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Investigation of antioxidant effects of the viscera extract of the marine cucumber Holothurian lessons

Abstract

Oceans are the origin of life and the source of various natural compounds incorporated in organisms. Bioactive compounds as sources of food and medicine have been isolated from diverse animal groups such as Corals (Cnidaria), Crabs, Tunics, Echinodermata, Bryozoans, Fish, sponges, jellyfish, and many other sea creatures. Sea cucumbers are one of the taxonomies of Echinodermata. These animals belong to the subphylum Espinoza, have leathery bodies, and live mainly on the seabed. Naming these animals is due to their cucumber shape. Free radicals are molecules with unpaired electrons in the outer layer that are unstable and highly active. Accumulating these oxidant substances mainly activates oxygenation in the human body, causing oxidative damage to DNA and other cell components and Cancer. On the other hand, antioxidants neutralize biomolecules by giving one of their electrons to prevent or delay oxidation, so antioxidants are irrefutable to protect the body against diseases like Cancer. The primary goal of this study is to evaluate the antioxidant effects of the viscera extract of the sea cucumber Holothurian lessons.

Keywords: *Holothurian- Bioactive compounds- Sea cucumbers- Free radicals- oxidative damage- DNA- Cancer- antioxidants- taxonomies- extract*

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1. Introduction

Marine cucumbers related to the class Holothuroidea of the phylum Echinodermata are cylindrical invertebrates that live in various seabed habitats, from tropical waters to cold holes deep in the sea. In the sea ecosystem, sea cucumber serves as scavengers based on their contributions to recycling nutrients and breaking down particles (1). New scientific documents confirming their value as functional foods and nutraceuticals have attracted the attention of nutritionists, pharmacists, and the public. From a nutritional point of view, sea cucumber is an excellent tonic food and folk medicine, as it carries a high level of protein and a lower level of fat than other foods (2, 3). Its body wall, composed of insoluble collagen, has been used as a dietary supplement for hematogenesis (4, 5). Since sea cucumbers have different types, each with specific breeding and medicinal properties, it is necessary to know them accurately. Sea cucumber has long been used as conventional medicine to treat weakness, constipation due to intestinal dryness, asthma, hypertension, rheumatism, anemia, improving body immunity, and sinus congestion in East Asia. Some unique pharmacological and biological properties have been ascribed to components isolated from sea cucumbers, such as acid mucopolysaccharides, triterpene glycosides, glycolipids, sphenoid bases, polysaccharides, fucosylated chondroitin sulfate, cerebrosides, and phospholipids, among others (3, 6).

Sea cucumber is rich in chondroitin sulfate, a pain reliever for osteoarthritis. Fucoidan is a sulfated L-fucose poly-saccharide present in the extracellular matrix of sea cucumber (7). Its Saponins (soap-like compounds) and polysaccharides have anticancer properties, including inhibition of lung and galactophore cancers, and the Mucoitin in sea cucumber is used as an anti-aggregation of the platelet (8, 9). Saponins are amphipathic compounds with triterpene, steroidal, or aglyconic structures. These chemical compounds, as antibacterial, restrain the growth of bacteria, viruses, and many marine organisms, such as barnacles, on the surface of other creatures (6). Sea cucumber saponins as derivatives from triterpenoids have a variety of beneficial medicinal properties in the cardiovascular disease, immune system, cytotoxic effects, anti-asthma, anti-eczema, anti-inflammatory, anti-arthritis, antioxidant, anti-diabetic, antibacterial, anti-viral, anti- Cancer, anti-angiogenic, anti-fungal, cytostatic hemolytic, reduces blood cholesterol and sugar and anti-dementia and forgetfulness (10, 11). In addition, sea cucumbers have bioactivities, including chemical preservatives (Frondanol A5), sulfated polysaccharides for reproduction, antihypertensive hydrolyzed gelatin, and antioxidant effects (12-14). Marine invertebrates, especially tropical invertebrates, can protect themselves against oxidative stress caused by prolonged exposure to the sun's UV rays due to the antioxidants in their bodies and UV-friendly bacteria that live inside their tissues. As a result, these invertebrates (such as sea cucumbers) can be considered a potential source of antioxidants (11). Hydrolysate protein from sea cucumbers exhibits antioxidant activities depending on amino acid

composition, peptide hydrophobicity, and molecular weight (15). Previous studies indicated that Fucosylated chondroitin sulfate derived from two sea cucumber species (A. molpadioidea and H. nobilis) exhibited moderate antioxidant properties (16).

