Indian Journal of Traditional Knowledge
Vol 18(2), April 2019, pp 272-280

Assessment of anticancer potential of selected *Holothuria* species

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Received 24 November 2018; revised 19 February 2019

All pharmaceutical fields are concerned about increase in cancer incidence throughout the world. Therefore, the discovery of new substances from natural origin to produce the cytotoxic drugs is required. For this purpose, we evaluated the anticancer activity of three *Holothuria* sea cucumbers species (*Holothuria scabra*, *H. parva* and *H. leucospilota*) from the Persian Gulf, Iran, of their extract from different organs, such as gonads (G), body wall (BW), intestine tract (IT), respiratory tree (RT), coelomic fluid (CF) and cuvierian tubules (CT) using organic solvents of n-Hexane (n-Hex), ethyl acetate (EtOAc) and Methanol (MeOH). Then cytotoxicity potential of each fraction was estimated using MTT assay to comparison of cell viability of human cancer (Caco-2) vs normal cell lines (HeLa). The data illustrated that toxicity toward cell lines (Caco-2) was only noticed for EtOAc extracts of BW organs of *H. parva* (up to 92% at 250 µg/mL, IC_{50}=16.78 µg/mL), followed by EtOAc extracts of CF organs of *H. scabra* (up to 88% at 250 µg/mL, IC_{50}=24.36 µg/mL). While, the more effective extracts was noticed against HeLa cells was detected for EtOAc extracts of IT organs of *H. Parva* (up to 80% at 250 µg/mL, IC_{50}=46.25 µg/mL). Significant cytotox potential were found in this study, which may be linked to the presence of possible anticancer compounds in chosen fractions and selective toxicity toward different cell lines.

Keywords: Cell-based assays, MTT, Persian Gulf, Sea cucumber, Toxic effect

IPC Code: Int. Cl.[19]: C12Q 1/6839, A61K 38/00, A61K 31/737, A01N 25/32

Cancer is one of the diseases characterized by out-of-control cell growth that is the leading cause of death in the developed world[1]. Occurrence of certain mutations in DNA leads to cancer by disrupting the programming regulating processes. The process of transformation of normal cells to cancer cells is known as carcinogenesis. It is characterized by a progression of changes at cellular and genetic level that reprogram a cell to form a malignant tumor[1]. Currently, the intervention in cancer diseases involves chemotherapy and surgery that eliminate cancer tissues[2]. There is an increase in the number of cancer drugs that exhibit a relatively very potent drugs show serious side effects[3]. Cytotoxic agents are the traditional therapies that damage cancer cells by interfering with DNA replication or its precursor, inhibiting the cellular division[4]. The anticancer molecular mechanisms include the induction of tumor cell apoptosis through 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 EGFR (epidermal growth factor receptor), Akt (protein kinase B), ERK (extracellular signal-regulated kinases), FAK (focal adhesion kinase), MMP-9 (matrix metalloproteinase-9) and others[5]. Although anticancer agents have the great drawback of killing healthy cells along with cancer cells and side effects associated with their usefulness[6]. Hence, biomedical researchers are trying to identify and develop new types of natural products contain effective and safe anticancer agents, therefore nowadays uses of sea cucumbers due to their potential health benefits to humans are attracting much attention

Studies on natural compounds from marine environment have been steadily increasing because global distribution of the marine biota far exceeds that of the terrestrial environment[1]. Among, sea cucumbers (class Holothuroidea), mostly have bioactive secondary metabolites and the medicinal potential for drug discovery[7]. Sea cucumbers are marine invertebrates of the phylum Echinoderm that found in the benthic areas and deep sea floor worldwide[8], which are important as food and folk medicine systems, particularly in some parts of Asia[7,9]. Sea cucumbers have an impressive profile of unique bioactive molecules and the medicinal

