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Research Article

Cytotoxic and antibacterial activities of *Holothuria* (*Mertenssiothuria*) *leucospilota* extracts

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Abstract

There are several studies on biological activities of echinoderms' secondary metabolites. Sea cucumbers are known to contain natural products with biological activities. Different sea cucumber species exist in Iranian waters of Oman Sea. We investigated cytotoxic and anti- bacterial activities of *Holothuria* (*Mertensiothuria*) *leucospilota* form Chabahar Bay. We derived four methanolic, chloroformic, n-hexane and aqueous extracts of sea cucumber, *H.* (*Mertensiothuria*) *leucospilota*. The cytotoxic activity of these extracts were evaluated by MTT and brine shrimp lethality assays. Disc diffusion method was used to evaluate antibacterial effect of *H.* (*Mertensiothuria*) *leucospilota* extracts against two gram negative pathogenic bacteria: *Escherichia coli* (PTCC 1399) and *E. coli K 12* (ATCC 23716), and two gram positive pathogenic bacteria: *Staphylococcus aureus* (ATCC 25923) and *Listeria monocytogenes* (ATCC 19117). Methanolic extract (100 mg/mL) was effective on *S. aureus* and *L. monocytogenes* while aqueous extract of *H.* (*Mertensiothuria*) *leucospilota* (100 mg/mL) demonstrated antibacterial effect against all tested strain. Chloroformic extract (100 mg/mL) was effective on both *E. coli K 12* and *S. aureus*, while n-hexane extract (100 mg/mL) was just effective on *E. coli*. Studying brine shrimp lethality assay indicated that all experimented extracts had strong cytotoxic activity with LC_{50} = 1.2–2.3 µg/mL. MEHL, ChEHL and n-hEHL showed cytotoxic effect on Caco-2 with 0/6, 0/8 and 1 mg/mL $LC_{50,}$ respectively. Due to our results, it can be said that *Holothuria (Mertensiothuria*) *leucospilota* extracts are potential antibacterial and cytotoxic agents.

Keywords: Sea cucumber, Cytotoxic, Antibacterial, Oman Sea

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Introduction

Marine environments are known as important source of bioactive agent. Diverse bioactive compounds have been isolated from marine organisms, such as algae, nudibranchs, echinoderms, sponges and corals (Carroll *et al*., 2021). Various biological activities of echinoderms have been reported including, anti-inflammatory and anti-proliferative (Kareh *et al*., 2018), antioxidant (Soleimani *et al*., 2017; Hapsari and Yasman, 2019), immunostimulating (Nurshid *et al*., 2021), antiherpetic (Keshavarz *et al*., 2021), antifungal (Adibpour *et al*., 2014), antimicrobial (Raj *et al*., 2021), cytotoxic (Mokhlesi *et al*., 2012; Klimenko *et al*., 2021), and anti-HIV-1 activity (Lian *et al*., 2013; Bahroodi *et al*., 2018). The sea cucumber *Holothuria* (*Mertensiothuria*) *leucospilota* is a marine invertebrate belonging to the phylum Echinodermata, class Holothuroidea, order Holothuriida and family Holothuriidae. Sea cucumbers live in shallow areas, among coral reefs, rocks, in muddy and sandy flats. These echinoderms usually live in intertidal zone but are also found in deeper waters (Dabbagh and Kamrani, 2011). Different species of sea cucumbers, like *Holothuria* (*Mertensiothuria*) *leucospilota, Stichopus herrmanni* (Afkhami *et al*., 2012), *H. impatiens, H.* (*Metriatyla*) *scabra* (Salarzadeh *et al*., 2013) exist in Persian Gulf and *H. arenicola, H.* (*Halodeima*) *atra, H.* (*Mertensiothuria*) *leucospilota, Stichopus horrens* are reported in Oman Sea. Sea cucumber *H.* (*Mertensiothuria*) *leucospilota* is distributed wildly in Persian Gulf and Oman Sea among corals and is identified as dominant species at Chabahar coastline. *Holothuria* is known as a resistant genus against unfavorable conditions (Shakouri *et al.*, 2009). Since these organisms are benthic, they are exposed to bacteria and fungus diseases and should possess mechanisms to survive (Haug *et al*., 2002). Therefore, they use secondary metabolites as chemical weapons. Some secondary metabolites such as triterpene glycosides (saponins), chondroitin sulfates, glycosaminoglycan (GAGs), sulfated polysaccharides, sterols (glycosides and sulfates), phenolics, cerebrosides, lectins, peptides, glycoprotein, glycosphingolipids and essential fatty acids were derived from various sea cucumber species (Yamada *et al*., 2001; Dang *et al*., 2007; Bordbar *et al*., 2011; Darya *et al*., 2020). There are a few studies on Persian Gulf sea cucumber natural products; ester saponin has been extracted from *Stichopus herrmanni* (Salari *et al*., 2017), α-cyanostilbene is an identified molecule in *H. parva* (Amidi *et al.*, 2017). Also three compounds were obtained from *H.* (*Mertensiothuria*) *leucospilota* in the Persian Gulf and characterized as holothurin A, echinoside A, and 24-dehydroechinoside A (Shushizadeh *et al*., 2019). Biological activities of the derived different extracts from Persian Gulf sea cucumbers, including cytotoxic (Mashjoor and Yousefizadi, 2019; Shushizadeh *et al*., 2019), antifungal (Ebrahimi *et al*., 2018), antibacterial

