




Antioxidant and antibacterial activities in 21 species of Indonesian sea cucumbers

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Revised: 11 January 2021 / Accepted: 26 January 2021 / Published online: 9 February 2021
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Abstract Sea cucumbers are marine organisms with uses in food, cosmetics, and medicine. This study aimed to identify Indonesian sea cucumbers with high antioxidant and antibacterial activities. Twenty-one sea cucumber species were used for this study. Antioxidant capacity was evaluated using the 2,2-diphenyl- β -picrylhydrazine assay. Antibacterial activity was assessed using the disk diffusion assay, whereas the resazurin-based assay was employed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Volatile compounds possibly related to the biological activity of sea cucumbers were analyzed via gas chromatography–mass spectrometry (GC–MS). *Holothuria atra* had the strongest antioxidant capacity ($IC_{50} = 14.22 \pm 0.87 \mu\text{g } \mu\text{L}^{-1}$). *Stichopus vastus* displayed the best antibacterial activity against *Staphylococcus aureus*, whereas *Stichopus ocellatus* extract was most potent against *Vibrio cholerae*. *Holothuria albiventer*, which controlled *Bacillus subtilis* most effectively while also being active against *S. aureus*

and *V. cholerae*, was the optimal antimicrobial species. *H. albiventer* and *Actinopyga echinites* inhibited *B. subtilis* growth at $12.5 \mu\text{g } \mu\text{L}^{-1}$. The MBC tests indicated that the antibacterial activities of sea cucumbers at the MIC were bacteriostatic, rather than bactericidal, in nature. GC–MS analysis uncovered long-chain fatty acids that might be associated with the antibacterial activities of sea cucumbers.

Keywords Antioxidant capacity · Disk diffusion · DPPH · Volatile compounds · Pathogenic bacteria · Long-chain fatty acids

Introduction

Indonesia, with its extensive coastlines based on its multiple islands, features a high diversity of marine species (Kasanah et al. 2015). Among the marine invertebrate species, sea cucumbers represent a potential source of new biologically active materials with uses in pharmaceutical applications, food supplements, and industrial applications. Sea cucumbers in Asian–Pacific waters display large biodiversity, with 1716 species identified in Indonesia alone. The region is host to as many as 350 species, including at least 26 species of known economic value (Pangestuti and Arifin 2017). The sea cucumber is important economically in various Asian countries such as China, in which it is used in both gourmet and traditional medical preparations with purported therapeutic benefits against disorders and diseases such as hypertension, eczema, arthritis, and tissue injury (Esmat et al. 2013; Dhinakaran and Lipton 2014). In particular, Holothuroidea species that sometimes reside in extreme environments produce various secondary metabolites that with beneficial effects (Pangestuti et al.

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2016) such as antifouling, antipredation, and UV-protective activity (Murniasih et al. 2015).

Extracts of subtropical sea cucumbers are known antioxidants against free radicals with positive effects on human health because they can protect against reactive species-induced damage at the cellular level (Pangestuti et al. 2016), and protective compounds been detected in sea cucumbers such as *Holothuria leucospilota* (Soltani and Baharara 2014), *H. scabra* (Nobsathian et al. 2017), and *Stichopus chloronotus* (Murniasih et al. 2015). In addition, sea cucumbers are also reported to possess antibacterial activity (Farjami et al. 2013), especially against pathogenic bacteria (Adibpour et al. 2014). Thus, they could be potential sources of novel antibiotics in view of several recent reports on the appearance of antibiotic-resistant pathogenic bacteria triggered by unregulated and excessive antibacterial agent use (Omar et al. 2012), in addition to naturally occurring resistance mechanisms (Spizek et al. 2010).

Reactive oxygen species (ROS) and free radicals have attracted increasing attention over the past decade. ROS, including superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals (OH^{\bullet}), and non-free radical species such as H_2O_2 and singlet oxygen (1O_2), comprise various forms of activated oxygen. These molecules exacerbate cellular injury and the aging process (Gulcin 2006). ROS are continuously produced during normal physiologic events, and they can easily initiate membrane lipid peroxidation, leading to the accumulation of lipid peroxides (Gulcin 2006). Moreover, ROS induce oxidative damage in biomolecules such as proteins, lipids, carbohydrates, and nucleic acids. This damage causes diseases including cancer and aging. ROS have been implicated in more than 100 diseases, including malaria, heart disease, acquired immunodeficiency syndrome, arteriosclerosis, stroke, cancer, and diabetes (Gulcin 2011). Thus, the development of safer antioxidants with natural origins has attracted increasing interest.

Although sea cucumbers represent a key non-fishing income stream for coastal peoples throughout the Indian and Pacific Oceans, information about biological activities such as antioxidant and antibacterial activities for some sea cucumber species is lacking. This study focused on the potential antioxidant and antibacterial activities of Indonesian sea cucumber species and characterization of their crude extracts regarding bioactive components using gas chromatography–mass spectrometry (GC–MS).

Materials and methods

Sample acquisition

In total, 21 sea cucumber species were collected in 2019 from Lampung Province and Lombok Island, Indonesia.

