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Antibacterial and anti-inflammatory potential of three sea cucumber species from Tukuran, Zamboanga del Sur, Mindanao, Philippines

MARY GRACE EREGUERO[1], CHONA GELANI[3], RODELYN DALAYAP[4], MARY ANNE CORDERO[5], SHARON ROSE TABUGO[1,2,♥]

[1]Department of Biological Sciences, Mindanao State University-Iligan Institute of Technology. Iligan City 9200, Philippines. Tel./Fax.: +63-917-1100599, ♥email: sharonrose.tabugo@g.msuiit.edu.ph
[2]Molecular Systematics and Conservation Genomics Laboratory, Center for Biodiversity Studies and Conservation, Premier Research Institute of Science and Mathematics, Mindanao State University-Iligan Institute of Technology. Iligan City 9200, Philippines
[3]Department of Chemistry, Mindanao State University-Iligan Institute of Technology. Iligan City 9200, Philippines
[4]Department of Biology, Sultan Kudarat State University. Tacurong City 9800, Philippines
[5]Basic Science Department, College of Medicine, Princess Nourah bint Abdulrahman University. Airport Road, Riyadh 11564, Kingdom of Saudi Arabia

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Abstract. *Ereguero MG, Gelani C, Dalayap R, Cordero MA, Tabugo SR. 2023. Antibacterial and anti-inflammatory potential of three sea cucumber species from Tukuran, Zamboanga del Sur, Mindanao, Philippines. Biodiversitas 24: 2527-2535.* The marine environment is a rich source of potential antibacterial and anti-inflammatory agents. This study investigated three sea cucumber species' antibacterial and anti-inflammatory activity from Tukuran, Zamboanga del Sur, Philippines. The methanol: ethyl acetate crude extracts of *Holothuria scabra*, *Stichopus* sp., and *Holothuria atra* were tested for their antibacterial activity against gram-negative (*Pseudomonas aeruginosa, Escherichia coli*) and gram-positive (*Staphylococcus aureus, Bacillus subtilis*) bacteria using modified microtiter plate method. Results revealed that sea cucumber extracts have MIC values of 0.0008-0.0944 mg/mL. One-way ANOVA results revealed a *p* value <0.05, and Tukey's pairwise result confirmed that the antibacterial activity of *H. scabra* extract is not comparable with Ciprofloxacin (*p* value <0.05). However, *Stichopus* sp., and *H. atra* (1) and *H. atra* (2) revealed a comparable antibacterial activity with Ciprofloxacin (*p* value >0.05). The crude extracts were also evaluated for *in vitro* anti-inflammatory activity using the albumin denaturation method. The results showed a percentage inhibition within the range from 44.50-49.69%, 44.74-51.44%, 31.82-49.48%, and 49.16-52.15% inhibition at 200-, 100-, 50-, and 25 ppm, respectively. One-way ANOVA and Tukey's pairwise result revealed that the sea cucumber extracts showed a comparable percent inhibition with the positive controls, Celecoxib and Diclofenac sodium, at 200 ppm. This study revealed sea cucumber crude extracts as a potential antibacterial and anti-inflammatory agent.

Keywords: Albumin denaturation, MIC, microtiter resazurin, sea cucumber

INTRODUCTION

Sea cucumbers belonging to phylum Echinodermata and class Holothuroidea are worm-like, leathery-bodied, benthic marine organisms (Siddiqui et al. 2022). They occupy a harsh environment wherein they are exposed to extreme conditions; thus, they must adapt to survive. These marine organisms have the greatest biodiversity in Asia, with 1716 species (Pangestuti and Arifin 2018). Most sea cucumbers are deposit feeders. They play an important role in marine environments as environmental cleaners since they feed on sediments, organic matter, benthic microalgae, protozoa, and detritus microalgae (Oh et al. 2017). There are 47 identified sea cucumber species across Mindanao (Arriesgado et al. 2022).

Over the years, these species have been consumed in Traditional Chinese Medicine (Siddiqui et al. 2022) and were believed to alleviate several ailments such as asthma, back pain, burns, cuts, constipation, impotence, hypertension, joint pain, kidney problems, reproductive disorders, rheumatism, and wound healing (Hossain et al. 2020). They are also a source of high-value-added compounds associated with health benefits to be utilized as functional ingredients. Examples of functional ingredients derived from sea cucumbers are amino acids, bioactive peptides, carotenoids, collagen, chondroitin sulfates, fatty acids, gelatin, minerals, vitamins, and other bioactive compounds (Pangestuti and Arifin 2018). Recent discoveries of the different chemicals and metabolites found in the sea cucumber species were believed to have pharmaceutical effects such as anti-cancer, antibacterial, anti-coagulant, antidiabetic, anti-inflammatory, antioxidant, and anti-thrombotic (Siddiqui et al. 2022).