(Jamali *et al*., 2009; Ebrahimi *et al*., 2018), hemolytic (Shadi and Oujifard, 2019) and antiviral (Bahroodi *et al*., 2018) activities were evaluated. Whereas colorectal cancer is one of the common carcinoma in developing countries, there is a prospective idea that there would be 1.1 million related deaths in 2030 and 60% increase in new cases (Arnold *et al*., 2017). So, finding effective compounds is an essential function. There is some evidence of presence of *E. coli* strains in colorectal cancer patients in Japan and Europe (Shimpo *et al*., 2017), and *E. coli* is known as a cofactor of colorectal cancer (Bonnet *et al*., 2014; Wassenaar, 2018). So both *E. coli* strains and a colon carcinoma cell line were applied in present experiment to evaluate cytotoxic effect of *H.* (*Mertensiothuria*) *leucospilota* extracts collected from Oman Sea against colorectal cancer. Due to high potential of bacteria mutations, finding new antibiotics and antibacterial agents with natural source to treat bacterial diseases (because of fewer side effects), has been noted by scientists worldwide.

Material and methods

Sampling and Identification

Samples of *Holothuria* (*Mertensiothuria*) *leucospilota* were collected by scuba diving in June at depth of 2-3 m from Beheshti wharf, Kalantari Jetty and Hotel Daryaei in Chabahar Bay, Oman Sea. The samples were frozen and transported to laboratory.

Extraction

To prepare the extracts, we followed the method of Lakshmi *et al*. (2008) and Jamali *et al*. (2009). The frozen sea cucumbers were defrosted, internal organs were removed and the body wall was cut into small pieces (approximately 1 cm) and soaked in 95% methanol (1g: 4 mL) for 7 days at room temperature. The solvent was evaporated in reduced pressure by rotary evaporator at 35°C and 120 rpm until the crude extract was gained. The concentrated methanol extract was departed into 3 sections. The first section was concentrated using vacuum in rotary evaporator; the residue was stored at -20°C as methanol extract. The same amount of n-hexane was added to the second part which was placed in decanter funnel and the mixture was shaken and the n-hexane layer was concentrated by rotary evaporator and the residue stored at -20°C as n-hexane extract. A similar procedure was performed for the chloroform extract on the third part (Lakshmi *et al*., 2008; Jamali *et al*., 2009). To prepare aqueous extract, 20 gram of the washed sea cucumbers was homogenized with the same amount of water. The homogenate centrifuged for 15 minutes, the above solution was separated and stored in at -20°C.