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potential for screening the source of valuable anticancer and anti-proliferative[10-11], antitumor[12-14], anti-angiogenic[15], antimicrobial[16-19], antioxidant[8], immunomodulatory[20] compounds. In sea cucumber medicinal benefits linked to the presence of a wide array of bioactive especially triterpeneglycosides (saponins), glycosaminoglycan (GAGs), sulfated polysaccharides, sterols (glycosides and sulfates), phenolics, cerberosides, lectins, peptides, glycoprotein, glycosphingolipids and essential fatty acids[7,8,21-26]. It has been shown that some of these natural products suppress the proliferation of various human tumor cell lines *in vitro* such as U-87-MG, HCT-8, leukemia P-388, KB, Schabel, Mel-28, A-549, MICF-1, HT-29, IA9, CAKI-1, SK-MEL, PC-3, lymphoidal leukemia L 1210, MCF-7, MKN-28, HCT-116, U87MG, HepG2, HeLa, THP-1, KB-VIN, HCT-8, C33A, and some others[5]. In this regard, the investigation showed that new active secondary metabolites isolated from polar extracts of sea cucumbers species that possess effective cytotoxicity against human tumor cell lines such as the human leukemia (HL-60, MOLT-4)[22], human lung cancer (A-549)[8,22], human hepatoma (BEL-7402) cells[22], human breast adenocarcinoma (MCF-7)[27], C33A (cervical cancer cells)[8] and increased stimulation of lysosomal activity in mouse macrophages[28]. Considering that sea cucumbers are an interesting source for newly marine natural products and since *Holothuria* species has a global distribution, in the present study, we have aimed to examine the anticancer potential of the organic crude extract of different tissues of *Holothuria* species (*H. leucospilota*, *H. parva* and *H. scabra*) by evaluating cytotoxicity in human cancer cell lines Caco-2 (heterogeneous human epithelial colorectal adenocarcinoma cells) and normal cells (HeLa).

Materials and methods

Collection of animals

H. leucospilota usually lives in quiet and deep areas on the sandy bottom or on coral rubble or under the rock from which alone exceeds the front. The specimens of *H. leucospilota* collected by scuba diving (6-9 m depth) from the seabed of Nakhiloo Island, Persian Gulf, Iran. *H. parva*, as morphological description has about 22 cm long, brownish colour skin and lighter colour in ventral side of its body and ventral mouth. Its habitat is rocky shores in the intertidal zone, as the specimen was usually hidden under stones[29]. On the northern coast of Qeshm Island (Persian Gulf), the most harvested sea cucumber species is the sand fish, *H. scabra*[30]. They are usually elongated, with a leathery skin and gelatinous body. Habitually, they tend to live in deep regions of sea.[31] 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[32] and transported in ice box to the laboratory of Hormozgan University to dissect the organisms and to separate the organs and tissues. Samples were rinsed with distilled water to remove debris, sand, salts, 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[33-35] using ossicles that were extracted from skin pieces of the mid-dorsal and mid-ventral body wall. In order to extract the ossicles a small piece of samples was placed into the commercial bleaching liquid for almost 30 min[35]. One drop of liquid was spread on the glass slide and photographs were taken to confirm the species[23]. Following identification, all of sea cucumber samples were discarded and their tissues divided into gonads (G), body wall (BW), intestine tract (IT), respiratory tree (RT), coelomic fluid (CF) and cuvierian tubules (CT). Different organs of *Holothuria* maintained at -20°C for subsequent lyophilization.

Processing and Extraction

Lyophilized samples of gonads, body wall, intestine tract, respiratory tree, coelomic fluid and cuvierian tubules were cut into small pieces and then extracted (at the ratio of 3:1 (v/w)) with solvents of different polarity: n-Hexane, ethyl acetate and methanol 99.99% (Merck, Darmstadt, Germany). The mixture was soaked and kept at room temperature for 4 days (48 h for each solvent). The crude 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 successively the dried extracts were weighed and the yield of each extract was calculated. For another analysis, samples were stored in the dark at 4°C.