Antioxidants are critical free radical scavengers that reduce the risk of cardiovascular disease, stroke, and cancer progression and protect cell membranes (17). Free radicals are molecules or molecular components comprising one or more unpaired electrons in atomic or molecular orbitals, such as reactive oxygen species (ROS) and active nitrogen species (RNS) (18). Due to their aggressive nature and by affecting different molecules and structures of the body, free radicals impede the proper functioning of biological systems and cause diseases such as Alzheimer's, Cancer, aging, heart failure, rheumatoid arthritis, cardiovascular disease fibrocystic, HIV, and other disorders (19, 20). In vivo studies using different animal models confirmed that injuries healed with sea cucumber extracts were better and more quickly treated when compared to those without therapy (21).

Unstable, ROS act as destructive molecules on cell membranes, mitochondria, and DNA. Oxidants can react with other inorganic molecules such as oxygen, intermediate metals, and superoxides. The potency of nitric oxide to react with DNA structures like pyrimidine bases and prosthetic groups such as heme or proteins leading to S-nitrosylation of the thiol group has also been identified. Furthermore, S-nitrosylation of specific proteins can produce protein function or structure changes. Nitrogenation of tyrosine roots or disruption of iron sulfide clusters or complexes has also been demonstrated (22-24). Recent epidemiological studies show that natural antioxidants have therapeutic effects against various diseases caused by oxidative stress like Cancer (25).

The properties of natural antioxidants are generally attributed to certain bioproducts, such as phenol, salts, sugars, carotenoids, ascorbic acid, glutathione, peptides, and pigments (26). Antioxidant ability in ovarian fluid and body extracts of sea cucumber species has been shown in previous studies (27). Phenols are an essential group of natural compounds with antioxidant properties with aromatic rings and can bear one or more hydroxyl (28). The extraction method is very effective on the amount of phenol. Despite several worldwide studies showing the potency of some sea cucumber species as possible sources of anticancer and antioxidant compounds, there is a lack of information about levels of these activities in most Persian Gulf and the Oman sea cucumber species. As organic extracts may contain additional amounts of saponins, this study aimed to investigate the antioxidant effects of the sea cucumber Holothurian lessons viscera extract and the presence of triterpene saponin compounds with potent antioxidant properties.

2. Methods

2.1. Chemicals

Ethanol, methanol, and Butylated hydroxytoluene (BHT) from Merck, free radical 2, 2 diphenyl 1-picrylhydrazyl (DPPH) from Sigma (St. Louis, MO, USA).

2.2. Sample Collection and Identification

Sea cucumber specimens were collected by the Persian Gulf and Oman Sea Ecology Research Institute from the north of Hengam Island, located in the Oman Sea with geographical position ″ 55′54 ° 55 - ″ 40′54 ° 55 east and ″ 15′41 ° 26 - ″ 26 ′ 36 ° 26'N at a depth of 15 to 20 meters (Figure 1A-B) in August and September 2017, and transferred to large containers containing seawater and sufficient aeration, live to the research institute.

Correctness of the genus and species of collected sea cucumber was determined using a 1 mm thick and 1 cm square piece of epidermal tissue removed with a surgical razor and stored in a tube containing 3 ml of bleach. A white deposit was collected after 20 minutes at the end of the tube. A drop of white residue was spread on a slide and observed with a 10x and 40x lens microscope. Then, the ossicles were compared with the FAO identification key for Holothuria lessoni (29).

