Antibacterial potential of crude extracts from sea cucumber *Holothuria fuscoscinerea* Jaeger, 1833

Genese Divine B. Cayabo and Jhonamie A. Mabuhay-Omar
College of Fisheries and Aquatic Sciences, Palawan, Philippines
Western Philippines University-Puerto Princesa Campus
Corresponding Author: jhonamie@gmail.com

ABSTRACT

This study was conducted to determine the antibacterial potential of the crude extracts of skin, gonad, Cuvierian tubules, Polian vesicles and intestine of the sea cucumber *Holothuria fuscoscinerea* collected from Rasa Island, Narra, Palawan, Philippines. The antibacterial potential of the extracts was determined against *Escherichia coli* and *Staphylococcus aureus* using filter paper disc diffusion method with Tetracycline as the positive control and distilled water as the negative control. The analysis of variance (ANOVA) of the results showed significant differences in the effects of the treatments when tested against *S. aureus* and *E. coli* ($p<0.05$). Tukey's test further proved that Polian vesicle was significantly highest in terms of antibacterial property among other extracts but not comparable to positive control against *S. aureus*. On the other hand, Tukey's test showed that Cuvierian tubules and Polian vesicles were not significantly different from each other in terms of antibacterial effect but not comparable to tetracycline when tested against *E. coli*. The extracts from skin, gonad and intestine did not show inhibitory effect on the test organisms. T-test showed that *E. coli* and *S. aureus* were not significantly different in terms of susceptibility towards the treatments. Based on the results, extracts from Cuvierian tubules and Polian vesicles of *H. fuscoscinerea* are potential sources of antibacterial compounds.

Keywords: sea cucumber, *Holothuria fuscoscinerea,* antibacterial potential, Cuvierian tubules, Polian vesicles

INTRODUCTION

Bacteria are one of the oldest and simplest organisms but they are more diverse than all other life-forms combined (Stainer et al. 1979). Bacteria are ubiquitous on earth as they play vital roles in many ecosystems. On the other hand, bacteria are also responsible for many serious human diseases that sometimes cause millions of deaths each year (Mitchel 1974). They are also causing problems in aquaculture where infectious diseases

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are always a hazard and may cause significant stock losses and problems in animal welfare (Romero et al. 2012).

Aquaculture is becoming a more concentrated industry in the country. It is still a fast-growing food production sector because it is an increasingly important source of protein for human consumption (Rodgers and Furones 2009). The Food and Agriculture Organization of the United Nation (UN FAO) estimates that half of the world's seafood demands will be met by aquaculture in 2020 (Moriarty 1999). In spite of this, aquaculture faces many problems that may hinder its increasing production. Diseases caused by bacteria and parasites are major factors that influence the production and quality of stocks (Frappado and Guest 1986). To counteract this problem, aquaculture industries use antibiotics to combat these fish diseases and parasites. Administration of low dose of antibiotics for growth promotion has also led to the increased use of antibiotics in aquaculture. However, the potential consequences of antibiotics use are the development of antibiotic-resistant microorganisms, multiple antibiotic resistance, and resistance transfer to pathogenic bacteria and reduced efficacy of antibiotic treatment for diseases caused by resistant pathogens (Khan 2008).

Antibiotic resistance of bacteria has led to the search for potential sources of bioactive compounds with antimicrobial properties. Some marine invertebrates successfully thrive in environments full of pathogenic microorganisms which develop their defense mechanisms and have made them prime candidates for extraction of antimicrobial compounds (Mydlarz et al. 2006). Sea cucumbers are presented as potential marine sources of antimicrobial compounds (Mokhlesi et al. 2012). They can be found throughout the world and have been used medicinally in Asian countries for years (Boadbar et al. 2011). The bioactive compounds in sea cucumbers make it a potent therapeutic food source. This echinoderm lives in the benthic areas and is a treasure chest of many vitamins and minerals, and contains anti- inflammatory, antimicrobial, antioxidant and anti- angiogenic properties (Mokhlesi et al. 2012). Some species of Family Holothuriidae exhibit antimicrobial activity like *Holothuria atra* (Boadbar et al. 2011), *H. leucospilota* (Mokhlesi et al. 2012), and *H. scabra* (Althunibat et al. 2009).

