is a species of sea cucumber that belongs to the family Stichopodidae. In Indonesia, *Stichopus hermanii is* often referred to as the golden sea cucumber [1]. *Stichopus hermanii* is commonly found in the western Indo-Pacific region, ranging from the coast of Africa to Malaysia, Indonesia, and Australia. Golden sea cucumbers have long been commercialized by local communities due to their numerous health benefits [2]. Sea cucumbers are invertebrate animals whose body walls are primarily composed of collagen, making them a potential source of collagen. Sea cucumbers are able to produce up to 72.2% collagen; this value is higher than collagen obtained from pigs (64.7%) or tilapia (67.33%) [3]. Sea cucumbers are an excellent source of protein. The protein content in dried sea cucumbers is 82 g per 100 g with a high digestibility value. Of that amount, around 80% is in the form of collagen. Collagen functions as a structural protein in bone and skin growth. In bone growth, calcium supplements alone are not enough because bones consist of calcium phosphate and collagen. Without collagen, bones will become brittle and break easily [4]. Collagen is the most abundant protein found in *Stichopus hermanii* extract. This highlights the potential of *Stichopus hermanii* as a collagen source [5]. *Virgibacillus chiguensis*, found in the intestines of sea cucumbers, has been identified as a bacterial symbiont [6]. Additionally, a consortium of bacterial symbionts was found in the stomach of sea cucumbers [7].

Bioactive compounds are chemical compounds that are naturally found in animals, plants and microorganisms. Bioactive compounds are researched extensively to be developed as health products, cosmetics, food, and so on. Bioactive compounds obtained from nature are generally non-toxic and safer to consume than synthetic compounds. Several bioactive compounds such as phenolic compounds, flavonoids, and carotenoids are known to have antioxidant, antibacterial, and antifungal activities [8-10]. Antioxidants are able to neutralize dangerous free radicals in the body, thereby preventing diseases such as cardiovascular disorders, cancer, and neurodegenerative diseases. Antibacterial and antifungal properties remain active research topics, especially in light of increasing antibiotic resistance levels. Nowadays, the use of bioactive compounds is not only focused on the pharmaceutical and health sectors but has also spread to the world of cosmetics and food. Ethanol extracts from plants exhibit SPF (Sun Protection Factor) properties and have the

potential to be developed as a cosmetic product [11]. The important role of bioactive compounds in all aspects of industry and life makes the exploration of bioactive compounds crucial and needs to be developed.

Collagen is a product that is now widely traded on the market because of its benefits in the fields of cosmetics, health, and wound healing. Collagen can moisturize the skin and can prevent aging. Research [12] stated that the hydrolyzate of collagen peptides from sea cucumbers was able to produce antioxidant activity of up to 41.2%, making it a potential natural antioxidant for food and medicine. The study [13] reported that enzymatically hydrolyzed collagen showed antibacterial activity against *Escherichia coli* and *Bacillus subtilis* and had antioxidant activity reaching 44%. Although research regarding the collagen content of *S. hermanii* has been conducted, the exploration of antioxidant, antibacterial, and antifungal activity of collagen extract derived from *S. hermanii* is still limited. This study aimed to evaluate the antioxidant, antibacterial, antifungal, and SPF properties of crude collagen extracted from *S. hermanii.*

2. MATERIAL AND METHOD

2.1 Collection of samples

The research material is collagen isolated from wild-caught golden sea cucumbers *S. hermanii* from the waters of Panjang Island, Jepara. The tools used in this research include an erlenmeyer, beaker, plastic filter, small funnel, hot plate, refrigerator, scissors, blender, white cloth, camera/cell phone, *centrifuge*, and a 50 ml tube. The ingredients used are distilled water, 70% alcohol, golden sea cucumber samples *S. hermanii*, tissue, NaOH 0.1 M, CH_3COOH 0.5 M, DMSO, aluminum foil, plastic ziplock, media *Nutrient Agar, Mueller Hinton Agar, Potato Dextrose Agar,* bacteria *Escherichia coli* and *S. aureus,* fungi *Candida albicans* and *Trichoderma sp.*, DPPH, etc.