Cytotoxicity assay

Cell culture

Human Colorectal Adenocarcinoma Caco-2 (ATCC®HTB-37™) was

obtained from Pasteur Institute of Iran. Cells were grown in DMEM (Dulbecco's Modified Eagle Medium (Sigma –Aldrich)) medium culture and 10% FBS (fetal bovine Serum (Gibco – Germany)) in cell culture flasks. Flasks were incubated in 37°C, 5% CO_2 and 95% of air. Medium was refilled every seven days (ATCC catalog of the cell line). Cell culturing was done for a month to reach fresh and healthy cells.

MTT assay was performed for screening of cytotoxicity activity of *H.* (*Mertensiothuria*) *leucospilota* different extracts. MTT salt (3-(4,5-dimethylthiazolyl-2)-2,5diphenyltetrazolium bromide) change color in exposure of viable cells. 5×10^4 Caco-2 cells were placed in a 96-well plate and exposed to different densities (50, 25, 12.5, 6.25, 3.125, 1.56 mg/mL) of the four prepared extracts. DMSO was used as negative control. All experiments were done in triplets. After 24 hours, 20 µl of 5 mg/mL MTT powder (Sigma –Aldrich) was added to each well and incubated in dark for four hours. Wells were suppressed with 100 µL DMSO as stop solution, optical density screened after 20 minutes using ELISA Reader (Anthos 2020 Austria) in 540 nm. Death cell percentage obtained by the following equation:

$$\text{Death Percentage} = \left(\frac{\text{OD of Negative Control} - \text{OD of Sample}}{\text{OD of Negative Control}}\right) \times 100$$

Brine shrimp lethality test (BSL)

Artemia urmiana cysts were hatched in artificial sea water for 48 hours (artificial sea water: 32 gL^{-1}). After hatching the eggs, light was used for gathering nauplii in a corner of the hatching tank. We used Pasteur pipette to separate the nauplii. 10, 100, and 1000 µg/mL of every extract were prepared using artificial sea water. 10 nauplii were introduced to a 96-well microplates and 200 µl of prepared extracts was added to them. This experiment was conducted with three repeats. DMSO was served as control. After 20 hours incubating under light at room temperature, the numbers of survived nauplii were counted. SPSS software, version 16 was applied to analysis descriptive statistic - frequency and determining cumulative percent and PROBIT analysis to find LC_{50}.

Antibacterial activities test

Bacteria source

Bacterial pathogens, *Escherichia coli* (PTCC 1399), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19117) were obtained from Iranian Research Organization for Science and Technology (IROST). *E. coli K 12* (ATCC 23716) was obtained from Pasteur Institute of Iran.

Disc diffusion test

In vitro antibacterial potential of 4 extracts of *H.* (*Mertensiothuria*) *leucospilota* was tested by disc diffusion method. The suspension of bacteria culture was prepared according to

McFarland standard 0.5. The overnight sub-cultured bacterial suspension (200 µL) was spread through nutrient agar plates. For each assay, 100 mg of each dry extract was solved in 1 mL of dimethyl sulfoxide (DMSO). The blank sterile discs (Whatman paper No. 1 filter paper) were inoculated by 20 µL of each extract. DMSO was used as negative control while the commercial antibiotic discs; Ampicillin 10 µg per disc, Cephalexin 30 µg per disc, Erythromycin 15 µg per disc and Amoxicillin 25 µg per disc were used as positive controls. For every extract, triplet discs were tested. The plates were incubated at 37°C and the inhibition zone screened after 20 hours. Antibacterial activity is measured as mean diameter of the inhibition zone.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum Inhibitory Concentration (MIC) was measured to identify the lowest concentration of extract which inhibits bacterial growth. MIC of *H.* (*Mertensiothuria*) *leucospilota* extracts was tested against *E. coli* and *E. coli K 12* using Clinical and Laboratory Standards Institute (NCCLS). Serial dilution made by the extracts and DMSO with 50, 25, 12.5, 6.25, 3.12 and 1.56 µg/mL concentration. 100 µL of all bacterial suspension, prepared according to McFarland standard 0.5, and was placed in 96-well microtiter plates. 40 µL of extracts of every dilution was added to 96-well microtiter plates. 100 µL of bacterial suspension plus 40 µL Lactose Broth was used as positive control, while 100 µL Lactose Broth plus 40 µL extract and DMSO was used as negative control. Optical density (OD) was measured at 630 nm using ELISA Reader. The plates were incubated at 37°C and bacterial growth was measured by OD at 630 nm using ELISA Reader after 24 h period of time. The test was repeated in triplets for every extract.