Holothuria fuscoscinerea is one among the sea cucumber species found in the Philippines (Kerr et al. 2006). It is usually found under rubble on the reef flat during the day and venturing out only at night. This species grows up to 30 cm long (Purcell et al. 2012). It readily releases large translucent Cuvierian tubules when disturbed (Kerr et al. 2006). However, this species has low commercial value in Western Central Pacific, though it is of commercial importance in China, Malaysia and Philippines (Purcell et al. 2012). According to Schoppe (2000), *H. fuscoscinerea* has a value of about PhP55 kg^{-1} or 1.38 US$ kg^{-1}. Since *H. fuscoscinerea* occurs in Rasa Island

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Wildlife Sanctuary in Narra, Palawan, Philippines, looking for other potential use of this organism such as antibacterial resource is deemed important.

This study aimed to determine the antibacterial potential of crude extracts from sea cucumber *Holothuria fuscocinerea*. Specifically, this study aimed to (1) determine the antibacterial effect of the crude extracts from gonad, intestine, Cuvierian tubules, Polian vesicle and skin of the sea cucumber against *Escherichia coli* and *Staphylococcus aureus*; (2) if antibacterial effect is present, determine which of the five extracts (gonad, intestine, Cuvierian tubules, Polian vesicle, skin) of sea cucumber would show the highest antibacterial potential and (3) determine which of the two test bacteria (*Escherichia coli* and *Staphylococcus aureus*) is more susceptible to the treatments if antibacterial potential is present.

MATERIALS AND METHODS

Sample Collection and Locale of the Study

This study was conducted from October to November 2015. Samples of *H. fuscoscinerea* were collected from Rasa Island, Narra, Palawan (Figure 1). The Island is characterized by having coastal forest, mangrove area, coconut plantation, barren areas and coral outcrops. Samples were collected during low tide in the reef areas. The collected samples were cleaned by rinsing with seawater, placed in a cooler box (with seawater) and immediately transported to the laboratory for analyses.

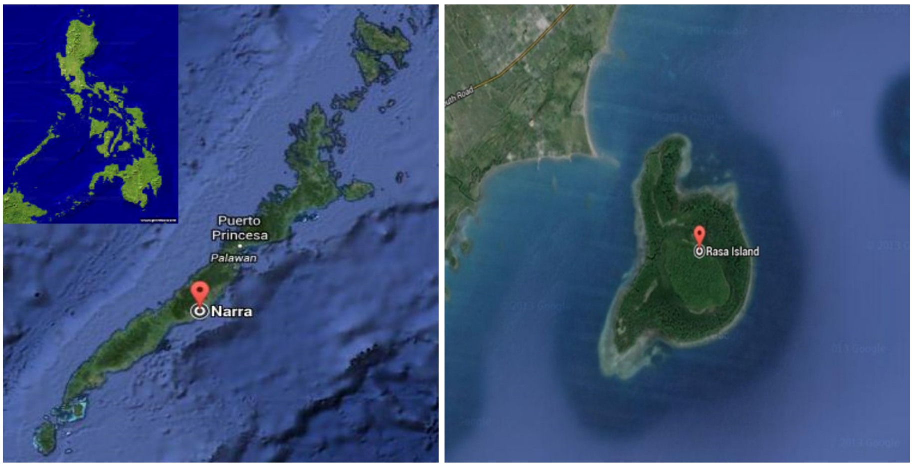

Figure 1. Map of Palawan, Philippines (left); location of Rasa Island, Narra, Palawan, Philippines (right) (Source: Google map).

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The intestine, gonads, Cuvierian tubules, Polian vesicles and skin were separated in the Microbiology Laboratory Room of Western Philippines University - Puerto Princesa Campus, Sta. Monica, Puerto Princesa City. These were placed in separated pre-labeled sterile containers and extraction immediately followed.

Inducement of Cuvierian Tubules

The expulsion of Cuvierian tubules was induced mechanically by pinching the dorsal integument of the sea cucumber with forceps. Five individuals were stimulated twice to expel about 10 tubules (Vandenspiegel et al. 1999). After tubule expulsion, Cuvierian tubules were placed in a sterile pre-labelled container.

