

In vitro study of antibacterial activities of ethanol, methanol and acetone extracts from sea cucumber *Holothuria parva*

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Abstract

Sea cucumbers are the most important medicinal group among echinoderms. The presence of a wide range of natural bioactive compounds from sea cucumbers has been confirmed in many studies. This study was aimed to evaluate the antibacterial activities of ethanol, methanol and acetone extracts from sea cucumber *Holothuria parva*. The sea cucumbers were collected during the low tide from Ola village, Bushehr, Iran. Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of the extracts were determined against five human pathogens. The different components in *H. parva* were determined by FTIR (Fourier Transform Infrared Spectroscopy) method. All of the crude extracts were shown antimicrobial activity against *E. coli*, *P. aeruginosa*, and *E. fecalis*. Also, methanol and ethanol extracts had inhibitory and bactericidal activities on the growth of the tested bacteria, respectively ($p < 0.05$). The FTIR spectra showed the presence of five components such as glycerol, gluconic acid, ouabain, spectinomycin and capreomycin in *Holothuria parva*. Our results showed that sea cucumber could be an appropriate marine source for antimicrobial compounds. Further, in vivo investigations need to be carried out to determine its potential application in other aspects of medicine.

Keywords: Antibacterial activity, Sea cucumber, *Holothuria parva*.

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Introduction

The high diversity of marine natural products and improved knowledge about the necessity of new anti-infective treatments has made a remarkable effort to move marine products into clinical applications (Donia and Hamman, 2003).

In recent years, a large number of bioactive compounds have been reported from different species of marine invertebrates (Mokhlesi *et al.*, 2011; Farjami *et al.*, 2014a; Kiani *et al.*, 2014). Sea cucumbers are marine invertebrates of the phylum of Echinodermata and the class of Holothuroidea (Althunibat *et al.*, 2013; Farjami *et al.*, 2014a). They produce a wide range of secondary metabolites (Mokhlesi *et al.*, 2011). There are more than 1500 Holothurian species in the world (Althunibat *et al.*, 2013). Among them, only 20 species have been identified in Iran (Dabbagh *et al.*, 2011). Different studies in the last two decades have been shown that sea cucumbers have a range of biological activities including antibacterial, antifungal, anticancer, cytotoxic (Farjami *et al.*, 2014a), antioxidant, wound healing (Kiani *et al.*, 2014), immunomodulatory (Salarzadeh *et al.*, 2012), cholesterol and lipid-reducing, anticoagulant and anti-thrombosis, antitumor (Farouk *et al.*, 2007), anti-inflammatory (Adibpour *et al.*, 2014), etc.

To date, various antimicrobial components have been extracted from the sea cucumbers such as steroidal glycosides, polyhydroxylated sterols, naphthoquinone pigments,

complement-like substances (Adibpour *et al.*, 2014), lysozymes and antimicrobial peptides (Canicatti *et al.*, 1989; Beauregard *et al.*, 2001).

The Persian Gulf is a broad coastal area with a rich biodiversity which makes it as an appropriate environment for marine studies (Mokhlesi *et al.*, 2011). Although there are a large number of investigations focusing on the new properties and applications of sea cucumbers worldwide, the Persian Gulf has not received much attention.

The present study was designed to investigate the antimicrobial activities of ethanol, methanol and acetone extracts from sea cucumber, *Holothuria parva*, collected from the Persian Gulf, Iran.

Materials and methods

Sample collection

Sea cucumbers (mean size of 10-15 cm) were collected during low tide from Ola village, Bushehr, Iran, from April 2014 to July 2014. All sea cucumbers were immediately transferred to the ecology laboratory at the Persian Gulf Research Institute, Bushehr and kept at 20°C. In this study, the specimens *H. parva* were identified using their ossicles as described by Kiani *et al.* (2014). Also, the results were compared with the identification keys of Food and Agriculture Organization of the United Nations (FAO) (Adibpour *et al.*, 2014).