In vitro cytotoxic activity

Cancer Cell Lines

Two human cancer cell lines of Caco-2 and normal cell lines of HeLa were obtained from National cell bank of Iran (Pasteur institute, Iran). Cells were cultured in 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 MTT assay

The tetrazolium-based colorimetric assay (MTT) was performed in the 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 were detached with 0.05% trypsin/EDTA and seeded in 96-well microtitre plate (200 µL/well) at a density of approximate 2 $\times 10^4$ cells/well in RPMI medium supplemented with 10% FBS and were subsequently incubated at 37°C in a 5% CO_2 humid incubator[36-37]. After reaching confluent (24 h), the cells were washed with PBS and then exposed at gradient final concentrations (2,4,8,15,31,62,125 and 250 µg/mL) of the n-Hexane, ethyl acetate and methanol extracts from different organs (G, BW, IT, RT, CF, and CT) of the three holothurians 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 ELISA Microplate reader[37]. The percentage of cell viability was calculated according to the formula:

Viability %= (Mean assay absorption test / Mean negative control absorption) ×100 … (2)

The cytotoxicity of the extract is expressed as the concentration of drug inhibiting cell growth by 50% (LC_{50}) 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 … (3)

Experimental design and statistical analysis

All experiments were conducted in triplicate. The SPSS 19.0 (IBM, SPSS) software package for Windows was used to analyze 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 were performed and Least Significant Difference (LSD) test. Values of $p<0.05$ were assumed significant. Lethal concentration 50 values, in the general toxicity assay, were calculated by linear regression analysis with Microsoft Excel program.

Results

The cytotoxicity activities of the n-Hexane (n-Hex), ethyl acetate (EtOAc) and Methanol (MeOH) extracts of different organs of holothurians sea cucumbers, *H. leucospilota*, *H. parva* and *H. scabra* by different concentration, were tested on human cancer and normal cell lines (Caco-2 and HeLa) (Fig. 1). In this study, the methanol extracts of three seacucumbers species showed relatively weak activity (Table 1). However, the study of the growth inhibitory effects of sea cucumbers EtOAc and followed by n-Hexfractions of three sea cucumbers species using the MTT assay showed relatively high potential anticancer activity on human cell lines, especially on Caco-2 cells (Table 1). Results of ANOVA analysis showed a significant difference ($p<0.05$) between the means of cytotoxic activity of n-Hex, EtOAc and MeOH extracts of various concentrations in three studied *Holothuria* species.

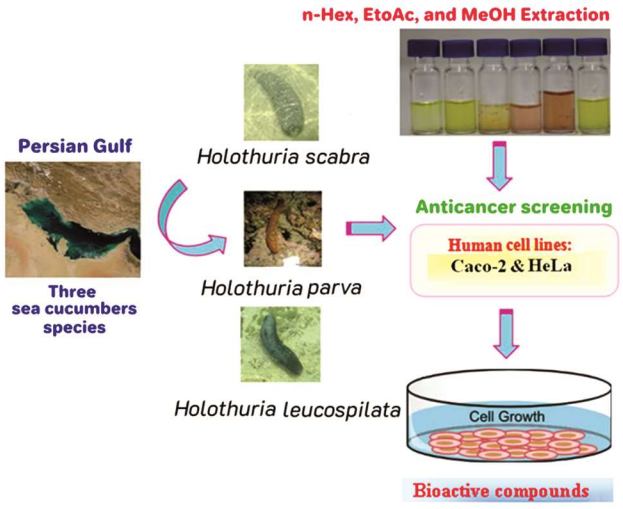

Fig. 1 — Schematic illustration of the *in vitro* cell based assay of anticancer agents in relation to *Holothuria* species from the Persian Gulf, Iran.

Table 1 — The LC_{50} (µg/mL) values for each organs fractions of *Holothuria* species against cell lines. Data represent, the inhibitory concentrations (µg/mL) of then-Hexane (n-Hex), ethyl acetate (EtOAc) and Methanol (MeOH) fractions of seacucumbers organ extracts: Body wall (BW), Intestine tract (IT), Gonads (G), Respiratory tree (RT), Coelomic fluid (CF) and Cuvierian tubules (CT).

