Antioxidant Capacities of *Holothuria* Sea Cucumbers

Tutik Murniasih*, MasteriaYunovilsa Putra, and Ratih Pangestuti

Research Center for Oceanography, Indonesian Institute of Sciences (LIPI), Indonesia

Abstract

Sea cucumbers are a potential source of biologically active metabolites that are widely used for nutraceutical, pharmaceutical and cosmeceutical products. In this study we investigated the antioxidant activity of several extracts of sea cucumbers, those are *Holothuria scabra*, *Holothuria atra, Holothuria leucospilota,* and *Holothuria excellens* collected from Lombok Island. The selected sea cucumber was then separated to get the active fractions and identified for profiling the secondary metabolites. Phytochemical screening reveals the presence of flavonoid, terpenoids, phenol, saponin, alkaloid, anthraquinone, and glycoside in the extracts of *Holothuria* sea cucumbers. Radical *s*cavenging effects on the DPPH (1,1-diphenyl-2-picryl-hydrazyl) model were evaluated. The previous work reported that the crude extract of *H. atra* showed the highest potential of antioxidant capacity. *H. atra* extract was separated by column chromatography on silica gel and eluted with a gradient system of increasing polarity. Fraction 7 of *H. atra* extract showed significantly inhibited radical scavenging activity (35.3487 %) at concentration 1 mg/ml. The result of Gas Chromatography-Mass Spectrometry analysis of *H. atra* fraction 2 showed that it has several active antioxidant compounds which are ethyl cetylate (3), hexadecyl-oxirane (5), 3-chloro-4-hydroxybenzoic acid (6), andoleyl alcohol, trifluoroacetate (9). Moreover, the active fraction 7 indicated several compounds, those are cycloicosane (17), pentadecyl trichloroacetate (18), 14β-pregnane (21) and cis-4-cyano-2-(2-hydroxycyclohexyl) pyridine. However, these are preliminary findings and further studies are needed to be focus on the purifications of unknown compounds and formulation for nutraceuticals.

Keywords: Holothurian sea cucumber, extract, antioxidant capacity

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*Corresponding author:
Jl. Pasir Putih I, Ancol Timur, Jakarta 14430, Indonesia
Tel. +21-64713850, Fax. +21-64711948
E-mail. tutiic_2000@yahoo.com

Introduction

Sea cucumber is marine organism that spread out fluid when their body is damaged or injured. Marine organism's chemical defense mechanism is reputed as marine natural product that has ecological function as antifouling, anti-predation and UV protection (Mc. Clintock & Baker, 2001). Marine natural products are known for its pharmaceuticals commodities. Sea cucumber is fascinating food despites it can be used for treating some diseases, such as hypertension, eczema, arthritis and wounds (Esmat *et al.*, 2013; Dhinakaran & Lipton, 2014). Holothurian is the most popular sea cucumber as bioactive substances producer. The methanol-water extract of *H. atra* from Indian Ocean was reported active against *Klebsiella pneumonia, Serratia liquaefacilusun*, and *Staphylococcus aureus*. (Dhinakaran & Lipton, 2014). Moreover, it is also confirmed to have antifungal activity also indicated by ethyl acetate-methanol extract (Septiadi *et al*., 2013), strong iron-chelating antioxidant activity (Esmat *et al*., 2012) and exhibit hepatoprotective activity, curative and antioxidant potential in rats model (Dakrory *et al*., 2015).

The presence of bioactive compounds with therapeutic properties in sea cucumber has made them an attractive bioactive source. Some important compounds include triterpene glycosides (saponins), chondroitin sulfates, glycosaminoglycans (GAGs), phenolic, and essential fatty acids (Soltani *et al*., 2014). The secondary metabolite compounds reported in *Holothuria* genus were some phenolic compounds such as chlorogenic acid, pyrogallol, coumaric acid, cathechin and ascorbic acid (Esmat *et al*., 2012), glycosides with three-sulphated branched pentasaccharide

carbohydrate (Avilov *et al*., 2003), nortriterpene and triterpene glycosides (Wang *et al*., 2012). Antioxidant activity is the most important element to natural product, since it will protect cell from the damage caused by unstable molecule such as free radical. As tropical country, so many diseases spread in Indonesia. Thus, supplement to increase human immune system is required. The search for novel source of antioxidant agent from marine natural resources is one of research strategy as solution of the issue. Althunibat *et al.* (2009) reported that aqueous extract derived from sea cucumber *H. leucospilota, H. scabra* and *Stichopus chloronotus* contain phenolic compounds and showed antioxidant capacity. This study was done to investigate the antioxidant agent from sea cucumber collected from East of Lombok (Indian Ocean area).