Eight species of sea cucumbers (*Actinopyga echinites* LPG, *H. atra*, *H. leucospilota* LPG, *H. scabra*, *S. hermanni*, *S. ocellatus*, *S. vastus*, and *Thelenota ananas*) were collected from Lampung waters by fishermen, and thirteen species (*Actinopyga echinites* LBK, *Bohadschia marmorata*, *H. albiventer*, *H. colluber*, *H. fuscogilva*, *H. hilla*, *H. impatiens*, *H. lesson*, *H. leucospilota* LBK, *H. pardalis*, *H. rigida*, *H. verrucosa*, and *Bohadschia* sp.) were collected in tidal waters along Lombok Island via direct sampling. Species-level identification was based on the morphological characteristics of the specimens.

Extraction

Samples (5 g wet weight) of each species were cut into small pieces and extracted with methanol at room temperature for 24 h. The maceration method followed that of Jones and Kinghom (2006). The samples were filtered and re-extracted with methanol. The filtrate was then concentrated under vacuum at a maximum temperature of 40 °C to obtain the extract.

2,2-Diphenyl-β-picrylhydrazine (DPPH) radical scavenging assay

The antioxidant potential of each sample was assessed at concentrations of 0.1–50 $\mu\text{g } \mu\text{L}^{-1}$. To 160 μL of each extract in the wells of a 96-well plate were added 40 μL of 1 mM DPPH solution (Sigma, D9132), and the mixture was incubated for 30 min at room temperature. The absorbance was then measured at a wavelength of 515 nm using a Tecan® Microplate Reader. Butylated hydroxytoluene (BHT, Sigma, W218405-1 KG-K) was used as a calibration standard at concentrations of 0.005–0.01 $\mu\text{g } \mu\text{L}^{-1}$. The measurement was repeated three times. Free radical inhibitory activity was calculated using the following formula:

$$\text{Scavenging effect} : [1 - A \text{ Sample}/A \text{ control}] \times 100\%,$$

where A Sample is the absorbance of the sample and A control is the absorbance of the control (Ak and Gulcin 2008). A straight-line equation based on the calibration curve was used to obtain the 50% inhibitory concentration (IC_{50}). Inhibitory activity was calculated using a previously reported formula (Sánchez-Moreno et al. 1998). The percent inhibition was also obtained at 10 $\mu\text{g } \mu\text{L}^{-1}$ using the regression equation $y = ax + b$, where y is the percent inhibition (%) and x is the targeted concentration (10 $\mu\text{g } \mu\text{L}^{-1}$). The concentration (10 $\mu\text{g } \mu\text{L}^{-1}$) was entered into the regression formula to obtain the percent inhibition ($y = \text{Inhibition } [\%]$).

Determination of the antibacterial activity of sea cucumber extracts

Disk diffusion assay

Inhibition zone testing was conducted using three replications of disposable Petri dishes containing Mueller–Hinton agar (Radjasa et al. 2001) and Mueller–Hinton broth enriched medium. All sample extracts were tested at the same concentration ($25 \mu\text{g } \mu\text{L}^{-1}$, $20 \mu\text{L}$) on paper disks (6 mm). Each agar plate contained a bacterial strain spread using a sterile cotton swab. The plates were incubated for 18–24 h at $37 \text{ }^\circ\text{C}$ as described previously (Sukmiwati et al. 2019). Four species of human pathogenic bacteria, namely the gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (ATCC 6633) and the gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Vibrio cholerae* (ATCC 14035), were used at $\text{OD}_{600\text{nm}}$ 0.3 in the antibacterial tests. The positive control wells contained $20 \mu\text{L}$ ($10 \mu\text{g } \mu\text{L}^{-1}$) of erythromycin, ampicillin, or kanamycin, and sterile dimethyl sulfoxide (DMSO, 10%) served as the solvent control. By measuring the zone of inhibition against the test organisms, antimicrobial activity was evaluated (Gulcin et al. 2010). The diameters of the zones of inhibition were measured using calipers. The method followed a previous report (Telahigue et al. 2020) with modifications.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) measurements

The serial dilution technique was performed in a 96-well microplate to determine the MIC of sea cucumber samples. Yield extracts were dissolved in DMSO to obtain a $100 \mu\text{g } \mu\text{L}^{-1}$ stock solution. Overnight cultures of each bacterium were diluted in sterile Mueller–Hinton broth. Following 18–24 h of incubation, the inocula were prepared by adjusting the turbidity of the suspension to $\text{OD}_{660\text{nm}}$ 0.3. For each of the bacteria used (*S. aureus*, *V. cholerae*, *E. coli*, and *B. subtilis*), $50 \mu\text{L}$ of each extract and antibiotic controls (erythromycin, ampicillin, and kanamycin) were serially diluted with $50 \mu\text{L}$ of sterile distilled water. Twenty microliters of bacterial culture and $30 \mu\text{L}$ of medium were added to each well, with some wells reserved for the sterile control (Mueller–Hinton broth), positive controls (antibiotics), and negative control (DMSO). The plates were aerobically incubated at $35 \pm 2 \text{ }^\circ\text{C}$ for 18–20 h, and the MIC was evaluated by observing the lowest concentration of the test extract that resulted in blue coloration following the addition of $20 \mu\text{L}$ of resazurin ($0.1 \mu\text{g } \mu\text{L}^{-1}$), indicating an antibacterial reaction. Conversely, pink coloration was indicative of bacterial activity and hence a negative antibacterial reaction. This method

followed that described by Sarker et al. (2007) with modifications.

