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Cytotoxic effects of three Persian Gulf species of Holothurians

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Summary

Background: Pharmaceutical industries around the world are struggling for finding new approaches to fight cancer and many researchers are involved in this process to find new drug candidates. **Aims:** The current study aimed at investigating the new marine natural products with anticancer potential from the three Persian Gulf *Holothuria* sea cucumbers. **Methods:** We evaluated the cytotox activity of different organs of three *Holothuria* sea cucumbers species (*H. scabra*, *H. parva*, and *H. leucospilota*) using organic extract (OE): n-Hexane (nH), ethyl acetate (E), and methanol (M). Cytotoxicity potential of three fractions was estimated using two toxicity models: brine shrimp (*Artemia salina*) lethality assay (BSA) and tetrazolium-based colorimetric assay (MTT) assay in human cancer cell lines (MCF-7) and normal cell lines (HeLa). **Results:** The data illustrated that toxicity depends on concentration but BSA was highest for the M extracts of cuvierian tubules (CT) organs of *H. leucospilota* (up to 95% at 1000 µg/ml, LC_{50} = 616.4 µg/ml) and respiratory tree (RT) organs of *H. parva* (up to 86% at 1000 µg/ml, LC_{50} = 607.2 µg/ml). Based on cell lines, the more effective extracts were noticed for E fractions of CT organs of *H. leucospilota* (up to 85% at 250 µg/ml, LC_{50} = 37.25 µg/ml) against MCF-7 and for E extracts of intestine tract (IT) organs of *H. parva* (up to 80% at 250 µg/ml, LC_{50} = 46.25 µg/ml) against HeLa cells. This variation indicates that the possible cytotoxic compounds in fractions are selective toxicity toward cell lines. **Conclusion:** The data demonstrated that *Holothuria* species are an interesting source for discovery of drugs.

Key words: Biological activity, Cell line, Persian Gulf, Sea cucumber, Secondary metabolites

Introduction

Research on the use of marine natural products as pharmaceutical agents has been steadily increasing because biodiversity of the marine environment far exceeds that of the terrestrial environment (Suarez-Jimenez *et al*., 2012). Among these, sea cucumbers (class Holothuroidea), mostly have bioactive secondary metabolites and the medicinal potential for drug discovery (Bordbar *et al*., 2011). Sea cucumbers are marine invertebrates of the phylum Echinoderm known as trepang, beche-de-mer, or gamat, found in the benthic areas and deep sea floor worldwide (Althunibat *et al*., 2009), and are important as food and folk medicine systems, particularly in some parts of Asia (Taiyeb-Ali, 2003; Bordbar *et al*., 2011). Sea cucumbers have an impressive profile of unique bioactive molecules and the medicinal potential for screening the source of valuable anticancer and anti-proliferative (Roginsky *et al*., 2004; Soltani and Baharara, 2014), antitumor (Wang *et al*., 2014; Seydi *et al*., 2015; Assawasuparerk *et al*., 2016), anti-angiogenic (Tian *et al*., 2005), antimicrobial (Mokhlesi *et al*., 2012; Mohammadizadeh *et al*., 2013a, b; Mashjoor and Yousefzadi, 2017) and antioxidant (Althunibat *et al*., 2009) compounds. In sea cucumber medicinal benefits are linked to the presence of a wide array of bioactive compounds, especially triterpene glycosides (saponins), glycosaminoglycan (GAGs), sulfated polysaccharides, sterols (glycosides and sulfates), phenolics, cerberosides, lectins, peptides, glycoprotein, glycosphingolipids and essential fatty acids (Nguyen *et al*., 2007; Han *et al*., 2009, 2010, 2012a, b; Bordbar *et al*., 2011; Caulier *et al*., 2013).

Cytotoxic agents are the traditional therapies that damage cancer cells by interfering with DNA replication or its precursor, inhibiting the cellular division (Mazzaferro *et al*., 2013). Although this types of agents has the great drawback of killing healthy cells along with cancer cells and side effects associated with their usefulness (Fitch *et al*., 2010). Hence, biomedical researchers are trying to identify and develop new types of natural products containing effective and safe anticancer agents, therefore nowadays uses of sea cucumbers due to their potential health benefits to humans are attracting much attention among consumers and scientists. According to previous studies, in an effort to search for new marine natural products with anticancer potential from Persian Gulf species of holothurian, in the present study, we have aimed to investigate cytotoxicity activities of the organic crude extract of different organs or tissues of *Holothuria* species (*H. leucospilota*, *H. parva*, and *H. scabra*) using cytotoxic brine shrimp (*Artemia salina*) assay (BSA) and *in vitro* cytotoxic methods against the MCF-7 (human breast adenocarcinoma) and HeLa cell lines. To the best of our knowledge, this is the first report that deals with the study of the anticancer potential of the Iranian *Holothuria* sea cucumbers for medicine and pharmaceutical development.