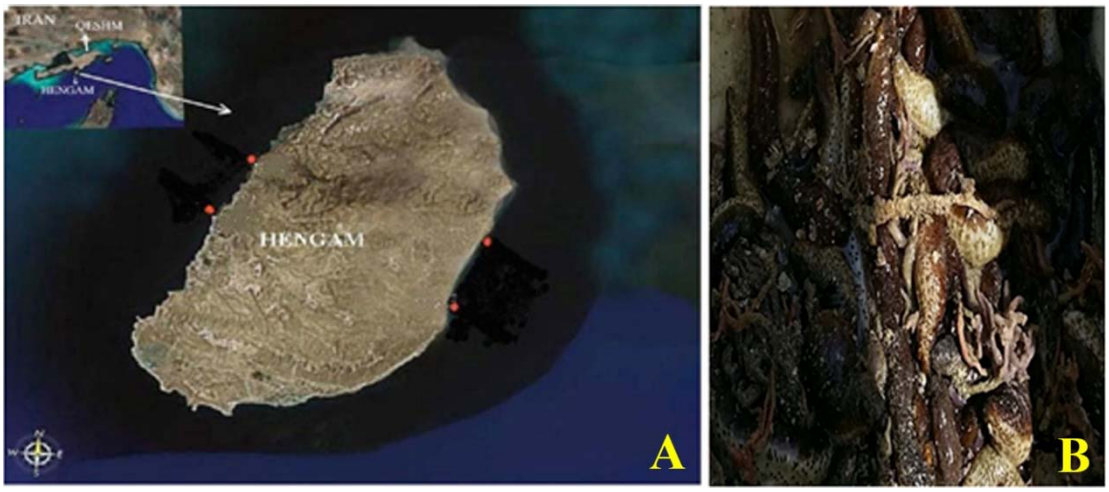

Figure 1. A: Sampling location marked with a red mark, B: H.lessoni sea cucumber specimen caught.

2.3. Preparation of extract

First, sea cucumber samples were cut longitudinally, the viscera and gonads were removed, and the body muscle was cut into 1 cm pieces (Figure 2-A). The samples (body muscles and gonads) were freezer-dried for 48 hours (Figure 2-B). The dried specimens were pulverized by a mill and placed in a -20 ° C freezer (Figure 2-C); the extraction was performed using the following soaking method. Powders were mixed at a weight/volume ratio of 5 g per 100 ml of ethanol solvent and placed at room temperature for 24 h. After 24 hours, the mixture was passed across a strainer to separate the particles. The obtained solution was stored in the refrigerator. Again, powder residue was soaked in equal proportions of ethanol at room temperature for 24 hours. Then, the soluble and solid phases were separated using a filter, and the soluble phase was kept in the refrigerator. Powder residues were extracted using the previous method for the third time. Ultimately, the solutions obtained from the previous steps were mixed, concentrated, and dried under a chemical hood at room temperature. The analogous steps were performed separately using methanol. The dried ethanoic and methanolic extracts were dissolved in ethanol and methanol, respectively, and then the antioxidant activity of the samples was evaluated as follows.

Figure 2. A: Prepared pieces of sea cucumber, B: Placed sea cucumber samples in the freeze-dryer, C: sea cucumber powder after freezer-drying and grinding.

2.4. Examination of antioxidant effects

2.4.1. Preparation of DPPH, BHT, negative control, and extract solutions

DPPH-methanol and BHT-ethanol solutions with a concentration of 0.004% and 0.005% were prepared daily and placed in a dark and cold environment before use. Also, dilutions (10, 20,40,60,80,100 µg / ml) were prepared using BHT solution as the main stock according to the formula C1V1 = C2V2. A mixture of methanol (1 ml) and DPPH (3 ml) solution was used as a negative control. It is noticeable that a negative control solution was also tested daily in addition to each BHT sample. In order to prepare, the initial stock of the extracted solution (with a concentration of 1000 µg / ml), 0.05 g of dry ethanolic extract, and the ethanol solvent were reached at a volume of 50 ml. Other dilutions (1000, 750, 500, 250, 125, 50, µg / ml) were prepared using this solution as the leading stock and according to C1V1 = C2V2. The above method was performed for dried methanol extract in the same order and concentrations using methanol solvent. All steps were performed with at least three repetitions.

2.4.2. Antioxidant properties of extracts using DPPH

The antioxidant content of the extracts was evaluated by the radical scavenging measurement method using 2-2-diphenyl-1-picrylhydrazyl (DPPH). This method is mainly based on electron transfer, and the most crucial factor in the antioxidant reaction with free radicals is spatial access. The advantages of this speedy method are no dependence on the polarity of the substrate, the ability to react in hydrophilic and lipophilic media, and easy access to the required equipment (30). The antioxidant potential of the extracts was estimated using DPPH free radicals and the IC50 index (concentration of the extract capable of inhibiting 50% of free radicals) for each of the methanolic and ethanolic extracts and compared with a synthetic antioxidant as standard.