Materials and Methods

Chemicals. Ethanol (EtOH), dichloromethane (DCM), *n*-buthanol (n-BuOH), ethyl acetate (EtOAc), *n*-hexane, methanol MeOH), silica gel (230-400 mesh) and Pre-coated silica-gel-60-F_{254} plates detected at 254 nm was purchased from Merck, Germany. Tecan Nano quant was used for measuring the antioxidant activity using DPPH (1,1-diphenyl-2-picryl-hydrazyl).

Animal Material, Extraction, and Isolation. Sea cucumbers, *H. scabra*, *H. atra, H. leucospilota* and *H. excellens,* extracts were obtained from our previous work (Pangestuti, *et al.,* 2016). These holothurians were collected in June 2015 in Jor Bay, East Lombok, Indonesia at the depth 1-2 m and the site sampling was S. 08^o 48. 212'; E. 116^o 30.031'. Fresh sea cucumbers were cut to separate its skin and viscera. The samples were wrapped with cotton, impregnated using alcohol and packed for transportation. In laboratory, samples were stored in -20 ^{o}C before extraction. Approximately 1.5 kg of *H. atra*, 0.5 kg of *H. leucospilota*, and 0.5 kg of *H. excellens* were repeatedly extracted with EtOH and DCM at room temperature and the combined extracts were concentrated to dry at reduced pressure using rotary evaporator. All of sea cucumber extracts were tested for antioxidant capacity. The selected extract of sea cucumber *H. atra* was partitioned between H_2O and EtOAc to give an ethyl acetate extract, while the water phase was further partitioned against *n*-BuOH, thus affording a butanol extract. The EtOAc extract was chromatographed using n-phase system. Silica gel (230-400 mesh) was used for column chromatography and eluted with n-hexane-EtOAc-MeOH gradiently. All fractions were tested for antioxidant capacity to get the potential fraction.

Phytochemical screening. All extracts of sea cucumber were subjected to phytochemical screening for the presence of secondary metabolites with standard conventional protocol as described by Harborne (1987).

Antioxidant activity test. The evaluation of antioxidant activity was done using the following method: Sea cucumber extract and fraction were dissolved in various concentrations of methanol. The negative control consists of 160 mL methanol mixed with 40 mL sample. The sample solution consists of 160 mL of DPPH and 40 mL sample (extract and fraction). The negative control and samples were incubated for 30 minutes in a dark room, then the absorbance was measured at a wavelength of 517 nm using a micro plate reader (TECAN). The inhibitory activity was calculated using the following formula (Sánchez-Moreno *et al*., 1998):

%Inhibition=([DPPH]0-[DPPH]s/[DPPH]0)×1000

[DPPH]0=concentration of initial DPPH
[DPPH]s=concentration of DPPH end of the remaining

Gas Chromatography - Mass Spectrometry (GC-MS) analysis. GC-MS analysis was conducted at The Medical Laboratory of Jakarta Province. The active fraction was injected to Agilent Technologies 7890 GC-MS with auto sampler and 5975 Mass Selective Detector and Chemstation Data System. This instrument was set as electron impact using ionization mode with electron energy 70 eV. Column for analysis was capillary column HP Ultra 2L, length (m) 30×0.25 (mm) I.D×0.25 (µm) Film thickness. Oven temperature was set as initial temperature at 70 ^{o}C, rising at 3

°C/min to 150 °C hold for 1 minute and finally rising 20 v/min to 280 °C hold for 26 minutes. Injection port temperature was 250 °C, ion source temperature 230 °C, interface temperature 280 °C and quadrupole temperature of 140 °C. The gas carrier was helium with the column flow 1.0 µL

Results and Discussion

Calculation of of crude extracts yield from sea cucumber *H. scabra*, *H. leucospilota H. excellens,* and *H. atra* were already done in our previous work, as described in Figure 1. Among 5 holothurians, the highest yield of ethyl acetate extract was *H. atra* with rendement about 6 % (Pangestuti *et al.*, 2016). The chemical analysis of crude extract of all sea cucumbers compound shows the presence of flavonoid, terpenoids, phenol, saponin, alkaloid, anthraquinone and glycoside. However, the tannin was not recognized in these organisms (Table 2).