Meanwhile, bacteriostatic activity was assessed using the MBC test as reported by Elshikh et al. (2016). To determine the bactericidal concentration, bacteria from the MIC wells were re-cultured on sterile Mueller–Hinton agar and incubated for 24 h at room temperature. The development of bacterial colonies indicated bacteriostatic activity, whereas the absence of bacterial colonies indicated bactericidal activity.

GC–MS analysis of sea cucumber extracts

Sea cucumber extracts were characterized by GC–MS at the Regional Health Laboratory (Labkesda), DKI Jakarta. Samples were injected into an Agilent Technologies 7890 gas chromatograph–mass spectrometer equipped with an autosampler and the 5975 Mass Selective Detector and linked to the Chemstation Data System. The HP Ultra 2L capillary column used in the analysis was $30 \text{ m} \times 0.25 \text{ mm}$ i.d. with a film thickness of $0.25 \mu\text{m}$. The instrument was set to the electron collision using ionization mode with an electron energy of 70 eV . After heating the oven to an initial temperature of $70 \text{ }^\circ\text{C}$, the temperature was increased by $3 \text{ }^\circ\text{C}/\text{min}$ to $150 \text{ }^\circ\text{C}$ and held for 1 min. The temperature was then increased by $20 \text{ }^\circ\text{C}/\text{min}$ to $280 \text{ }^\circ\text{C}$ and held for 26 min. The settings of the system were as follows: gas carrier, helium; column flow rate, $1.0 \mu\text{L}$; injection port temperature, $250 \text{ }^\circ\text{C}$; interface temperature, $280 \text{ }^\circ\text{C}$; ion source temperature, $230 \text{ }^\circ\text{C}$; and quadrupole temperature, $140 \text{ }^\circ\text{C}$. GC–MS analysis was performed using sea cucumber samples with bioactive components based on the highest antibacterial activity against *S. aureus*, *V. cholerae*, *B. subtilis*, and *E. coli*.

Statistical analysis

All data analyses were performed in triplicate, and data are expressed as the mean \pm SD. Statistical significance was determined using one-way ANOVA. SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for the analysis. Differences were considered statistically significant at $P < 0.05$.

Results and discussion

DPPH radical scavenging assay

Sea cucumbers as natural products contain valuable ingredients including health-promoting substances (Qi et al. 2017). Knowing the differences in antioxidant capacity among various sea cucumber species is necessary

to determine the source of the product with the best potential for utilization. In this study, antioxidant activity was tested to select the species of sea cucumbers with the best capacity using the DPPH method (Blois 1958; Abdille et al. 2005). Briefly, antioxidants react with stable free radicals such as 2,2-diphenyl- β -picrylhydrazyl, which is converted into DPPH, with a loss of color indicating antioxidant potential and scavenging of free radicals in the test sample (Hossain and Shah 2015). The use of DPPH in this study had advantages of minimal cost, simple procedures, and the ability to analyze large numbers of samples (Sveinsdottir et al. 2014).

The results of the revealed that *H. atra* has the strongest antioxidant activity ($IC_{50} = 14.22 \pm 0.87 \mu\text{g } \mu\text{L}^{-1}$), and the percent inhibition was $36.36 \pm 1.58\%$ at $10 \mu\text{g } \mu\text{L}^{-1}$ (Table 1). Meanwhile the IC_{50} of *H. leucospilota* LBK was $23.37 \pm 0.37 \mu\text{g } \mu\text{L}^{-1}$, and the percent inhibition at $10 \mu\text{g } \mu\text{L}^{-1}$ was $23.39 \pm 0.21\%$. BHT, as an indicator of antioxidant capacity, had stronger effects than all tested sea cucumber extract, and based on research conducted by Murniasih et al. (2015), the antioxidant capacity of fractionated compounds is much better than that of the crude

extract. In this research, the ethyl acetate extract of *H. leucospilota* had the strongest antioxidant activity, with a percent inhibition of 11.12% at $1 \mu\text{g } \mu\text{L}^{-1}$. Meanwhile, the antioxidant activity of *H. atra* was verified using GC–MS. In other studies, antioxidant activity has reported for other sea cucumbers such as *H. scabra* (Nobsathian et al. 2017), *H. leucospilota* (Soltani and Baharara 2014), *H. atra* (Dhinakaran and Lipton 2014), and *S. chloronotus* (Althunibat et al. (2009)). In the future, it is strongly recommended to perform phytochemical screening to clarify the relationships of the observed biological activity with the detected bioactive compounds.

