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ANTIOXIDANT AND CYTOTOXIC PROPERTIES OF TWO SEA CUCUMBERS, *HOLOTHURIA EDULIS* LESSON AND *STICHOPUS HORRENS* SELENKA

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Sea cucumbers are marine invertebrates of the phylum of Echinodermata that have been used in Asian traditional medicine since ancient times. This study was conducted to investigate the antioxidant and cytotoxic properties of aqueous and organic extracts from two sea cucumber species, *Holothuria edulis* Lesson (Holothuriidae) and *Stichopus horrens* Selenka (Stichopodidae). Antioxidant activities of the extracts were evaluated by DPPH• and β-carotene bleaching assays, while MTT and trypan blue exclusion assays were used to demonstrate the cytotoxic effects of the extracts against two human cancer cell lines, non-small cell lung cancer cells (A549) and esophageal cancer cells (TE1). The results showed that both aqueous and organic extracts of *H. edulis* were able to scavenge DPPH radical (IC_{50} at 2.04 mg/ml and 8.73 mg/ml, respectively). Aqueous and organic extracts of *S. horrens* inhibited 79.62% and 46.66% of β-carotene oxidation by linoleate free radical. On the other hand, the organic extract of *S. horrens* exhibited the highest cytotoxic effects against A549 and TE1 cancer cells giving IC_{50} at 15.5 and 4.0 µg/ml, respectively. In conclusion, the present study revealed that *H. edulis* and *S. horrens* contain promising levels of antioxidant and cytotoxic natural products that might be used for cancer prevention and treatment.

Keywords: Antioxidant – cytotoxic – sea cucumber – *Holothuria edulis* – *Stichopus horrens*

INTRODUCTION

Cancer is a leading cause of death worldwide and it is estimated by world health organization (WHO) that without intervention 180 million people will die of cancer during the time between 2005 and 2015. However, cytotoxic drug therapy (chemotherapy) is one of the main methods of modern cancer treatment and it is the only

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approach to treat the metastastatic cases. On the other hand, the development of resistance to cytotoxic chemotherapy is one of the most challenging problems in cancer treatment [13].

Experimental evidences have revealed that the free radicals, mainly reactive oxygen species (ROS), are involved in initiation, promotion and progression of carcinogenesis. The role of oxidative stress has been established in several types of cancers like lung cancer, leukemia, breast, ovary and rectum cancers [21]. However, recent studies have shown that the intake of natural antioxidants could have great effect as therapeutic agents in several diseases mediated by oxidative stress [8]. Likewise, some epidemiological studies have demonstrated the protective effect of dietary antioxidants on reducing risks of cancer [24].

For the past few decades, marine natural products have been an attractive area worldwide for developing different pharmaceutical products. Sea cucumbers are marine invertebrates of the phylum of Echinodermata and the class of Holothuroidea, with an elongated body and leathery skin. There are more than 1500 species of sea cucumbers distributed on the sea floor worldwide, and around 49 species have been identified in Malaysia costal water. Sea cucumbers are used traditionally as food stuff for their favorable flavor, and they are also eaten to treat a number of diseases such as eczema, arthritis and hypertension. Furthermore, several scientific studies conducted in the last two decades came in support of these medicinal purposes and showed multiple biological activities of sea cucumber extracts as wound healing promoters in addition to demonstrating antimicrobial, immune-modulatory and antinociceptive properties [19].

In our previous study, three different Malaysian sea cucumber species have showed varied antioxidant and cytotoxic properties [2]. In addition, antioxidant properties of coelomic fluid from three different Malaysian sea cucumbers have been reported [9]. Recent reports have demonstrated the phenolic contents and antioxidant capacity of tissues of Atlantic sea cucumber, *Cucumaria frondosa* Gunnerus (Cucumariidae), and Far-Eastern sea cucumber *Stichopus japonicus* Selenka (Stichopodidae) [10, 15, 27]. Other reports showed the potential cytotoxic effects of some sea cucumber species against cancer cell lines [18]. Therefore, this study was conducted to investigate the cytotoxic and antioxidant activities of extracts from two Malaysian sea cucumbers, *Holothuria edulis* Lesson (Holothuriidae) and *Stichopus horrens* Selenka (Stichopodidae).