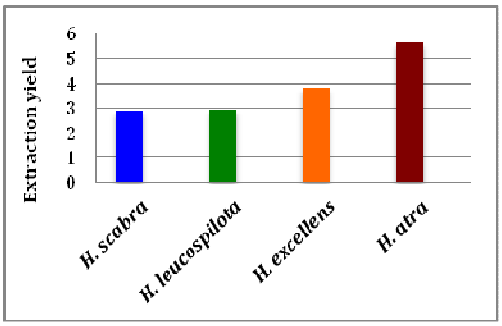

Figure 1. The yield of crude extracts (%) from Holothurian sea cucumbers (Pangestuti *et al*., 2016).

The crude extracts of Holothurian sea cucumber were tested for its antioxidant capacity by their ability to scavenge free radicals using DPPH method. Ethyl acetate was chosen for extracting antioxidant substances from sea cucumber. The most potent antioxidant was observed in ethyl acetate extract (Mamelone *et al.*, 2011).

As shown in Figure 2, the antioxidant activity of crude extract of Holothurian sea cucumbers shows dose dependent manner and the *H. leucospilota* extract shows the highest antioxidant capacity with the DPPH scavenging effect is 11.12 % at concentration of 1 mg/mL. Although *H. leucospilota* extract shows potential antioxidant activity, further separation was not done on this sample because the amount of the extract was smaller than *H. atra*. The yield of *H. leucospilota* extract and its natural stock was very low.

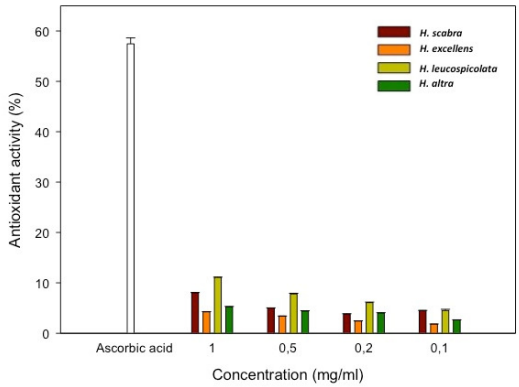

Figure 2. The antioxidant activity of Holothurian sea cucumber extracts

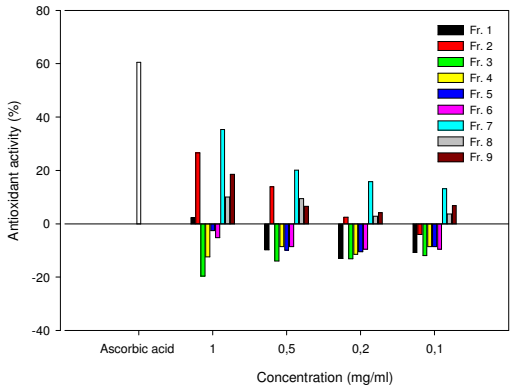

Figure 3. Antioxidant activity of fractions from *Holothuria atra.* Ascorbic acid (0.2 mg/ml) was used as positive control.

Based on the amount of the extract samples of Holothurian sea cucumbers,we continued investigation on *H atra* extract. The column chromatography of ethyl acetate extract from *H. atra* resulted 9 fractions. All fractions were then evaluated for antioxidant capacity. Figure 3 shows the major fraction with the highest yield was owned by semi polar fraction number 7 (25 mg) which was eluted with EtOAc:MeOH (9:1). Among 9 fractions, fraction 7 of *H. atra* extract had a moderate inhibition of radical scavenging activity (35.3487 %) using DPPH at concentration 1 mg/mL. Fraction 7 was evaluated for phytochemical screening of secondary metabolites and shows the presence of saponin, glycoside and alkaloid. Saponins are the majority of compounds in Holothurian sea cucumbers, generally known as holothurins, are usually triterpene glycosides, which belong

to the holostane type group rather than nonholostane (Avilov *et al*., 2004). However, since these are preliminary findings, further studies will be conducted and should focus on isolation of bioactive substances derived from *H. atra* and identify other health benefit effects of *H. atra*.

Another fraction, which has moderate antioxidant activity using DPPH method, is fraction 2. Characterization of active fractions using GC-MS is described in Table 3.