Inflammation is a pathophysiological response to harmful stimuli that disrupt homeostasis, such as damaged cells, irradiation, pathogens, or toxic compounds, and acts by removing injurious stimuli and initiating the healing process. Therefore, inflammation is a defense mechanism that is essential to health. However, uncontrolled acute inflammation may result in chronic inflammation, contributing to various chronic inflammatory diseases. It is said that inflammation is an integral part of several diseases, such as autoimmune and chronic metabolic diseases, wherein both became a global problem due to the increasing cases (Olivera-Castillo et al. 2018). Thus, nonsteroidal anti-inflammatory drugs (NSAIDs) are

utilized to treat and manage inflammation (Parvin et al. 2015). However, the current inflammatory treatments pose numerous problems. Common drugs such as NSAIDs are said to irritate the gastrointestinal tract and disrupt blood cells. In addition, steroid drugs have more complex and dangerous adverse effects (Moelyono et al. 2018).

Also, the dramatic increase in emerging antibiotic resistance coupled with poor infection control practices results in easily disseminating resistant bacteria to other patients and the environment. Antimicrobial resistance of bacterial pathogens has become a worldwide challenge and is linked to high morbidity and mortality (Frieri et al. 2016). Infectious diseases are responsible for the death of approximately fourteen million individuals per annum (Akbar et al. 2019). The current shortage of effective drugs, lack of successful prevention measures, and only a few new antibiotics in the clinical pipeline are the challenges in battling bacterial infections and their accompanying diseases. Multidrug resistance patterns of the gram-positive and gram-negative bacteria have resulted in difficult-to-treat or, even worse, untreatable infections with conventional antimicrobials (Frieri et al. 2016).

Therefore, new anti-inflammatory agents with relatively low or no side effects and novel antibacterial agents derived from natural ingredients are needed. Moreover, one of the most common physiological responses to bacterial infection is the inflammatory response, often triggered by changes in humoral and cellular components after tissue injury. Since bacterial infection can often elicit a problematic inflammatory response, medicines that can provide antibacterial and anti-inflammatory responses would be of therapeutic interest (Shu et al. 2016). The present study reports the *in vitro* antibacterial activity using the modified microtiter plate method and *in vitro* anti-inflammatory activity using albumin denaturation of selected sea cucumber species extracts from Brgy. Sugod, Tukuran, Zamboanga del Sur, Philippines, the results will serve as baseline information for further studies.

MATERIALS AND METHODS

Specimen collection

Before the study, the institutional review board (IRB) approved the research, and biosafety clearance was obtained. Informed consent and permits were also acquired from the Mayor and the Department of Agriculture, Bureau of Fisheries and Aquatic Resources (DA-BFAR). A total of three different sea cucumber species were collected from Brgy. Sugod, Tukuran, Zamboanga del Sur, Mindanao, Philippines (7°50'11.5"N, 123°36'30.6"E) (Figure 1). The samples were morphologically identified using the identification guide of Purcell et al. (2012). The collected samples were transferred into a clean container and placed in an ice chest for transport. The samples were brought to Molecular Systematics and Conservation Genomics Laboratory, Center for Biodiversity Studies and Conservation (CBSC), Premier Research Institute of Science and Mathematics (PRISM), MSU-IIT, for the experiment to be carried out.

Preparation of sea cucumbers and crude extracts

The collected sea cucumber samples were washed and cleaned with distilled water to remove the unwanted materials on their epidermal surface. Twelve (12) specimens of sea cucumbers were grouped morphologically. Prior to the extraction procedure, the samples were cut into small pieces and soaked separately in methanol:ethyl acetate (50:50) for one week. Methanol is frequently used as an extraction solvent because of its high polarity, which produces high extraction yields (Hassim et al. 2014). Ethyl acetate is also considered a good solvent due to its high vitality and efficacy (Yeung and Stanley 2011). A total of four (4) extracts were obtained, since extracts from *Holothuria atra* has two separate layers, upper (1) and lower (2) layer, *Holothuria scabra*, and *Stichopus* sp. To obtain the crude extracts, the extracts were filtered and concentrated *in a vacuo* using a rotary evaporator then exposed to pressurized nitrogen to remove the residues. Extracts of *H.* scabra, *Stichopus* sp., *H. atra* (1), and *H. atra* (2) appeared to be colored white, golden yellow, orange and pale yellow, respectively.