MATERIAL AND METHODS

Sample collection and extraction

The two sea cucumber species, *H. edulis* and *S. horrens,* were collected from Terengganu coastal area in Malaysia. Samples were dissected to remove internal organs, and packed immediately with ice prior sending to the laboratory to be kept at –80 °C until extraction. Voucher specimens have been deposited at the museum at Faculty of Science, International Islamic University Malaysia. About 200 g of frozen

Table 1
Yields of aqueous and organic extracts from sea cucumber species

Species	Locations	% Yield of aqueous extracts*	% Yield of organic extracts*
Stichopus horrens	Terengganu	4.40	0.15
Holothuria edulis	Terengganu	4.75	0.40

*%Yield = (weight of dried extract/weight of frozen sample) *100.

samples were homogenized with deionized water and the mixture was continuously stirred in the dark at 4 °C for 24 h. Then it was filtrated by using four layers of gauze before it was centrifuged at 3500 rpm for 15 min, and the supernatant was then collected and filtrated again through Whatman filter paper No. 4. The collected aqueous extracts were freeze-dried and kept at –80 °C, while the insoluble solid materials were re-extracted with MeOH–CH_2Cl_2 (1 : 1 v/v), followed by methanol (100%). The organic extracts were combined and the solvents were removed by rotary evaporation at 40 °C under vacuum. The yield of each extract was calculated as ratio of weight of dried extract to weight of frozen sample (Table 1).

Evaluation of antioxidant activities

Determination of total phenolic content

Total phenolic contents of aqueous and organic extracts were assessed approximately by using Folin–Ciocalteu phenol reagent according to the methods described before, with some modifications [15]. All determinations were performed in triplicates, and samples' total phenolic contents were expressed as gallic acid equivalents (GAE) in milligrams per gram of the extract.

DPPH scavenging assay

Radical scavenging abilities of the extracts were measured by using the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to the method described previously [4]. The extract concentration which gave 50% radical scavenging (IC_{50}) was determined after calculating radical scavenging activity of different extracts concentrations according to the following formula:

$$\% \text{ Radical scavenging} = (\text{Control OD} - \text{Sample OD}/\text{Control OD}) * 100$$

β-Carotene bleaching assay

Oxidation induced β-carotene bleaching method was used to evaluate the total antioxidant activities of sea cucumber extracts according to the method described earlier in [11]. The effects of the samples on the time-dependent β-carotene bleaching were monitored spectrophotometrically at 470 nm, for 3 h at 30 min intervals. Total antioxidant activities (% AA) of the extracts were measured based on their ability to inhibit a bleaching of β-carotene by using the following formula:

$$\%AA = (1 - [(A0 - At) / (A°0 - A°t)]) * 100$$

A0 and A°0 are the absorbance values measured at initial time of the incubation for samples and control, respectively, while At and A°t are the absorbance values measured in the samples and control at t = 180 min. All determinations were performed in triplicate, and the extracts' antioxidant effects were compared with negative control and α-tocopherol effect.

Evaluation of cytotoxic effect

Cell lines

Two human cancer cell lines; A549 (human non-small cell lung carcinoma) and TE1 (human esophageal carcinoma) were kindly provided by Dr. Masa-aki Ikeda; Department of Molecular and Craniofacial Embryology, Tokyo Medical and Dental University. DMEM medium supplemented with 10% heat inactivated FBS and 1% penicillin-streptomycin was used for cell maintenance in a humidified atmosphere containing 5% CO_2 at 37 °C.

MTT assay

Cytotoxic effect of the extracts against the cancer cell lines was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), according to the method described previously [17]. The experiment was repeated three times and the extract concentrations required for inhibition of 50% of cell viability (IC_{50}) were determined.