Culture Media Preparation

The nutrient agar was prepared by dissolving 23 g of nutrient agar in 1000 ml distilled water while the nutrient broth was prepared by dissolving 13 g of nutrient broth in 1000 ml distilled water as prescribed by the manufacturers. Both preparations were done in Erlenmeyer Flasks aided by hot water bath to hasten the dissolution with constant stirring until the liquid became transparent. Sterilization at 121°C, 15 psi for 15 minutes then followed (Mabuhay 2015).

Preparation of Microorganisms

Pure cultures of *E. coli* and *S. aureus* were obtained from the culture collection of DOST-Palawan, Sta. Monica, Puerto Princesa City, Palawan. These were the bacteria used in the study because they were readily available to represent the two physiologic groups of bacteria, the Gram-positive and the Gram-negative bacteria.

Extraction of Samples

Each of the samples of intestines, gonads, Cuvierian tubules, Polian vesicles and skin was soaked in 10 ml distilled water for 3 min and then the water was discarded. This procedure was repeated five times to remove the salt content of the samples. After soaking, the samples were crushed separately using a mortar and pestle with the ratio of 4 g of sample is to 1 ml distilled water. Four replicates were prepared for each sample. The extracted samples were then placed in pre-labelled sterile containers. The samples were stored at refrigerator for at 0°C until usage (Mokhlesi et al. 2012).

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Antibacterial Assay

The filter paper disc diffusion method was used to determine the antibacterial potential of the extracts from sea cucumber. For the positive control, a 500 mg Tetracycline, which was dissolved in 10 ml sterile distilled water, was used. Tetracycline is one of the popular antibiotics, which are most extensively used in aquaculture (Neela et al. 2008). On the other hand, 10 ml sterile distilled water was used as the negative control. The treatment designations were the following; T1 (Gonad), T2 (Skin), T3 (Cuvierian Tubules), T4 (Polian vesicle), T5 (Intestine), T6 (Positive control) and T7 (Negative control).

A loopful of test organisms, *E. coli* and *S. aureus*, from the subcultures were inoculated into the sterile nutrient agar (20 ml) by direct seeding before pouring it into Petri dishes and allowed to solidify. The previously sterilized filter paper discs (cut by paper puncher to 6mm diameter) were soaked to saturation in different extracts (gonad, intestine, Cuvierian tubules, Polian vesicle, skin) and in the positive and negative control using sterile forceps. These impregnated discs were placed on the designated areas (3 discs on 1 plate) (Figure 2). Four replicates for each treatment were prepared. The Petri plates were incubated for 24 hours to allow bacterial growth. After 24 hours, the plates were examined and zones of inhibition were measured using standardized transparent ruler (in mm).

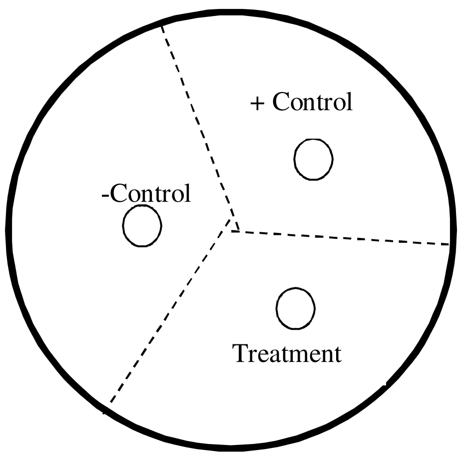

Figure 2. Illustration of antibacterial assay.

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Statistical Analyses

The data on the zones of inhibition of the treatments against *Escherichia coli* and *Staphylococcus aureus* were analyzed using one- way analysis of variance (ANOVA) to test the significant differences. The data were subjected to Post Hoc test (Tukey's Test) to compare the means. T-test was used to test the significant difference in the susceptibility between the two test organisms.

RESULTS

Antibacterial Effect of Treatments against *E. coli*

In this study, extracts from the skin, Cuvierian tubules and Polian vesicle showed zones of inhibition when tested against *E. coli* while gonad and intestine did not (Table 1). ANOVA proved that there were significant differences in the effects of the treatments when tested against *E. coli.* Tukey's test showed that the antibacterial property of Cuvierian tubules (Figure 3) and Polian vesicles (Figure 4) did not differ significantly from each other but significantly higher compared to other treatments. However, positive control is still significantly higher compared to Cuvierian tubules and Polian vesicles (Table 1). Although extract from skin showed zones of inhibition in 2 out of 4 replicates against *E. coli*, it was not significantly different from negative control.