Extract	Organs	Caco-2			HeLa		
		H. leucospilota	*H. parva*	*H. scabra*	*H. leucospilota*	*H. parva*	*H. scabra*
n-Hexane (n-Hex)	BW	107.62±7.25^{b}	34.1±2.39^{e}	30.5±1.74^{b}	189.09±11.31^{a}	139.23±10.85^{a}	116.5±9.92^{a}
	IT	30.5±1.92^{e}	106.7±8.32^{a}	28.31±1.21^{b}	60.0±2.78^{e}	68.66±3.74^{d}	59.3±2.41^{d}
	G	42.69±2.23^{d}	98.5±4.92^{b}	-	110.2±7.36^{b}	92.30±6.21^{b}	-
	RT	132.60±9.95^{a}	49.33±2.79^{c}	42.30±2.74^{a}	108.95±8.92^{b}	146.66±12.87^{a}	109.16±8.46^{b}
	CF	91.78±6.36^{c}	24.93±1.71^{f}	30.88±1.65^{b}	84.11±5.95^{d}	89.09±5.92^{b}	95.8±5.67^{c}
	CT	43.80±2.31^{d}	40.72±2.65^{d}	-	100.83±8.46^{c}	73.84±5.49^{c}	-
Ethyl acetate (EtOAc)	BW	112.44±7.92^{a}	16.78±0.9^{d}	59.28±4.31a	120.2±9.10^{a}	129.5±10.78^{a}	101.11±6.48^{a}
	IT	30.93±1.01^{e}	27.67±2.32^{c}	53.16±3.20^{b}	45.6±2.39^{d}	46.25±2.12^{e}	60.3±3.24^{d}
	G	45.61±2.75^{d}	32.45±2.52^{b}	-	61.42±3.23^{d}	70.90±4.33^{b}	-
	RT	63.1±3.69^{b}	62.2±3.14^{a}	51.5±2.92^{b}	100.9±7.14^{b}	127.5±9.34^{a}	91.81±4.16^{b}
	CF	55.84±2.23^{c}	>250	24.36±2.01^{c}	61.3±3.36^{c}	59.23±3.41^{c}	85.38±5.86^{c}
	CT	45.20±2.87^{d}	>250	-	115.5±7.34^{a}	78.5±5.79^{c}	-
Methanol (MeOH)	BW	>250	44.78±2.45^{b}	60.25±4.74^{a}	221.5±12.74^{a}	197.5±12.32^{a}	142.3±9.41^{c}
	IT	>250	44.91±3.74^{b}	55.90±3.78^{b}	154.6±10.87^{c}	189.0±15.41^{b}	120.2±8.68^{d}
	G	115.4±7.24^{b}	44.64±2.92^{b}	-	172.85±11.36^{b}	160.2±10.35^{c}	-
	RT	192.2±9.87^{a}	56.79±4.21^{a}	57.02±3.36^{b}	>250	>250	167.5±11.25^{a}
	CF	>250	51.03±3.85^{a}	60.52±4.11^{a}	>250	>250	153.74±9.10^{b}
	CT	>250	26.48±1.31^{c}	-	>250	>250	-

Results are mean ± SD of three replications. Inactive (-), the values are significantly different ($p<0.05$) by different letters in the same column

Inhibitory effect of seacucumbers extracts on the growth of Caco-2 cells

The possible cytotoxicity effect of the sea cucumbers organic extracts was also evaluated using normal human cell lines of HeLa and heterogeneous human epithelial colorectal adenocarcinoma cells (Caco-2). The cells were treated at gradient final concentrations (2–250 µg/mL) n-Hex, EtOAc and MeOH extract of three studied *Holothuria* species of different organs: body wall, intestine tract, gonads, respiratory tree, coelomic fluid, cuvierian tubules 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 n-Hex, EtOAc and MeOH extracts of different organs of sea cucumbers, *H. leucospilota*, *H. parva* and *Holothuria scabra*, the viability of Caco-2 cells decreased rapidly with concentrations.