Determination of antibacterial activity

Bacterial strains of *Staphylococcus aureus* BIOTECH 1582 (gram-positive), *Bacillus subtilis* BIOTECH 1679 (gram-positive), *Pseudomonas aeruginosa* BIOTECH 1335 (gram-negative), and *Escherichia coli* BIOTECH 1634 (gram-negative) which are considered opportunistic pathogens, were obtained from the University of the Philippines - Los Baños Biotechnology Laboratory. Bacterial strains were sub-cultured on appropriate media as prescribed and incubated overnight at room temperature.

Mueller Hinton Broth (MHB) was used for the assay to determine the minimum inhibitory concentration (MIC) for FDA and DOH recommend it for antimicrobial susceptibility testing of the most often encountered aerobic and facultative anaerobic bacteria in food and clinical substances (NCCLS).

Figure 1. Three sea cucumber species collected from Brgy. Sugod, Tukuran, Zamboanga del Sur, Mindanao, Philippines. (A. *Holothuria scabra*; B. *Stichopus* sp.; C. *Holothuria atra*)

For the preparation of bacterial culture, aseptic techniques were employed. A single colony was transferred into a 100 mL of Nutrient Broth (NB) capped, and incubated overnight at 35°C. The Optical Density of the Bacterial Strain at 0.5-1 absorbance reading at 600 nm was measured using a photopette, a handheld fixed-wavelength spectrophotometer, for rapid analysis.

The desired extract of 100 ppm was made by dissolving 1 mg of the crude extract into 10 mL of sterile distilled water to obtain 0.1 mg/mL extract.

The procedure of Sarker et al. (2007) was employed in this study with minor modifications. Resazurin solution was prepared by dissolving a 67.5 mg tablet in 10 mL sterile distilled water. A vortex mixer was used to ensure a well-dissolved and homogenous solution. Resazurin is used as an indicator that detects microbial growth in minute volumes.

Each sterile 96-well plate was appropriately labeled and prepared under aseptic conditions. A volume of 100 µL of the sea cucumber extract was prepared at 0.1 mg/mL, and pipetted into the first row (row A) in columns 1-4 and 7-10 of the plate. A volume 100 µL Ciprofloxacin (positive control) was pipetted into column 6 and column 12 of row A. Then, 50 µL of sterile saline was added to all wells except for column 5 and column 11 of row A. Serial dilution was performed using a multichannel pipette discarding the last 50 µL from the last row. Then, 30 µL of MHB was added to all wells, and 10 µL of the respective bacterial suspension (5×10^6 CFU/mL) was added to each well. Separate plates were utilized for each of the extracts. The plates were then incubated for 24 hours.

After 24 hours, each well was added 10 µL of resazurin indicator solution and was observed for color change. Results were assessed visually by observing changes to a stable color from blue to pink. Color change from blue to pink or colorless was recorded as positive (there is bacterial growth; no inhibition). Blue color/purple indicates negative (no bacterial growth; inhibition). Observation was done for 30 min and photographs were taken every 5 min. The lowest concentration where the color remains blue/purple was observed and taken as the Minimum Inhibitory Concentration (MIC) value. The plates were prepared in triplicate, and the average values were calculated and considered as the MIC value for the sea cucumber extract for each bacterial strain (Sarker et al. 2007).

Microplate-based inhibition of albumin denaturation assay

The method of Mizushima and Kobayashi (1968), and Sakat et al. (2010) were employed with some modifications. The sea cucumber extracts and the anti-inflammatory drugs Celecoxib and Diclofenac sodium were prepared at 25-, 50-, 100-, and 200 ppm concentrations. Celecoxib and Diclofenac sodium were used as the positive control. A volume of 100 mL of 1% Bovine Serum Albumin (BSA) was also prepared. A reaction mixture with a total volume of 250 µL was prepared in each well by adding 222.50 µL of 1% BSA and 27.5 µL of the sea cucumber extract or the positive control, Celecoxib and Diclofenac sodium. The 1% BSA was used as the negative control. The Corning Costar 96-well plate (Sigma Aldrich) containing the reaction mixtures was incubated for 20 min at 37°C and was heated for another 20 min at 57°C. Then, the absorbance was measured at 660nm using the Clariostar Plus microplate reader (BMG LABTECH). The inhibition percentage of protein denaturation was calculated using the following formula:

$$\%\ Inhibition = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

Where; $Abs_{control}$ is the absorbance of the denatured protein albumin and Abs_{sample} is the absorbance of the reaction mixture of the extract or the positive control.