Trypan blue exclusion test

Effects of different extract concentrations on the cell viability and treated cells membrane integrity were evaluated by trypan blue exclusion test according to the method described in the previous study [14].

RESULTS

Total phenolic content

Total phenolic compounds in tested extracts varied according to the species and extracting solvents (Table 2). Phenolic compounds were found to be more concentrated in the aqueous extracts of both tested species compared to their organic extracts. Aqueous extract of *H. edulis* showed the highest level of total phenols, with 7.33 mg/g GAE, being 3.4-fold higher than the organic extract of the same species, and 1.4-fold higher than the aqueous extract of and *S. horrens*. However, the lowest amount of phenolic compounds was obtained from the organic extract of *S. horrens,* 1.49 mg/g of GAE.

Table 2
Total phenolic contents, DPPH• scavenging capacities and antioxidant activities of sea cucumbers extracts

Species	Total phenols*		DPPH-IC_{50}**		β-Carotene bleaching***	
	Aq. ext.	Org. ext.	Aq. ext.	Org. ext.	Aq. ext.	Org. ext.
Stichopus horrens	5.24 ± 0.01	1.49 ± 0.07	>10	>10	79.62 ± 1.91	46.66 ± 1.13
Holothuria edulis	7.33 ± 0.02	2.17 ± 0.10	2.03 ± 0.06	8.73 ± 0.13	42.69 ± 1.25	28.52 ± 1.31
α-Tocopherol	X		0.01 ± 0.001		86.87 ± 2.60	

* Data expressed as gallic acid equivalents (GAE) mg/g extract, mean ± SD ($n = 3$).

** Data expressed as IC_{50} (the concentration of the extract required for scavenging of 50% of DPPH•) in mg/ml, mean ± SD ($n = 3$).

*** Data expressed as % antioxidant activity (A.A) according to the formula in methods, mean ± SD ($n = 3$).

Antioxidant activity

Antioxidant activities of the extracts were evaluated by demonstrating their ability for direct scavenging of a synthetic free radical DPPH•. The extract concentrations required for scavenging of 50% of radical DPPH were determined and the results were presented in (Table 2). An aqueous extract of *H. edulis* exhibited higher radical scavenging activity than the organic extract, giving $IC_{50} = 2.03$ vs. 8.73, respectively. On the other hand, even at high extract concentrations (>10 mg/ml), both aqueous and organic extracts of *S. horrens* were unable to scavenge 50% of radical DPPH. However, all the tested extracts showed significantly lower radical scavenging capacities than α-tocopherol which showed IC50 in microgram scale ($IC_{50} = 9.02$ µg/ml).

Antioxidant activities of sea cucumber extracts were also detected by β-carotene-linoleate model and compared with positive and negative controls. In opposite of the results obtained by DPPH assay, *S. horrens* extracts showed higher antioxidant capacity than *H. edulis* extracts by the β-carotene-linoleate model. Aqueous extract of