Materials and Methods

Collection of sea cucumbers

Holothuria leucospilota usually lives in quiet and

deep areas on the sandy bottom or on coral rubble or under the rock. The specimens of *H. leucospilota* were collected by scuba diving (6-9 m depth) from the seabed of Nakhiloo Island, Persian Gulf, Iran. *Holothuria parva*, is morphologically described as about 22 cm long, brownish in colour and lighter colour in ventral side and ventral mouth and its habitat is rocky shores in the intertidal zone, as the specimen was usually hidden under stones (Dabbagh, 2011). On the northern coast of Qeshm Island (Persian Gulf), the most harvested sea cucumber species is the sand fish, *H. scabra* (Afkhami *et al*., 2012). They are usually elongated, worm-like organisms, with a leathery skin, soft flexible gelatinous body, looking like a cucumber (Figs. 1A-C). Habitually, they tend to live in deep regions of sea (Conand, 1990). Live specimens of the sea cucumber, *H. parva* and *H. scabra* were obtained at the low tide time (according to the tide time table obtained from www.tides4fishing.com, www.tide-forecast.com) by catching from the coast of Bandar-e Lengeh and Qeshm Island, respectively. For anaesthetization and rapid killing sea cucumber samples were sacrificed in chilled water (AVMA, 2013) and transported in ice box to the laboratory of University of Hormozgan to dissect the organisms and to separate the organs and tissues. Samples were rinsed with distilled water to remove debris, sand, salt, epiphytes, and foreign particles, then dried with filter paper, weighed and finally anatomized for the collection of target organs. All samples were taxonomic identified according to the characteristics and identification keys in the taxonomic publications (Hickman, 1998; Kerr and Kim, 2001; Samyn *et al*., 2006) using ossicles that were extracted from skin pieces of the mid-dorsal and mid-ventral body wall (BW). In order to extract the ossicles a small piece of each sample was placed into the commercial bleaching liquid for almost 30 min (Hickman, 1998). One drop of liquid was spread on the glass slide, and photographs were taken to confirm the species (Samyn *et al*., 2006). Following identification, all organs and tissues of samples were discarded (Figs. 1D-E) and divided into gonads (G), BW, intestine tract (IT), respiratory tree (RT), coelomic fluid (CF), and cuvierian tubules (CT) and maintained at -20°C for subsequent lyophilization.

Processing and extraction

Lyophilized samples of G, BW, IT, RT, CF, and CT were cut into small pieces and then extracted (at the ratio of 3:1 (v/w)) with solvents of different polarity: n-Hexane (nH), ethyl acetate (E), and methanol (M) 99.99% (Merck, Darmstadt, Germany). The mixture was soaked and kept at room temperature for 4 days (48 h for each solvent). The tissue extract of sea cucumbers was removed after squeezing and filtered through a 0.45 µm sterile Whatman filter paper (CamLab, Cambridge, UK). After filtration, the extracts were evaporated at low pressure by using a rotary evaporator at 35°C. Then the supernatant residue of each sample was concentrated and the dried extracts were weighed successively and the yield of each extract was calculated. For another analysis, samples were stored in the dark at 4°C.

Brine shrimp cytotoxicity

Cytotoxicity using BSA lethality assay, as a convenient monitor for screening the most effective extracts was used to determine the toxicity effects of the