Statistical analysis

Paleontological Statistics (PAST) v.2.17 software was used to analyze the data. Statistical differences between the concentrations were analyzed using a one-way Analysis of Variance (ANOVA). Tukey's pairwise test was employed to check if the groups examined were comparable with each other and the positive control.

RESULTS AND DISCUSSION

Determination of antibacterial activity

The crude extracts of *H. scabra, Stichopus* sp., and *H. atra* were tested for antibacterial activity against four (4) microorganisms (*P. aeruginosa*, *E. coli*, *B. subtilis*, and *S. aureus*) using modified microtiter plate-based *in vitro* screening, antibacterial assay (Figure 2 and 3).

The minimum inhibitory concentration (MIC) of crude methanol:ethyl acetate extracts of *H. scabra* against *P. aeruginosa*, *E. coli*, *S. aureus*, and *B. subtilis* were 0.0889, 0.1, 0.0944, and 0.0241 mg/mL, respectively. As for the crude extracts of *Stichopus* sp., the MIC against *P. aeruginosa*, *E. coli*, *S. aureus*, and *B. subtilis* were 0.0556, 0.0833, 0.0236, and 0.0008 mg/mL, respectively. The MIC of the *H. atra* (1) crude extracts against *P. aeruginosa*, *E. coli*, *S. aureus*, and *B. subtilis* were 0.0377, 0.0835, 0.1, and 0.0365 mg/mL, respectively. Lastly, the MIC of *H. atra* (2) crude extracts against *P. aeruginosa*, *E. coli*, *S. aureus*, and *B. subtilis* were 0.0490, 0.0613, 0.0611, and 0.0008 mg/mL, respectively (Table 1).

Analysis of Variance (ANOVA) was used to compare the antibacterial activity of sea cucumber crude extracts and positive control as shown in Table 2. The one-way ANOVA analysis revealed a p value of 0.03338, where $p<0.05$, indicating a significant difference between the groups examined. Thus, the antibacterial activity exhibited is not comparable between groups and with respect to the positive control. However, we need to confirm where the differences lie between groups.

Table 1. Determination of MIC using modified microtiter antimicrobial assay of sea cucumber crude extracts

Bacterial strains	Minimum Inhibitory Concentration (mg/mL)				
	Extracts/Test materials				Positive control
	H. scabra	*Stichopus* sp.	*H. atra* (1)	*H. atra* (2)	Ciprofloxacin
P. aeruginosa (-)	0.0889	0.0556	0.0377	0.0490	0.0055
E. coli (-)	0.1	0.0833	0.0835	0.0613	0.0040
S. aureus (+)	0.0944	0.0236	0.1	0.0611	0.0049
B. subtilis (+)	0.0241	0.0008	0.0365	0.0008	0.0014

Table 2. One-way Analysis of Variance of sea cucumber extracts and positive control, Ciprofloxacin

	Sum of sqrs	df	Mean Square	F	P-value
Between groups:	0.0124	4	0.0031	3.485	0.0334
Within groups:	0.0133	15	0.0009		
Total	0.0257	19			

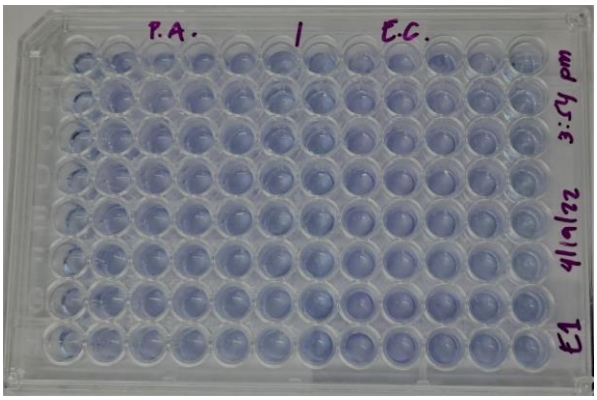

Figure 2. Plate incubated for 24 hrs. After 24 hrs, wells turned blue after adding 10 µL of resazurin dye; further change in color was then observed and noted

