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# Antibacterial, antifungal and cytotoxic effects of a sea cucumber *Holothuria leucospilota*, from the north coast of the Persian Gulf

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*Bioactive compounds of gonad, respiration tree, cuvierian organ, and body wall of the sea cucumber* Holothuria leucospilota *collected from the north coast of the Persian Gulf were extracted using ethyl acetate, methanol and water–methanol solvents. 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 determined by brine shrimp lethality assay. Results demonstrated that the* A. niger *was shown to be the most sensitive microorganism followed by* C. albicans. *The inhibition zone against* A. niger *and* C. albicans *had minimum inhibitory concentrations ranging from 3 to 7 μg/ml. The highest antifungal activity was found in G (water–methanol) with an inhibition zone of 50 mm against* A. niger *at 18 μg/ml extract concentration continuing with CT (methanol) with 46 mm inhibition zone against* A. niger *at 18 μg/ml extract concentration. The highest cytotoxic effect of methanol extract was found with $LC_{50}$ values of about 40.31 μg/ml in the gonad organ from* H. leucospilota *continuing with the respiration tree organ with $LC_{50}$ values of about 72.49 μg/ml.*

**Keywords:** antibacterial, antifungal, cytotoxic, *Holothuria leucospilota*, Persian Gulf

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## INTRODUCTION

Sea cucumbers belong to the phylum Echinodermata, meaning that; they are spiny-skinned, under the class Holothuridea (Ridzwan, 2007). The name holothuroid was given by the Greek philosopher, Aristotle (384 BC–322 BC) (*holos*: whole and *thurios*: rushing). The scientific name *Cucumis marimus* which means 'sea cucumber' was coined by the Roman natural philosopher, Pliny the Elder (23 AD–25 August 79 AD; among many of his talents, he was an invertebrate taxonomist) (Ridzwan, 2007). Although there are many cultured and harvestable sea cucumber species, only around 20 species are reported with relatively high economic and food value. A multitude of harvestable sea cucumbers species have been exploited in response to growing global demand due to their food and pharmaceutical uses (Mehmet *et al.*, 2011). Sea cucumbers, commonly known as trepang, beche-de-mer, or gamat, have long been utilized in the food and folk medicine systems of Asia and Middle East communities (Yaacob *et al.*, 1997; Huizeng, 2001).

During the past three to four decades many efforts have been devoted to isolating numerous biologically active novel compounds from marine sources. Many of such naturally occurring compounds are of great interest for potential drug development as well as an ingredient of new leads and commercially successful products for various industrial applications, especially, pharmaceuticals, agrochemicals, functional foods and nutraceuticals (Venugopal *et al.*, 2009). Sea cucumbers are one of the potential marine animals with high food and medicinal value. The medicinal properties of these animals are ascribed to the presence of functional components with promising multiple biological activities (Bordbar *et al.*, 2011).

Sea cucumbers have been well recognized as a tonic and traditional remedy in Chinese and Malaysian literature for their effectiveness against hypertension, asthma, rheumatism, cuts and burns, impotence and constipation (Wen *et al.*, 2010). These medicinal benefits and health functions of sea cucumbers can be attributed to the presence of appreciable amounts of bioactive compounds, especially the triterpene glycosides (saponins), chondroitin sulphates, glycosaminoglycan, sulphated polysaccharides, sterols (glycosides and sulphates), phenolics, peptides, cerberosides and lections (Bordbar *et al.*, 2011).

*Holothuria leucospilota* usually lives in quiet and deep areas on the sandy bottom or on coral rubble. It is observed often under the rock from which alone exceeds the front. It is supposed that *H. leucospilota* is the dominant species in the Persian Gulf (Afkhami *et al.*, 2012). Among commercial

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species *Holothuria leucospilota* has a low value (Toral-Granda, 2006). The first report of successful *H. leucospilota* larval development in Iran was conducted by Dabbagh *et al.* (2011).

In this report, we describe the screening of antibacterial, antifungal and cytotoxic activities of *H. leucospilota*, a sea cucumber species collected from the coastline of the Persian Gulf against five human pathogenic microorganisms and using the brine shrimp lethality assay.

# MATERIALS AND METHODS

## Sample collection

Sea cucumber samples were caught near Hamoon jetty on the north coast of Qeshm Island (Persian Gulf) in May 2011 by SCUBA diving in depths of 5–12 m. The samples were dissected to remove internal organs, and packed immediately with ice prior to sending to the laboratory and kept at −20°C until extracted. The taxonomic identity of the samples was confirmed by Dr Gustave Pauly, the chairman of the Invertebrate Department of Florida University.

