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Antibacterial Activity Test of Etanol Extract of Black Sea Cucumber (*Holothuria atra*) against the Bacteria *Staphylococcus aureus* and *Escherichia coli*

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Abstract

Black Sea cucumber (*Holothuria atra*) contain chemical compounds that has antibacterial activity. One of the bacteria that is pathogenic to humans and at risk of death are *Staphylococcus aureus* and *Eschericia coli.* The purpose of this study is to ascertain whether black sea cucumber ethanol extract in Pataomeme waters has antibacterial activity against the microorganisms *E. coli* and *S. aureus*. The type of research used is quantitative research with quasi laboratory experiments. Antibacterial activity test using disc diffusion method. Black Sea cucumber extract was made in 3 concentrations, consisted of 7.5%, 10% and 12.5%. The results showed that black sea cucumber extract has antibacterial activity seen through the inhibition zone formed. The maximum inhibition zone is at a concentration of 12.5% sea cucumber extract, which is 28.59 mm against *S. aureus* bacteria and 21.02 mm against *E. coli* bacteria. According to our results, sea cucumber may be a suitable marine source of antibacterial substances. To ascertain its potential use in other areas of medicine, more in vivo research must be conducted.

Keywords: Sea Cucumber, Antibacterial, *Staphylococcus aureus*, *Escherichia coli*.

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1. INTRODUCTION

Bacterial resistance to antibiotics is nothing new. The overuse of antibiotics is the main cause of many disease-causing bacteria that are resistant to antibiotics. The increasing resistance of bacteria to antibiotics is a serious threat to the healthcare sector (Mamangkey et al., 2022). With this increase in resistance, it is necessary to discover and develop new types of antibiotics that are effective against the occurrence of resistance in bacteria. This also provides a great opportunity in utilizing the potential of natural resources, especially animals in Indonesian waters as potential antibacterial agents such as sea cucumbers (Nimah et al., 2012; Aba, & Rusliadi, 2020; Abdulkadir, Suleman, & Hasan, 2021).

The black sea cucumber is a tropical marine species that has long been utilized in traditional practices as a natural remedy for various health conditions (Lee et al., 2019). Black Sea cucumber (*Holothuria atra*) belong to the holothuroide class of marine organisms that are included in echinoderms, spineless and soft-bodied (Roihanah, Sukoso, & Andayani, 2012; Umboh, Wewengkang, & Yamlean, 2018). Based on research Husain et al., (2022), black sea cucumbers taken in *Patoameme* Village, *Boalemo* Regency have antioxidant compounds, namely phenols with total phenolic levels of 1.88 ± 0.065 mg / extract and flavonoids with total flavonoid levels of 1.01 ± 0.013 mg / extract. Black sea cucumbers have very high protein and collagen content (Bordbar et al., 2011). In addition, black sea cucumbers also contain flavonoids, phenols, saponins, triterpenoids and alkaloids, which act as antiangiogenic, anticoagulant, antiviral, antitumor, antithrombocyte, anticancer and antibacterial (Roihanah, Sukoso, & Andayani, 2012; Dwicahyani et al., 2018; Oktaviani et al., 2015). An antibacterial agent damages the metabolism of bacteria, preventing them from growing or perhaps killing them (Kano et al., 2022). The compounds that act as antibacterials are flavonoids, phenols, saponins and alkaloids (Direktorat Jenderal Kefarmasian dan Alat Kesehatan, Kementerian Kesehatan Republik Indonesia, 2017). Black sea cucumber has bioactive compounds as potential antibacterial agents, antibacterial activity in black sea cucumber which has proven potential as an antibacterial including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus cereus* (Sari et al., 2014; Manoppo, Wewengkang, & Kojong, 2017).

Flavonoid compounds work against bacteria by rupturing the cell wall and interfering with metabolic functions. The mechanism of antibacterial action of phenolic compounds is to denature proteins and damage cell membranes. The antibacterial properties of saponin compounds work by decreasing the cell wall's surface tension. By dissolving the peptidoglycan components in bacterial cells, alkaloid compounds function as antibacterials (Dwicahyani et al., 2018). Chloramphenicol is used as a comparison of antibacterial compounds because it has a broad spectrum and has a mechanism of action that inhibits bacterial protein synthesis. Bacteria are one of the causes of various infectious diseases. One of the bacteria that is pathogenic to humans and at risk of death is *S. aureus* and *E. coli*.