The results in Tables 1 and Fig. 2 revealed a significant cytotoxic behavior in the cancer cell cultures in maximum concentrations (250 µg/mL) was only noticed for the seacucumbers EtOAc extracts of BW organs of *H. parva* (up to 92%, IC_{50} = 16.78 µg/mL) as compared to other extracts of holothurians organs. As seen in Fig. 2,5 this extract among other seacucumbers organ extracts against two cell lines (Caco-2 and HeLa) caused highest cytotoxic effect.

Hence, this fraction was identified as exhibiting profound anticancer effects on Caco-2 cells. But also after that, the EtOAc extracts of *H. parva* gonads organs (up to 90%, IC_{50}=32.45 µg/mL), intestine tract organs (up to 89%, IC_{50}=27.67 µg/mL), the n-Hexextracts of coelomic fluid organs (up to 88%, IC_{50}=24.93 µg/mL), body wall organs (up to 89%, IC_{50}=34.1 µg/mL), and the n-Hexcuvierian tubules fractions of *H. leucospilota* (up to 89%, IC_{50}=43.8 µg/mL), as compared to other extracts of holothurians organs exhibited a maximum cytotoxic activity (Table 1; Fig. 2). The less cytotoxic effects of the holothurians extracts against the Caco-2 cell line were recorded in MeOH extracts of cuvierian tubules organs of *H. leucospilota* (22.9% at 250 µg/mL and, IC_{50}=1611.1 µg/mL) (Table 1; Fig. 2). All of the n-Hex, EtOAc and MeOH extracts of *H. scabra* G and CT organs did not show any cytotoxic activity on Caco-2 cells with increasing the concentration of the extract (Tables 1). So, there might be low levels of toxic compounds present in this species.

Toxicity assessment based on the species showed that the most effective organ extracts of species,

H. leucospilota on Caco-2 cells by the half maximal inhibitory concentration (IC_{50}) belongs to the n-Hex and EtOAc extracts of intestine tract organs (IC_{50}=30.5±1.92 and 30.93±1.0 µg/mL, respectively) as compared to control (Table 1; Fig. 2,5). The most promising of the IC_{50} values for the *H. parva* organ extracts, on Caco-2 cells was obtained from the EtOAc extracts of body wall organs (IC_{50}=16.78±0.9 µg/mL) (Table 1; Fig. 2,5), followed by then-Hex extracts of coelomic fluid and MeOH extracts of cuvierian tubules organs (IC_{50}=24.93±1.71 and 26.48±1.31 µg/mL, respectively) (Tables 1). In this regard, for the *H. scabra* species, this pick belongs to the EtOAc extracts of coelomic fluid organs (IC_{50}=24.36±2.01 µg/mL) (Table 1; Fig. 2,5), followed by the n-Hex extracts of intestine tract organs (IC_{50}=28.31±1.21 µg/mL) (Tables 1). As shown Fig. 3, there was a significant loss of viable Caco-2 cells when treated with organic extracts of *H. parva* dissolved in DMSO. At high concentration of seacucumbers organic extracts, the cell proliferation was inhibited and morphological changes seen in the cells after one day of exposure (Fig. 3b).

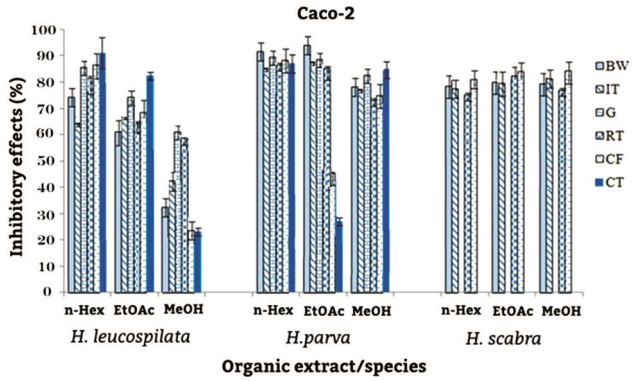

Fig. 2 — The inhibitory effect of then-Hexane (n-Hex), ethyl acetate (EtOAc) and Methanol (MeOH) fractions of each *Holothuria* species organ extracts: Body wall (BW), Intestine tract (IT), Gonads (G), Respiratory tree (RT), Coelomic fluid (CF) and Cuvierian tubules (CT)at maximum concentrations (250 µg/mL) againstCaco-2cell line. Values expressed as mean ±SD of triplicate experiments.