Samples included:

- a positive control (BHT),
- negative control (1 ml of solvent (ethanol or methanol) with 3 ml of DPPH solution),
- blank (1 ml of diluted extracts with 3 ml of solvent (ethanol/methanol) and
- Extract (1 ml of the ethanolic or methanolic extract with 3 ml of DPPH solution).

The absorbance of each replicate of the samples was measured at 517 nm using a spectrophotometer. After calculating the mean, the percentage of free radical scavenging of DPPH was determined using the following formula. Wherein: ODC Average absorbance of control solution, ODS Average absorbance of extract solution, ODB Average absorbance of Blank.

DPPH scavenging effect %= [ODC (ODS-ODB) / ODC] * 100

3. Results

The findings of this study authenticate a wide range of new and unique saponins with high structural diversity of both sulfated and non-sulfated saponins and different sugar and aglycone poles in the viscera compared to the sea cucumber's body wall.

In addition, the high bio-structural diversity of these metabolites enables them to have significant functional variety. The number of saponins ranged from 460 to 1600 Da. We have recognized more than ten aglycone structures in this species. Our results highlight a more significant number of novel saponins in the viscera than in the body wall, showing the viscera as an important source of these compounds. This paper reports the presence of several novel saponins in the viscera of Holothuria lessons. It shows the highest number of saponin congeners identified in the viscera of any sea cucumber species. The extensive content of saponins in Holothurian lessons may increase its antioxidant properties. Sea cucumber's triterpene glycosides (saponins) can be attributed to compounds with the chemical structure of glucocorticoids, chondroitin sulfate, glucose aminoglycans, and sulfated polysaccharides glycoproteins, glycosphingolipids, and fatty acids.

Evaluation of antioxidant activity was determined according to the suggested protocol based on changes in adsorption at 517 nm (maximum wavelength). Antioxidant activity was compared with BHT as a positive control. According to the previous studies' results, the best volume ratio of the sample to the DPPH was determined to be 1 3. Extract antioxidant properties change DPPH's color from purple to yellow (Figure 3).

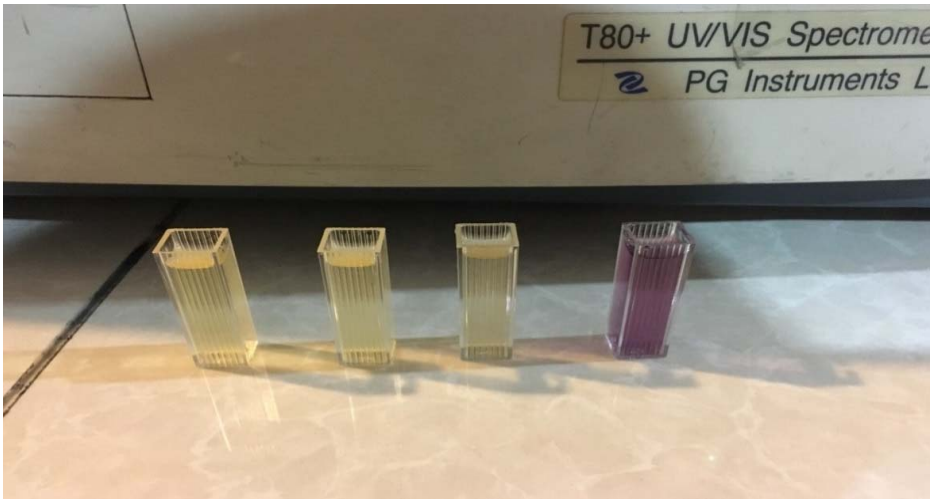

Figure 3. Color changes in the positive control and no change in the negative control.

To evaluate the amount of positive control adsorption, the absorbance of BHT was studied (Table 1). The absorbance rate of ethanolic samples (50 to 4000 µg/ml) was assessed. Also, the absorbance of the methanolic specimens in the concentration of 50 to 4000 µg/ml was determined (Table 2). The DPPH scavenging effect of different concentrations of Holothuria lessons ethanolic and methanolic extracts is summarized in Table 2. The scavenging effect of ethanolic extract at all concentrations was significantly higher than H.lessoni methanolic extract ($P<0.05$), indicating more antioxidant properties of ethanolic extract (Table 2). However, the scavenging effect of BHT samples (positive control) was substantially greater than both ethanolic and methanolic extracts of H.lessoni, indicating the poor antioxidant capacity of the studied extracts (Fig 4).