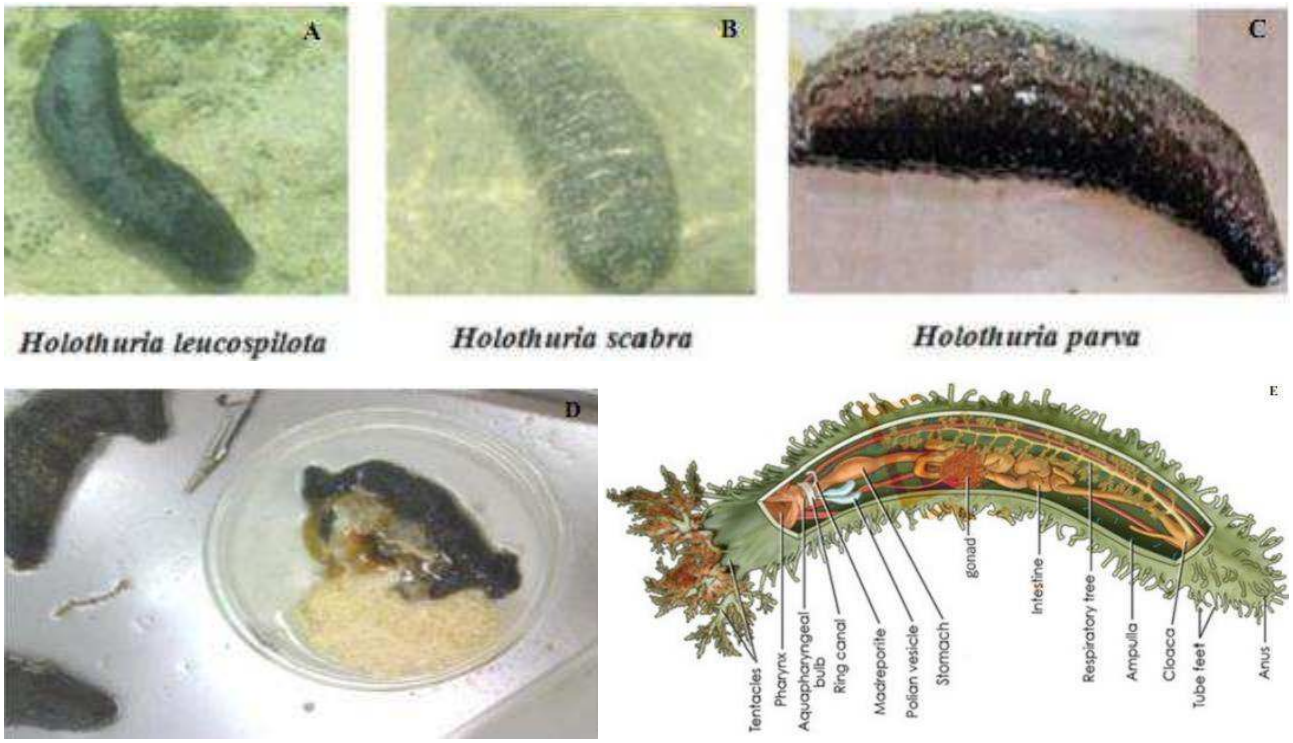

Fig. 1: Three species of marine *Holothuria* species, in the Persian Gulf (**A-C**), dissection their internal organs (**D**), and anatomy of the *Holothuria* sea cucumbers (**E**) (http://www.interiordesign.live/holothuroidea-anatomy.html)

nH, E and M extracts from different organs of the three *Holothuria* sea cucumbers (*H. leucospilota*, *H. parva*, and *H. scabra*) against zoological organism, BSA according to the method (Meyer *et al*., 1982). For this purpose, BSA cysts (Oji Art Industries, Japan) were hatched in artificial seawater (3.5% NaCl solution) for 48 h. The freshly hatched phototrophic larvae, called nauplii were collected with a pipette and transferred (20 nauplii) to vials filled with sea water (2 ml) in the 24-well plates. Different concentrations (125, 250, 500, and 1000 mg/ml) of each extract were prepared by dissolved sea cucumbers extracts in dimethyl sulfoxide (DMSO) and incorporated into the vials containing sea water (pH = 8.8; salinity = 35%) and *A. salina.* Four concentrations of each extract were tested thrice and a control DMSO was done each time and vials were maintained under illumination. The number of surviving nauplii were counted after 24 h and the mortality percentage at each dose and control (DMSO and seawater) was determined as the absence of controlled forward motion during 30 s of observation. Percentage mortality was calculated by the following formulae:

% Mortality = Number of dead nauplii/Initial number of live nauplii × 100

The mean lethal concentration (LC_{50}) after 24 h, for various test concentrations of different sea cucumbers extracts was calculated by plotting the results as a log of % mortality vs log concentration.

In vitro cytotoxic activity

Cell cultures

One human cancer cell lines, MCF-7 and normal cell lines of HeLa, were obtained from National Cell Bank of Iran (Pasteur Institute, Iran). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco Grand Island, NY) and 1% penicillin streptomycin, at 37°C, in humidified air containing 5% CO_2.

Determination of cell viability by tetrazolium-based colorimetric assay (MTT) assay

The MTT was performed in each cancer cell line to determine cell viability. The colorimetric assay is based on the conversion of the yellow tetrazolium bromide to the purple formazan derivative by mithochondrial succinate dehydrogenase in living cells. The mitochondrial metabolism of 3-(4, 5-dimethylthiazol-2-y1)-2, diphenyltetrazolium bromide (MTT) salt into formazan took place and the amount of produced formazan was correlated with the number of viable cells present. When the cell lines reached ~90% confluency they were detached with 0.05% trypsin/EDTA and seeded in 96-well microtitre plate (200 µL/well) at a density of approximately 2×10^4 cells/well in RPMI medium supplemented with 10% FBS and were subsequently incubated at 37°C in a 5% CO_2 humid incubator (Shokrgozar *et al*., 2007; Ming *et al*., 2008). After reaching confluence (24 h), the cells were washed with PBS and then exposed to different concentrations (µg/ml) of the nH, E and M extracts from different organs of the three *Holothuria* sea cucumbers (each group was done in triplicate wells) for 24 h. At the end of incubation, l0 µL (5 mg/ml in PBS) of MTT dye solution was added to each well for 4 h at 37°C. After removal of the MTT dye medium, cells were treated with 100 µL DMSO and eventually, the absorbance at 570 nm was quantified using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Ming *et al*., 2008). The percentage of cell viability was calculated according to the formula:

Viability % = (Mean assay absorption test/Mean negative control absorption) × 100

The cytotoxicity of the extract is expressed as the concentration of drug inhibiting cell growth by 50% (LC_{50}) and was calculated after comparing with the control (treated with 0.1% DMSO) and measured using the formula:

% Cell inhibition (CI) = [1-(optical density of sample/optical density of control)] × 100

Experimental design and statistical analysis

All experiments were conducted in triplicate. The SPSS 19.0 (IBM, SPSS) software package for Windows was used for analysis of a variance of the raw data. All data are reported as mean±SD and by using the Duncan's multiple range tests in ANOVA, comparisons among multiple groups and least significant difference (LSD) test were performed. Values of $P<0.05$ were assumed significant. Lethal concentration of 50 values, in the general toxicity assay, was calculated by linear regression analysis with Microsoft Excel program.