Determination of the antibacterial activity of sea cucumber extracts

Disk diffusion assay

Because of its convenience, efficiency, and cost, the disk diffusion method, also termed the Kirby–Bauer test, is the most widely used method globally for assessing antibacterial activity, especially antimicrobial resistance. The

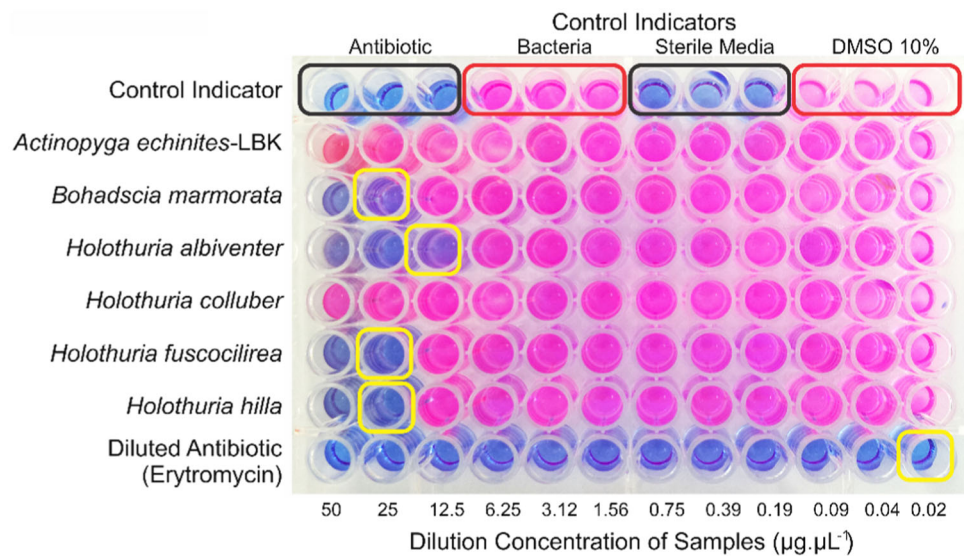
Table 1 Antioxidant activity (IC_{50} and percent inhibition) of the extracts of 21 sea cucumber species

No	Species	Inhibition at $10 \mu\text{g } \mu\text{L}^{-1}$ (%)	Regression	IC_{50} ($\mu\text{g } \mu\text{L}^{-1}$)
1	<i>Actinopyga echinites</i> LPG	14.68 ± 0.34	$y = 1.1021x + 3.6561$	42.05 ± 2.40
2	<i>Holothuria atra</i>	36.36 ± 1.58	$y = 3.2314x + 4.0452$	14.22 ± 0.87
3	<i>Holothuria leucospilota</i> LPG	17.17 ± 0.26	$y = 1.7687x - 0.5188$	28.56 ± 0.53
4	<i>Holothuria scabra</i>	12.31 ± 0.76	$y = 1.1223x + 1.088$	43.58 ± 4.25
5	<i>Stichopus hermanni</i>	8.34 ± 0.39	$y = 0.6969x + 1.3744$	69.77 ± 0.40
6	<i>Stichopus ocellatus</i>	17.26 ± 0.17	$y = 1.1051x + 6.2097$	39.63 ± 2.08
7	<i>Stichopus vastus</i>	13.95 ± 0.35	$y = 0.6323x + 7.6311$	67.01 ± 0.74
8	<i>Thelenota ananas</i>	11.12 ± 0.20	$y = 0.7978x + 3.1443$	58.73 ± 12.83
9	<i>Actinopyga echinites</i> LBK	19.33 ± 0.12	$y = 1.7171x + 2.1573$	27.86 ± 0.31
10	<i>Bohadschia marmorata</i>	6.23 ± 0.22	$y = 0.4828x + 1.4017$	100.66 ± 7.16
11	<i>Holothuria albiventer</i>	18.64 ± 0.58	$y = 1.9569x - 0.9313$	26.03 ± 0.72
12	<i>Holothuria colluber</i>	7.01 ± 0.38	$y = 9.1797x + 4.6075$	95.40 ± 5.22
13	<i>Holothuria fuscogilva</i>	9.37 ± 0.27	$y = 0.8854x + 0.5208$	55.88 ± 4.87
14	<i>Holothuria hilla</i>	17.47 ± 1.34	$y = 1.6317x + 1.151$	29.94 ± 3.06
15	<i>Holothuria impatiens</i>	9.29 ± 0.48	$y = 0.8039x + 1.2519$	60.64 ± 3.13
16	<i>Holothuria lesson</i>	7.71 ± 0.21	$y = 0.8352x - 0.6412$	60.63 ± 1.21
17	<i>Holothuria leucospilota</i> LBK	23.39 ± 0.21	$y = 0.8352x - 0.6412$	23.37 ± 0.37
18	<i>Holothuria pardalis</i>	7.27 ± 0.15	$y = 0.6828x + 0.439$	72.58 ± 1.77
19	<i>Holothuria rigida</i>	7.79 ± 0.26	$y = 0.7732x + 0.0592$	64.59 ± 2.22
20	<i>Holothuria verrucosa</i>	11.48 ± 0.59	$y = 0.9305x + 2.173$	51.40 ± 2.83
21	<i>Bohadschia</i> sp.	11.46 ± 0.43	$y = 1.2415x - 0.9575$	41.05 ± 2.85
22	Butylated hydroxytoluene	$> 100 \pm 105.88$	$y = 1236x + 5.5851$	0.04 ± 0.00

Collection location codes: LBK: Lombok, LPG: Lampung

IC_{50} , 50% inhibitory concentration

Fig. 1 Resazurin-based assay to determine minimum inhibitory concentration



advantages of the disc diffusion method include its simplicity and the absence of special equipment (Sandle 2016). In this research, the antibacterial properties of the methanol extracts of sea cucumber species were tested against four human pathogenic bacteria.