The wells, in which no growth occurred, were noted to specify the Minimum Bactericidal Concentration (MBC), 24 hours' light wells were used to culture in Mueller Hinton agar plates, and incubated overnight at 37°C. Bacterial colony observation, demonstrated the inhibitory effect of extracts on bacterial growth while absence of bacterial colony supported mortal effect.

SPSS software, version 16 was used to analyze data. One-way ANOVA test was applied to identify significant differences within data. Probit value analysis from the software was applied for LC_{50} values of *H.* (*Mertensiothuria*) *leucospilota* extracts against Caco-2 cell line. For BSL assay Descriptive statistics, Frequency and determining Cumulative Percent and Probit analysis was applied to find $LC_{50.}$

Results

Identification

Identification of the samples was done by comparing ossicles with Holothuroidea FAO key, (Carpenter and Niem, 1998; Jamali *et al*., 2009; Mashjoor *et al*., 2019) and also comparing with a survey that mentioned

genomic identification (Kamarudin and Rehan, 2015). Other characters of collected specimen were investigated on Marine Species Identification Portal. Species *Holothuria* (*Mertensiothuria*) *leucospilota* (Brandt, 1835) was confirmed.

Cytotoxic assay

Effectiveness of *H.* (*Mertensiothuria*) *leucospilota* extracts against Caco-2 cell line in concentrations: 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/mL was applied by MTT assay and cell death percentages are mentioned in Table 1.

Table 1: Death percentages of *H.* (*Mertensiothuria*) *leucospilota* extracts on Caco-2 cell line.

Extract	Concentration (mg/mL)						*p*-value
	50	25	12.5	6.25	3.12	1.56	
MEHL	68.847	67.738	73.728	60.22	61.498	59.82	<0.0001
ChEHL	72.2	67.97	56.546	71.163	60.779	59.98	<0.001
n-hEHL	62.377	68.383	71.603	56.865	55.52	54.988	<0.021
AEHL	48.238	32.66	36.42	51.353	29.71	10.26	<0.001

The most effective extract was MEHL, which inhibited cell growth by LC_{50} 0.6 mg/mL, ChEHL made the same action by LC_{50} 0.8 mg/mL, n-hEHL was less effective by LC_{50}: 1 mg/mL, while no cytotoxicity effect was observed by AEHL. LC_{50} of extract is shown in Table 2.

Table 2: LC_{50} of *H.* (*Mertensiothuria*) *leucospilota* extracts on Caco-2 cell line.

Extract	LC_{50} (mg/mL)
MEHL	0.6±0.06
ChEHL	0.8±0.08
n-hEHL	1±0.06
AEHL	-

Brine Shrimp lethality assay (BSLA)

All of *H.* (*Mertensiothuria*) *leucospilota* extracts showed cytotoxic activity. They all made 100% of mortality in dose 1000 µg/L. MEHL, ChEHL and n-hEHL were the most effective ones and AEHL made this in fewer levels. The LC_{50} results are mentioned on Table 3. While there were dead Artemia in all experimented extracts, we obtained Cumulative Percent of dead *Artemia urmiana* and the results are shown in Table 4.

Table 3: LC_{50} determined by *H.* (*Mertensiothuria*) *leucospilota* extracts on brine shrimp (µg/mL).