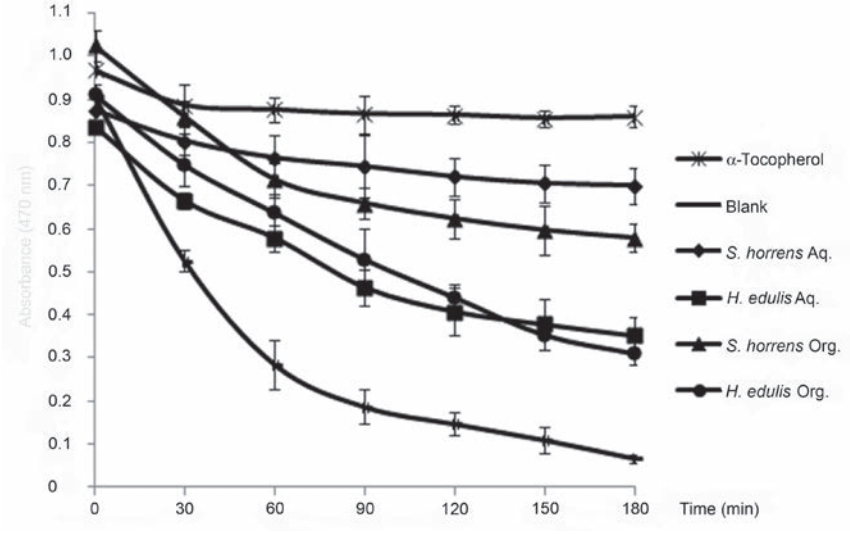

Fig. 1. Reaction kinetics of sea cucumber extracts (50 mg/ml) with β-carotene in comparison with α-tocopherol (50 μg/ml) and negative control. Data are presented as mean ± SD of triplicate experiments

S. horrens exhibited the highest antioxidant activity (AA = 79.62%) through inhibition of β-carotene oxidation showing reaction curve close to that of α-tocopherol effect (Fig. 1). On the other hand, an organic extract of *H. edulis* showed the lowest antioxidant activity against linoleate-free radical, giving AA = 28.52%.

Cytotoxic effects

The dose-dependent inhibitory effects of the extracts were plotted against the percentage of cell viability in order to determine the inhibitory concentrations required to reduce 50% of cell viability (IC_{50}) compared to vehicle treated control. As shown in (Table 3), among the two aqueous extracts only *H. edulis* exhibited cytotoxic effects against the cancer cells. However, *H. edulis* aqueous extract was more active against TE1 cells (IC_{50} = 78.0 μg/ml) than A549 (IC_{50} = 132.0 μg/ml). On the other hand, the two organic extracts inhibited growth of the cancer cell lines, where organic extract of *S. horrens* was found to be the most active cytotoxic extract against both A549 and TE1 cell lines, giving IC_{50} equal to 15.5 μg/ml and 4.0 μg/ml, respectively.

In accordance with the results obtained from MTT test, the dose-dependent changes in the viability of cancer cells were observed by using trypan blue exclusion test, which depends on the cell membrane integrity (Figs 2, 3). The results showed that organic extracts are more cytotoxic, and TE1 cell is more sensitive to sea cucumber extracts than A549 cell.

Table 3
Inhibitory effects of Malaysian sea cucumbers extracts on growth of two cancer cell lines (TE1 and A549)

Species	TE1 IC_{50} (μg/ml)*		A549 IC_{50} (μg/ml)*	
	Aq. ext.	Org. ext.	Aq. ext.	Org. ext.
Stichopus horrens	ND	4.0±0.5	ND	15.5±2.0
Holothuria edulis	78.0±3.0	17.0±1.5	132.0±9.0	22.5±1.0

* Data represent IC_{50}, the inhibitory concentrations (μg/ml) of the extract required to reduce 50% of cell viability by using MTT assay, mean ± SD (n = 3).

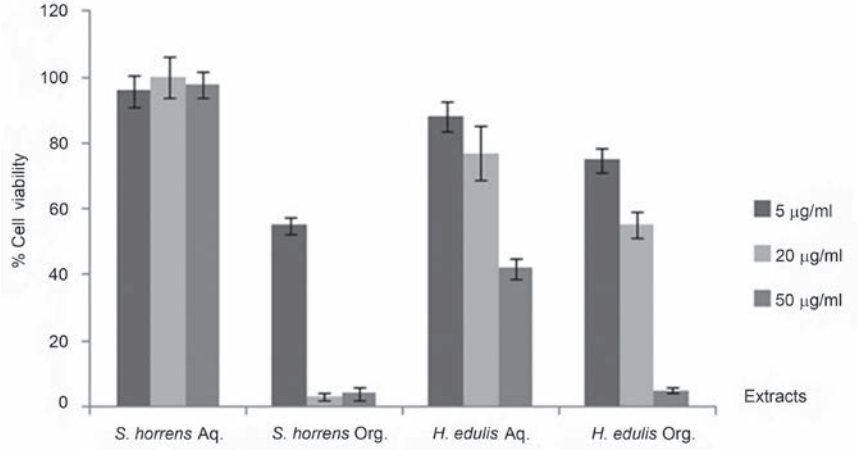

Fig. 2. Dose-dependent inhibitory effect of sea cucumbers' extracts on growth of TE1 cell line by trypan blue exclusion assay. Data are presented as mean ± SD of triplicate experiments

