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Apoptosis inducing capacity of *Holothuria arenicola* in CT26 colon carcinoma cells *in vitro* and *in vivo*

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ABSTRACT

Objective(s): Sea cucumber is one of the classes of echinoderms, which is considered as a health marine product and possess various biological characteristics with therapeutic application. The present investigation attempted to evaluate the potential of anti-cancer Persian Gulf sea cucumber species *Holothuria arenicola (H. arenicola)* aqueous extract on mice colon carcinoma cells *in vitro* and *in vivo*.
Materials and Methods: The CT26 carcinoma cells were treated with various concentrations of extract in 24 and 48 hr, and then its anti-proliferative effect was measured by MTT assay and morphological observations. The apoptotic effect was examined by fluorescence microscopy (DNA fragmentation assay), Flow cytometry, caspase-3 and -9 colorimetric assays. The *in vivo* anti-tumor efficacy of sea cucumber extract on CT26 tumor cells transplanted in BALB/c mice was also investigated.
Results: The results showed that the water extract of sea cucumber revealed remarkable anti-proliferative effect on CT26 tumor cells with IC_{50}= 31 µg/ml with recruitment of intrinsic apoptotic pathway *in vitro*. In addition, the colon tumor volume in treated groups remarkably reduced in homozygous mice. Histopathological examination elucidated that sea cucumber extract attenuated tumor size and volume along with apoptosis characteristics. Moreover, RT-PCR analysis revealed that sea cucumber extract induced intrinsic apoptosis *in vivo* through suppression of Bcl-2 expression.
Conclusion: Our data confirmed this notion that sea cucumber administrates anti-cancer effect that can be used as complementary in preclinical experiments, so further characterization are recommended for detection sea cucumber metabolites and clinical application.

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Introduction

Cancer is one of the deadly cause of morbidity in the worldwide that affected people's lives and health (1). Colorectal carcinoma is the third most common form of cancer and the second leading reason of mortality among men and women globally which possess potency of extension to other parts of body including liver, lung, ovaries and other organs (2). The increasing rate of colorectal cancer undertake because high smoking, lack of physical activities, overweight, obesity, processed meat consumption, and excessive alcohol consumption (3).

The conventional therapeutic methods for colon cancer therapy have been included surgery, chemotherapy with 5-fluorouracil alone or concurrent with irinotecan, oxaliplatin, panitumumab, cetuximab or bevacizumab and other remedies such as radiotherapy, targeted therapy and immunotherapy. Meanwhile, limited antitumor activity of chemotherapeutic agents beside high incidence rate and recurrence rate of colon cancer have been proposed new approaches to the treatment of colorectal carcinoma (4, 5).

In this subject, combinational treatment in the presence of multiple current therapeutic strategies against colorectal cancer using chemotherapeutic lead derived from natural products have been offered alternative adjuvant related to colorectal cancer modalities (6). In recent years, the pharmacological application of natural products as a complementary or replacement therapy has been increased greatly (7). In the extensive oncological researches, the isolation and purification of biological active compounds from marine organisms

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has been increased due to discovery of efficient anti-tumor drugs with high biodiversity and minimum side effects, however, it is demonstrated that the whole natural extract provides the synergistic and antagonistic function of bioactive constituents in the extract which enhances the therapeutic efficacy (8).

Natural products indicate crucial function as chemotherapeutic agents by inducing apoptosis, which is a valuable defense mechanism and is characterized by obvious hallmarks such as reducing cell volume, membrane blebbing, DNA fragmentation and caspase activation (9, 10). Hence, researchers attempt to find novel apoptosis inducers against cancer cells (11).

Echinoderm are marine invertebrate composed of wide array of bioactive metabolites that play an important role in organism maintenance as defense system (12). Sea cucumbers are dietary food ingredients belonging to diverse class of echinoderm, found on the sea floor worldwide which proposed as rich source of substances with medicinal efficacy in folk medicine (13).