Table 1. Mean zones of Inhibition (in mm) of the treatments against *E. coli*, its ANOVA and Tukey's test.

TREATMENTS	MEAN (mm)	Tukey's test (α=0.05)	ANOVA
T1 (Gonad)	**0**	**A**	**F=** 63.49** *p*=**.**000
T2 (Skin)	**6.5**	**A**	
T3 (Cuvierian Tubules)	**18.25**	**B**	
T4 (Polian vesicle)	**21.75**	**B**	
T5 (Intestine)	**0**	**A**	
T6 (Positive control)	**32.95**	**C**	
T7 (Negative control)	**0**	**A**	

**highly significant at α=0.01; Different letters signify significant differences at α=0.05

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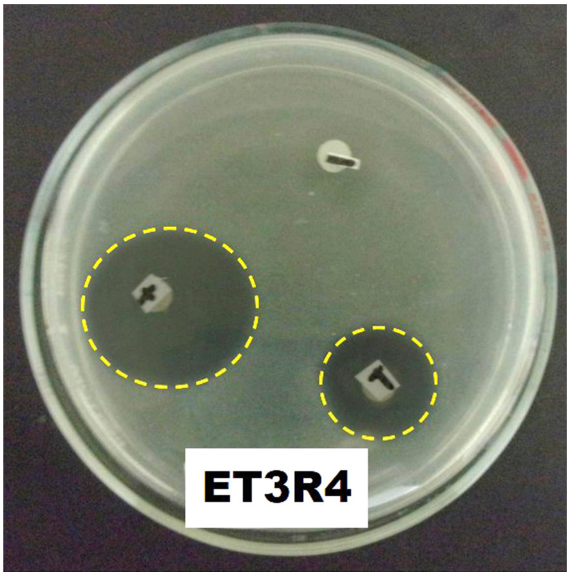

Figure 3. A representative replicate of Cuvierian Tubules extract against *E. coli*. Circles highlight the zones of inhibition. T for treatment, + for positive control, - for negative control.

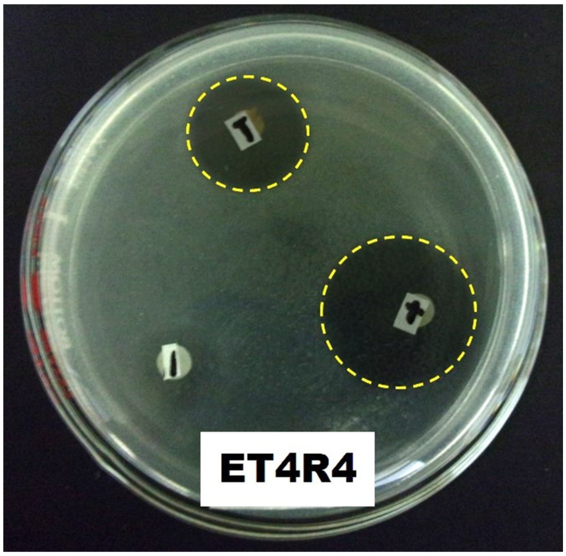

Figure 4. A representative replicate of Polian vesicle extract against *E. coli*. Circles highlight the zones of inhibition. T for treatment, + for positive control, - for negative control.

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Antibacterial Effect of Treatments against *S. aureus*

The gonads, Cuvierian tubules and Polian vesicle extracts showed zones of inhibition when tested against *S. aureus* while the skin and intestine did not (Table 2). ANOVA showed that there were significant differences in the effects of the treatments when tested against *S. aureus.* Tukey's test further proved that Polian vesicle (Figure 5) was significantly highest in terms of antibacterial property among other treatments next to positive control as shown in Table 2. Although the extracts of Cuverian tubules and gonad showed zones of inhibition in 2 of 4 replicates against *S. aureus*, they were not significantly different from negative control.

Table 2. Mean zones of inhibition (in mm) of the treatments against *S. aureus*, its ANOVA and Tukey's test.