The results revealed that fourteen extracts had antibacterial activity against *S. aureus* and *V. cholerae*, four species were active against *B. subtilis*, and no extract was active against *E. coli*. Regarding *S. aureus*, *S. vastus* had the strongest growth-inhibitory effects (10.38 ± 0.09 mm), whereas *S. ocellatus* (14.60 ± 0.21 mm) displayed the strongest effects against *V. cholerae*. Meanwhile, *H. albiventer* (10.60 ± 0.57 mm) most strongly inhibited the growth of *B. subtilis*. Four species (*A. echinites* LPG, *H. albiventer*, *H. fuscogilva*, and *H. hilla*) exhibited antimicrobial activity against *S. aureus*, *V. cholerae*, and *B. subtilis*. However, none of the crude extracts exhibited commensurate inhibitory activity as ampicillin, erythromycin, and kanamycin despite the use of lower concentrations for the antibiotics ($1.25 \mu\text{g } \mu\text{L}^{-1}$). It is likely that the result would differ if the test subject were a single compound opposed to the entire extract.

In addition to its strong inhibitory effects on *S. aureus*, *S. vastus* also exhibited activity against *Enterobacter* and *Klebsiella* spp. as ethyl acetate and methanol extracts and against *E. coli* only as an ethyl acetate extract in prior research (Pringgenies et al. 2017). Moreover, the n-butanol, ethyl acetate, and n-hexane extracts of *S. vastus* exhibited activity against *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosa*, and saponins, terpenoids, and steroid compounds were reportedly contained in the extracts (Sukmiwati et al. 2018). In other bacterial tests, *S. ocellatus* displayed antibacterial activity against *V. eltor* and *E. coli* (Rasyid et al. 2018), and saponins, terpenoids, steroids, and

fatty acid compounds (Chasanah et al. 2016) were reportedly present in the extract of *S. ocellatus*. The antibacterial activity of *H. albiventer* has not been examined previously. Concerning its beneficial compounds, saponins have the ability to induce the leakage of proteins and certain enzymes in cells. Terpenoids can dissolve the cell wall of microorganisms by weakening the membranous tissue. Moreover, steroids are known for their effects on membrane lipids and ability to cause liposome leakage (Mujeeb et al. 2014).

Escherichia coli is gram-negative pathogenic bacteria that can cause diseases by its ability to invade tissues and induce toxigenesis (Manu et al. 2011). The bacterium is becoming highly resistant to conventionally used antibiotics (both newer and older medicines) as evidenced by many studies, perhaps because of the unregulated use of antibacterial agents (Omar et al. 2012). Antimicrobial resistance in *E. coli* in developing countries including Ethiopia is reported as a major cause of treatment failure in patients with infectious diseases. (Tuem et al. 2018). Thus, new therapeutic compounds are urgently needed (Manilal et al. 2009; Moran et al. 2011), prompting the inclusion of *E. coli* in the panel of bacteria treated with various sea cucumber extracts in the present study. Unfortunately, none of the sea cucumber species tested in the present study appeared to have any antibacterial effects on *E. coli*.

MIC and MBC assays

The MIC is a primary indicator of the antibacterial potential of agents (Elshikh et al. 2016). Based on their activity, antibiotics can be classified as bactericidal or bacteriostatic. Bactericidal antibiotics can kill bacteria, whereas bacteriostatic agents only inhibit their growth.

Table 2 Potential antibacterial activity of sea cucumbers against four bacteria tested using the disk diffusion method