Preparation of the sea cucumber extracts

Briefly, the tissue and whole body wall muscle of the sea cucumber were rinsed with distilled water and cut into small

pieces. The prepared samples were soaked in different solvents (ratio 1:4) such as 94% ethanol, methanol, acetone, and maintained at room temperature for about 72 to 96 h. Then, different extracts were filtered through Whatman filter paper No. 1. (Cam Lab, Cambridge, UK) and concentrated under vacuum in a rotary evaporator (LARK, Model: VC-100A) at 45 °C (Periyasamy *et al.*, 2012). Sterilization of the extracts was performed using filters with 0.45 and 0.22 µm pore sizes.

Antibacterial assay

The antibacterial activity of *H. parva* extracts was evaluated against five human pathogens: *Staphylococcus aureus* (ATCC 25923), *S. epidermidis* (ATCC 14990), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), and *Enterococcus fecalis* (ATCC 129212).

Briefly, a concentration of 250 mg mL⁻¹ of different dried extracts was prepared and added in a tube containing Mueller Hinton broth (Merck, Germany) and bacterial suspensions were adjusted with 0.5 McFarland standards. In this study, a tube containing Mueller Hinton broth and bacterial suspension was considered as a growth control. Also, another tube of the Mueller Hinton broth containing the same concentration of the extract was used as a negative control. After incubation at 37°C for 24 hours, antibacterial effects of the extracts were evaluated by the lack of turbidity in the test tubes for each bacterium (Tajbakhsh *et al.*, 2015).

Minimum inhibitory concentration and minimal bactericidal concentration determination

Since various extracts of *H. parva* showed antibacterial activity against *E. coli*, *E. fecalis* and *P. aeruginosa*, the MIC of the extracts was assayed using broth dilution method following Tajbakhsh *et al.* (2011). In this study, the extract concentrations ranged from 100 to 250 mg/ml. At the end of the incubation period (24 hours at 37°C), plates were evaluated for growth.

Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the extracts at which there was no bacterial growth. For the determination of minimal bactericidal concentration (MBC), 100 µL from those tubes that showed no growth were cultured on Mueller-Hinton agar (Merck, Germany). After incubation at 37°C for 24 hours, the MBC value of the extracts was determined as the lowest concentration that revealed no visible bacterial growth (Kang *et al.*, 2011). The tests were carried out three times for each bacterium.

Fourier transform infrared spectroscopy (FTIR) spectral analysis

In this study the frequencies of different components in each sea cucumber *H. parva* sample were determined by FTIR method (Periyasamy *et al.*, 2012). The disc was evaluated with spectrophotometry (Bio-Rad FTIR-40-model, USA).

Statistical Analysis

In this study, all the experiments were replicated three times. Analysis of

Variance (ANOVA) test was applied to data analysis. The differences in means were evaluated by a post-hoc multiple comparisons (Fisher's LSD) test. The data were statistically analyzed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ were considered to be statistically significant.

Results

Antibacterial assay

In the present study, all three different crude extracts from one species of sea cucumber, *H. parva*, had antimicrobial activity against three human pathogenic bacteria such as *E. coli*, *P. aeruginosa* and *E. fecalis* (Table 1). However, *S. aureus* and *S. epidermidis* were

resistant to all the extracts studied. The Fisher's LSD test at the confidence level of 0.95 showed that methanol extract was the most effective extract to inhibit the visible growth of bacteria. But acetone and ethanol extracts had the lowest inhibitory effects. Also, ethanol and acetone extracts had the highest and lowest bactericidal activity, respectively at the confidence level of 0.5 ($p < 0.05$) (Table 2).

In addition, all the extracts had the most inhibitory and bactericidal activities on *P. aeruginosa*. But *E. fecalis* was the most resistant bacteria to the studied extracts ($p < 0.05$).

Table 1: Antibacterial activity of different extracts of Sea cucumber *Holothuria parva*.