Hexadecyl-oxirane was reported active and very effective as anticancer (Perumal *et al*., 2014). Compound (z)-octadec-9-en-1-ol is active sunblock and commercially used for cosmetic ingredient. (*https://pubchem.ncbi.nlm.nih.gov/compound/oleyl_alcohol*). Carbonyl group in 7,9-di-tert-butyl-1-oxaspiro (4, 5) deca-6,9-diene-2,8-dione plays an important role in radical scavenging activity (Halogen attached in organic compound pentadecyl trichloroacetate and oleyl alcohol, trifluoroacetate). Halogen is highly reactive free radical, the deprotonated form of this group could act as reactive scavenger to the toxic ROS as its function as antioxidant agent. The other study reported that several compounds with halogen functional group determined as antioxidant activity (Kumar *et al*., 2014).

Table 2. Phytochemical constituents of extracts of Holothurian sea cucumbers

Phytochemical constituents	*H. scabra*	*H. atra*	*H. leucospilota*	*H. excellens*
Tannins	-	-	-	-
Flavonoid	+	+	+	+
Terpenoids	+	+	+	+
Phenols	+	+	+	+
Saponins	+	+	+	+
Glycoside	+	+	+	+
Alkaloids	+	+	+	+
Anthraquinones	+	+	+	+

+ = Presence; -- = Absence

Table 3. The Compounds in active fractions 2 and 7 analyzed using GC-MS

Fraction number	% area	Retention time (minute)	Molecular weight (g/mol)	Compound
2	0.92	30,609	256.2	Ethyl myristate
2	1.61	31,389	228	Dodecanoic acid, ethyl ester
2	7.51	32,499	284.1	Etyl cetylate
2	6.23	32,347	282.0	Ethyl 9-hexadecenoate
2	0.38	33,299		Hexadecyl-oxirane
2	15.97	33,685	172	3-Chloro-4-hydroxybenzoic acid
2	12.65	34,505	338.3	unknown
2	1.26	35,484	366.3	Ethyl 13-docosenoate
2	3.29	36,739	364	Oleyl alcohol, trifluoroacetate
7	1.10	3,135	106.1	p-Xylol
7	3.36	26,269	182.0	alpha-oxoditane
7	1.31	31,626	276.1	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
7	1.05	32,075	280.2	Cycloicosane
7	2.02	32,116	210	pentadecyl trichloroacetate
7	6.27	33,998	292.3	unknown
7	2.78	34,067	287.1/208	2-tridecyloxirane
7	3.99	34,364	532.5; 570	14B-pregnane
7	2.30	34,605	202	Cis-4-cyano-2-(2-Hydroxycyclohexyl)pyridine
7	5.81	49,623	647.5	6H,16H,31H-5;15,19-dimethano-10,14-metheno-26,30-nitrilo-5H,25H-dibenzo[b,s][1,21,4,8,14,18]dioxatetraazacyclooctanecosine-34,36-dione,7,8,17,18-tetrahydro-35-methoxy-1,3,21,23-tetramethyl-

Approximately 15.97 % was the highest percentage of compound found in fraction 2, which was identified as 3-chloro-4-hydroxybenzoic acid. This phenolic compound plays an important role for antioxidant properties. Study of several plant and marine organisms concluded that the antioxidant activity was related to phenolic constituent (Rice-Evans *et al*., 1997). On the other hand, the highest component in fraction 7 was unknown compound with the molecular weight 292.3.

The similar previous work confirmed that methanolic extract of *H atra,* which was collected from Kanyakumari, contained 1,1-dicloroethane, 1,2 benzene dicarboxylic acid diethyl ester, cyclopropane, 1,3,7 octatriene (Dinakaran & Lipton, 2014). The use of different solvent for extraction, so does different sampling location, would present different isolated substances. Further purification and detailed structural elucidation is needed.

Conclusion

In the present study, we investigated some Holothurian sea cucumbers from Lombok Island. All extracts of Holothurian sea cucumbers were evaluated for its antioxidant capacity. *H. leucospilota* and *H. atra* have potent antioxidant capacity especially for radical scavenging activity using DPPH method. Phytochemicals constituents in *H. leucospilota* and *H. atra* were flavonoid, terpenoid, phenols, saponins and glycoside. The result of GC-MS analysis shows that the active antioxidant compounds of *H atra* fractions in ethyl acetate extract were (3), hexadecyl-oxirane(5),3-chloro-4-hydroxy benzoic acid (6), andoleyl alcohol, trifluoroacetate (9) which were collected from fractions 2; on the other hand, cycloicosane (17), pentadecyl trichloroacetate (18), 14β-pregnane (21) and cis-4-cyano-2-(2-hydroxycyclohexyl) pyridine were gathered from fraction 7.

Further study to find the unknown antioxidant compounds derived from the active fractions will be very recommended.

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