2.2 Crude collagen extraction

Dried samples of golden sea cucumber *S. hermanii* are cut and cleaned from dirt. Samples of dried sea cucumber meat were then cut and blended. Dried sea cucumber meat (1000 g) was treated with 0.1 M NaOH (10:1 ratio, w/v) for 48 hours at 4℃ to remove non-collagen proteins within the sample, with solution changes every 6 hours [14]. After 48 hours, the sea cucumber meat was washed with running water until the pH was neutral. Once neutral, then given 0.5 M CH_3COOH Acetic Acid (1:10, ratio w/v) for 48 hours at a refrigerator temperature of 4℃ without changing the solution. Acetic acid was used to solubilize the collagen and break down the bonds in the collagen structure, thus separating it from other raw material components [15]. After 48 hours, the sample was washed again until the pH was neutral. Once the pH and temperature are neutral, extraction is carried out with distilled water (1:2, ratio w/v) for 2 hours with a temperature of 40℃, a maximum of 45℃, and stirred occasionally. Distilled water is used throughout the extraction process to wash and rinse the materials [16]. Once finished, the solution is poured into a white bag/cloth for filtering and squeezed to separate the collagen. The extraction results are then centrifuged for 30 minutes per 45 ml tube at a speed of 6000 rpm and freeze-dried to preserve collagen [17].

2.3 Antioxidant

Antioxidant test using the DPPH method [18] by dissolving the sample in ethyl acetate and then carrying out graded dilutions. The sample was dissolved in ethyl acetate pa 10000 µg/ml then a concentration series of 50, 100, 150, and 200 µg/ml was made. A total of 2 ml of sample was reacted with 2 ml of 0.1 mM DPPH solution and incubated for 30 minutes in the dark. Absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm. Percent inhibition is calculated using the following formula:

$$\%Inhibition = \frac{Ac - As}{And} \times 100\% \quad (1)$$

where,

Ac = Control absorbance

As = Sample absorbance

The inhibition concentration (IC50) value was determined graphically using a plot of the inhibition value and extract concentration. The form of the linear regression equation obtained is y = bx + a and the line equation obtained is used to calculate the 50% inhibition concentration (IC50).

2.4 Antibacterial activity

Pathogenic bacteria *S. aureus* (ATCC 29213) *and E. coli* (ATCC 25922) were cultured in nutrient broth media for 1 x 24 hours. The bacteria that had grown were inoculated on new media and the density was equalized to the McFarland standard of 0.5. The crude extract was diluted using 10% DMSO with variable concentrations of 2000 µg/ml, 1000 µg/ml, 500 µg/ml, and 250 µg/ml. The prepared Mueller Hinton Agar media is inoculated with pathogenic bacteria. Paper discs were placed on the media and inoculated with the extract. The positive control is amoxicillin 10% (100 mg/ml), and the negative control is DMSO 10%. Petri dishes were incubated for 2 days, with observations every 24 hours [19].

2.5 Antifungal test

The antifungal activity test used the disk diffusion method using paper disks. Pathogenic fungi *Candida albicans* (ATCC 90028) and *Trichoderma* sp. (strain TV-15) are inoculated in liquid media (PDB) and shaker 1×24 hours. The pathogenic fungus is then inoculated into the test medium of Potato Dextrose Agar (PDA). This test is carried out by dissolving the collagen extract in 10% DMSO (Dimethyl Sulfoxide). The extract is injected inside a paper disk with concentrations of 2000 µg/ml, 1000 µg/ml, 500 µg/ml, and 250 µg/ml. The positive control is ketoconazole 10% (100mg/1ml) and the negative control is DMSO 10%. The test medium was incubated for 2×24 hours at 37°C and observed every 1×24 hours [20].

2.6 Sun protection factor (SPF)

The determination of sunscreen value was carried out in vitro using the UV-Vis spectrophotometry method. A total of 0.1 g of collagen was dissolved in an appropriate solvent to obtain a solution with a concentration of 1000 µg/ml for each extract. Next, graded dilutions were carried out until concentrations of 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml, and 1000 µg/ml were then measured, and the absorbance value

was then measured at a wavelength of 290-320 nm at 5 nm intervals using the formula [21].

$$SPF\ spectrophotometric = CF \times f(x) = \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda) \quad (2)$$

where,

EE: Erythemal effect spectrum
I: Solar intensity spectrum
Abs: Absorbance of sunscreen product
CF: Correction factor (=10)

3. RESULTS AND DISCUSSION

3.1 Collagen

Collagen was isolated from sea cucumber meat *Stichopus hermanii* through a series of chemical extraction processes (1 kg sample) and collagen 37.5 g. Dried sea cucumber meat is soaked in NaOH to dissolve non-collagen protein. The swelling of sea cucumber tissue by NaOH causes collagen telopeptides to open so that water can dissolve non-collagen proteins out of the collagen matrix [22]. The remains of non-collagen components are removed by soaking in distilled water. The collagen produced from this extraction process is crude collagen that has not been purified.