TREATMENTS	MEAN (mm)	Tukey's test (α=0.05)	ANOVA
T1 (Gonad)	**5.5**	**A**	F= 38.204** *p*=.000
T2 (Skin)	**0**	**A**	
T3 (Cuvierian Tubules)	**5.75**	**A**	
T4 (Polian vesicle)	**16.25**	**B**	
T5 (Intestine)	**0**	**A**	
T6 (Positive control)	**35.15**	**C**	
T7 (Negative control)	**0**	**A**	

** highly significant at α=0.01, Different letters signify significant differences at α=0.05

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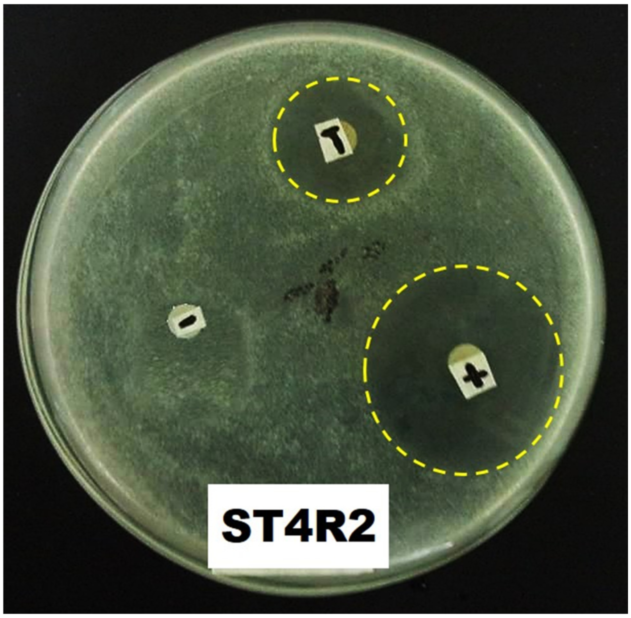

Figure 5. A representative replicate of Polian vesicle extract against *S. aureus.* Circles highlight the zones of inhibition. T for treatment, + for positive control, - for negative control.

Susceptibility of *E. coli* and *S. aureus* towards the Different Treatments

The T-test showed that *E. coli* and *S. aureus* were not significantly different in terms of susceptibility towards the treatments (Table 3).

Table 3. T- test on the susceptibility of *E. coli* and *S. aureus* towards the different treatments.

Pair	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
E. coli – S. aureus	2.40	7.215	1.363	-.397	5.197	1.76	27	.090[ns]

ns – not significant

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DISCUSSION

Among the five extracts tested, Polian vesicles showed the highest inhibitory effects against *E. coli* and *S. aureus*. According to Smith (1978) as cited by Boadbar et al. (2011), Polian vesicles of *Holothuria cinerascens* are known to be the organ attributing inflammatory (including immunologic) receptiveness of this species. Polian vesicles are "muscular" sacs arising interradially from the water ring. They accept excess water-vascular fluid when the animal contracts in an emergency and maintain pressure in the system (Baccetti and Rosati 1968). Echinoderms, like sea cucumbers, are able to give a cell- mediated response against pathogens and parasites (Canicattì et al. 2009). It is due to the presence of humoral substances such as agglutinins, lysins and other compounds that exert antibiotic effects. In some holothurians, Coelomocyte aggregates form around the foreign materials. They are referred to as brown bodies and represent an efficient structure related to the defense mechanism of the host (Canicattì et al. 2009). It is mainly produced in the coelomic cavity but they are also produced by Polian vesicles (Canicattì et al. 2009). Brown bodies from both coelomic cavity and Polian vesicles are composed of phagocytes, cells (as a white blood cell) that engulf and consume foreign material such as microorganisms and debris (Canicattì et al. 2009). They are probably elicited in response to natural invaders.

In this study, Cuvierian tubules extract showed inhibitory effect on *E. coli* but not to *S. aureus*. These results were different from the study conducted by Mokhlesi et al. (2012) wherein Cuvierian extracts of *Holothuria leucospilota* did not show antibacterial activity against *E. coli* and *S. aureus* (Table 4). However, it showed antifungal activity against *Candida albicans* and *Aspergillus niger*. Adhesion plays an important role in many invertebrates for a variety of different functions. Some species of holothuroids possess a special defense system involving adhesion, called the Cuverian tubules, which are mainly activated when mechanically stimulated. These white sticky filaments can entangle and immobilize potential predators (Baranowska et al. 2011). They are present in several species of order Aspidochirotida. Cuverian tubules are attached to the base of the respiratory trees and can be expelled in some *Holothuria* and *Bohadschia* species as a response to irritation (Mokhlesi et al. 2012). Cuverian tubules are made up of 60% protein and 40% carbohydrates. They are considered to be highly insoluble.