Based on the research of Dwicahyani et al., (2018), it shows that the ethanol extract of black sea cucumbers from the waters of *Menjangan Kecil* Island, *Karimunjawa* Islands, Central Java has the ability as an antibacterial against *S. aureus* bacteria with a concentration of 2.5% producing an inhibition zone of 3.32 ± 0.27 mm and *E. coli* with a concentration of 2.5% producing an inhibition zone of 2.40 ± 0.15 mm. Another study showed that at a concentration of 10 ppm, fraction A of Holothuria atra exhibited antibacterial activity comparable to tetracycline at a concentration of 5 ppm. However, its antibacterial activity against E. tarda was weak (Kartikaningsih et al., 2018). The purpose of this study is to ascertain whether black sea cucumber ethanol extract in Pataomeme waters has antibacterial activity against the microorganisms *E. coli* and *S. aureus.*

2. RESEARCH METHOD

This research was an experimental laboratory study using a completely randomized design (CRD) with five treatment groups: three different concentrations of black sea cucumber extract (7.5%, 10%, and 12.5%), a positive control (chloramphenicol), and a negative control (aquadest). Each treatment was tested against two bacterial strains, *Staphylococcus aureus* and *Escherichia coli*, in triplicate.

Sample Extraction. Fresh black sea cucumbers (*Holothuria atra*) were thoroughly cleaned, and the internal organs were removed. The samples were cut into small pieces to increase the surface area for extraction. The extraction process involved maceration, where 136 grams of the sample were soaked in 3000 mL of 96% ethanol for 72 hours. This process was repeated three times, and the maceration was carried out in a dark environment to prevent exposure to direct sunlight. Following maceration, the mixture was filtered using filter paper. The filtrate was then concentrated using a rotary evaporator at 50°C until a thick paste formed. This crude extract was weighed, stored in vial bottles, and refrigerated at 4°C for subsequent antibacterial analysis (Husain et al., 2022).

Antibacterial Activity Testing. The antibacterial activity of the 95% ethanol extract was tested at concentrations of 7.5%, 10%, and 12.5%. Aquadest was used as the negative control, while chloramphenicol served as the positive control. Paper discs were impregnated with each treatment solution by immersing them in the respective extracts and control solutions. A 50 µL aliquot of actively growing bacterial culture (*S. aureus* and *E. coli*) was pipetted into sterile Petri dishes containing nutrient agar and evenly spread. The treated paper discs were then placed onto the agar surface using sterile forceps. The plates were incubated at 37°C for approximately 48 hours. After incubation, the inhibition zones were measured using a caliper. The diameter of the inhibition zone was calculated by subtracting the diameter of the paper disc and the solvent inhibition zone (if any) from the total diameter (disc + inhibition zone) (Arifin et al., 2013).

Statistical Analysis. The inhibition zone data were first tested for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. The Shapiro-Wilk test indicated that the data were normally distributed ($p > 0.05$), while the Levene's test indicated non-homogeneous variance ($p < 0.05$). Given these results, a One-Way ANOVA was conducted to determine significant differences among treatment groups, followed by a Post Hoc Least Significant Difference (LSD) test for pairwise comparisons.

The ANOVA test revealed significant differences among the treatment groups ($p = 0.000 < 0.05$). Post Hoc LSD analysis showed that the 12.5% concentration did not differ significantly from the 10% concentration but differed significantly from the 7.5% concentration, positive control, and negative control. The 10% concentration was not significantly different from the 12.5% and 7.5% concentrations but showed significant differences compared to the positive and negative controls. The 7.5% concentration was significantly different from the 12.5% concentration, positive control, and negative control but not from the 10% concentration. Both the positive and negative controls showed significant differences from all extract concentrations and each other. This research has also received Ethical Approval from the Ethics Commission of the Poltekkes Kemenkes Gorontalo with Number: DP.01.01/KEPK/104/2023.