Results

The cytotoxicity activities of the nH, E, and M extracts of different organs of *Holothuria* sea cucumbers, *H. leucospilota*, *H. parva*, and *H. scabra* (Figs. 1A-C), by different concentration, were tested in different models: BSA as a convenient monitor for the cytotoxic screening and two human cancer cell lines (MCF-7 and HeLa). Among the extracts under this study, the M extracts of three sea cucumbers species showed relatively active cytotoxic effect in BSA assay (Table 1), but potential anticancer activity on different cell lines displayed weak activity (Table 2). However, the study of the growth inhibitory effects of sea cucumbers E and followed by nH fractions of three sea cucumbers species using the MTT assay showed relatively high potential anticancer activity on human cell lines (Table 2). Results of ANOVA analysis showed a significant difference ($P<0.05$) between the means of cytotoxic activity of nH, E, and M extracts of various concentrations in three studied *Holothuria* species.

Brine shrimp toxicity

Brine shrimp lethality test was performed. Newly hatched *A. salina* was incubated for 24 h, with 4 different

Table 1: Cytotoxic activity of *Holothuria* sea cucumbers species extracts of different organs by brine shrimp lethality assay. Control group (treated with DMSO+0 µg/ml of solvent) with no cytotoxic effect

Organism	Extract	Organ	Concentrations (µg/ml)				LC_{50}
			125	250	500	1000	
H. parva	nH	BW	1.44 ± 2.51^{b}	4.04 ± 3.50^{b}	9.51 ± 1.50^{b}	54.26 ± 2.22^{a}	987.9 ± 10.67
H. parva	E	BW	4.76 ± 8.25^{b}	7.54 ± 4.53^{b}	5.65 ± 2.90^{b}	49.05 ± 2.85^{a}	-
H. scabra	M	BW	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	6.06 ± 1.05^{b}	32.44 ± 1.27^{a}	-
H. leucospilota	M	RT	0.0 ± 0.0^{b}	2.08 ± 3.60^{b}	6.06 ± 2.25^{a}	84.24 ± 1.41^{a}	567.46 ± 2.47
H. parva	M	RT	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	39.37 ± 2.58^{b}	86.67 ± 2.31^{a}	607.26 ± 2.89
H. leucospilota	M	CF	1.59 ± 2.74^{b}	3.19 ± 2.87^{b}	9.04 ± 1.19^{b}	83.82 ± 1.41^{a}	724.73 ± 1.75
H. leucospilota	M	CT	1.59 ± 2.74^{d}	4.42 ± 3.97^{c}	32.34 ± 2.08^{b}	95.23 ± 8.25^{a}	616.46 ± 4.32

nH: n-Hexane, E: Ethyl acetate, M: Methanol, BW: Body wall, RT: Respiratory tree, CF: Coelomic fluid, and CT: Cuvierian tubules. Data expressed as mean value±SD (n=6). Inactive or non-lethal by LC_{50}>1000 (-), the values are significantly different (P<0.05) by different letters in the same column

Table 2: The LC_{50} (µg/ml) values for each organs fractions of *Holothuria* sea cucumber species against cell lines. Data represent the inhibitory concentrations (µg/ml) of nH, E, and M fractions of sea cucumbers organ extracts: BW, IT, G, RT, CF, and CT

Extract	Organs	MCF-7			HeLa		
		H. leucospilota	*H. parva*	*H. scabra*	*H. leucospilota*	*H. parva*	*H. scabra*
n-Hexane	BW	121.2 ± 9.74^{a}	88.63 ± 5.74^{d}	182.5 ± 12.41^{a}	189.09 ± 11.31^{a}	139.23 ± 10.85^{a}	116.5 ± 9.92^{a}
	IT	81.4 ± 4.36^{d}	120.2 ± 9.14^{b}	109.9 ± 8.36^{c}	60.0 ± 2.78^{e}	68.66 ± 3.74^{d}	59.3 ± 2.41^{d}
	G	109.5 ± 8.16^{b}	93.92 ± 6.24^{d}	-	110.2 ± 7.36^{b}	92.30 ± 6.21^{b}	-
	RT	82.17 ± 5.36^{d}	166.25 ± 12.14^{a}	153.07 ± 9.14^{b}	108.95 ± 8.92^{b}	146.66 ± 12.87^{a}	109.16 ± 8.46^{b}
	CF	95.11 ± 5.11^{c}	106.6 ± 8.32^{c}	142.5 ± 10.65^{b}	84.11 ± 5.95^{d}	89.09 ± 5.92^{b}	95.8 ± 5.67^{c}
	CT	75.27 ± 3.87^{e}	94.61 ± 5.16^{d}	-	100.83 ± 8.46^{c}	73.84 ± 5.49^{c}	-
Ethyl acetate	BW	121.5 ± 11.92^{a}	74.61 ± 4.32^{c}	166.85 ± 12.36^{a}	120.2 ± 9.10^{a}	129.5 ± 10.78^{a}	101.11 ± 6.48^{a}
	IT	60.35 ± 2.28^{d}	92.5 ± 5.18^{a}	61.7 ± 3.21^{c}	45.6 ± 2.39^{d}	46.25 ± 2.12^{e}	60.3 ± 3.24^{d}
	G	76.56 ± 3.33^{c}	53.6 ± 2.45^{d}	-	61.42 ± 3.23^{d}	70.90 ± 4.33^{b}	-
	RT	83.5 ± 4.87^{b}	86.47 ± 4.13^{b}	124.25 ± 9.92^{b}	100.9 ± 7.14^{b}	127.5 ± 9.34^{a}	91.81 ± 4.16^{b}
	CF	37.25 ± 1.92^{e}	65.3 ± 3.36^{c}	>250	61.3 ± 3.36^{c}	59.23 ± 3.41^{c}	85.38 ± 5.86^{c}
	CT	79.25 ± 3.77^{b}	83.4 ± 4.11^{b}	-	115.5 ± 7.34^{a}	78.5 ± 5.79^{c}	-
Methanol	BW	>250	>250	>250	221.5 ± 12.74^{a}	197.5 ± 12.32^{a}	142.3 ± 9.41^{c}
	IT	>250	>250	>250	154.6 ± 10.87^{c}	189.0 ± 15.41^{b}	120.2 ± 8.68^{d}
	G	154.08 ± 11.41^{a}	191.33 ± 10.25^{b}	-	172.85 ± 11.36^{b}	160.2 ± 10.35^{c}	-
	RT	98.66 ± 7.36^{b}	204.5 ± 12.36^{a}	>250	>250	>250	167.5 ± 11.25^{a}
	CF	>250	>250	>250	>250	>250	153.74 ± 9.10^{b}
	CT	>250	>250	-	>250	>250	-