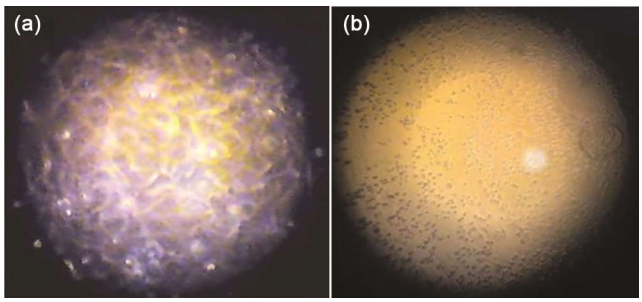

Fig. 3 — Morphology of unexposed control cultures of human Caco-2 cells (a), and exposed Caco-2 cultures only for EtOAc extracts of BW organs of *H. parva* after 24 h exposure (b).

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

Cytotoxic activity of the different organs of treated *Holothuria* species on proliferation of normal cells such as HeLa cells was tested by ELISA reader. The results showed a dose-dependent decrease in viability of HeLa cells compared with the control group. As seen in Fig. 5, the holothurians sea cucumbers extracts did not markedly affect to HeLa cell, where a caused relatively weaker cytotoxic effect than the other studied human cancer cell lines (Caco-2) at high concentrations. The

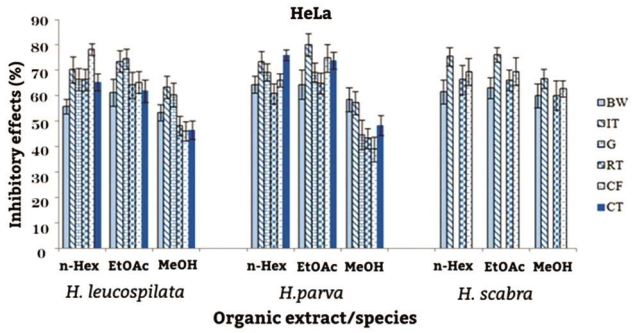

Fig. 4 — The inhibitory effect of then-Hexane (n-Hex), ethyl acetate (EtOAc) and Methanol (MeOH) fractions of each *Holothuria* species organ extracts: Body wall (BW), Intestine tract (IT), Gonads (G), Respiratory tree (RT), Coelomic fluid (CF) and Cuvierian tubules (CT) at maximum concentrations (250 µg/mL) against HeLa cell line. Values expressed as mean ±SD of triplicate experiments.

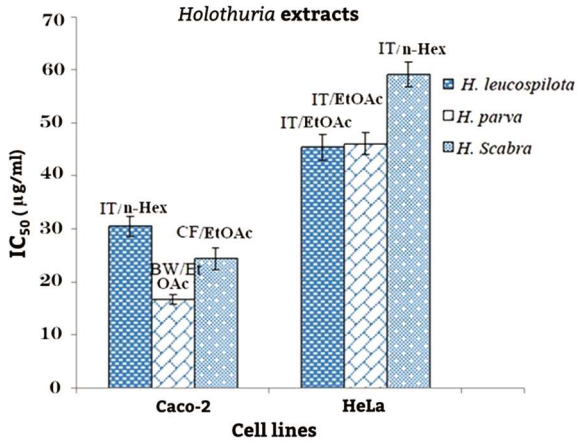

Fig. 5 — The LC_{50} values for the most active cytotoxic fractions of each *Holothuria* species against cell lines. Data represent the inhibitory concentrations (µg/ml) of the ethyl acetate (EtOAc) and n-Hexane (n-Hex) fractions of seacucumbers organ extracts: Intestine tract (IT), Gonads (G) and Coelomic fluid (CF).Values expressed as mean ±SD of triplicate experiments.

data were exhibited that the highest cytotoxic effect in maximum concentrations (250 µg/mL) of organic holothurians extract against HeLa cell lines, dependent to the EtOAc extracts of IT organs of *H. parva* (up to 80%, LC_{50}=46.25 µg/mL), followed by the n-Hex extracts of CF organs of *H. leucospilota* (up to 78%, LC_{50}=84.11 µg/mL), as compared to other sea cucumbers extracts (Table 1; Fig. 4).