## Extraction of the samples and isolation

Bioactive compounds were extracted as a function of their polarity using water and organic solvents according to the method of Mamelona *et al.* (2007). The samples of gonad (G), respiration tree (RT), cuvierian organ (CO) and body wall (BW) were defrosted before use. The recuperated species was cut into small pieces. The samples were homogenized using a blender and suspended followed by extraction with ethyl acetate, methanol and water–methanol (50%) successively by percolation (72 hours for each solvent) at room temperature. After filtration and centrifugation (15 minutes, 30,000 × g, 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.

## Assay of cytotoxicity effect

Cytotoxic activity of extracts was determined by the brine shrimp lethality assay (BSA) as described by Meyer *et al.* (1982). A simple zoological organism (*Artemia saline*) was used as a convenient monitor for the screening. The cysts of the brine shrimp were hatched in artificial seawater (3.8% NaCl solution) for 48 hours to mature into shrimp called nauplii. 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. Twenty nauplii were added to each concentration of the extracts in 24 well chamber slides. The number of nauplii alive after 24 hours was noted. The mortality end point of the bioassay was determined as the absence of controlled forward motion during 30 seconds of observation. Seawater and berberine hydrochloride ($LC_{50}$ = 26 μg/ml) were used as controls. Lethality percentage was determined and $LC_{50}$ calculated based on probit analysis with 95% confidence interval using the computer software 'BioStat-2007'.

## Antibacterial and antifungal assay

The antibacterial and antifungal activities of the *H. leucospilota* extracts were assessed against *Escherichia coli* (ATCC 1763), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 25853), *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404) by the disc diffusion susceptibility method triplicates. Minimum inhibitory concentrations (MIC) of the extracts were tested in the lowest concentration at which no growth was observed. Gentamycin, fluconazole and ciprofloxacin were used as positive controls (Mokhlesi *et al.*, 2011).

# RESULTS

Rather powerful cytotoxic effects in some tests were observed in water–methanol, ethyl acetate and methanol extracts. Based on this the higher cytotoxic activity was for gonad methalonic which continue with respiration tree methanolic and body wall methanolic extractions (Table 1). The highest cytotoxic effect was found in methanol extract ($LC_{50}$ values of about 40.31 μg/ml) in the gonad organ from *Holothuria leucospilota* continuing with the respiration tree organ ($LC_{50}$ values of about 72.49 μg/ml).

All concentrations of the three extracts from gonad (G), respiration tree (RT), cuvierian organ (CO), and body wall (BW) did not show antibacterial activity against *S. aureus*,

**Table 1.** Antifungal activity of *Holothuria leucospilota* extracts: gonads (G); respiration tree (RT); cuvierian organ (CO); body wall (BW).

| Organism | Extract | Zone of inhibition (mm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | MIC μg/ml | 4 μg/ml | 8 μg/ml | 10 μg/ml | 14 μg/ml | 16 μg/ml | 18 μg/ml |
| *Candida albicans* | BW(methanol) | 7 | 0 | 15 | 16 | 17 | 17 | 20 |
| *C. albicans* | BW(methanol–water) | 3 | 13 | 23 | 25 | 27 | 27 | 30 |
| *C. albicans* | G (methanol) | 7 | 0 | 17 | 19 | 20 | 22 | 25 |
| *C. albicans* | G (methanol–water) | 3 | 1 | 18 | 21 | 25 | 27 | 30 |
| *C. albicans* | CT (methanol) | 3 | 12 | 20 | 20 | 24 | 30 | 34 |
| *Aspergillus niger* | BW (methanol) | 3 | 12 | 18 | 20 | 21 | 21 | 25 |
| *A. niger* | BW (methanol–water) | 3 | 15 | 23 | 24 | 26 | 28 | 30 |
| *A. niger* | G (methanol) | 7 | 0 | 22 | 28 | 30 | 35 | 40 |
| *A. niger* | G (methanol–water) | 3 | 22 | 30 | 35 | 43 | 45 | 50 |
| *A. niger* | CT (methanol) | 3 | 25 | 30 | 32 | 34 | 34 | 46 |
| *A. niger* | CT (methanol–water) | 3 | 25 | 30 | – | – | – | – |
| *A. niger* | RT (methanol) | 3 | 15 | 15 | 16 | 18 | 20 | 24 |
| *A. niger* | RT (methanol–water) | 3 | 15 | 17 | 20 | 24 | 25 | 28 |