H. (Mertensiothuria) leucospilota extracts	LC_{50}
MEHL	1.2±0.07
ChEHL	1.2±0.08
n-hEHL	1.5±0.06
AEHL	2.3±0.06

Disc diffusion method

Antimicrobial activity of *H.* (*Mertensiothuria*) *leucospilota* extracts against different tested organisms by disc diffusion is shown in Table 4. The inhibition zone was observed to be in rang of 8-11 mm. MEHL and AEHL exhibited the most antibacterial activity on the experimental bacteria; however, MEHL had no inhibitory effect on *E. coli.* The lowest effect was made by n-hEHL, it had antimicrobial activity only against *E. coli*. ChEHL displayed moderate inhibitory on *E. coli K 12* and

S. aureus (Table 5). Significant statistical activity was defined for all experimented extracts (p<0.05). Comparing with commercial antibiotics, *H.* (*Mertensiothuria*) *leucospilota* extracts indicated less antibacterial activity. The most antibacterial activity was determined by MEHL and AEHL against gram negative and gram positive bacteria.

Table 4: Percentage of mortality of *Artemia urmiana* exposed to *H.* (*Mertensiothuria*) *leucospilota* extracts.

Concentration (µg/mL)	MEHL	ChEHL	n-hEHL	AEHL
1000	100	100	100	100
100	66.7	66.7	66.7	62.5
10	33.3	33.3	33.3	25

Table 5: Antimicrobial activity of *H. (Mertensiothuria) leucospilota* extracts: comparison with antibiotics.

Bacteria strain	Inhibition zone in mm								
	MEHL	ChEHL	n-hEHL	AEHL	Amoxicillin	Ampicillin	Cephalexin	Erythromycin	DMSO
E coli	-	-	8.33±0.47	8.4±0.56	8.03±0.15	10.13±0.15	8.06±0.11	-	11±0.1
E. coli K 12	9±0	8.06±0.2	-	9.06±0.16	12.06±0.11	9±0.07	10.06±0.08	-	9.3±0.15
S. aureus	11.1±0.21	9.3±0.2	-	10.96±0.12	9.1±0.1	13.03±0.15	8.03±0.25	8.03±0.1 5	11±0.1
L. monocytogenes	11.06±0.16	-	-	10.96±0.12	10.16±0.15	13.06±0.11	10.1±0.1	10.1±0.06	13.03±0.2

Values are expressed as Mean ± SD " –" indicates no inhibition zone

Due to some problems which happened during the experimental period, MIC and MBC just applied on *E. coli* strains.

Escherichia coli K 12

MEHL inhibited bacterial growth (MIC) in 6.25 mg/mL concentration (MIC = 7.15 mg/mL) at time 4 (MIC = 17.96 mg/mL), MBC indicated no bacterial death. Significant relation exists within OD and time (p<0.002). The other three extracts made no inhibition effect. Table 6 shows MIC result on *E. coli* strains.

Escherichia coli

At time 8 bacterial growths was inhibited by MEHL in 50 (MIC = 8.25 mg/mL) and 25 mg/mL (MIC = 3.57 mg/mL) concentrations. 1.56 mg/mL concentration made inhibition activity at times 4 and 8 (MIC = 11.47 mg/mL). Results of one-way ANOVA analysis showed significant statistical relationship within time and OD (p<0.0001), by passing time the OD rate increased. ChEHL inhibited bacterial growth in 50 and 25 mg/mL concentration. Significant relation identified between time and OD (p<0.26). n -hEHL and AEHL made no effect (Table 6).

Table 6: MIC of *H. (Mertensiothuria) leucospilota* extracts against Escherichia coli K 12 and Escherichia coli.