In normal cells the results of LC_{50} differed from those obtained with cancer cell lines (Table 1), and in all fractions the rate of viable cells was markedly higher than in cancer cell lines. The lowest rate of viable HeLa cells was found in MeOH fraction of CF organs of *H. parva* (39.07% at 250 µg/mL and, LC_{50}=372.47 µg/mL) (Tables 1; Fig. 4). The organic extracts of *H. scabra* gonads and cuvierian tubules organs did not show any cytotoxic activity on HeLa cells with dose dependently of extract relative to the untreated control groups (Tables 1). The LC_{50} rely on the species showed that the most effective organ extracts of *H. leucospilota* and *H. parva* normal cells proliferation belongs to the EtOAc 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 to n-Hex extracts of intestine tract organs (LC_{50}=59.3±2.41 µg/mL) (Table 1; Fig. 4, 5).

Discussion

A large number of natural compounds that may be function as anticancer drugs for human cancer treatment have been identified from marine invertebrates. 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.

Colorectal cancer (CRC) is a *polyp*or *tumor* inside the colon or rectum that starts as small, noncancerous clumps of cells. Over time some of these polyps can turn into cancer. The accession of colorectal tumors is 30-50% after the age of 65 years, but patients with removed polyps is associated with developing new cases of colorectal polyps yet[38].

In effort to search for newly marine natural products from Persian Gulf holothurians sea cucumbers, in this study, three types of organic solvents used to perform the crude extraction. The results concerning cytotoxicity of organic crude extract of different organs of *Holothuria* species (*H. leucospilota*, *H. parva* and *H. scabra*) showed that EtOAc and followed by n-Hex fractions of *Holothuria* species was able to inhibit cell viability in studied colorectal cancer cell lines. The results in the current study exhibited that the viability of Caco-2 and HeLa cells decreased rapidly with concentrations of the n-Hexane, ethyl acetate and methanol extracts of different organs of holothurians seacucumbers, *H. leucospilota*, *H. parva* and *Holothuria scabra*. Layson et al[39]. suggested the chloroform extract of *H. nobilis* that demonstrated cytotoxic 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 or less than 30 ppm (30 µg/mL). Their result showed from aqueous, methanol, chloroform and hexane extracts of seven Philippine echinoderms, only sea cucumber samples exhibited antitumor activity.

The more effective sea cucumbers tissue organic extracts by cytotoxicity activity was only noticed for EtOAc extracts of body wall (BW) organs of *H. parva* (IC_{50}=16.78 µg/mL) on cell growth Caco-2 cells as compared among other seacucumbers organ extracts against HeLa cell lines. The result indicated that this fraction caused to highest cytotoxic effect in studied cancer cell lines and reinforcing the notion of the presence of compounds with profound anticancer potential. As mentioned, this extract also appears to have potent toxic and close value (IC_{50}=>30 µg/mL) to the standard anticancer drugs such as Taxol that was introduced by NCI.

The cytotoxic properties of glycosides, specifically saponin and saponin-like components isolated from sea cucumbers extracts were mostly focused in previous studies[7]. Toxicity assessment result in the current study based on Caco-2 cell lines by efficiency of LC_{50} value below the 30 µg/mL standard showed that after EtOAc extracts of BW of *H. parva*, the most effective organ extracts belongs to the EtOAc extracts of CF organs of *H. scabra* (IC_{50}=24.36 µg/mL), n-Hex extracts of CF and MeOH fraction of CT organs of *H. parva* (IC_{50}=24.93 and 26.48 µg/mL, respectively), n-Hex extracts of IT organs of *H. scabra* (IC_{50} = 28.31 µg/mL), n-Hex and EtOAc extracts of IT organs of *H. leucospilota* (IC_{50}=30.5 and 30.93 µg/mL, respectively) as compared to control (Table 1; Fig. 2). So the data showed the holothurians extract effects on Caco-2 cells exhibited an effective and hopefulness antitumor activity is less or equal than NCI standard.