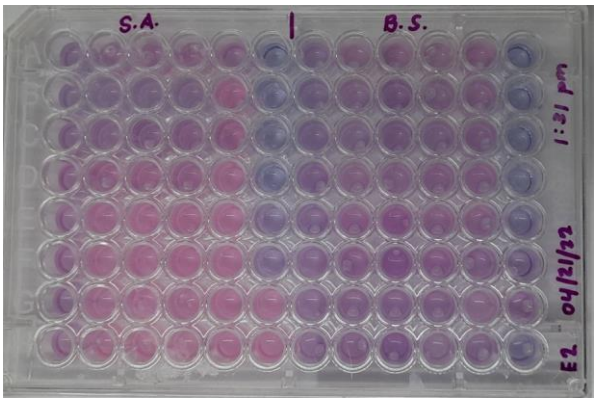

Figure 3. Color transition as observed in plates in modified microtiter plate assay after adding resazurin as an indicator. Pink color indicates growth and blue/purple means inhibition of growth. The test orgnisms were *S. aureus* and *B. subtilis*. C1 and C7, sterility control (sea cucumber extract in serial dilution + broth + saline + indicator), no bacteria; C2-C4 and C8-C10, sea cucumber extract (in serial dilution in wells 1-12 + broth + indicator + bacteria); C5 and C11, negative control (bacteria + broth + indicator); C6 and C12, positive control (ciprofloxacin in serial dilution + broth + indicator + bacteria)

Tukey's pairwise result as shown in Table 3, confirmed that the antibacterial activity of *H. scabra* is not comparable with Ciprofloxacin with *p*-value of 0.02473. Except for *H. scabra*, the antibacterial activity of other extracts is comparable with Ciprofloxacin with a $p>0.05$. The results suggest that *H. scabra* extract is not that highly effective against the test organisms. On the other hand, *Stichopus* sp., *H. atra* (1) and *H. atra* (2) extracts are comparable with Ciprofloxacin, which implies that these extracts are as effective as the positive control. The results also highlighted that the antibacterial activity of *Stichopus* sp. and *H. atra* (2) crude extracts are comparable with each other with *p* value of 1. This implies that they are highly active against test organisms, especially against *B. subtilis*.

It was found that *Stichopus* sp. and *H. atra* (2) extracts were able to inhibit the growth of *B. subtilis* with MIC value of 0.0008 mg/mL. As for the remaining extracts, they still showed activity against test organisms with MIC value of 0.0241-0.1 mg/mL but not as potent as the positive control, Ciprofloxacin with MIC value of 0.0014-0.0055 mg/mL; considering that the extracts required a higher concentration to inhibit the growth of the bacteria. It is worth highlighting that the extracts inhibited a high antimicrobial activity on *B. subtilis*, a gram-positive bacterium.

Gram-negative bacteria contain an outer membrane; thus, they are less susceptible to antibiotics. Therefore, gram-negative bacteria are more pathogenic than gram-positive bacteria. On the other hand, gram-positive bacteria are more susceptible to antibiotics due to the lack of an outer membrane (Lakna 2017). Nevertheless, several infections are caused by gram-positive strains, such as strep throat, skin infections, and foodborne botulism (Nunez 2019). *B. subtilis* is associated with infectious diseases such as bloodstream infection, cholangitis, chronic osteomyelitis, meningitis, panophthalmitis, pneumonia, and ventriculoperitoneal shunt infections (Bratcher 2018). Based on the results, the crude extracts of the three sea cucumber samples show a considerably good potential against *B. subtilis* (gram-positive bacteria).