nH: n-Hexane, E: Ethyl acetate, M: Methanol, BW: Body wall, IT: Intestine tract, G: Gonads, RT: Respiratory tree, CF: Coelomic fluid, and CT: Cuvierian tubules. Results are mean±SD of three replications. Inactive or non-lethal by LC_{50}>1000 (-), the values are significantly different (P<0.05) by different letters in the same column

dose levels and six replicates per dose. The data in Table 1 illustrate that toxicity towards BSA larvae in maximum concentrations (1000 µg/ml) were highest for the sea cucumbers methanolic extracts of CT organs of *H. leucospilota* (up to 95%, LC_{50} = 616.4 µg/ml), RT organs of *H. parva* (up to 86%, LC_{50} = 607.2 µg/ml), and RT organs of *H. leucospilota* (up to 84%, LC_{50} = 567.4 µg/ml). The larvae were less sensitive to the E extracts of BW organs of *H. parva* (LC_{50} = 2724 µg/ml) between all the sea cucumbers organic extracts (OEs). These would have been traceable only if present in the OEs at a concentration of about >1000 µg/ml. The results displayed the nH, E, and M extracts of other different organs of *Holothuria* species, had no toxicity by BSA at the concentrations tested and caused no deaths at 1000 µg/ml, this fraction was only toxic at higher concentrations with the assay method [as noted, data from higher extract concentrations (>1000 µg/ml) is not mentioned in the Table 1]. Of the toxins tested, methanolic extracts of CT and RT organs of *H. leucospilota* were more toxic to the larvae than other fractions; however, the LC_{50} values obtained for RT fractions of *H. leucospilota* were lower than another. In the BSA bioassay, the present data indicated the sea cucumbers extract by dose-dependent manner, exhibited a moderate cytotoxic activity.

Inhibitory effect of sea cucumbers extracts on the growth of MCF-7 cells

To study the possible toxic effect of the sea cucumbers OEs on cell growth, in the first set of experiments, MCF-7 cells were treated at gradient final concentrations (2-250 µg/ml) nH, E and M extract of three studied *Holothuria* species of different organs: BW, IT, G, RT, CF, CT, and DMSO as control for 24 h incubation period. Experiments were conducted to assess the cell survival using MTT assay. In the all of the nH, E, and M extracts of different organs of *Holothuria* sea cucumbers, *H. leucospilota*, *H. parva*, and *H. scabra*, the viability of MCF-7 cells decreased rapidly in a dose-dependent manner. The results in Table 2 and Fig. 2 revealed a significant cytotoxic behavior in the cancer

cell cultures that in maximum concentrations (250 µg/ml) was only noticed for the sea cucumbers E extracts of CF organs of *H. leucospilota* (up to 85%, LC_{50} = 37.25 µg/ml), as compared to other extracts of *Holothuria* organs. As seen in Fig. 3, this extract among other *Holothuria* species organ extracts against two cell lines (MCF-7 and HeLa) caused the highest cytotoxic effect. Hence, this fraction was identified as exhibiting moderately strong anticancer effects on MCF-7 cells. But also after that, the nH extracts of CT organs (up to 89%, LC_{50} = 75.27 µg/ml), and the nH extracts of BW organs of *H. parva* (up to 88%, LC_{50} = 88.63 µg/ml), as compared to other extracts of *Holothuria* organs (Table 2; Fig. 2), caused the cytotoxic effect.

The less inhibitory effects of the sea cucumber extracts against the MCF-7 cell line were recorded in methanolic extracts of RT organs of *H. parva* (33.31% at 250 µg/ml and, LC_{50} = 481.25 µg/ml) (Table 2; Fig. 2).

