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Biological Activities of the Sea Cucumber *Holothuria leucospilota*

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ABSTRACT

Sea cucumbers are potentially presented as the marine sources of antimicrobial and cytotoxic compounds. In this study, we focused on the sea cucumber, *Holothuria leucospilota* which has not been evaluated until now for antibacterial, antifungal and toxicity of its main organs. Ethyl acetate, methanol and water-methanol extracts were prepared from cuvierian organ, coelomic fluid and body wall of *H. leucospilota* collected from Persian Gulf. Extracts were evaluated for their antibacterial and antifungal activities against *Aspergillus niger*, *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. The activity was determined using the disc diffusion test. Cytotoxic activities of the extracts were assessed by *Artemia salina* larvae. Results showed that methanol extract of cuvierian organ (Minimum inhibitory concentration (MIC/disc) <1 mg against *A. niger*) and water-methanol extract of coelomic fluid (MIC/disc = 1 mg against *C. albicans*) showed significant antifungal activities but no inhibitory effect of the extracts against bacteria was observed. MIC for water-methanol extract of coelomic fluid was 3 mg against both *C. albicans* and *A. niger*. Significant inhibitory effect was observed in cytotoxic assays which was highest in body wall methanol extract (LC_{50} = 0.4 μg mL^{-1}).

Key words: Antibacterial, antifungal, cytotoxic, *Holothuria leucospilota*

INTRODUCTION

Marine biotechnology is the science in which marine organisms are used in full or partially to make or modify products, to improve plants or animals or to develop microorganisms for specific uses (Jha and Zi-rong, 2004). In recent years, many bioactive compounds have been extracted from various marine animals. The search for new metabolites from marine organisms has resulted in the isolation of more or less 10,000 metabolites. Many of which are endowed with pharmacodynamic properties. Marine natural products represented a potential large resource of new active compounds could have antibacterial activity (Faulkner, 1993; Wainwright, 1996). Bioactive compounds have been isolated from a number of groups, including corals (Jensen *et al.*, 1996; Koh, 1997), crabs (Chattapadhyay *et al.*, 1996), tunicates (Findlay and Smith, 1995), bryozoans (Laycock *et al.*, 1986), echinoderms (Bryan *et al.*, 1992), fish (Cole *et al.*, 1997) and sponges (Fusetani, 1996). Sea cucumbers are holothurian belonging to the phylum Echinodermata, class Holothuroidea. There are about 1,200 holothurian species in the world (McElroy, 1990). Among marine organisms, sea cucumbers are a large and diverse group of organisms from which a wide range of secondary metabolites have been isolated. A number of these compounds possess biological activity such as toxicity, antibacterial, antifungal, antiviral, anti-tumor and other specific activities (Bryan *et al.*, 1992; Villasin and Pomory, 2000). Sea cucumbers are well known to exert beneficial effects on human health. These echinoderms are used in Asian traditional medicine to maintain fitness during long fishing travels or to prevent, reduce or cure several ailments like constipation, renal deficiency or arthritis. Several papers published in the last two decades came in support to these medicinal purposes showing multiple biological activities of sea cucumber extracts as wound healing promoter and exhibiting antimicrobial, anticancer and immunomodulatory properties (Fredalina *et al.*, 1999; Aminin *et al.*, 2001). Their antioxidant properties have been recently reported from coelomic fluid of three species (*Bohadschia marmorata vitiensis, Stichopus variegatus, Stichopus badionotus*) (Hawa *et al.*, 1999). Research on the medicinal properties of sea cucumbers is restricted because studies were only limited to their physiological and ecological aspects (Bakus, 1973; Nitisewojo *et al.*, 1993) and also due to interest in other marine resources, especially the algae (Fenical *et al.*, 1973; Hashimoto, 1997). Heding (1940) recorded 17 species of holothurians found in the waters around Iran. Among commercial species *Holothuria leucospilota* has a low value (Toral-Granda, 2006). The objective of this study was to determine the cytotoxic, antibacterial and antifungal activities of the body wall, cuvierian organ and coelomic fluid of the *H. leucospilota,* a species found along the north coastal of Persian Gulf, Iran.

MATERIALS AND METHODS

Sample collection: The sea cucumber *H. leucospilota* were collected from the Persian Gulf, around the sandy shore of the Bostaneh, Iran in low tide time, in June 2009. Identification of the species was based on the studies of Heding (1940). The collected samples were cleaned by rinsing with seawater and distilled water and transported in cool box to the laboratory where, the cuvierian organ were removed; then the cuvierian organ, coelomic fluid and body wall were recuperated in separated labeled plastic bags and kept frozen at -20°C until extraction.

Extractions of the samples: The samples of cuvierian organ, coelomic fluid and body wall were defrosted before use. The coelomic fluid recuperated was homogenized with stirring using the magnetic stirrer for 15 min and filtered using some cotton wool followed by passage through a Whatman filter paper, after centrifugation (15 min, 30,000 xg, 4°C). The body wall recuperated was cut into small pieces (about 2 cm). Cuvierian organ and the body wall samples were homogenized using a blender and suspended followed by extraction with ethyl acetate, methanol and water-methanol (50%) successively by percolation (72 h for each solvent) at room temperature.