The results coincide with several studies on the antibacterial potential of sea cucumber species. Adibpour and colleagues (2014) studied the body wall, Cuvierian organs, and coelomic fluid of *Holothuria leucospilota* extracts against *S. aureus*, *Staphylococcus typhi*, *E. coli*, and *P. aeruginosa* and revealed antibacterial activity at 2000- and 1000 µg/mL. The antibacterial activity of the active fractions obtained from column chromatography of *H. atra* against various gram-positive and gram-negative bacteria: *Klebsiella pneumonia*, *Serratia liquefaciens*, *S. aureus*, *Listeria monocytogenes*, and *E. coli*. The fractions collected from 50 to 100% showed maximum effect (Dhinakaran and Lipton 2014). The extracts from the skin, Cuvierian tubules, and Polian vesicle of *Holothuria fuscoscinerea* exhibited antibacterial activity against *E. coli* while the gonads, Cuvierian tubules and Polian vesicle extracts inhibited the growth of *S. aureus* (Cayabo and Mabuhay-Omar 2016). In the study of Shakouri et al. (2017), the aqueous methanol extracts of the *Stichopus variegatus* body wall affected the *E. coli* strains with a zone of inhibition of 12.26 mm at 8 mg/mL. The methanol extract of the said species inhibited the growth of *E. coli* at a concentration of 1 mg/mL with a 1.36 mm zone of inhibition. Lastly, the chloroform extract affected the *S. aureus* strain with a zone of inhibition of 9.30 mm at 8 mg/mL. At 100% concentration, the hexane fraction of *H. atra* has antibacterial activity against *P. aeruginosa* with a diameter of inhibitory zone of 14.61±0.02mm (Sukmiwati et al. 2019). *H. leucospilota* was also evaluated for its antibacterial activity using the disk diffusion method. The results revealed that the n-hexane and ethyl acetate extract of the body wall and the ethyl acetate extract of the gonad exhibited strong antibacterial activity against *S. aureus* (Darya et al. 2020). Different organs of *Holothuria forskali* ethyl acetate extracts were tested for antibacterial activity against four bacterial strains, *E. coli*, *Bacillus cereus*, *B. subtilis*, and *P. aeruginosa*. At the concentration of 2 mg/mL, the body wall extract inhibited the growth of all bacterial strains, while the muscle extract exhibited antibacterial activity except against *P. aeruginosa*. The respiratory tree extract inhibited the growth of *E. coli* at 2 mg/mL concentration with a zone of inhibition of 11.3±1.1 mm. The digestive tract extract inhibited the growth of *E. coli* and *B. subtilis* at a concentration of 0.4 mg/mL while at 2 mg/mL concentration, the extracts inhibited the growth of *E. coli*, *B. subtilis*, and *P. aeruginosa*. Lastly, the gonad extract could only inhibit the growth of *B. subtilis* at 0.4 mg/mL, and *B. cereus*, and *B. subtilis* at 2 mg/mL concentration (Telahigue et al. 2020). The Kirby-Bauer disc diffusion assay screened the sea cucumber *Muelleria lecanora* for its antibacterial activity against the bacterial strains *E. coli*, *Salmonella typhi*, and *S. aureus*. The methanol extract exhibited a zone of inhibition of 6.2 mm, 7.34 mm, and 7.02mm; the acetone extracts displayed a zone of inhibition of 6.22 mm, 7.26 mm, and 6.88 mm; and the hexane extracts showed a zone of inhibition of 6.48mm, 7.87 mm, and 7.22 mm, against *E. coli*, *Salmonella typhi*, and *S. aureus*, respectively (Yusuf et al. 2020). At 100 mg/mL concentration, *Stichopus ocellatus* (1:5 w/v ratio) extract had an inhibition zone diameter of 14.66±0.37mm and 15.45±0.17 mm against *B. cereus* and *E. coli*, respectively (Sukmiwati et al. 2021).

The coastal marine sediment is believed to be a reservoir for numerous pathogenic and antimicrobial-resistant bacterial strains. Thus, deposit-feeding feeding and sedentary bottom-dwelling organisms such as sea cucumbers are incessantly exposed to many harmful pathogens. As a defense mechanism, several benthic marine invertebrates constitute secondary metabolites with antimicrobial activities to protect themselves against microbial infections and surface-fouling microorganisms (Telahigue et al. 2020).

Microplate-based inhibition of albumin denaturation assay

The anti-inflammatory activity of the sea cucumber crude extracts has been investigated by inhibiting albumin denaturation. The results presented in Table 4 showed that *H. scabra* exhibited a percent inhibition ranging from 44.74%-49.76%, the extract has highest percent inhibition at 25 ppm concentration. *Stichopus* sp. showed a percent inhibition ranging from 46.57%-51.44% with highest percent inhibition at 100 ppm concentration. *H. atra* (1) and *H. atra* (2) demonstrated a percent inhibition ranging from 29.03%-44.98% and 35.21%-46.41%, respectively. *H. atra* (1) has highest percent inhibition at 100 ppm while *H. atra* (2) at 200 ppm concentration. The positive control, Celecoxib exhibited a percent inhibition ranging from 49.16%-52.15% while Diclofenac sodium showed a percent inhibition ranging from 44.78%-50%. Celecoxib has highest percent inhibition at 100 ppm while Diclofenac sodium at 50 ppm.

One-way ANOVA was performed to determine if there was a significant difference between the sea cucumber extracts and the positive control. Results indicate no significant difference between sea cucumber extracts and the positive control, Celecoxib and Diclofenac sodium at 200 ppm concentration (p=0.2253) (Table 5). This implies that the percent inhibition of albumin denaturation of the sea cucumber extracts is comparable to the positive control. However, one-way ANOVA results revealed that there was a statistically significant difference between the sea cucumber extracts and reference drugs at concentration 100 ppm (p=0.049) (Table 6), 50 ppm (p=7.96E-06) (Table 7), and 25 ppm (p=8.287E-08) implying that at this concentrations it yield noncomparable results to the positive controls (Table 8).