No	Sea cucumber species	Zone of inhibition (mm)			
		<i>S. aureus</i>	<i>V. cholerae</i>	<i>B. subtilis</i>	<i>E. coli</i>
1	<i>Actinopyga echinites</i> LPG	8.75 ± 0.18	9.77 ± 0.42	8.48 ± 0.19	0.00 ± 0.00
2	<i>Holothuria atra</i>	0.00 ± 0.00	13.72 ± 0.22	0.00 ± 0.00	0.00 ± 0.00
3	<i>Holothuria leucospilota</i> LPG	8.02 ± 0.37	10.55 ± 0.33	0.00 ± 0.00	0.00 ± 0.00
4	<i>Holothuria scabra</i>	0.00 ± 0.00	13.95 ± 0.28	0.00 ± 0.00	0.00 ± 0.00
5	<i>Stichopus hermanni</i>	0.00 ± 0.00	13.47 ± 0.22	0.00 ± 0.00	0.00 ± 0.00
6	<i>Stichopus ocellatus</i>	8.17 ± 0.26	14.60 ± 0.21	0.00 ± 0.00	0.00 ± 0.00
7	<i>Stichopus vastus</i>	10.38 ± 0.09	8.60 ± 0.31	0.00 ± 0.00	0.00 ± 0.00
8	<i>Thelenota ananas</i>	8.17 ± 0.26	12.55 ± 0.51	0.00 ± 0.00	0.00 ± 0.00
9	<i>Actinopyga echinites</i> LBK	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10	<i>Bohadschia marmorata</i>	7.50 ± 0.29	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
11	<i>Holothuria albiventer</i>	8.30 ± 0.38	10.48 ± 0.14	10.60 ± 0.57	0.00 ± 0.00
12	<i>Holothuria colluber</i>	7.97 ± 0.38	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
13	<i>Holothuria fuscogilva</i>	9.90 ± 0.21	8.63 ± 0.15	9.15 ± 0.32	0.00 ± 0.00
14	<i>Holothuria hilla</i>	7.30 ± 0.10	9.40 ± 0.15	9.17 ± 0.37	0.00 ± 0.00
15	<i>Holothuria impatiens</i>	8.05 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
16	<i>Holothuria lessoni</i>	8.08 ± 0.09	9.15 ± 0.62	0.00 ± 0.00	0.00 ± 0.00
17	<i>Holothuria leucospilota</i> LBK	7.98 ± 0.24	8.73 ± 0.44	0.00 ± 0.00	0.00 ± 0.00
18	<i>Holothuria pardalis</i>	0.00 ± 0.00	9.60 ± 0.10	0.00 ± 0.00	0.00 ± 0.00
19	<i>Holothuria rigida</i>	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20	<i>Holothuria verrucosa</i>	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
21	<i>Bohadschia</i> sp.	8.35 ± 0.32	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Antibiotic (control, 1.25 µg µL ⁻¹)					
22	Ampicillin	14.20 ± 0.40	23.70 ± 0.33	37.70 ± 0.06	19.67 ± 0.28
23	Erythromycin	20.26 ± 0.31	25.20 ± 0.41	23.90 ± 0.21	11.83 ± 0.72
24	Kanamycin	20.85 ± 0.15	19.43 ± 0.12	29.13 ± 0.33	20.60 ± 0.31

Collection location codes: LBK: Lombok, LPG: Lampung

Both definitions indicate that bactericidal antibiotics have stronger effects than bacteriostatic antibiotics (Nemeth et al. 2014). The MIC is considered a basic reference in determining the susceptibility of organisms to antimicrobials, supporting its wide use in vitro to identify novel therapies (Andrews 2001). Thus, the MIC was tested in this study identify sea cucumbers with the strongest antibacterial activity.

Despite the standard and easy use of the disk diffusion assay (Jorgensen and Ferraro 2009), minor technical problems can occur, such as the inaccurate diffusion of a test sample because of differences in polarity between the test compound and the agar medium (Sánchez and Kouznetsov 2010). For this reason, a second assay was employed in which the blue resazurin indicator would change in color to pink when reduced by oxidoreductase in bacterial cells (Sarker et al. 2007). The MIC results demonstrated that eleven species had antibacterial activity against only *B. subtilis*: *A. echinites* LPG (12.5 µg µL⁻¹), *T. ananas* (50 µg µL⁻¹), *B. marmorata* (25 µg µL⁻¹), *H. albiventer* (12.5 µg µL⁻¹), *H. fuscogilva* (25 µg µL⁻¹), *H.*

hilla (25 µg µL⁻¹), *H. impatiens* (50 µg µL⁻¹), *H. lessoni* (50 µg µL⁻¹), *H. leucospilota* LBK (25 µg µL⁻¹), *H. verrucosa* (50 µg µL⁻¹), and *Bohadschia* sp. (25 µg µL⁻¹). Of these, the strongest effects against *B. subtilis* were recorded for *A. echinites* LPG and *H. albiventer* (Table 3 and Fig. 1). The concentration was generally started at 50 µg µL⁻¹, but the concentration was started at 25 µg µL⁻¹ in some instances because of the limited yield.

Based on the results using the MIC and disk diffusion assays, seven sea cucumber species exhibited antibacterial activity against three pathogenic species: *A. echinites* LPG, *T. ananas*, *H. albiventer*, *H. fuscogilva*, *H. hilla*, *H. lessoni*, and *H. leucospilota* LBK. In line with our previous results, none of the sea cucumber samples displayed stronger antibacterial activity than the control antibiotics. Nonetheless, it should be noted that the active antibacterial factors were not purified and concentrated from the sea cucumber extracts to make an objective comparison. Overall, *H. albiventer* exhibited the greatest potential as an antibacterial agent in the two assays. This species was

Table 3 Potential antibacterial activity tested using the resazurin-based minimum inhibitory concentration method