The inhibitory effects of holothurians sea cucumbers extracts on the growth of HeLa cells showed that the most effective organ extracts belongs to the ethyl acetate extracts of IT organs of *H. leucospilota* and *H. parva* (LC_{50}=45.6 and 46.25 µg/mL, respectively), and the n-Hexane extracts of IT organs of *H. scabra* (LC_{50}=59.3 µg/mL) (Table 1; Fig.4). However the data indicated IT organs of each three *Holothuria* species have anticancer activity potent on HeLa cells by LC_{50} value to some extent close to NCI standard.

This indicates that the cytotoxic compounds are present in Persian Gulf holothurians sea cucumbers contain a range of natural anticancer levels. But, according to this study, various cell lines were not markedly affected to the same extent when exposed to the same fraction. 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[40] and this 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[41]. These results were in conformity with others findings of sea cucumber natural products and their effects were discrepant in activities against various cancers *in vitro* and *in vivo* models[42]. Ehsanpour et al.[27] reported aqueous extracts of whole body of sea cucumbers, *H. parva* on human MCF-7 cancer cells showed cytotoxicity activity[27]. Furthermore, Soltani and Baharara[11] reported that the dichloromethane fraction of *H. leucospilota* exhibited antiprolifereative capacity against MCF-7 and A549 cancer cell lines and their found MCF7 cell was more sensitive to the dichloromethan extracts than A549 cells. In addition, Souhaly and Rahayu[43] found that extracts of sea cucumber, *Bohadschia argus* have many other cytotoxic activity on human breast cancer cell line T47D with optimum concentration of water extraction at 480 µg/mL and 145 µg/mL for methanol extraction. Althunibat et al[8]. investigated the cytotoxic effects of aqueous and organic extracts from three species of sea cucumber, *H. scabra, H. leucospilota* and *S. chloronotus* on the proliferation of two human cancer cells: A549 and C33A. Their finding showed only aqueous extract (AE) of *S. chloronotus* showed 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. However, the organic extract (OE) of this species offered higher antiproliferative action against cancer cells, so that the OE cytotoxic action of *H. scabra* and *S. chloronotus* on A549 and C33A cells (LC_{50}= 15.5, 3.0, 21.0 and 6.0 µg/mL, respectively). Althunibat et al[8]. considered that the antitumor functionality of sea cucumber extracts might be ascribed to the presence of considerable amounts of phenols and flavonids compounds[12]. In a study, Wang[12] examined cytotoxic and apoptosis-inducing activity of triterpene glycosides of sea cucumber, *H. scabra* on HepG2 cells. Their results showed this componds could affect their cytotoxicity towards tumor cells by significantly inhibited cell viability and induced apoptosis in HepG2 cells. The presence of triterpene glycosides has been confirmed in other marine cucumber, such as Vietnamese sea cucumber *Stichopus horrens*[44]. As reported by Cuong et al[44]., four new triterpene diglycosides namely stichorrenosides A-D, were isolated from a methanol extract of *S. horrens*. This new compounds showed strong cytotoxicity against five human cancer cell lines, Hep-G2, KB, LNCaP, MCF7, and SK-Mel2. Therefore, high functionality of sea cucumber extracts might be ascribed to considerable amounts of natural-products such as phenols and triterpene glycosides in their body.

Conclusions

This study was conducted to discover new marine natural products of *Holothuria* species (*H. leucospilota*, *H. parva* and *H. scabra*) from Persian Gulf for consideration in therapeutic studies. Significant cytotoxic effects were found in the EtOAc extracts of body wall of *H. parva* against the tumor cells (Caco-2). Based on diversified chemicals with bioactivities, in continue, higher purification of possible active compounds present in the EtOAc and n-Hex fractions of selected sea cucumber species are necessary.

Acknowledgments

The authors are thankful to the Iran National Science Foundation (INSF, 92041203) for financial support of this project.

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