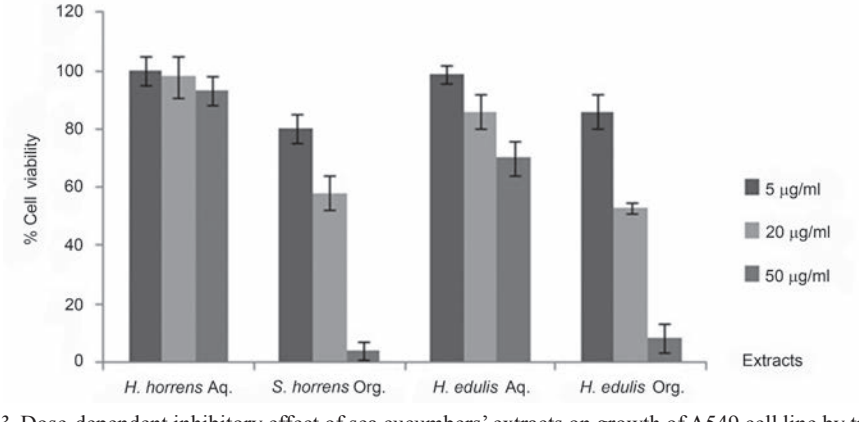

Fig. 3. Dose-dependent inhibitory effect of sea cucumbers' extracts on growth of A549 cell line by trypan blue exclusion assay. Data are presented as mean ± SD of triplicate experiments

DISCUSSION

Free radicals are the molecules that have unpaired electrons in the outermost shell and therefore are unstable and highly reactive. Accumulation of such oxidizing agents, mainly reactive oxygen species (ROS), in the human body induces oxidative damage for DNA and other cellular components leading to cancer initiation and propagation [23]. In contrast, antioxidants neutralize the free radicals by donating one of their own electrons to prevent or delay oxidation of the biomolecules. Thus, antioxidant supplements have been recommended to protect a human body from cancers and other diseases mediated by oxidative stress [8]. Moreover, the use of cytotoxic drug therapy is the most common way in current cancer treatments since it offers the only approach to treat the metastasized cancer cases. Marine organisms produce some of the most cytotoxic compounds that have been found in the anticancer area [3]. Accordingly, the present study was conducted to evaluate antioxidant and cytotoxic properties of two sea cucumber species, *S. horrens* and *H. edulis*.

Two different free radical systems were used to evaluate the antioxidant activities of the tested extracts, stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH•) and linoleic acid free radical-mediated-β-carotene bleaching. Radical DPPH produces violet solution in methanol and it is reduced in the presence of antioxidant molecules to form a light yellow solution. The extent of violet color reduction is proportional to antioxidant potencies of the tested materials, thus DPPH assay is used to evaluate the antioxidant activity of both synthetic and natural compounds [1, 4]. In the β-carotene bleaching system, β-carotene molecules are oxidized by linoleate free radical resulting in loss of their characteristic orange color. Active antioxidant molecules neutralize linoleate-free radical and then delay the extent of β-carotene bleaching [11].

Findings of this study revealed that sea cucumbers contain a range of natural antioxidants levels, in which the aqueous extracts exhibited higher antioxidant effects than the corresponding organic extracts. Antioxidant properties of natural extracts are generally ascribed to the presence of some bioproducts including phenolic compounds, salt, sugars, carotenoids, ascorbic acid, glutathione, peptides and pigments [7]. The presence of various antioxidant capacities in sea cucumber species came in support to the antioxidant properties previously reported in coelomic fluid and body extracts from other Malaysian sea cucumber species [2, 9]. The results of the present study showed variation in scavenging capacities of the same extract against different radicals. This difference might be explained by the different mechanisms involved in the radical-antioxidant reactions. Other factors, such as selectivity of the radicals or the solubility of the extracts in different testing systems might also affect the capacity of the extract to react and quench the different radicals. These results are in accordance with the previous reports which showed fluctuation of the antioxidant effects of marine natural products against various radicals [16].