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**Table 2.** Brine shrimp assay (BSC) (cytotoxic effects) of *Holothuria leucospilot a* extracts: gonads (G); respiration tree (RT); cuvierian organ (CO); body wall (BW).

| Extract | Dose (µg/ml) | Log dose | Total | Alive | Death | (%) | LC50 | Chi-square | 95% Confidence limits | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Lower | Upper |
| BW (methanol) | 10 | 1 | 20 | 14 | 6 | 30 | | | | |
| | 100 | 2 | 20 | 10 | 10 | 50 | 73.646 | 0.216 | 13.769 | 204.914 |
| | 500 | 2.7 | 20 | 6 | 14 | 70 | | | | |
| | 1000 | 3 | 20 | 4 | 16 | 80 | | | | |
| BW (water–methanol) | 10 | 1 | 20 | 18 | 2 | 10 | | | | |
| | 100 | 2 | 20 | 15 | 5 | 25 | 681.47 | 1.871 | 272.66 | 5283.93 |
| | 500 | 2.7 | 20 | 13 | 7 | 35 | | | | |
| | 1000 | 3 | 20 | 7 | 13 | 65 | | | | |
| BW (ethyl acetate) | 10 | 1 | 20 | 17 | 3 | 15 | | | | |
| | 100 | 2 | 20 | 15 | 5 | 25 | 981.7 | 0.489 | — | — |
| | 500 | 2.7 | 20 | 12 | 8 | 40 | | | | |
| | 1000 | 3 | 20 | 9 | 11 | 55 | | | | |
| RT (methanol) | 10 | 1 | 20 | 15 | 5 | 25 | | | | |
| | 100 | 2 | 20 | 11 | 9 | 45 | 72.494 | 1.364 | 26.085 | 151.617 |
| | 500 | 2.7 | 20 | 4 | 16 | 80 | | | | |
| | 1000 | 3 | 20 | 2 | 18 | 90 | | | | |
| RT (water–methanol) | 10 | 1 | 20 | 17 | 3 | 15 | | | | |
| | 100 | 2 | 20 | 13 | 7 | 35 | 145.89 | 8.48 | — | — |
| | 500 | 2.7 | 20 | 9 | 11 | 55 | | | | |
| | 1000 | 3 | 20 | 0 | 20 | 100 | | | | |
| G (methanol) | 10 | 1 | 20 | 14 | 6 | 30 | | | | |
| | 100 | 2 | 20 | 9 | 11 | 55 | 40.311 | 3.015 | 15.536 | 78.225 |
| | 500 | 2.7 | 20 | 2 | 18 | 90 | | | | |
| | 1000 | 3 | 20 | 0 | 20 | 100 | | | | |
| G (water–methanol) | 10 | 1 | 20 | 18 | 2 | 10 | | | | |
| | 100 | 2 | 20 | 8 | 12 | 60 | 92.05 | 1.133 | 41.958 | 172.376 |
| | 500 | 2.7 | 20 | 5 | 15 | 75 | | | | |
| | 1000 | 3 | 20 | 2 | 18 | 90 | | | | |
| CO (methanol) | 10 | 1 | 20 | 17 | 3 | 15 | | | | |
| | 100 | 2 | 20 | 10 | 10 | 50 | 72.046 | 0.795 | 34.691 | 128.286 |
| | 500 | 2.7 | 20 | 2 | 18 | 90 | | | | |
| | 1000 | 3 | 20 | 1 | 19 | 95 | | | | |
| CO (water–methanol) | 10 | 1 | 20 | 18 | 2 | 10 | | | | |
| | 100 | 2 | 20 | 11 | 9 | 45 | 139.11 | 1.407 | 66.066 | 266.114 |
| | 500 | 2.7 | 20 | 7 | 13 | 65 | | | | |
| | 1000 | 3 | 20 | 2 | 18 | 90 | | | | |

*P. aeruginosa* and *E. coli* and nor was any inhibition zone observed for these tests. Altogether, no antifungal activity of ethyl acetate extracts was seen while the *A. niger* was shown to be the most sensitive microorganism followed by *C. albicans*. The result showed an inhibition zone against *A. niger* and *C. albicans* with MIC ranging from 3 to 7 µg/ml. The highest antifungal activity was found in G (water–methanol) with an inhibition zone of about 50 mm against *A. niger* at 18 µg/ml extract concentration continuing with CT (methanol) which had a 46 mm inhibition zone against *A. niger* at 18 µg/ml extract concentration. Table 2 summarizes the screening test for biological activity of methanol and water–methanol extracts isolated from *H. leucospilota*.