Tukey's pairwise comparison revealed no statistically significant difference in the activity of the four (4) extracts: *H. scabra*, *Stichopus* sp., *H. atra* (1), *H. atra* (2) compared to the positive control: Celecoxib and Diclofenac sodium with $p>0.05$ (Table 9). The results imply that the percent inhibition of albumin denaturation of the sea cucumber extracts at the 200 ppm was comparable to the positive controls.

Table 4. Percent inhibition rate of *in vitro* anti-inflammatory activity using albumin denaturation method of sea cucumber crude extracts and positive control (Celecoxib and Diclofenac sodium) in four different concentrations

Concentration (ppm)	Percent Inhibition (%)					
	H. scabra	***Stichopus* sp.**	***H. atra* (1)**	***H. atra* (2)**	**Celecoxib**	**Diclofenac sodium**
200	46.77	49.96	44.50	46.41	51.95	44.78
100	44.74	51.44	44.98	45.22	52.15	48.09
50	49.36	49.28	31.82	35.21	50	50
25	49.76	46.57	28.03	39.23	49.16	48.80

Table 5. One-way Analysis of Variance (ANOVA) of sea cucumber extracts and positive controls (Celecoxib and Diclofenac sodium) at 200 ppm concentration

	Sum of sqrs	**df**	**Mean Square**	**F**	**P-value**
Between groups:	254.014	5	50.8028	1.488	0.2253
Within groups:	955.793	28	34.1355		
Total	1209.81	33			

Table 6. One-way Analysis of Variance (ANOVA) of sea cucumber extracts and positive controls (Celecoxib and Diclofenac sodium) at 100 ppm concentration

	Sum of sqrs	**df**	**Mean Square**	**F**	**P-value**
Between groups:	288.935	5	57.787	2.616	0.049
Within groups:	552.3	25	22.092		
Total	841.235	30			

Table 7. One-way Analysis of Variance (ANOVA) of sea cucumber extracts and positive controls (Celecoxib and Diclofenac sodium) at 50 ppm concentration

	Sum of sqrs	**df**	**Mean Square**	**F**	**P-value**
Between groups:	1787.32	5	357.463	12.11	7.95E-06
Within groups:	678.642	23	29.5062		
Total	2465.96	28			

Table 8. One-way Analysis of Variance (ANOVA) of sea cucumber extracts and positive controls (Celecoxib and Diclofenac sodium) at 25 ppm concentration

	Sum of sqrs	**df**	**Mean Square**	**F**	**P-value**
Between groups:	2112	5	422.4	17.82	8.287E-08
Within groups:	639.962	27	23.7023		
Total	2751.96	32			

Table 9. Tukey's pairwise comparison of four sea cucumber extracts and positive controls (Celecoxib and Diclofenac sodium at 200 ppm concentration.

	H. scabra	***Stichopus* sp.**	***H. atra* (1)**	***H. atra* (2)**	**Celecoxib**	**Diclofenac sodium**
H. scabra	-					
Stichopus sp.	0.93	-				
H. atra (1)	0.99	0.64	-			
H. atra (2)	1	0.91	0.99	-		
Celecoxib	0.65	0.99	0.31	0.63	-	
Diclofenac sodium	0.99	0.65	1	0.99	0.3	-

Based on the results, Diclofenac sodium has 44.78% inhibition of albumin denaturation at 200 ppm, while extracts of *H. scabra*, *Stichopus* sp., and *H. atra* (2) have higher percent inhibition on albumin denaturation of 45.57%, 49.96%, and 46.41%, respectively. From these, it is inferred that sea cucumber extracts exhibit anti-inflammatory effect against denaturation of the protein.