High therapeutic and economic values of sea cucumbers caused to name them various names such as ginseng in the sea, trepang, bechedemer, or gamat in the world (14). Numerous effects against hypertension, asthma, rheumatism and other injuries characterized their importance in traditional medicine (15). Bio-medicinal profits of sea cucumbers are attributed with the presence of valuable constituents include sterols, triterpene glycosides (saponin), sulfated polysaccharides, chondroitin sulfates, glycosaminoglycan, peptides and other secondary metabolites (16). There are two species of sea cucumber (*Holotheria leucospilata* and *Holotheria arenicola)* in sublittoral zone of Persian Gulf (17). Therefore, exclusive biological and pharmacological properties of sea cucumbers encouraged us to investigate the antitumor potential of Persian Gulf sea cucumber species of *H. arenicola* (with grey to yellow color dorsally with dark brown spots) against colorectal carcinoma *in vitro* and *in vivo*.

Materials and Methods

Reagents

CT-26 colorectal carcinoma cancer cells were purchased from NCBI (National Cell Bank of Iran). RPMI-1640 Medium, FBS (Fetal Bovine Serum), PBS, trypsin-EDTA and antibiotic (penicillin-streptomycin) were obtained from Gibco-USA. MTT (3-[4, 5- dimethyl thiazol-2-yl]-2, 5-diphenyl tetrazolium bromide), acridine orange, propodium iodide was purchased from sigma (USA). DAPI solution prepared from Applichem, USA. Caspase-3 and caspase-9 colorimetric assay kit were purchased from Abcam (England). The RNA isolation kit was purchased from Roche, Germany. The C-DNA synthesis kit and RT-PCR kit were purchased from Pars Tous, Mashhad, Iran. Ketamine/xylazine was prepared from Sigma (USA). Specimens of the sea cucumber (*H. arenicola*) were obtained from rocky intertidal flats of Persian Gulf waters. Methanol, were purchased from Merck (Germany). Homozygous BALB/c mice were purchased from Pasteur institute of Tehran, Iran.

Preparation of sea cucumber extract

H. arenicola collected from Bandar Abbas and transported to Research Center Applied Biology of Mashhad Islamic Azad University. After being sliced open and its internal organs removed, the body wall of Sea cucumber were washed with tap water and stored at −80 ˚C. For extract preparation, sea cucumber samples (about 20 g) dried, minced with distilled water. The mixture was continuously stirred in the dark at 4 ˚C for 24 hr. Then, it was filtered and supernatant mixed with 200 ml methanol. Then, the extract constantly stirred (72 hr) to room temperature, refluxed, filtered through an 11 µm Whatman filter and concentrated under vacuum evaporator (Heidolph, Germany) and stored in −20 ˚C (18).

Cell culture

The CT26 Cell line obtained from NCBI were cultured in RPMI-1640 medium supplemented with 10 % FBS, 1 % penicillin/streptomycin and were incubated at 37°C in a humidified 5 % CO_2 incubator.

MTT assay

CT26 cells (2×10^4) were seeded in a 96- well plate and incubated for 24 hr. The cells were then were treated with various concentrations of sea cucumber extract (15, 31, 62, 125, 250 µg/ml) for 24 hr and 48 hr, then MTT solution (5 mg/ ml in phosphate buffered saline) was added to each well and maintained at 37 ˚C in the dark for 4 hr. Finally, DMSO was added to dissolve the formazan crystals. The absorbance of each well was measured at a wavelength of 560 nm with an Epoch spectrophotometer (USA) and the cell viability was considered according to the next formula:

Cell viability (%) = Absorbance in test wells/ Absorbance in control wells×100

The IC_{50} (50% inhibition dosage) of extract was determined as the concentration which reduced 50% cell growth (19).

Acridine Orange/ Propodium Iodide Staining

For morphological assessment, the CT26 cancer cells treated with 20, 40, 100 µg/ml of extracts for 48 hr were visualized under fluorescence microscope. To perform this method, the untreated and the treated cells were harvested, rinsed with PBS and then 10 µl acridine orange (100 µg/ml) and propodium iodide (100 µg/ml) (1:1) was added. Then the cells visualized under a fluorescence microscope (Olympus, Japan) for determination of the cells undergoing apoptosis (20).

DAPI staining

DAPI (4', 6-diamidino-2-phenylindole dihydrochloride) staining conducted to evaluate the morphology of the nuclei. The cells (1.5×10^5) were seeded in the 6-well plate and treated with different concentrations of extracts and were incubated for 48 hr. Cells were observed with inverted microscope for evaluating morphological changes. Then, the cells were washed twice with PBS and DAPI was added and incubated for 10 min in the dark. Finally, the cells were washed twice with PBS and suspended in 1000 µl of methanol and the morphology of nuclei was observed under fluorescence microscopy (Olympus, Japan) (21).