Bacteria	Extracts	Antibacterial Activity	MIC (mg mL ⁻¹)	MBC (mg mL ⁻¹)
<i>S. aureus</i> ATCC 25923	Ethanollic extract	-	-	-
	Methanolic extract	-	-	-
	Acetonic extract	-	-	-
<i>S. epidermidis</i> ATCC 14990	Ethanollic extract	-	-	-
	Methanolic extract	-	-	-
	Acetonic extract	-	-	-
<i>E. fecalis</i> ATCC 29212	Ethanollic extract	+	200	-
	Methanolic extract	+	175	-
	Acetonic extract	+	175	-
<i>P. aeruginosa</i> ATCC 27853	Ethanollic extract	+	100	200
	Methanolic extract	+	100	225
	Acetonic extract	+	125	-
<i>E. coli</i> ATCC 25922	Ethanollic extract	+	150	200
	Methanolic extract	+	150	-
	Acetonic extract	+	150	-

+: Shows activity; -: No activity.

Table 2: Fisher's LSD test results for the studied bacteria and extracts.

	Mean for MIC	Mean for MBC
Extracts		
Acetone	150 ± 21.65 ^{a*}	0.1 ^c
Ethanol	150 ± 43.3 ^a	133.33 ± 100 ^a
Methanol	141.66 ± 33.07 ^b	75 ± 112.5 ^b
Bacteria		
<i>E. coli</i>	150 ± 0 ^b	66.6 ± 100 ^b
<i>P. aeruginosa</i>	108.33 ± 12.5 ^c	141.66 ± 106.80 ^a
<i>E. fecalis</i>	183.33 ± 12.5 ^a	0.1 ^c

** Values marked with different superscripts are significantly different from each other ($p < 0.05$)

FTIR spectral analysis

The FTIR spectra of the different extracts of *H. parva* showed a wide peak at 3000 cm⁻¹ and a sharp peak at 1675 cm⁻¹ (Fig. 1). The results showed the stretching vibration of OH and C=O

links, respectively. In this study, five components such as glycerol, gluconic acid, ouabain, spectinomycin and capreomycin in sea cucumber *H. parva* were detected.

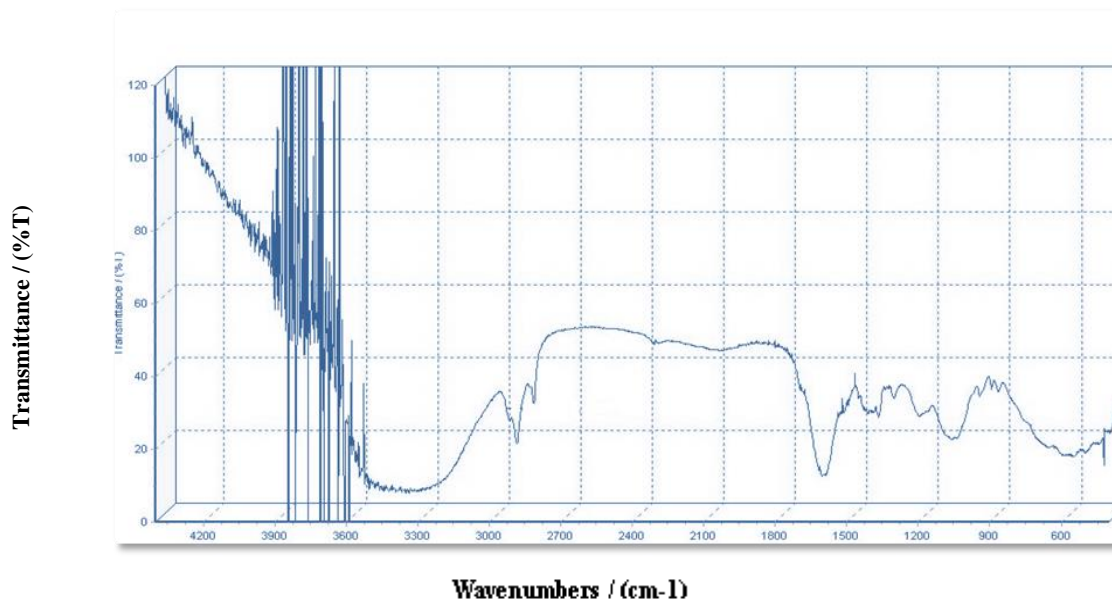


Figure 1: The FTIR spectra of the methanolic extract of *Holothuria parva*.