## DISCUSSION

In recent years, great attention has been paid to study of the bioactivity of natural products and their potential pharmacological utilization. The rationale of searching for drugs from the marine environment stems from the fact that marine plants and animals have adapted to all sorts of marine environments and these creatures are constantly under tremendous selection pressure including space competition, predation, surface fouling and reproduction (Kumaravel *et al.*, 2010). The development of safe and effective antimicrobial drugs has revolutionized medicine in the past 70 years (Franklin & Snow, 2005). However, the widespread use of antibiotics has promoted the emergence of antibiotic-resistant pathogens (Normark & Normark, 2002). The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including natural sources from any terrestrial or marine source (Blunt *et al.*, 2007).

Our study was designed to cover a broad range of polarities and consequently a wide range of the active substances present in the various extracts used in this study. Although there was significant antifungal and cytotoxic activity for methanol and methanol–water extracts of the isolated organs of *Holothuria leucospilota*, no antibacterial activity of these extracts was observed. As shown in Table 1, methanolic and water–methalonic extracts have indicated antifungal activity against *A.*

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*niger* and *C. albicans* and also these effects were higher on *A. niger*. None of the ethyl acetate extracts showed any antifungal activity.

In the present study the negative result of *H. leucospilota* against S*taphylococcus aureus* and *E. coli* is highly supported by the investigations of Rafat *et al.* (2008).

Our results show that *H. leucospilota* is rarely exposed to the fungal contact and its mediated infection. There is growing evidence that pathogens have significant impacts on marine systems (Harvell *et al.*, 1993). Extracts from the sea cucumber *H. leucospilota* show full inhibition of fungal growth at the lowest concentration tested, making this species a promising candidate for further studies. Methanol extract of the sea cucumber, *Actinopyga lecanora* also showed promising antifungal activity, *in vitro* (Kumar *et al.*, 2007). Farouk *et al.* (2007) have isolated some bacterial strains from various tissues of the sea cucumber species, *Holothuria atra*. Yuan *et al.* (2009) have isolated antifungal activity from the sea cucumber species, *Bohadschia marmorata*.

Because the majority of the positive results have been recorded in the methanol and water–methanol fractions, this shows that these fractions are good solvent systems for the solubility of bioactive compounds present in the sea cucumber.

In the future our research will focus on the analysis of possible organic compounds present in the sea cucumber extract, and compound purification and isolation will be attempted in order to elucidate the exact target moiety which is responsible for inducing the antifungal effect against the tested pathogenic organisms.

The highest toxic activity was found in methanolic extracts and the highest cytotoxic effect was found in methanol extract ($LC_{50}$ values of about 40.31 μg/ml) in the gonad organ from *H. leucospilota* continuing with the respiration tree ($LC_{50}$ values of about 72.49 μg/ml).

Sea cucumbers are reported to contain several compounds with anticancer and antiproliferative properties (Bordbar *et al.*, 2011). Silchenko *et al.* (2007) also studied the anticancer activity of three new triterpene oligoglycosides, okhotosides B1, B2 and B3, isolated from sea cucumber (*Cucumaria okhotensis*). Antiproliferative and anticancer functionality of sea cucumber extracts might be ascribed to the presence of considerable amounts of total phenols and flavonids which are regarded as effective antioxidants to protect from oxidative stress and degenerative diseases (including certain cancers: Althunibat *et al.*, 2009).

## CONCLUSION

Antifungal activity in crude extracts of the sea cucumber *Holothuria leucospilota* suggests a possible ecological function for their secondary metabolites. The antifungal property of the *H. leucospilota* extracts reveals that they are high enough to induce the effect against fungal pathogens rather than against the bacterial pathogens. It may due to the incidence of fungal presence in their habitat that induces the sea cucumber to produce the antifungal compounds. These problems coupled with the toxicity effect of continued use of several antifungal drugs highlights the need to search for new drugs to treat opportunistic fungal infections. Sea cucumbers might be in the future an appropriate source of cytotoxic natural compounds.

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