After filtration and centrifugation (15 min, 30,000 xg, 4°C), extracts were evaporated under vacuum at 45°C by a rotary evaporator. The powdered extracts of each sample were obtained by freeze dryer and stored at -20°C (Mamelona *et al.*, 2007).

Antibacterial and antifungal assay: The antibacterial and antifungal activities of the *H. leucospilota* extracts were assessed against *Staphylococcus aureus* (ATCC 29737), *Pseudomonas aeruginosa* (ATCC), *Escherichia coli* (ATCC 8739), *Candida albicans* (ATCC 14053) and *Aspergillus niger* (ATCC 16404) by the Disc Diffusion Susceptibility method (Gohari *et al.*, 2010).

The extracts were tested in the lowest concentration at which no growth was observed, recorded as Minimum Inhibitory Concentration (MIC). Culture media with different concentrations of Gentamycin and Fluconazole were used as positive controls. Antibacterial and antifungal assays were performed in triplicates.

Brine Shrimp Lethality Assay (BSA): Cytotoxic activities of the *H. leucospilota* extracts were assessed by *Artemia salina* according to modified Mongelli method described by Saeidnia *et al.* (2009). Brine shrimp (*Artemia salina*) eggs were hatched in flask containing 300 mL artificial seawater made by dissolving distilled water in 29-30°C temperature and aerate condition. Different concentrations of each extract dissolved in normal saline were obtained by serial dilution. Four concentrations of each extract were prepared with 10, 100, 500 and 1000 μg mL^{-1}. Ten to twenty nauplii were added to each concentration of the extracts in 24 well chamber slides. Number of nauplii alive noted after 24 h. The mortality end point of the bioassay was determined as the absence of controlled forward motion during 30 sec of observation. Seawater and berberine hydrochloride (LC_{50} = 26 μg mL^{-1}) were used as controls. Lethality percentage was determined and LC_{50} calculated based on Probit Analysis with 95% of confidence interval (Saeidnia *et al.*, 2009).

RESULTS

Results of antibacterial and antifungal assay: Three extracts from cuvierian organ, coelomic fluid and body wall of *H. leucospilota* were tested against three types of bacteria and one filamentous fungi and one type of yeast. All concentrations of three extracts from cuvierian organ, coelomic fluid and body wall were not showed antibacterial activity against *S. aureus*, *P. aeruginosa* and *E. coli* and no inhibition zone observed for these tests. The results of the other screening test are summarized in Table 1.

Results of cytotoxic assay: Such as results of antibacterial and antifungal tests for ethyl acetate extracts, no inhibitory effects of the ethyl acetate extracts were observed in Cytotoxic assays on *H. leucospilota*. However, powerful inhibitory effects in some tests were observed. Results of BSA assays are summarized in Table 2. Cytotoxic activity of the extracts is ordered below (up to down): Body wall methanolic > cuvierian organ methanolic > Body wall water-methanohc > Coelomic fluid methanolic.

DISCUSSION

Although there was considerable antifungal and cytotoxic activity for some extracts of the isolated organs of *H. leucospilota*, no antibacterial activity of these extracts was observed. As shown in Table 1, methanolic extract of cuvierian organ and water- methanol extract of coelomic fluid indicated antifungal activity against *A. niger* and *C. albicans* and also this effect was higher on *A. niger*. All the body wall extracts (consist of ethyl acetate, methanol and water-methanol) showed no antifungal activity. Dabbagh *et al.* (2011) presented the first report of successful *H. leucospilota* larval development in Iran (Dabbagh *et al.*, 2011), so results of this study can be a potential application for wide culture of this species.

Numerous chemical and pharmacological studies carried out on several species of sea cucumbers indicated that these invertebrate contain triterpene glycoside with antifungal, antibacterial and cytotoxic properties. Other species of sea cucumber have been examined for antibacterial activities

Table 1: Selected antifungal activity of the effective extracts of *H. leucospilota*

Fungi	Extracts	MIC/disc (mg)	8 mg	4 mg	2 mg	1 mg
A. niger	Cuverian organ (methanol)	<1	20	16	13	10
A. niger	Coelomic fluid (water-methanol)	3	22	8	0	0
C. albicans	Cuverian organ (methanol)	1	13	10	7	0
C. albicans	Coelomic fluid (water-methanol)	3	9	7	0	0