Phenolic compounds form an integral part of the human diet and are of great current interest due to their antioxidative and possible anticarcinogenic activities [6]. The daily food of sea cucumbers is composed of phytoplankton and particles of marine macro-algae which are rich in phenolic compounds [19]. Therefore, it is expected to

find these compounds and their derivatives in the tissues of sea cucumbers. However, only a few studies have been conducted to demonstrate the presence of phenolic compounds in marine invertebrates and specifically in sea cucumber species. Levels of total phenols and flavonoids from an Atlantic sea cucumber, *C. frondosa,* were reported by Mamelona and colleagues [15]. In the present study, water was used first to extract the polar (hydrophilic) phenols and other bioactive compounds from the tested samples. Thereafter, the non-soluble compounds (hydrophobic) were successively extracted by organic solvents with different polarities. The higher antioxidant potential observed in the aqueous extracts compared to the corresponding organic extracts indicated that most of the extracted antioxidant molecules were hydrophilic in nature.

The effects of aqueous and organic extracts of the tested sea cucumber species on the growth of two human cancer cell lines, i.e. human non-small cell lung carcinoma (A549) and human esophageal carcinoma (TE1) were evaluated by MTT assay. The principle of this colorimetric assay depends on the reduction cleavage of the yellow tetrazolium salt (MTT) into formazan blue by mitochondrial succinate dehydrogenase. Since the reduction of MTT takes place only in the living cells, the amount of the formazan produced is proportional to the number of the viable cells present [26]. Thus, the MTT assay is reliable in order to evaluate the cytotoxic activities of various synthetic and natural products.

The cytotoxic effect of the aqueous extract of *H. edulis* resulted from the presence of hydrophilic cytotoxic compounds which might be either absent or present in minute concentration in *S. horrens* aqueous extract. Crude saponins extracted from *S. badionotus* showed potent cytotoxic effects against leukemia cells [12]. In addition, Rodriguez and colleagues isolated five saponin triterpenes from sea cucumber species, *Holothuria forskalii* Chiaje (Holothuriidae), and reported their cytotoxic and antiviral activities [20]. As saponins are water soluble compounds, therefore, they might be the main active compounds in the aqueous extract of *H. edulis*. Cytotoxic activity of the saponin triterpene glycosides is a result of their ability to form complexes with membrane sterols that lead to formation of ion channels and pores. This disturbs the cellular osmosis and in consequence damages the cellular membrane [5].

The organic extracts of both sea cucumber species, which contained water non-soluble compounds, exhibited potent cytotoxic effects against the two cancer cell lines. The organic extracts in this study might be rich with sphignoid bases which were isolated previously from sea cucumber species and showed cytotoxic properties [22]. In addition, Yang and his colleagues isolated branched-chain fatty acid, 12-methyltetradecanoic acid, from sea cucumber extract and demonstrated its anti-proliferative effect against prostate cancer cells (PC3) via induction of apoptosis [25]. Furthermore, the organic extracts might contain additional amounts of saponins which could be extracted efficiently by a polar organic solvent such as methanol.

In conclusion, this study revealed that the two Malaysian sea cucumber species, *Holothuria edulis* and *Stichopus horrens*, contain promising levels of natural antioxidant and cytotoxic compounds being more efficient as cytotoxic materials which may be used for cancer treatment. Antioxidant activity was demonstrated higher by the

water-soluble fractions, while the cytotoxic activity was found to be higher in the water-insoluble fractions indicating that the two activities were exhibited by different components and independent from each other. Isolation and identification of the active compounds from these extracts are recommended.

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