Extract type	Time (h)	*E. coli*						*E. coli K 12*					
		Concentrations (mg/mL)						Concentrations (mg/mL)					
		50	25	12.5	6.25	3.12	1.56	50	25	12.5	6.25	3.12	1.56
MEHL	0	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	11.47	-	-	-	7.15	-	-
	8	8.25	3.57	-	-	-	11.47	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-	-	-	-	-	-
ChEHL	0	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-	-	-	-	-	-
n-hEHL	0	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-	-	-	-	-	-
AEHL	0	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-	-	-	-	-	-

Discussion

Plenty of surveys have assessed biological activity of marine organisms' natural products. Cytotoxic effects of natural products isolated from marine organisms, such as sponges (Dhinakaran *et al*., 2012), hard corals (Mohammadizadeh *et al*., 2014) and holothurians (Sugawara *et al*., 2006) are assessed. Some antitumor and cytotoxic compounds are reported from sea cucumbers, nobiliside D, a kind of triterpene glycosides, extracted from *Holothuria* (*Microthele*) *nobilis* (Selenka) made inhibitory effect on different cell lines and induced apoptosis (Zhang and Zhu, 2017). Also, triterpene glycosides, named as philinopsides A, B, E and F, from sea cucumber *Colochirus quadrangularis* made significant antitumor action (Hua *et al*., 2009); the other triterpene glycoside, frondoside A, obtained from Atlantic sea cucumber *Cucumaria frondosa* is proved to make synergistic effect with some conventional chemotherapeutic agents (Adrian and Collin, 2018). Organic extracts of body wall of *Holothuria (Halodeima) atra* in southeast coast of India represented effective antitumor and antifungal activity (Dhinakaran and Lipton, 2015). In the other study 55-98% inhibition values of Caco-2 cell line; colon carcinoma was evaluated from hot water extract of *Apostichopus japonicus* (Ogushi *et al*., 2005).

Potency of Persian Gulf sea cucumbers to contain biological activity is

investigated in several studies through their extracts. Cytotoxic efficiency of ethyl acetate extracts of cuvierian tubules of *H.* (*Mertensiothuria*) *leucospilota* and intestine tract of *H. parva* are proved (Mashjoor and Yousefzadi, 2019). Antifungal effect of methanol and chloroform extracts of *H.* (*Mertensiothuria*) *leucospilota* collected from Larak Island of Persian Gulf on *Candida albicans* and *Aspergillus niger* is demonstrated (Farjami *et al*., 2014). Different extracts of *Holothuria* sp. isolated from Hengam Island of Persian Gulf demonstrated antibacterial impact against several *E. coli* strains; K 12, TG1 and Top10F' (Jamali *et al*., 2009); also cytotoxic activity of methanolic extract of the Persian Gulf sea cucumber, *H.* (*Mertensiothuria*) *leucospilota*, is approved (Mokhlesi *et al*., 2012).

Holothurins A and B, leucospilotasides A, B and C and echinoside B are other natural products that are identified in *H.* (*Mertensiothuria*) *leucospilota* (Pangestuti and Arifin, 2018). Furthermore, three ganglioside molecular species are isolated from chloroform/methanol extract of the sea cucumber *H.* (*Mertensiothuria*) *leucospilota* and HLG-1, HLG-2, and HLG-3, and their neuritogenic effect against rat pheochromocytoma cell line (PC-12 cell) in the presence of nerve growth factor is considered (Yamada *et al*., 2001). Methanol extract of *H. arenicola* collected from Persian Gulf, induced apoptosis in colon carcinoma cell line: CT 26 (Baharara *et al*., 2016). Saponin of *H.* (*Mertensiothuria*) *leucospilota* proved to be an antioxidant and antitumor compound (Soltani *et al*., 2015).