3. RESULTS AND DISCUSSION

Antibacterial activity is influenced by several factors, namely extract concentration, antibacterial compound content, extract diffusion power and the type of bacteria inhibited. (Rahman, Haniastuti, & Utami, 2017). Sea cucumber identification is currently developing by utilizing sea cucumber meat. Sea cucumber bodies generally contain secondary metabolites while the utilization of sea cucumber innards is currently still not widely used. The process of processing sea cucumbers in the innards in the form of intestines, gonads and other organs are simply thrown away. This situation encourages efforts to utilize it.

An antibacterial activity test can be used to identify pure compounds with antibacterial activity as well as the degree to which bacteria are susceptible to antibacterial agents. Antibacterial activity test can be done by disc diffusion method. The 95% ethanol extract is used in the antibacterial activity test, with concentration variations of 7.5%, 10%, and 12.5%. Aquadest is the negative control, and chloramphenicol is the positive control.

This study uses black sea cucumber samples with the aim of testing the inhibition in inhibiting the growth of *S. aureus* and *E. coli* bacteria using the disc diffusion method. The disc diffusion method is done by using disc paper as a medium to absorb samples or antibacterial ingredients. Subsequently, the disc paper is positioned on top of agar medium that has been infused with a test bacterial culture, and it is incubated for a full day at 37°C. It was found that the area surrounding the disc paper, or the clear inhibition zone, indicated whether or not there was bacterial growth. The results of the antibacterial test of ethanol extract of black sea cucumber against the growth of *S. aureus* and *E. coli* bacteria as shown in Table 1.

Table 1. Antibacterial Test Results of Black Sea Cucumber Ethanol Extract against the Growth of *S. aureus* and *E. coli* Bacteria

Bacteria	Extract	Diameter of Inhibition (mm)				Inhibition category
		R1	R2	R3	Average	
Staphylococcus aureus	7.5%	22.96	21.09	16.45	20.16	Strong
	10%	25.75	24.01	20.73	23.50	Very strong
	12.5%	31.16	28.55	26.06	28.59	Very strong
	Positive control	33.39	33.31	31.39	32.70	Very strong
	Negative control	0	0	0	0	None
Escherichia coli	7.5%	17.43	17.00	13.08	15.83	Strong
	10%	18.60	18.37	17.15	18.04	Strong
	12.5%	21.52	23.42	18.14	21.02	Very strong
	Positive control	31.61	26.55	23.08	27.08	Very strong
	Negative control	0	0	0	0	None

Abbrev: R = Repeat of diameter measurements

Table 1 shows that the inhibition test of S. aureus shows that the average inhibition category is very strong at extract concentrations of 10% and 12.5%, while at 7.5%, it is in the strong category. The results of the antibacterial test on E. coli showed that the average inhibition category was very strong at extract concentrations of 12.5%, while at 7.5% and 10%, it was in the strong category.

Table 2. One-Way ANOVA Results of Inhibitory Activity among Treatment Groups

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1927,042	4	481,761	94,396	,000
Within Groups	51,036	10	5,104		
Total	1987,078	14			

Table 2 shows that the One Way Anova test findings showed a p-value of 0.000 <0.05, indicating a significant difference in the sample, then continued with the Post Hoc LSD further test to determine the difference.

Table 3. LSD Post Hoc Test Results for Differences in Inhibitory Activity between Treatment Groups

Concentration (I)	Concentration (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
12.5%	10%	5.09333*	1.84456	.020	.9834	9.2033
	7.5%	8.42333*	1.84456	.001	4.3134	12.5333
	Positive Control	-4.10667	1.84456	.050	-8.2166	.0033
	Negative Control	28.59000*	1.84456	.000	24.24801	32.6999
10%	12.5%	-5.09333*	1.84456	.020	-9.2033	-.9834
	7.5%	3.33000	1.84456	.101	-.7799	7.4399
	Positive Control	-9.20000*	1.84456	.001	-13.3099	-5.0901
	Negative Control	23.49667*	1.84456	.000	19.3867	27.6066
7.5%	12.5%	-8.42333*	1.84456	.001	-12.5333	-4.3134
	10%	-3.33000	1.84456	.101	-7.4399	.7799
	Positive Control	-12.53000*	1.84456	.000	-16.6399	-8.4201
	Negative Control	20.16667*	1.84456	.000	16.0567	24.2766
Positive Control	12.5%	4.10667	1.84456	.050	-.0033	8.2166
	10%	9.20000*	1.84456	.001	5.0901	13.3099
	7.5%	12.53000*	1.84456	.000	8.4201	16.6399
	Negative Control	32.69667*	1.84456	.000	28.5867	36.8066
Negative Control	12.5%	-28.59000*	1.84456	.000	-32.6999	-24.4801
	10%	-23.49667*	1.84456	.000	-27.6066	-19.3867
	7.5%	-20.16667*	1.84456	.000	-24.2766	-16.9567
	Positive Control	-32.69667*	1.84456	.000	-36.8066	-28.5867