Discussion

The increasing of resistance to current antimicrobial drugs is one of the most challenging problems in the treatment of infectious diseases (Nazarian *et al.*, 2015). Marine organisms are emerging

resources of bioactive natural products with a high impact on human pathogens (Kang *et al.*, 2015).

Antimicrobial activities of several species of sea cucumbers from around the world such as Iran have been

confirmed (Farjami *et al.*, 2014b), Mexico (Moguel-Salazar *et al.*, 2013), Norway (Haug *et al.*, 2002), Egypt (Ibrahim, 2012; Omran and Allam, 2013), Italy (Schillaci *et al.*, 2013), etc. On the contrary, there are few studies indicating no antibacterial activity from different species of sea cucumbers (Dobretsov *et al.*, 2009; Lawrence *et al.*, 2010).

Minimal inhibitory concentrations (MICs) ranging from 100 to 250 mg mL⁻¹ were determined against the studied bacteria. In the present study, methanol extract exhibited the most antibacterial effects with a mean of 141.66 mg mL⁻¹. This finding is the same as other studies in Iran (Mokhlesi *et al.*, 2011) and worldwide (Ibrahim, 2012). However, it is in contrast to those noted in some previous studies which indicated that methanol extracts had no appreciable effect on the tested bacteria (Adibpour *et al.*, 2014; Farjami *et al.*, 2014b).

In this investigation, we examined the antimicrobial activity of the whole bodies of sea cucumber, in accordance with other studies (Bano and Ayub, 2012; Periyasamy *et al.*, 2012). However, there are few reports from antimicrobial activities of the egg (Stabili and Pagliara, 1994; Salarzadeh *et al.*, 2012), gonad, intestine (Farjami *et al.*, 2014a), cuvierian organs, and coelomic fluid of sea cucumber (Adibpour *et al.*, 2014).

In addition to antimicrobial activities, the presence of bioactive natural products with antioxidant (Mokhlesi *et al.*, 2011; Pishehvarzad *et al.*, 2014), anticancer (Aminin *et al.*, 2015),

antiangiogenic and antitumor (Tian *et al.*, 2005), cytotoxic (Althunibat *et al.*, 2013; Ehsanpour *et al.*, 2015) properties have been reported from different solvents (such as hexane, chloroform, methanol, ddH₂O) of sea cucumbers species.

Previous studies have shown that triterpene glycosides are the most prevalent structural configuration of antibacterial agents in holothurians (Chludil *et al.*, 2002; Silchenko *et al.*, 2002; Farouk *et al.*, 2007). Among them, saponins are water-soluble glycosides that pose a broad-spectrum of pharmacological effects (Kelly, 2005; Caulier *et al.*, 2011; Farjami *et al.*, 2014a).

The results of the FTIR spectra revealed the presence of two glucosidal components including ouabain, spectinomycin and a cyclic peptide, capreomycin. In this study, the antibacterial activity of different extracts might be related to these antimicrobial substances in Holothuria. Therefore, more in-depth studies are needed to identify and evaluate their potential strength for novel drugs in biomedical research.

In the present study, all three different crude extracts from *H. parva* had antibacterial effects against both the gram-negative and with a weaker ratio on gram-positive bacteria. Sea cucumber could be a particular marine source for antimicrobial components. Therefore, further in vivo investigations need to be carried out on its potential application in other aspects of medicine.

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