Flow cytometry analysis

The cells (1.5×10^5) were cultured in 6-well plates. After 48 hr treatment, were removed culture medium and was added 700 µl PI solution to untreated and treated cells and then maintained in the incubator for 20 min in the dark. Eventually supernatant transferred into separate microtubes and then analyzed by FACS caliber flow cytometry instrument (Bekton Dickinson, USA) (22).

Caspase-3 and Caspase-9 assay

In order to detect apoptotic pathway induced, caspase-9 or caspase-3 colorimetric Bio Assay Kit (Abcam-UK) were used on the basis of the company's protocol. CT26 cells ($2\text{-}5\times10^6$) were treated with cytotoxic concentrations 31, and 62 µg/ml of extract for 48 hr. The control and the experimental cells harvested and mixed with 50 µl of cell lysis buffer and kept on ice for 10 min. Subsequently, 50 µl of 2X reaction buffer (containing 10 mM DTT) was added to each the supernatants cytosolic extract. Finally, to complete reaction, 5 µl of substrates (4 mM) (200 µM final concentration) were added and after 1-2 hr incubation at 37 ˚C, the absorbance of samples were read at 405 nm (23).

Experimental animals

Male Balb/C mice (Five-week-old) were purchased from Pasteur Institute of Iran. The mice were maintained under standard conditions (temperature of 22-26 ˚C, 45-50% relative humidity with water and food *ad libitum* sterile diet under pathogen-free environment and maintained on a 12 hr light/dark photo period) (6).

In vivo anti-tumor assessment

To create an allograft colon carcinoma model, CT-26 mouse colon cancer cells were grown under appropriate conditions. 1.5×10^6 cells were suspended in 0.2 ml PBS and were injected subcutaneously into the right flank of BALB/c mice (n=6). After tumor inoculation (approximately 7 days) the animals randomly divided into 4 groups of 6 mice.

The mice were assigned into group A (control) and treatment groups received sea cucumber extract dissolved in PBS and were divided into group B (50 mg/kg sea cucumber extract), group C (100 mg/kg sea cucumber extract), group D (400 mg/kg sea cucumber extract). Control group administrated PBS orally duration treatment period and experimental groups received different concentration of sea cucumber extract via oral administration (gavage) once daily for 2 weeks. The mice were euthanized at the end of 14 days treatment period and the tumors were excised and weighted. The tumor volumes were measured according to below formula: $a\times b^2 \times 0.5$ where "a" is length, "b" is a width. The histopathological examination was conducted by H&E staining to confirm sea cucumber treatment effect (24).

Assessment the mRNA expression of Bcl-2

RNA was isolated from untreated and treated cells using High Pure RNA Isolation kit (Roche, Germany) following the manufacturer's protocol. Easy cDNA Synthesize (Pars Tous, Iran) Kit was used to synthesize cDNA from 100 ng of RNA. RT- PCR was performed using Taq PCR master mix (Pars Tous, Iran) following the fast thermal cycling conditions: 95 ˚C for 5 min and 40 cycles of 95 ˚C for 15 sec and 60 ˚C for 1 min. The sequences of primers used are as follows: B2M Forward5' TGGTGCTTGGCTCACTGACC 3', Reverse 5' TATGTTCGGCTTCCCATTCT 3' was used as the housekeeping gene. The forward primer and reverse primer Bcl-2 was designed as 5'CATGTGTGT GGAGAGCGTCAAC 3' and 5' CAGATAGGCACCCAGGG-TGAT3 3', respectively. Following amplification, The PCR products were subjected to electrophoresis in a 2% agarose gel and visualized by green viewer staining.

Statistical analysis

All results expressed as mean ± SD and performed at least triplicate. The statistical significance was evaluated by SPSS software, one-way ANOVA, Tukey test and $P \leq 0.05$ was considered significant.

Results

Cytotoxic assay

Assessment of the growth inhibitory effect of sea cucumber aqueous extract on CT26 cell proliferation revealed that increasing dosages of sea cucumber extract were disruptive against colon carcinoma. As shown in Figure 1, sea cucumber aqueous extract suppressed cancer cell survival in a concentration dependent manner in 24 and 48 hr, so that treatment concentration of 40 µg/ml considered IC_{50} value.