Numerous chemical and pharmacological studies were done on several species of sea cucumbers indicated that they contain triterpene glycoside with antifungal, antibacterial and cytotoxic properties (Mokhlesi et al. 2012; Aminin et al. 2015). A lot of these organisms produce their antibacterial factors as a first line of defense against pathogenic

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microorganisms (Ibrahim 2012). Huag et al. (2002) studied the antibacterial activity of different parts of sea cucumber *Cucumaria frondosa*. They found that antibacterial activity was detected but mainly on coelomycetes and body wall of the organism. According to Layson et al. (2014), *Holothuria nobilis, Bohadschia marmorata* and *Stichopus chloronotus* have antibacterial activity in all extracts (aqueous, chloroform and hexane), except in methanol. The body wall of *Stichopus hermanii* showed high antifungal activity when tested against the fungus *Aspergilus niger* but it did not show antibacterial property against *E. coli* and *S. aureus* (Sarhadizadeh et al. 2014) (Table 4).

Table 4. List of sea cucumber species and their body parts extracts tested against *E. coli* and *S. aureus.*

Sea Cucumber Species	Author/s	Body Parts	*E. coli*	*S. aureus*
Holothuria. leucospilota	Mokhlesi et al. 2012	Coelomic fluid	-	-
		Cuvierian organ	-	-
		Body wall	-	-
	Ibrahim HA, 2012	Coelomic fluid	-	+
		flesh	-	+
	Shakouri et al. 2014	Body wall	+	+
		Guts	+	+
		White strings	+	+
	Adibpour et al. 2014	Body wall	+	+
		Cuvierian organ	+	+
		Coelomic fluid	+	+
H. scabra	Ibrahim HA, 2012	Coelomic fluid	-	+
		flesh	-	+
H. atra	Ibrahim HA, 2012	Coelomic fluid	-	+
		flesh	-	+
Stichopus hermanni	Sarhadizadeh et al. 2014	Gonad	-	-
		Respiratory tree	-	-
		Cuvierian organ	-	-
		Body wall	-	-

+ = presence of antibacterial property: **-** = absence of antibacterial property

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This study proved that extracts from Cuvierian tubules and Polian vesicles of sea cucumber *Holothuria fuscoscinerea* have antibacterial property. This species contains triterpene glycosides (saponin) bioactive compound (Boadbar et al. 2011) which could probably be present in Cuvierian tubules and Polian vesicles which explains their antibacterial effects.

The susceptibility or resistance of the test microorganisms representing two physiologic groups, the Gram positive and Gram negative bacteria, gives a picture of the spectrum of the antibacterial potential of the treatments. Since the two test organisms did not vary in their susceptibility, the antibacterial potential of the treatments, especially Polian vesicle, can be applied to both Gram positive and Gram negative bacteria.

Due to emergence of a number of drug- resistant bacteria, fungi and viruses, extraction of different sea cucumber species and other potential sources of antimicrobial compounds were conducted. *Holothuria fuscoscinerea* is one of the potential species of sea cucumber to be a good source of antibacterial compounds. In this study, its Polian vesicles extract showed highest antibacterial property when tested against *E. coli* and *S. aureus*. Cuvierian tubules extracts showed only antibacterial property against *E. coli*. *S. aureus* and *E. coli* have similar susceptibility towards the treatments. These findings are significant in medical, pharmacological and allied health fields as *Holothuria fuscoscinerea* can be a source of potent antimicrobial drugs within the reach.

With the findings of the study, it is recommended to isolate and identify bioactive compounds from the active extracts (Cuvierian tubules and Polian vesicles) of sea cucumber *Holothuria fuscoscinerea*. Also, a similar study can be conducted using the same species but will be collected from other areas of Palawan and use other parts of the sea cucumber such as Coelomic fluid and respiratory tree for antibacterial property. The antibacterial potential of the sea cucumber extracts was proven in this study through the use of Filter Paper Disc Diffusion Method but is recommended to countercheck results by using other method such as Minimum Inhibitory Concentration and viability assays.

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