No	Sea cucumber species	Bacteriostatic concentration($\mu\text{g} \cdot \mu\text{L}^{-1}$)			
		<i>S. aureus</i>	<i>V. cholerae</i>	<i>B. subtilis</i>	<i>E. coli</i>
1	<i>Actinopyga echinites</i> LPG	> 50	> 50	12.5	> 50
2	<i>Holothuria atra</i>	> 50	> 50	> 50	> 50
3	<i>Holothuria leucospilota</i> LPG	> 50	> 50	> 50	> 50
4	<i>Holothuria scabra</i>	> 25	> 25	> 25	> 25
5	<i>Stichopus hermanni</i>	> 25	> 25	> 25	> 25
6	<i>Stichopus ocellatus</i>	> 50	> 50	> 50	> 50
7	<i>Stichopus vastus</i>	> 50	> 50	> 50	> 50
8	<i>Thelenota ananas</i>	> 50	> 50	50	> 50
9	<i>Actinopyga echinites</i> LBK	> 50	> 50	> 50	> 50
10	<i>Bohadschia marmorata</i>	> 50	> 50	25	> 50
11	<i>Holothuria albiventer</i>	> 50	> 50	12.5	> 50
12	<i>Holothuria colluber</i>	> 50	> 50	> 50	> 50
13	<i>Holothuria fuscogilva</i>	> 50	> 50	25	> 50
14	<i>Holothuria hilla</i>	> 50	> 50	25	> 50
15	<i>Holothuria impatiens</i>	> 50	> 50	50	> 50
16	<i>Holothuria lessoni</i>	> 50	> 50	50	> 50
17	<i>Holothuria leucospilota</i> LBK	> 50	> 50	25	> 50
18	<i>Holothuria pardalis</i>	> 50	> 50	> 50	> 50
19	<i>Holothuria rigida</i> *	–	–	–	–
20	<i>Holothuria verrucosa</i>	> 50	> 50	50	> 50
21	<i>Bohadschia</i> sp.	> 50	> 50	25	> 50
<i>Antibiotic (control)</i>					
22	Ampicillin	1.25	0.625	< 0.0048	< 0.0048
23	Erythromycin	< 0.0048	< 0.0048	< 0.0048	< 0.0048
24	Kanamycin	< 0.0048	< 0.0048	< 0.0048	< 0.0048

*Insufficient sample for tests

Collection location codes: LBK: Lombok, LPG: Lampung

active against three bacterial species, producing inhibitory zones in the disk diffusion assay against *S. aureus* (8.30 ± 0.38 mm), *V. cholerae* (10.48 ± 0.14 mm), and *B. subtilis* (10.60 ± 0.57 mm) (Table 2), and this sea cucumber species was also bacteriostatic (MIC) against *B. subtilis* ($12.5 \mu\text{g} \mu\text{L}^{-1}$) in the resazurin-based assay. A similar result also achieved for *A. echinites* LPG as the most potent antibacterial agent with activity against *S. aureus* (8.75 ± 0.18 mm), *V. cholerae* (9.77 ± 0.42 mm), and *B. subtilis* (8.48 ± 0.19 mm) in the disk diffusion assay (Table 2) and bacteriostatic activity (MIC) against *B. subtilis* at $12.5 \mu\text{g} \mu\text{L}^{-1}$ (Table 3). Another study conducted by Abraham et al. (2002) explained that *A. echinites* has antibacterial activity against *S. aureus*, and its bioactive compounds were triterpene glycosides (saponins) (Bordbar et al. 2011). In line with the previous discussion about *H. albiventer*, no related study of this species has been conducted, especially concerning antibacterial activity. This study could be the first evidence of the value of *H.*

albiventer, especially regarding its antibacterial activity and antioxidant capacity.

The MBC test is performed to determine whether compounds are bactericidal or bacteriostatic. *S. aureus*, *V. cholerae*, and *E. coli* were not included in the MBC test because the findings were negative for these microbes in the MIC test. Seven species exhibited potential activity against *B. subtilis* in the MIC test. Of these species, all were revealed to have bacteriostatic effects opposed to bactericidal activity.

GC–MS analysis of sea cucumber extracts

In recent years, GC–MS studies have been increasingly applied to analyze medicinal plants, as this technique has proven valuable for analyzing non-polar components and volatile essential oils, fatty acids, lipids, and alkaloids. It has also been exclusively used for the analysis of esters, fatty acids, alcohols, aldehydes, terpenes, and other compounds. Based on the principle adsorption and partition of

Table 4 Gas chromatography–mass spectrometry analysis of antibacterial activity in sea cucumbers

No	RT (min)	Compounds	Molecular formula	Molecular weight (g/mol)	Peak area (%)
<i>Holothuria albiventer</i> extract					
1	29.093	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270.50	4.06
2	29.265	7-Hexadecanoic acid, methyl ester	C ₁₇ H ₃₂ O ₂	268.4	1.47
3	29.727	Hexadecanoic acid, 15-methyl-, methyl ester	C ₁₈ H ₃₆ O ₂	284.48	1.29
4	29.893	Octadecanal	C ₁₈ H ₃₆ O	268.47	1.34
5	30.279	Methyl stearate	C ₁₉ H ₃₈ O ₂	298.50	7.66
6	30.389	Cis-13-octadecanoic acid, methyl ester	C ₁₉ H ₃₆ O ₂	296.49	3.03
7	30.417	11-Octadecanoic acid, methyl ester	C ₁₉ H ₃₆ O ₂	296.49	4.18
8	30.761	Nonadecanoic acid, methyl ester	C ₂₀ H ₄₀ O ₂	312.53	2.50
9	31.230	Methyl nonadecanoate	C ₂₀ H ₄₀ O ₂	312.5	4.55
10	31.327	Methyl 9-eicosenoate	C ₂₁ H ₄₀ O ₂	324.5	7.04
11	31.865	5,8,11,14-Eicosatetraenoic acid, methyl ester	C ₂₁ H ₃₄ O ₂	318.5	5.59
<i>Stichopus ocellatus</i> extract					
1	29.08	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270.50	7.03
2	30.26	Methyl stearate	C ₁₉ H ₃₈ O ₂	298.50	3.60
3	32.17	Hexadecanoic acid, bis (2-ethylhexyl) ester	C ₂₂ H ₄₂ O ₄	370.56	28.57
<i>Stichopus vastus</i> extract					
1	29.093	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270.50	10.18
2	30.272	Methyl stearate	C ₁₉ H ₃₈ O ₂	298.50	4.82
3	30.382	9-Octadecanoic acid, methyl ester	C ₁₉ H ₃₆ O ₂	296.48	11.43