The total yield of collagen produced was 3.75%, this amount of yield is higher compared to the results of collagen extraction from sea cucumbers *Stichopus variegatus* amounting to 2.43% [22] and the ultrafiltration method affords a higher yield of collagen (11.39%) than that of the dialysis (5.15%) [23]. but is lower than *Stichopus horrens* with a yield reaching 16.7% [24]. Collagen extraction in *H. scabra* using HCl solution has a yield reaching 20.76% [23], while extraction on *H. atra* can produce a yield of up to 61.60 ± 0.57% [25]. The difference in yield results is caused by several factors, including the solvent used and its ratio, temperature, length of time, and so on.

3.2 Antioxidant activity

The antioxidant activity of collagen crude extract can be observed in Table 1 and Figure 1. The lowest percent inhibition is shown by a concentration of 50 µg/ml with a value of 49.86%, while the highest percent inhibition is shown by a crude collagen extract with a concentration of 200 µg/ml with a value of 61.35%. The IC50 value of collagen crude extract is 56.82 µg/ml, this value is in the strong category.

Table 1. Antioxidant activity of collagen crude extract *Stichopus hermanii* and antioxidant activity of ascorbic acid

Antioxidant Activity	Concentration (µg/ml)	Inhibition (%)	IC50 (µg/ml)
Collagen	50	49.86	56.82
	100	51.76	
	150	60.68	
	200	61.35	
Ascorbic acid	1.25	3.2	4.0
	2.5	14.3	
	5	25.6	
	10	33.7	
	20	83.94	

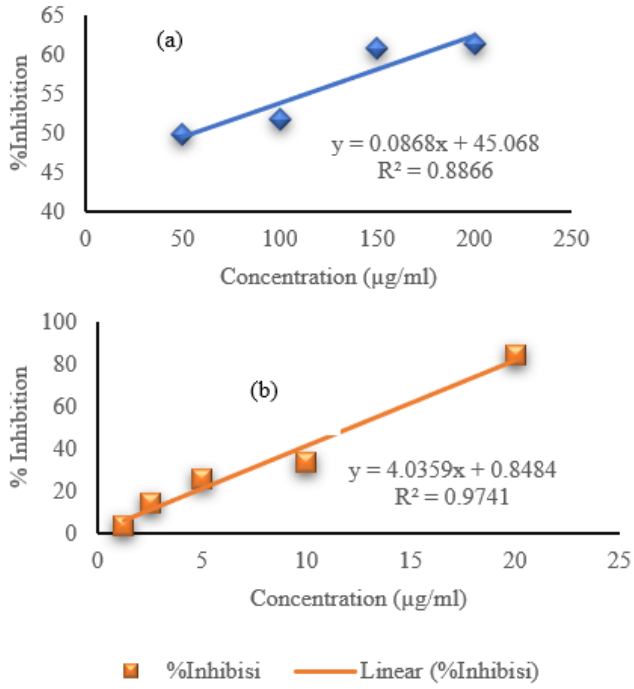

Figure 1. Antioxidant activity of (a) collagen crude extract and (b) ascorbic acid

The principle of measuring the antioxidant activity of the DPPH method is based on the color change that occurs when the sample reacts with DPPH. The antioxidant content of the compound will cause a hydrogen atom donating reaction to the DPPH free radical compound so that the compound becomes more stable. This donation will change purple Diphenylpicryhydrazyl (free radical) into yellow Diphenylpicryhydrazyl [26].

The results show that crude collagen from golden sea cucumbers has quite significant antioxidant activity; this is shown by the IC50 value, which reaches 56.82 µg/ml. Apriani and Pratiwi [27] reported that antioxidant activity with an IC50 value of <50 µg/ml is categorized as very strong, an IC50 value of 50-100 µg/ml is categorized as strong, an IC50 value of 100-150 is moderate, while an IC50 value of >150 µg/ml belongs to the weak category [28]. Based on these categories, the antioxidant activity of collagen crude extract *S. hermanii is* included in the strong category.

The IC50 value shows the concentration required to inhibit half or 50% of free radicals. The lower the IC50 value, the stronger the inhibitory activity of a compound. The IC50 value of crude sea cucumber collagen extract *S. hermanii* reached a value of 56.82 µg/ml, this value shows stronger antioxidant activity than the antioxidant activity of *Stichopus horrens* hydrolysate collagen with an IC50 value of 5250 µg/ml [28]. Percent inhibition of crude collagen extract *S. hermanii* increases with increasing concentration. The results of statistical analysis showed that differences in extract concentrations had a significant influence ($p<0.05$) on antioxidant activity. Similar results were also reported by study [29], where increasing the extract concentration results in a decrease in absorbance so that the percent inhibition increases.