In the present experiment, cytotoxic effect of *H.* (*Mertensiothuria*) *leucospilota* extracts was determined by more concentration but in less time treatment. Methanolic, chloroformic and n-hexane extracts showed moderate cytotoxicity against human colorectal adenocarcinoma cells with LC_{50} = 0.6-1 mg/mL. In a similar study, different organs of three Holothurians; *H.* (*Mertensiothuria*) *leucospilota, H. parva* and *H.* (*Metriatyla*) *scabra* collected from Persian Gulf, were extracted by distinct solvents and their toxicity were evaluated against Caco-2 cell line. The ethyl acetate extract of *H.* (*Metriatyla*) *scabra* coelomic fluid, and the same solvent of *H. parva* body wall, showed potent cytotoxic activity with LC_{50} 24.36 and 16.78 µg/mL, respectively, but no cytotoxic activity was observed by *H.* (*Mertensiothuria*) *leucospilota* extract (Mashjoor and Yousefzadi, 2019), that contradicted the result of the present experiment about n-hexane and methanol extracts of *H.* (*Mertensiothuria*) *leucospilota.* Lc_{50} of twelve antitumor pharmaceutical drugs, such as Doxorubicin, epirubicin, idarubicin, raltitrexed, cisplatin, oxaliplatin, irinotecan, doxorubicin, etc. against Caco-2 cell line are evaluated (Fohlen *et al*., 2021). Cytotoxicity of n-hEHL of the present research was approximately equal with irinotecan (LC_{50} = 0.95 mg/mL) and paclitaxel (LC_{50} = 0.10 mg/mL), while ChEHL of the current research was more effective

than streptozocin (LC_{50} = 22.93 mg/mL) and gemcitabine (LC_{50} = 3.03 mg/mL) but was less than that of ethyl acetate extract of *H. parva* and *H.* (*Metriatyla*) *scabra* and completely in contradiction with *A. japonicus* hot water extract.

Brine shrimp lethality test is a trusted method to evaluate cytotoxicity of different chemicals and natural products (Wu, 2014). Cytotoxicity effect of isolated lectin from *H.* (*Metriatyla*) *scabra* on brine shrimp was less than that of commercial agents (Mojica and Merca, 2005). In the present study cytotoxic activities of experimented extracts; MEHL, ChEL, n-hEHL and AEHL were revealed and dose related relevance were performed in all of the extracts. MEHL and ChEL represented the same effect with equal LC_{50}, so these two extracts may contain the same substance. Results of the present assay supported the obtained result of the same sea cucumber species (n-Hexane extract LC_{50} 0.389 mg/mL and methanol extract LC_{50} 0.098 mg/mL) in Persian Gulf that revealed the cytotoxic activity of different extracts of the *Holothuria* against brine shrimp (Darya *et al*., 2020).

Disc diffusion method and MIC are common methods to determine antibacterial effects (Balouiri *et al*., 2016). Sea cucumber antibacterial activity has been studied worldwide (Park *et al*., 2011; Mokhlesi *et al*., 2012; Moguel-Salazar *et al*., 2013, Sottorff *et al*., 2013; Darya *et al*., 2020). A few previous studies specified antibacterial effects of sea cucumbers. The effects of the sea cucumber *Apostichopus parvimensis* methanol/acetone extract against *E. coli* and *Bacillus subtilis* in comparison with antibiotics is investigated (Villasin and Pomory, 2000). Among different sea cucumber extracts that were assessed for antibacterial activities, only the methanol one was defined as bacterial growth inhibitor for gram positive pathogens, *S. aureus* and *Streptococcus pyogenes* (Al-Haj *et al*., 2010). The alcoholic extract of the sea cucumber *Actinopyga miliaris* and non saponin fraction of *H.* (*Metriatyla*) *scabra* had less effect against *E. coli* in 200 μg/disc than Ampicillin, while it was not effective on *S. aureus* (Abraham *et al*., 2002). The antibacterial assay of ethanol and hot water extracts of *A. japonicus* showed growth inhibition on two gram positive strains (*S. aureus* and *Staphylococcus epidermidis*). Water extract of this species was more effective (Park *et al*., 2011).