Table 1. Scavenging effect (%) of BHT with different concentration on (DPPH) radical

Concentration (µg/ml)	**Absorption (mean ± SD)**	**Scavenging effect (%) (mean ± SD)**
100	0.5517 ± 0.025	60.60 ± 2.74
80	0.5878 ± 0.030	55.98 ± 2.86
60	0.5613 ± 0.055	51.66 ± 5.06
40	0.6390 ± 0.048	47.50 ± 3.56
20	0.6613 ± 0.015	45.00 ± 1.02
10	0.8266 ± 0.025	31.66 ± 0.95

Table 2. Scavenging effect (%) of ethanolic and methanolic H.lessoni extracts on (DPPH) radical

Concentration (µg/ml)	Ethanol extract		Methanol extract		P*
	Absorption (mean ± SD)	Scavenging effect (%) (mean ± SD)	Absorption (mean ± SD)	Scavenging effect (%) (mean ± SD)	
4000	0.828 ± 0.038	24.72 ± 1.13	0.829 ± 0.039	30.91 ± 1.45	0.003
3000	0.835 ± 0.039	24.09 ± 1.12	0.835 ± 0.037	30.41 ± 1.34	0.003
2000	0.849 ± 0.061	22.81 ± 1.63	0.849 ± 0.061	29.25 ± 2.10	0.013
1000	0.872 ± 0.022	20.72 ± 0.52	0.875 ± 0.022	27.08 ± 0.68	<0.001
750	0.996 ± 0.015	10.00 ± 0.15	1.003 ± 0.005	16.00 ± 0.08	<0.001
500	1.013 ± 0.015	8.18 ± 0.12	1.012 ± 0.001	15.80 ± 0.01	<0.001
250	1.038 ± 0.044	6.36 ± 0.27	1.061 ± 0.023	11.50 ± 0.24	<0.001
125	1.063 ± 0.046	3.63 ± 0.15	1.125 ± 0.021	6.66 ± 0.12	<0.001
50	1.086 ± 0.032	1.81 ± 0.05	1.146 ± 0.029	5.00 ± 0.12	<0.001

* Scavenging effect values were used for statistical analysis

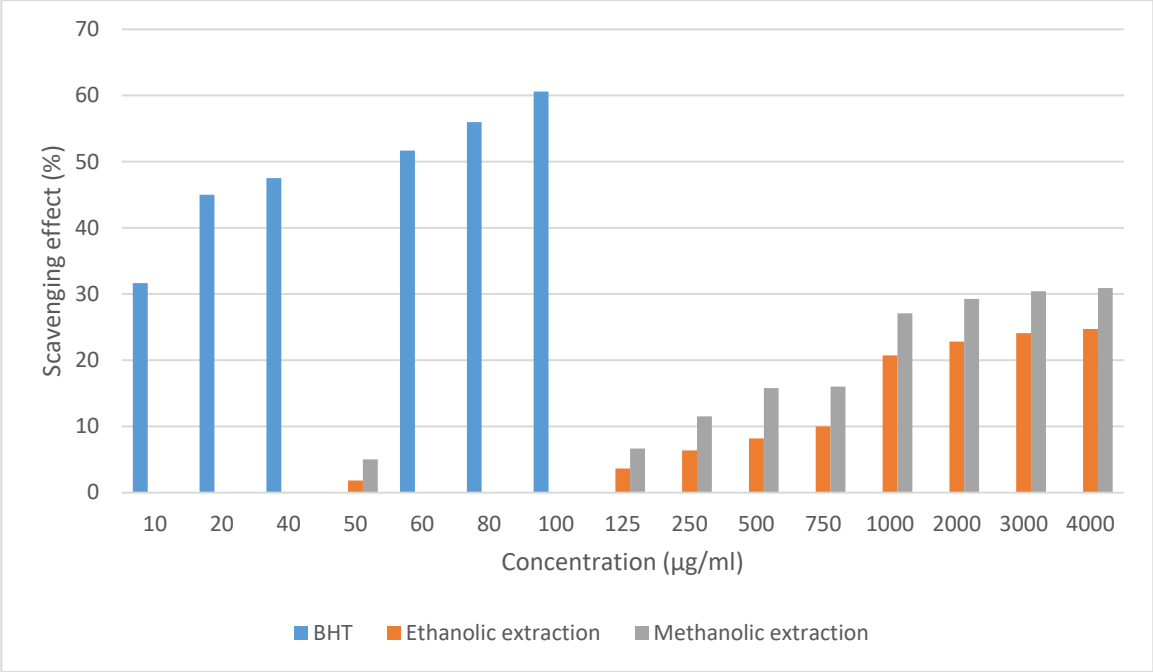

Figure 4. Comparison of the Scavenging effect of the studied samples in different concentrations on (DPPH) radical