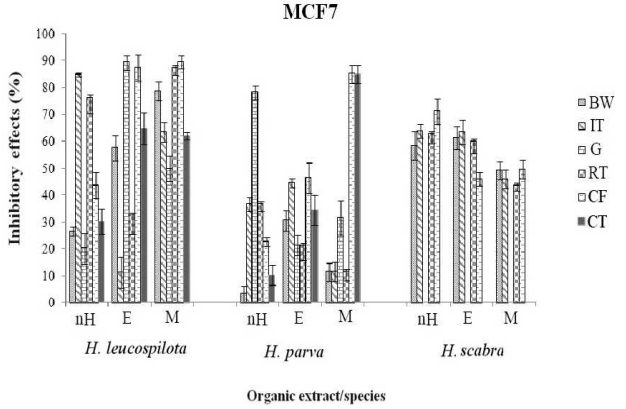

Fig. 2: The inhibitory effect of the n-Hexane (nH), ethyl acetate (E), and methanol (M) fractions of each *Holothuria* sea cucumber species organ extracts: body wall (BW), intestine tract (IT), gonads (G), respiratory tree (RT), coelomic fluid (CF), and cuvierian tubules (CT) at maximum concentrations (250 µg/ml) against MCF-7 cell line. Values expressed as mean±SD of triplicate experiments

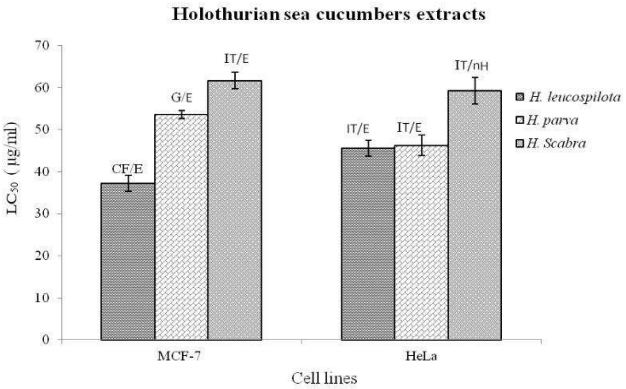

Fig. 3: The LC_{50} values for the most active cytotoxic fractions of each *Holothuria* sea cucumber species against cell lines. Data represent the inhibitory concentrations (µg/ml) of the ethyl acetate (E), and n-Hexane (nH) fractions of sea cucumbers organ extracts: intestine tract (IT), gonads (G), and coelomic fluid (CF). Values expressed as mean±SD of triplicate experiments

All of the OEs of *H. scabra* G and CT organs did not show any antiproliferative activity on MCF-7 cells with increasing the concentration of the extract (Table 2). Toxicity assessment based on the species showed the most effective organ extracts of species, *H. leucospilota* on MCF-7 cells by LC_{50} values belongs to the E extracts of CF organs (LC_{50} = 37.25 ± 1.92 µg/ml), for *H. parva* dependent on the E extracts of G organs (LC_{50} = 53.6 ± 2.45 µg/ml), and for the *H. scabra* species belongs to the E extracts of IT organs (LC_{50} = 61.7 ± 3.21 µg/ml) (Table 2; Fig. 2).

Inhibitory effect of sea cucumbers extracts on the growth of HeLa cells

Investigation the dose-dependent effects of *Holothuria* sea cucumbers extracts on proliferation of HeLa cells measured spectrometrically by ELISA reader, demonstrated that all of the OEs of different organs of treated *Holothuria* species showed a dose-dependent decrease in viability compared with the control group. The data were exhibited that the highest cytotoxic effect in maximum concentrations (250 µg/ml) of organic *Holothuria* extract against HeLa cell lines, dependent on the E extracts of IT organs of *H. parva* (up to 80%, LC_{50} = 46.25 µg/ml), followed by the nH extracts of CF organs of *H. leucospilota* (up to 78%, LC_{50} = 84.11 µg/ml), as compared to other sea cucumbers extracts (Table 2; Fig. 4). As seen in Fig. 3, the *Holothuria* sea cucumbers extracts caused relatively weaker cytotoxic effect against HeLa cell lines, than the other studied human cancer cell lines (MCF-7) at high concentrations. The lowest cytotoxic effects of the sea cucumber extracts against the HeLa cell line were recorded in methanolic extracts of CF organs of *H. parva* (39.07% at 250 µg/ml and, LC_{50} = 372.47 µg/ml) (Table 2; Fig. 4). The OEs of *Holothuria scabra* G and CT organs did not show any cytotoxic activity on HeLa cells dose dependently of extract relative to the untreated control groups (Table 2). The LC_{50} reliance on the species showed that the most

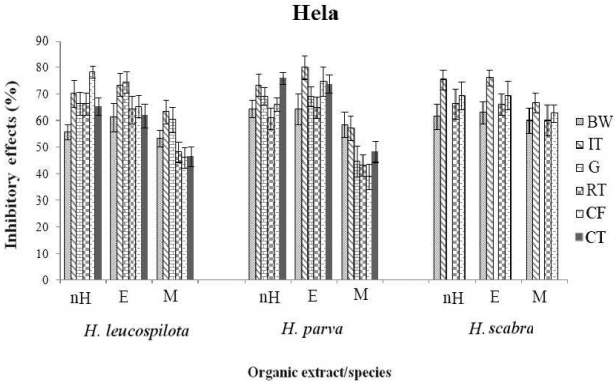

Fig. 4: The inhibitory effect of the n-Hexane (nH), ethyl acetate (E), and methanol (M) fractions of each *Holothuria* sea cucumber species organ extracts: body wall (BW), intestine tract (IT), gonads (G), respiratory tree (RT), coelomic fluid (CF), and cuvierian tubules (CT) at maximum concentrations (250 µg/ml) against HeLa cell line. Values expressed as mean±SD of triplicate experiments

effective organ extracts of species, *H. leucospilota* and *H. parva* on HeLa cells proliferation belongs to the E extracts of IT organs (LC_{50} = 45.6 ± 2.39 and 46.25 ± 2.12 µg/ml, respectively), and for the *H. scabra* species dependent on nH extracts of IT organs (LC_{50} = 59.3 ± 2.41 µg/ml) (Table 2; Fig. 4).