The extracts of *H.* (*Metriatyla*) *scabra* made no bacterial growth inhibition effect versus *S. aureus, Pseudomonas aeroginosa and E. coli*, although made antifungal and cytotoxic impacts (Mohammadizadeh *et al*., 2013). Inhibitory effect of various *H.* (*Mertensiothuria*) *leucospilota* extracts (ethyl acetate, methanol and water–methanol), collected from north of Persian Gulf against *S. aureus, P. aeroginosa and E. coli* was examined and no antibacterial effect was observed (Mokhlesi *et al*., 2012), which is variant with the present study results and may be caused by different extraction methods of bioactive compound. Our findings were in line with Jamali *et al*. (2009)

results who evaluated the antibacterial activities of *Holothuria* sp. methanol and chloroform extracts against *E. coli K 12*, although the aqueous extract of *Holothuria* sp. increased the bacterial growth, the current experiment indicated inhibition impacts against all bacteria. In comparison with Cephalexin and erythromycin, antibacterial activity of AEHL was more intense, but it made less effect with Ampicillin on *E. coli* K 12. Although the methanol extract of different organs of *H.* (*Mertensiothuria*) *leucospilota*, harvested from 25-30 m depth of Larak Island in Persian Gulf, displayed antibacterial activity against *P. aeroginosa*, it was not effective versus *S. aureus* and *B. subtilis.* Chloroform and Hexane body wall extracts made inhibitory effects on *S. aureus*, although chloroform extract of body wall was not effective against *P. aeroginosa* (Farjami *et al*., 2013). Results of the present investigation were approximately opposite to those of Darya *et al*. (2020), both studies confirmed antibacterial activities of the sea cucumber species, but n-hexane and methanol extracts of *H.* (*Mertensiothuria*) *leucospilota* body wall, collected from Hengam Island in Persian Gulf, inhibited *E. coli* and *S. aureus* growth (Darya *et al*., 2020), however in the current assay n-hexane extract inhibited growth of *E. coli* and was not effective on *S. aureus* that was the same for methanol extract against *E. coli.* In the present experiment maximum antibacterial activity was observed for MEHL and AEHL against *S. aureu*s and *L. monocytogenes*, which exhibited existing of antibacterial active compounds in these extracts. MEHL and AEHL were more effective than Cephalexin, Erythromycin and Amoxicillin antibiotics, while less effective than Ampicillin. The lowest antibacterial effect revealed by n-hEHL, represented less existence of antibacterial substance in the non-polar *H.* (*Mertensiothuria*) *leucospilota* extract. With regards to wide spreading distribution in nature, *L. monocytogenes* is one of the most important food-borne pathogens. We reported significant antibacterial activity of sea cucumber *H.* (*Mertensiothuria*) *leucospilota* substances versus *L. monocytogenes,* for the first time, although this was less efficient than that of Ampicillin as a specific antibiotic treatment of this bacteria (Noll *et al*., 2018), but considering high level of resistance to antibiotics in this bacteria strains, sea cucumber extracts could be applied as new source of antibiotics. Different efficiency character of MEHL on *E. coli* strains in the current study could be due to their genome differential. The nonequivalent results obtained from the present experiment and *H.* (*Mertensiothuria*) *leucospilota* antibacterial activities studied by Mokhlesi *et al*. (2012), Farjami *et al*. (2013) and Darya *et al*. (2020) may be as a result of different extraction methods or different particular conditions of Oman Sea, Red Sea and Persian Gulf coastlines, or even anesthetizing the organism in $MgCl_2$ before dissection. With regards to close extraction method of the present investigation and that of

Yamada *et al*. (2001), we can mention that may be the gangliosides responsible for biological activity of *H.* (*Mertensiothuria*) *leucospilota.* In conclusion, the obtained results from the current study, suggest that the extracts isolated from *H.* (*Mertensiothuria*) *leucospilota*, the domestic sea cucumber species of Chabahar Bay in Oman Sea, contain cytotoxic and broad-spectrum of antibiotic substances (gram positive cocci, gram negative and gram positive bacilli). Our findings suggest further biological assays and isolating and purification of active compounds of this species.

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