The results of the anti-inflammatory activity of the selected sea cucumber species in the present study was supported by numerous studies. Wijesinghe et al. (2015) reported that sea cucumber consumption benefits the body and has anti-inflammatory properties. Sea cucumber extract can suppress lipopolysaccharide (LPS), which induces and suppresses prostaglandins. Sea cucumber extract has an anti-inflammatory effect by reducing nitric oxide (NO) and/or prostaglandin E2 (PGE2) by reducing the production of tumor necrosis factor α (TNF-α). Interleukin-β and interleukin-6 cytokines in LPS stimulated by macrophages or suppress the expression of proinflammatory mediators such as inducible nitric oxide synthase (iNOS) and/or COX-2. The *H. leucospilota* extract showed a changed expression of COX-2 and vascular endothelial growth factor (VEGF) genes following the increasing concentration of the extract from 10 to 100 µg/mL. The expression level of the COX2 and VEGF following the exposure at 100 µg/mL of the body wall extract exhibited a remarkable down-regulation (Taghdiri et al. 2018). In the study of Moelyono and colleagues (2018), the *Stichopus horrens* extract demonstrated a highest percentage of anti-inflammatory effects occurs at fifth hour for the dosage of 250 mg/kg BW, 500 mg/kg BW, and 1000 mg/kg BW with percentages of 43.37%, 53.70% and 78.90%, respectively. The anti-inflammatory effects of *Stichopus japonicus* sea cucumber extracts were examined against LPS-stimulated RAW 264.7 cells, by evaluating the NO production, as excess NO production initiates allergic inflammatory diseases. It was found that NO levels drastically decreased after treatment with *S. japonicus* fractions, and *S. japonicus* water extract inhibited NO release (Song et al. 2013). In the study of Kareh et al. (2018), they reported that the effect of *H. scabra* extract in reducing the inflammation might be attributed to the ability of *H. scabra* extract to reduce levels of IL-6, NO, and matrix metalloproteinase 9 (MMP9). In the study of Shahrulazua (2013), *Stichopus* sp1 demonstrated healing of fracture in rabbits due to the fatty acids (EPA and DHA) in sea cucumber extract. EPA and DHA are known as n3 fatty acids, inhibiting prostaglandin synthesis by suppressing COX-2 and 5-LOX in inflammatory conditions and accelerating wound healing (Bordbar et al. 2011). The anti-inflammatory effect was demonstrated by the inhibitory effect of carrageenan-induced paw edema is depicted in the *H. atra* extract which has high activity at 100 mg/kg and 200 mg/kg. The body wall extracts of *H. atra* suppressed the acute and chronic inflammation strikingly in rats. The release of histamine, serotonin, and prostaglandin could be related to reducing inflammation. The *H. atra* extract had various compounds such as flavonoids, phenolic components, terpenoids, saponins, and alkaloids (Dhinakaran and Lipton 2014).

Several studies have reported sea cucumbers to have active ingredients, including chondroitin sulphate, flavonoids, glucose sulphate triterpenes, glucoproteins, glycosaminoglycan, hydrosalicylate, lectins, peptides, phenols, proteins, sterol, sulphate polysaccharides, and triterpene glycosides (saponin) (Arifin et al. 2013). Various antimicrobial components including steroidal glycosides, polyhydroxylated sterols, napthoquinone pigments, lysozymes, complement-like substances, and antimicrobial peptides have been isolated from the sea cucumbers (Adibpour et al. 2014). As most tropical sea cucumbers feed on live microorganisms together with the organic contents found in sand or slime, there is the possibility that some pathogenic microorganisms are taken together with the food substances. The presence of some form of active antibacterial substances for body defense is only to be expected (Kiani et al. 2013). Phenol compounds can damage cell's membranes, deactivate enzymes, and denature proteins, thus decreasing membrane permeability.

Changes in permeability of the cytoplasmic membrane disrupt the transportation of essential ions into the cells, which causes inhibition of cell growth and even cell death. The terpenoid compound can inhibit transport across the thicker cell membrane bacteria because of the large polysaccharide groups and some sulfates from the triterpene antibacterial agents. Saponins inhibit or kill microbes by reacting with membrane sterols. The main effect of saponins in bacteria is the release of proteins and enzymes from the cells. Thus, saponins are active in inhibiting cell growth (Sukmiwati et al. 2021).

On the other hand, saponins, chondroitin sulfate, and flavonoids are the compounds that are likely to be anti-inflammatory compounds in sea cucumber extract. These compounds can inhibit the cyclooxygenase enzyme by converting arachidonic acid to prostaglandins as an inflammatory mediator. This mechanism is almost the same as the mechanism of action of NSAIDs, which have inflammation-inhibiting activity by inhibiting prostaglandin biosynthesis through inhibition of the cyclooxygenase enzyme activity (Burhan et al. 2019; Deligiannaki and Sarigiannis 2019; Rasyid 2017).

In conclusion, *Stichopus* sp., *H. atra* (1), and *H. atra* (2) possessed an antibacterial effect against gram-positive and gram-negative bacteria. Moreover, all sea cucumber extracts have a comparable albumin inhibitory effect with respect to the positive controls at 200 ppm concentration. These results imply that the mentioned species can be a potential antibacterial and anti-inflammatory source. However, further investigation is needed to precisely identify the active constituents responsible for its antibacterial and anti-inflammatory effects. Additional *in vivo* and *in vitro* studies should also be considered to further verify its antibacterial and anti-inflammatory potential.

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