Discussion

Identification and development the natural products that exist in marine invertebrates function as anticancer drugs, especially cytotoxic agents, led to the development of anticancer therapeutics for several decades. Anticancer drugs are divided into two categories: cytotoxic (cell killing), cytostatic (biological and hormonal agents as cell stabilizing) drugs and both agents lead to a reduction in the size of the tumour and inhibit one or more stages of carcinogenesis by preventing or delaying cancer development. The molecular mechanisms of anticancer compounds include the induction of tumor cell apoptosis by the activation of intracellular caspase cell death pathways, arrest of the cell cycle at S or G2/M phases, influence on nuclear factors, NF-κB, and up-down regulation of certain cellular receptors and enzymes participating in cancerogenesis, such as epidermal growth factor receptor (EGFR), Akt (protein kinase B), extracellular signal-regulated kinases (ERKs) or classical mitogen activated protein (MAP) kinases, focal adhesion kinase (FAK), matrix metalloproteinase-9 (MMP-9) and others (Aminin *et al*., 2015).

Sea cucumbers are reported to contain several compounds with anticancer and antiproliferative properties (Bordbar *et al*., 2011). This functionality of sea cucumber extracts might be ascribed to the presence of notable amounts of total phenols and flavonid compounds which are important as effective antioxidants to protect from oxidative stress and degenerative diseases such as certain cancers (Althunibat *et al*., 2009). To our knowledge and through our effort to search for newly marine natural products from Persian Gulf *Holothuria* sea cucumbers, in this study, we have reported the investigation of the anticancer activity of the organic crude extract of different organs of *Holothuria* species (*H. leucospilota*, *H. parva*, and *H. scabra*) using *in vitro* cytotoxicity models: BSA as a convenient monitor for the cytotoxic screening and different human cancer cell lines (MCF-7 and HeLa) are shown in Tables 1 and 2. Of the three types of solvents used to perform the crude extraction, M extracts only in BSA test and E, followed by nH fractions, showed high potential concerning cytotoxicity activities in all studied cancer cell lines. In the first step of this study, for the primary screening of the most effective cytotoxic extracts using BSA lethality assay (as a rapid and simple method), we found that methanolic extracts of CT (LC_{50} = 616.4 µg/ml) and RT (LC_{50} = 607.2 µg/ml) organs of *H. leucospilota* by depending on concentrations were more toxic to the larvae than other fractions and overall exhibited a moderate cytotoxic activity. These findings agreed with the report of Mohammadizadeh *et al*. (2013a, b) that exhibited the M extract of the G of *H. scabra* and *H. leucospilota* from Persian Gulf (Qeshm Island) by BSA test showed the highest cytotoxic effect (LC_{50} = 50.5 and 40.31 mg/ml, respectively) continuing with RT, M extract (LC_{50} = 69.96 and 72.49 mg/ml, respectively). Their suggested methalonic extraction is a good solvent system to the solubility of the bioactive compounds present in *H. scabra.* Furthermore, Sarhadizadeh *et al*. (2014) reported water-methanol and M extracts of BW (LC_{50} = 109.76 and 144.82 µg/ml, respectively) continuing with E extract from cuvierian organ (LC_{50} = 196.29 µg/ml) extracts of sea cucumber, *S. hermanni* from Persian Gulf (Qeshm Island) exhibited cytotoxic activities on *A. salina*. Albuntana *et al*. (2011) showed cytotoxic effects of sea cucumber, *H. leucospilota* crude extracts from Jakarta (Seribu Islands) on BSA demonstrated water fraction is the most active fraction by LC_{50} 50.968 µg/ml. Layson *et al*. (2014) suggested the chloroform extract of *H. nobilis* that demonstrated cytotoxic (*Artemia*) activity by LC_{50} of less than 10 ppm may be considered to contain antitumor agents since the standard set by the National Cancer Institute (NCI) of the US for a bioactive compound to be an effective antitumor agent is equal to or less than 30 ppm (30 µg/ml). Their result showed from aqueous, M, chloroform and hexane extracts of seven Philippine echinoderms, only sea cucumber samples exhibited antitumor activity. In case of *in vitro* cytotoxicity activity the results in the current study exhibited that, of all the nH, E, and M extracts of different organs of *Holothuria* sea cucumbers, *H. leucospilota*, *H. parva*, and *H. scabra*, the viability of MCF-7 and HeLa cells decreased rapidly with concentrations. Previous researches on cytotoxic properties of *Holothuria* sea cucumbers extracts were mostly focused on the M fraction but the result of present study showed the more effective extracts were noticed for E fractions, especially for E extracts of CF organs of *H. leucospilota* (up to 85%, LC_{50} = 37.25 µg/ml) on cell growth MCF-7 cells as compared with other sea cucumbers organ extracts against three cell lines. The result indicated that this fraction caused the highest cytotoxic effect in studied cancer cell lines and reinforced the notion of the presence of compounds with profound anticancer potential. As mentioned, this extract appears to also have potent toxic and close value (LC_{50} = >30 µg/ml) to the standard anticancer drugs that were introduced by NCI.