gas chromatography, this technique is the most widely used technique for this purpose (Al-Rubaye et al. 2017).

GC–MS analysis was performed using the three sea cucumber extracts with the strongest growth-inhibitory effects against each bacterial strain, revealing the diversity of the bioactive molecules. *H. albiventer*, which had the strongest effects against *B. subtilis*, featured eleven active compounds, and three compounds each were detected in the extracts of *S. vastus* and *S. ocellatus* (Table 2). The eleven compounds detected in *H. albiventer* were hexadecanoic acid, methyl ester (C₁₇H₃₄O₂); 7-hexadecanoic acid, methyl ester (C₁₇H₃₂O₂); hexadecanoic acid, 15-methyl-, methyl ester (C₁₈H₃₆O₂); octadecanal (C₁₈H₃₆O); methyl stearate (C₁₉H₃₈O₂); cis-13-octadecanoic acid, methyl ester (C₁₉H₃₆O₂); 11-octadecanoic acid, methyl ester (C₁₉H₃₆O₂); nonadecanoic acid, methyl ester (C₂₀H₄₀O₂); methyl nonadecanoate (C₂₀H₄₀O₂); methyl 9-eicosenoate (C₂₁H₄₀O₂); and 5,8,11,14-eicosatetraenoic acid, methyl ester (C₂₁H₃₄O₂). The bioactive compounds in *S. ocellatus* were hexadecanoic acid, methyl ester; methyl stearate; and hexadecanoic acid, bis (2-ethylhexyl) ester (C₂₂H₄₂O₄). The active constituents of *S. vastus* were hexadecanoic acid, methyl ester; methyl stearate; and 9-octadecanoic acid, methyl ester (Table 4).

GC–MS analysis revealed the presence of long-chain fatty acids (LCFAs) without other secondary metabolites

with potential antibacterial activity such as phenolic compounds or terpene compounds. LCFAs display interesting biological activities such as antibacterial activity, and they are the key ingredients of some antibacterial herbs and antimicrobial food additives. C16–C20 LCFAs are known to actively inhibit bacterial growth (Zheng et al. 2005). In other words, the detected compounds possess strong correlations with the inhibitory effects on bacterial growth. In a deep analysis, each compound has unique abilities to inhibit bacterial growth, but generally, the primary mechanisms include increased membrane permeability and cell lysis, disruption of the electron transport chain and uncoupling of oxidative phosphorylation, and inhibition of the activity of bacterial enzymes (Yoon et al. 2018). Other studies described LCFAs in other sea cucumber species such as *S. horrens* (Rasyid et al. 2018), *H. atra* (Murniasih et al. 2015), *S. hermanni*, *Thelenota anax*, *H. fuscogilva*, *H. leucospilota*, and *H. scabra* (Pangestuti and Arifin 2017).

Conclusion

The results in this study could represent an effective introduction to the antioxidant capacities and antibacterial activities of sea cucumbers, especially rare species. Antimicrobial and antioxidant activities were detected in

extracts from various species of sea cucumbers collected from Lombok Island and Lampung, Indonesia. The most promising sea cucumber regarding antioxidant activity was *H. atra*, whereas the most promising antibacterial species was *H. albiventer*. Based on this research, those species could be further studied to obtain valuable information for researchers or industries with interest in developing functional foods or medicinal ingredients from sea cucumbers. Further studies are needed for other biological activities such as cytotoxic and antifungal activity as well as to characterize bioactive components such as phenolic, terpenoid, and saponin compounds.

Acknowledgements The authors would like to thank Enago™ English Editing Service for editing a draft of this manuscript.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by AN, IAH, AB, ARA, AS, MYP. The first draft of the manuscript were written by AN, AB, TM and MYP and all authors reviewed and approved the final manuscript.

Funding This research was supported by a research grant awarded to Masteria Yunovilsa Putra by the Coral Reef Rehabilitation and Management Program-Coral Triangle Initiative (COREMAP-CTI) 2019 and National Research Priority (PRN), Indonesian Institute of Sciences.

Data availability All data generated or analysed during this study are included in this published article.

Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

Human and animal rights This article does not contain any studies with human or animal subjects.

Informed consent This article does not contain any individual person's data in any form.

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