**The mean difference is significant at the 0.05 level*

Table 3 shows that the considering the Post Hoc LSD test results, the 12.5% concentration does not have a significant difference to the 10% concentration, but is significant to the 7.5% concentration, positive control and negative control. There is no discernible difference between the 12.5% and 7.5% concentrations and the 10% concentration, but is a significant to the positive control and negative control. 7.5% concentration has no significant difference to the 10% concentration, but is significant to the 12.5% concentration, positive control and negative control. Positive control had a significant difference against 12.5%, 10%, 7.5% concentration and the negative control. Negative control had significant difference against 12.5%, 10%, 7.5% and positive control concentrations.

DISCUSSION

As shown in Table 1, the results of this study support the findings of Dwicahyani et al. (2018), which demonstrated that ethanol extract of black sea cucumber (*Holothuria atra*) from the waters of Menjangan Kecil Island, Karimunjawa, Central Java exhibited antibacterial activity. At a 7.5% concentration, the extract produced an inhibition zone of 6.98 mm against *Staphylococcus aureus* and 5.93 mm against *Escherichia coli*, both of which fall within the moderate antibacterial activity category.

In comparison, Septiani et al. (2017) reported that black sea cucumber extract from the Sepanjang waters of Yogyakarta showed strong antibacterial activity at a higher concentration of 15%, with inhibition zones of 5.84 mm for *S. aureus* and 5.63 mm for *E. coli*. These findings indicate that the antibacterial potency of sea cucumber extracts may vary depending on geographical origin, extraction concentration, and phytochemical composition.

Previous studies by Husain et al. (2022) confirmed the presence of phenolic and flavonoid compounds in black sea cucumbers collected from the waters of Patoameme. Additionally, other studies have reported the presence of bioactive compounds such as saponins, triterpenoids, and alkaloids (Roihanah, Sukoso, & Andayani, 2012; Dwicahyani et al., 2018; Oktaviani et al., 2015). These secondary metabolites are known to contribute to antibacterial activity.

Flavonoids exert their antibacterial effects by damaging bacterial cell walls, inhibiting nucleic acid synthesis, disrupting cytoplasmic membrane function, and interfering with metabolic pathways. Phenolic compounds act by denaturing proteins and disrupting bacterial cell membranes. Saponins reduce surface tension on the bacterial cell wall, while alkaloids interfere with the integrity of bacterial peptidoglycan layers, leading to cell lysis (Dwicahyani et al., 2018).

For comparison, chloramphenicol was used as a positive control due to its broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria. Chloramphenicol functions by inhibiting bacterial protein synthesis through binding to the 50S ribosomal subunit, effectively serving as a standard for evaluating the antibacterial potential of natural compounds (Wendersteyt et al., 2021). This study has several limitations. First, the antibacterial tests were limited to only two bacterial strains, *S. aureus* and *E. coli*, which may not fully represent the extract's broad-spectrum potential. Second, only three concentrations of the extract were tested, without exploring minimum inhibitory concentrations (MIC) or minimum bactericidal concentrations (MBC), which could provide a more accurate measure of antibacterial potency. Third, the extraction process used ethanol only; using other solvents or purification steps could potentially yield different or enhanced antibacterial activity. Lastly, no phytochemical quantification was conducted in this study to correlate compound concentration with antibacterial activity.

4. CONCLUSION

Based on the results demonstrated that the ethanol extract exhibited inhibitory effects against both bacterial strains. The minimum inhibitory concentration was observed at 7.5%, with an inhibition zone of 20.16 mm for S. aureus and 15.83 mm for E. coli, indicating strong antibacterial activity. These findings suggest that the black sea cucumber has potential as a natural marine-based antibacterial agent. However, to explore its therapeutic applications further, additional studies involving compound isolation, phytochemical quantification, and in vivo evaluations are necessary.

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