Results are presented by the diameter of the inhibition zones (mm)

but the results were different. Kuznetsova *et al.* (1982) reported the evaluation of *H. atra, H. scabra* and Bohadshia argus against seven species of bacteria and found that lipid and methanolic extracts had no inhibitory activity, while a phosphate buffered saline extract showed inhibitory activity Another study revealed that the extract of Parastichopus parvimensis did not inhibit bacteria compaired to Tetracycline and Ampicillin (Villasin and Pomory, 2000). T-antigen binding lectin with antibacterial activity from *H. scabra* showed strong broad spectrum antibacterial activity against both gram-positive and gram-negative bacteria (Gowda *et al.*, 2008). In addition, antibacterial activity of the extracts from the body wall of *P. parvimensis* was showed (Villasin and Pomory, 2000). Farouk *et al.* (2007) reported new bacterial species isolated from Malaysian sea cucumbers with optimized secreted antibacterial activity (Farouk *et al.*, 2007). Anyhow, antibacterial activity was reported in *Strongylocentrotus droebachiensis* (Echinoidea), *Cucumaria frondosa* (Holothuroidea) and *Asterias rubens* (Asteroidea) (Haug *et al.*, 2002). In the present study antibacterial activity of *H. leucospilota* (against *S. aureus, P. aeruginosa* and *E. coli*) was not observed and this is in agreement with the antibacterial result of a report on *P. parvimensis* (Villasin and Pomory, 2000).

Batrakov *et al.* (1980) have isolated a complex of antifungal triterpene glycosides (from the skin muscular sac of the sea cucumber, *C. japonica*) which inhibited the growth of *C. albicans* and *C. tropicalis* in concentration of 60 mg mL^{-1}. The fractionation of this complex resulted in isolation of its two main components, cucumariosides I and II (Batrakov *et al.*, 1980). Ismail *et al.* (2008) studied on antifungal activity of aqueous and methanolic extracts from the Mediterranean sea cucumber, (*H. polii*). They reported that both aqueous and methanolic extracts were found to produce a significant antifungal activity (Ismail *et al.*, 2008). Other study reported that twenty-seven species of marine filamentous fungi were isolated from all organs of Holothurians collected from the Sea of Japan (Pivkin, 2000). These fungi isolated from the holothurian surface were more diverse and abundant than those from internal organs and coelomic fluids (Ismail *et al.*, 2008).

Triterpene glycosides are the predominant secondary metabolites of the sea cucumber, exhibiting wide spectra of biological activities, such as antifungal, cytotoxic, hemolytic, cytostatic and immunomodulatory activities (Chludil *et al.*, 2002). Yuan *et al.* (2008) reported the antifungal activities of two triterpene glycosides isolated from the sea cucumber *H. axiloga* against three strains, *C. albicans, Cryptococcus neoformans* and *A. fumigates*.

In the other study antifungal activity of *Actinopyga lecanora* was studied and reported the bioassay-guided fractionation of methanol extract of *A. lecanora* which led to the isolation of a new triterpene glycoside (Kumar *et al.*, 2007).

More studies were carried out on cytotoxic effect of the sea cucumbers too. Two triterpene glycoside isolated from the sea cucumber *Pseudocolochirus violaceus* exhibited significant cytotoxicity against cancer cell lines MKN-45 and HCT-116 (Zhang *et al.*, 2007). Three new cytotoxic triterpene glycosides were reported from the sea cucumber *Mensamaria intercedens* by

Table 2: Brine shrimp cytotoxicity of the extracts of *H. leucospilota*

		Concentrations (μg mL^{-1})				
Organs	Extracts	1000	500	100	10	LC_{50}
Cuverian organ	Ethyl acetate	14*	14	15	15	>1000
	Methanol	0	1	4	5	3
	Water-methanol	14	14	14	15	>1000
Body wall	Ethyl acetate	14	15	15	15	>1000
	Methanol	1	1	1	4	0.4
	Water-methanol	1	2	3	8	9
Coelomic fluid	Ethyl acetate	14	15	15	15	>1000
	Methanol	0	3	8	10	44
	Water-methanol	15	14	14	14	>1000

* Number of live larvae

Zou *et al.* (2005). Present results showed higher cytotoxic effect from methanol extracts with LC_{50} values about 0.4 μg mL^{-1} for the body wall, 3 μg mL^{-1} for cuvierian organ and 44 μg mL^{-1} for coelomic fluid (Table 2). It seems that further studies need to prove the anticancer or anti-tumor effects.

CONCLUSION

In the current study only methanol and water-methanol extracts of cuvierian organ and coelomic fluid exhibited a significant antifungal activity. This indicates that the active compound(s) which are responsible at least in part, for the antifungal activity of both extracts from sea cucumber, *H. leucospilota* is locate in cuvierian organ and coelomic fluid. Considering this and previous studies, the active fractions should be considered for further studies in order to the isolation and determination of the chemical structures of antifungal or cytotoxic compounds. Authors concluded that sea cucumbers might be in the future an appropriate source of antifungal and cytotoxic natural compounds. This benthic organism deserves much more interest in marine natural products as its antifungal and cytotoxic properties. Its potential application in nutraceutical and medicinal products needs to be studied.

In conclusion, results obtained from the present study suggest that the sea cucumber (*H. leucospilota*) may be an interesting source of antifungal and cytotoxic compounds. *H. leucospilota* could be a lead source in the development of the potent antifungal and cytotoxic drugs.

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