3.3 Antibacterial activity

The results showed that the crude extract of sea cucumber

collagen *S. hermanii* has antibacterial activity, both against pathogens *S. aureus* and *E. coli* (Table 2 and Figure 2). However, antibacterial activity was only observed at a concentration of 2000 µg/disc, while at concentrations of 250 µg/disc, 500 µg/disc, and 1000 µg/disc, no antibacterial activity was observed. Amoxicillin with a concentration of 30 µg/disc was used as a positive control; the clear zone produced was larger than the diameter of the clear zone produced by the sample.

Table 2. Clear zone diameter of crude collagen extract against *S. aureus* and *E. coli*

Concentration	*S. aureus*		*E. coli*	
	24 hours	48h	24 h	48h
250	0	0	0	0
500	0	0	0	0
1000	0	0	0	0
2000	7.7±0.1	6.3±0.1	5.4±0.01	5.4±0.01
K+	14.4±2.4	11.4±1.8	16.2±1.4	10.2±0.6
K- (DMSO 10%)	0	0	0	0

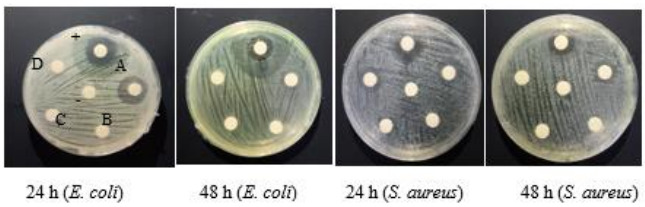

Figure 2. Antibacterial test results of crude collagen extract *S. hermanii*
Note: (A) 2000 µg/disc; (B) 1000 µg/disc; (C) 500 µg/disc; (D) 250 µg/disc; (+) K positive; (-) Negative control

Antibacterial activity is indicated by the formation of a clear zone around the paper disc. The wider the diameter of the clear zone, the stronger the antibacterial activity. This is due to the activity of the compound inoculated on paper discs, which is able to inhibit the growth of pathogenic bacteria. At the end of the study, the diameter value of the clear zone against *S. aureus* (6.3±0.1 mm) was larger than the clear zone against *E. coli* (5.4±0.01 mm). This is caused by differences in the cell wall composition of the test bacteria. Gram-negative bacteria such as *E. coli* have a more complex cell wall layer, consisting of 3 layers, namely lipoprotein, lipopolysaccharide, and peptidoglycan. This makes the cell walls difficult for foreign compounds to penetrate from outside. Gram-positive bacteria, such as *S. aureus,* although they have thicker peptidoglycan than gram-negative bacteria, have a simpler cell wall structure and are mostly polysaccharides, so they are more easily penetrated by compounds [30].

Table 2 provides the diameter of the clear zone from the crude collagen extract *S. hermanii* to *S. aureus* and *E. coli.* The diameter of the clear zone against *S. aureus* experienced a decline initially of 7.7 ± 0.1 mm at 24-h observation, which became 6.3 ± 0.1 mm. On the other hand, the diameter of the clear zone is *E. coli* did not change, namely 5.4 ± 0.01 mm. Antibacterial activity in this study is only exhibited by the concentration of 2000 µg/disc, this suggests that the collagen extract might have concentration-dependent activity [30, 31], requiring higher amounts to be effective or it could indicate low bioavailability, where the extract isn't sufficiently absorbed or distributed at lower concentrations [32]. Based on this, it can be seen that the antibacterial activity of collagen crude extract is included in the bacteriostatic category. Bacteriostatic antibacterial activity was characterized by a slightly cloudy clear zone and the appearance of dots of bacterial colonies in the clear zone, although faint [33]. This shows that the compound tested was able to inhibit the growth of the test bacteria but was not completely able to eliminate or kill the test bacteria. The diameter of the clear zone against *S. aureus* and *E. coli* has a value of >5 mm and is included in the medium category [34].

3.4 Antifungal activity

The results show that the crude collagen extract of *S. hermanii* has antifungal activity against *C. albicans,* however, it was not observed to have antifungal activity against *Trichoderma* sp. (Figure 3). Antifungal activity is indicated by the clear zone around the paper disc. The largest clear zone towards *C. albicans was* shown by an extract concentration of 500 µg/disc with a clear zone value of 19.7 ± 2.5 mm at 24 hours of observation, then decreased to 17.2 ± 1.6 mm at 48 hours of observation. The smallest clear zone diameter was shown by an extract concentration of 250 µg/disc with a clear zone value of only 5.9 ± 0.5 at 24 hours of observation and 5.1 ± 0.1 at 48 hours. The positive control used was 30 µg/disc ketoconazole with a clear zone value greater than the sample.