Breast cancer is a malignant tumor that starts in the cells of the breast and the second leading cause of cancer death in women with greater than 1,300,000 cases and 450,000 deaths each year worldwide (Cancer Genome Atlas Network, 2012). Breast tumors are classified according to the location of origin to ductal tumors that develop in breast ducts (80%), lobular tumors that develop inside the lobes (10-15%) and other subtypes which represent less than 10% of cases diagnosed per year (Vargo-Gogola and Rosen, 2007). Toxicity assessment results in the current study based on cell lines (human breast adenocarcinoma, MCF-7) exposed to

Holothuria OEs, due to efficiency of LC_{50} value near the 30 µg/ml standard showed the most active cytotoxic fractions recorded for E extracts of CF organs of *H. leucospilota* (LC_{50} = 37.25 µg/ml), E extracts of G organs of *H. parva* (LC_{50} = 53.6 µg/ml), and the E extracts of IT organs of *H. scabra* (LC_{50} = 61.7 µg/ml) as compared to control (Table 2; Fig. 2). Hence, only E extracts of CF organs of *H. leucospilota* fraction were identified as exhibiting profound anticancer effects on MCF-7 cells by LC_{50} value close to NCI standard.

The inhibitory effects of *Holothuria* sea cucumbers extracts on the growth of HeLa cells showed that the most effective organ extracts belong to the E extracts of IT organs of *H. leucospilota* and *H. parva* (LC_{50} = 45.6 and 46.25 µg/ml, respectively), and the nH extracts of IT organs of *H. scabra* (LC_{50} = 59.3 µg/ml) (Table 2; Fig. 4). However, the data indicated IT organs of each of the three *Holothuria* species have anticancer activity potent on HeLa cells by LC_{50} value to some extent close to NCI standard. Overall findings demonstrated that Persian Gulf *Holothuria* sea cucumbers contain a range of natural anticancer levels in the OEs, but this study exhibited various cancer cell lines were not markedly affected to the same extent when exposed to the same fraction and, for example, the greatest impact of the anticancer activity was shown in the MCF-7 cells. This variation indicates that the possible cytotoxic compounds that are present in fractions are selective toxicity toward different cell lines and in the case of preferable cancer treatment suggested a specific mode of action (Alves *et al*., 2015). These differences between species can result from a variety of their natural habits, geographical ecological locations and may be due to adaptation strategy to thrive in the sea environment (Ridzwan *et al*., 1995). These results were in agreement with others' findings of sea cucumber natural products and their effects were discrepant in activities against various cancers *in vitro* and *in vivo* models (Janakiram *et al*., 2015). Ehsanpour *et al*. (2015) reported aqueous extracts (AEs) of sea cucumbers *H. parva* on human MCF-7 cancer cells showed cytotoxicity activity. Furthermore, Soltani and Baharara (2014) reported that the dichloromethane fraction of *H. leucospilota* exhibited antiprolifereative capacity against MCF-7 and A549 cancer cell lines and they found MCF7 cell was more sensitive to the dichloromethan extracts than A549 cells. Althunibat *et al*. (2009) investigated the cytotoxic effects of aqueous and OEs from three species of sea cucumber, *H. scabra*, *H. leucospilota* and *S. chloronotus* on the proliferation of two human cancer cells: A549 (human non-small lung carcinoma) and C33A (cervical cancer cells). Their finding showed only AE of *S. chloronotus* exhibited antiproliferative activity against C33A cells (LC_{50} = 10.0 µg/ml) and AE fraction from *H. leucospilota* and *H. scabra* displayed no notable action on the growth of the tested cancer cell lines. Based on this evidence, the OE of this species offered higher antiproliferative action against cancer cells. The LC_{50} values as a parameter for cytotoxic action of OE from *H. scabra* and *S. chloronotus* on A549 and C33A cells were determined as 15.5, 3.0, 21.0, and 6.0 µg/ml, respectively. Althunibat *et al*. (2009) considered that the antitumor functionality of sea cucumber extracts might be ascribed to the presence of considerable amounts of phenols and flavonoids compounds. Wang (2014) examined cytotoxic and apoptosis-inducing activity of triterpene glycosides of sea cucumber, *H. scabra* on HepG2 cells and the results showed these compounds could affect their cytotoxicity towards tumor cells by significantly inhibited cell viability and induced apoptosis in HepG2 cells.

The data demonstrated that these *Holothuria* species (*H. leucospilota*, *H. parva*, and *H. scabra*) from Persian Gulf are an interesting source of natural compounds with anticancer potential and pave the way for discovery of new marine natural products. Further studies are already in progress for identification and purification of possible active compounds present in the most effective fractions: E extracts of CF organs of *H. leucospilota* (against MCF-7) and E extracts of IT organs of *H. parva* (against HeLa) and other more cytotoxic E and nH fractions of these three studied species that may be responsible for selective toxicity against the tumor cells.

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