4. Result and Discussion

Several studies are interested in sea cucumbers because of their great potential in producing bioactive natural reagents, especially antioxidant agents (31). Therefore, free radicals are molecules with unpaired electrons in their outer layer and are highly unstable and reactive. Accumulation of specific oxidizing agents, primarily reactive oxygen species (ROS) derived from normal essential metabolic processes in the body, causes the destruction of DNA and other cell organs, which initiates Cancer (32, 33). In contrast, antioxidants prevent free radicals from oxidizing biomolecules by giving them one of their electrons. Marine organisms are rich in cytotoxic compounds with anticancer properties; therefore, studying the biological properties of sea cucumber species to obtain precursors of natural drugs is beneficial.

Althunibat et al. Reported significant antioxidant properties for organic and aqueous extracts of Holothuria edulis lesson and Stichopus horrens Selenka, two sea cucumbers obtained off the coast of Malaysia (34). However, not all sea cucumbers have favorable antioxidant properties. A study in Turkey showed that the inhibition of free radicals using methanol and aqueous extracts of Holothuria tubulosa is significantly less than vitamin C and BHT (35). Saponins are substances with proven antioxidant properties. Bahrami et al.(36) reported that the amount of saponins in H.lessoni viscera was higher than in its body wall. This led us to extract and evaluate the viscera and body wall in the present study.

A recent study of 5 sea cucumbers in Indonesian waters reported an acceptable antioxidant property for H.lessoni extract with a scavenging effect of 63.81% at a concentration of 250 µg/ml (37). In the present study, at the highest concentration (4000 µg/ml), H.lessoni extract inhibited 30.91% of free radicals, less than half of the antioxidant capacity reported in the mentioned study with lower extract concentrations. This difference may be due to differences in the preparation of extracts. In the present study, the body wall was prepared for extraction along with the viscera, while in the Indonesian study, only the body wall of H.lessoni was used for extraction.

Various studies have reported the antioxidant activity of many metabolites extracted from marine invertebrates. For example, in a study, Motohashi et al. recognized two peptides with high antioxidant activity called JBIR-34 and JBIR-35 from sponge-related actinobacteria (38). In this regard, Poongodi et al. reported the IC50 level of antioxidant activity of actinobacterium extracted metabolite called Nocardiopsis sp. isolated from Manna Bay sediments equal to 2.58 µg/ml (39). In another study by Gozari et al., the antioxidant activity of metabolites extracted from a new actinobacteria strain called Streptomyces sp. strain SC 156 was reported to inhibit DPPH free radicals at 2.211 µg/ml (40). Consequently, the present study evaluated the antioxidant properties of the sea cucumber species Holothuria lessoni. The evaluation of the methanolic extracted metabolites' antioxidant activity showed more DPPH free radicals inhibiting than extracted metabolites. Also, based on the obtained pattern of antioxidant activity, the presence of actinobacteria as an active part of the microbiota related to the species of Holucuria lessons in the Persian Gulf was confirmed. Antioxidant compounds produced by the obtained isolates can be further identified and studied in future research. In general, contrary to expectations and previous studies' results, this species' weak antioxidant properties were obtained. In contrast, the positive control sample BHT results were favorable, indicating the validity of the raw materials, reagents, and experimental protocol. These unexpected findings can be attributed to a variety of reasons, such as differences in the saponin content of H.lessoni in the Australian and Oman Seas, the age of the samples, the negative effect of some organs such as gonads on the antioxidant activity of the extract and the time interval between sea cucumber fishing and analysis.

5. Conclusion

As mentioned, in this research, the antioxidant effect of the sea cucumber species of the Oman Sea from the Holothuridea family, including H. lessoni, was investigated in different concentrations. DPPH antioxidant test The results of the antioxidant effect test of the methanolic extract of sea cucumber organs, including: body wall, digestive tube, gonad, respiratory tree, coelomic fluid, and coelomic tube, were studied for H.lesson species.

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