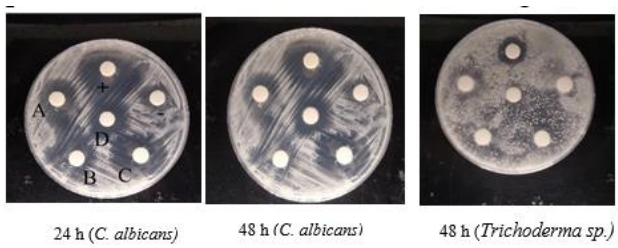

Figure 3. Antifungal test results of collagen crude extract *S. hermanii*

Antifungal activity was divided into 3 categories based on the diameter of the clear zone [22]. The length of the clear zone diameter > 21 mm is categorized as very strong, 11-20 mm is in the strong category, 6-10 mm is in the medium category, and < 5 mm is in the very weak category. Based on this category, collagen crude extract has strong antifungal activity against *C. albicans* except for the extract with a concentration of 250 µg/disc. The results of the statistical analysis show that the p-value is < 0.05, meaning that the difference in treatment has a significant impact on the diameter of the clear zone produced. This is thought to be because at low concentrations, active compounds more easily penetrate fungal cells and damage them.

3.5 Sun protection factor

The results of determining the SPF value of the crude extract of sea cucumber collagen can be seen in Figure 4. The variations in concentration used were 1000 µg/ml, 800 µg/ml, 600 µg/ml, 400 µg/ml, and 200 µg/ml. The higher the extract concentration, the higher the SPF value. The highest SPF value was shown by the collagen extract with a concentration of 1000 µg/ml with an SPF value of 1.46. The lowest SPF value was shown by the collagen extract with a concentration of 200 µg/ml worth 0.2.

The Sun Protection Factor is a value that shows the ability

of a compound to protect the skin from the effects of solar radiation [35]. A high SPF value indicates high-quality sunscreen. According to the FDA (Food Drug Administration), the SPF value of sunscreen is divided into 5, namely minimum (SPF between 2-4), medium (SPF between 4-6), Extra (SPF between 6-8), maximum (SPF between 8- 15) and ultra (SPF > 15). The results of determining the SPF value show that the highest SPF value is the crude extract of collagen *S. hermanii* is 1.46 at a concentration of 1000 µg/ml. Based on these results, it can be said that the crude extract of sea cucumber collagen *S. hermanii* has no potential as a sunscreen. This could be because collagen lack the UV protection properties, since the primary focus of collagen from sea cucumbers is on its structural and bioactive properties, such as enhancing skin elasticity, providing antioxidant benefits, and supporting wound healing. However, it should also be noted that the SPF value is significantly influenced by the extract concentration ($p < 0.05$), so it is necessary to carry out further testing with higher concentrations to better understand the SPF value of collagen crude extract.

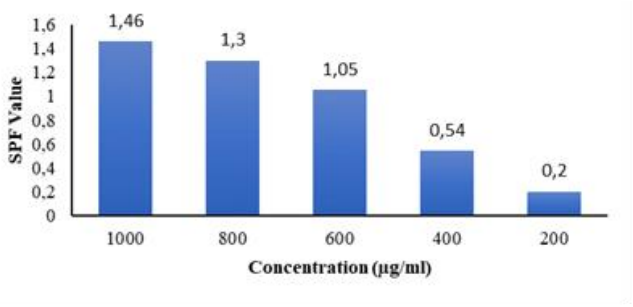

Figure 4. SPF value of collagen crude extract *S. hermanii*

4. CONCLUSION

Wet collagen crude extract was successfully extracted from *S. hermanii* with a total yield of 3.75%. The results showed that collagen crude extract had antioxidant activity reaching IC50 56.82 µg/ml, and positive antibacterial activity against *S. aureus* and *E. coli* with clear zone diameters at the end of the study respectively 6.3 ± 0.1 mm and 5.4 ± 0.01 mm, positive for antifungal activity against *C. albicans* (17.2±1.6 mm), but does not have antifungal activity against *Trichoderma* sp. Collagen crude extract has no potential as a sunscreen because of its low SPF value. This study is limited to crude collagen extract; further studies using purified collagen and more in-depth analysis are needed to better understand the antioxidant, antibacterial, and antifungal activity of collagen derived from *S. hermanii.*

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