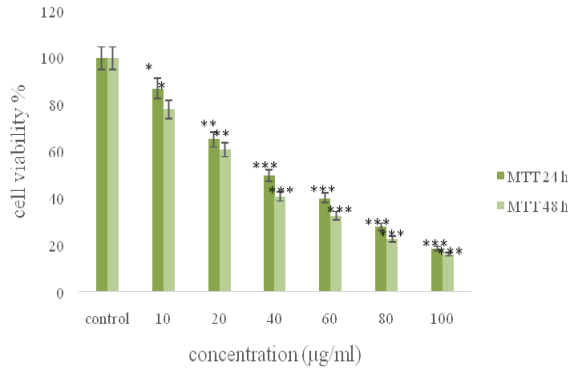

Figure 1. The effect of sea cucumber aqueous extract on cell viability of CT26 cells within 24, 48 hr treatment which examined by MTT assay. Values provided as mean±SD and **P*<0.05, ***P*<0.005 and ****P*<0.001 were considered significant

Morphological evaluations

Morphological assessment of CT26 cells exhibited the morphological characteristics of apoptosis cell death such as reduction of cell volume, chromatin condensation, cell membrane blebbing, and apoptotic bodies formation so that CT26 cells lost elongated shape and converted to circle forms visualized by inverted microscopy (Figure 2).

After incubation of cells with inhibitory concentration of extract, treated cells stained with DAPI or AO/ PI to distinguish type of cell induced. As shown in the figure 3 treated cells exhibited apoptotic features in IC_{50} value and necrotic cell death occurred in higher concentration (Figure 3b). DAPI staining displayed DNA fragmentation and nuclear apoptosis and confirmed apoptotic cell death in IC_{50} concentration in CT26 treated cells (Figure 3a).

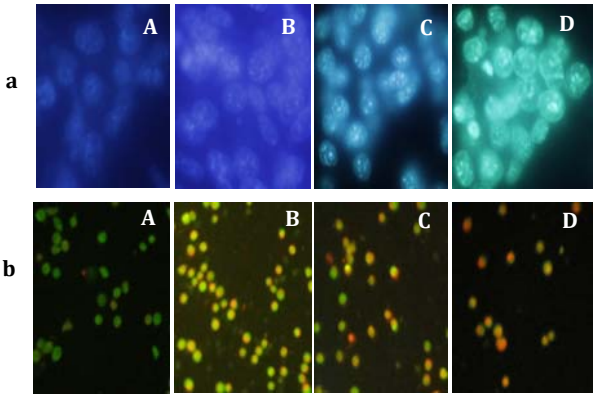

Figure 3. a) Fluorescence images of AO / PI stained CT26 colon cancer cells treated with sea cucumber extract. A) Untreated cells with intact nuclei (green color) B) treatment with 20 µg/ml, C) treatment with 40 µg/ml and D) treatment with 100 µg/ml sea cucumber extract. As shown, yellow, orange color is signs of apoptosis. b) DAPI staining exhibited chromatin fragmentation and nuclear disintegration. A) Untreated cells with normal nuclei, B) 20 µg/ml, C) 40 µg/ml and D) 100 µg/ml treatment with sea cucumber extract

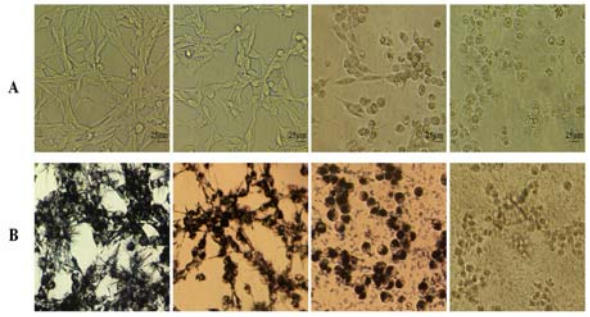

Figure 2. Morphological evaluation of CT26 colon cancer cells treated with sea cucumber extract. A) left to right; untreated cells, CT26 cells treated with 20, 40, 100 µg/ml extract for 48 hr, respectively. B) The same classification after MTT assay, as shown in Figure 2B, formazan crystals are abundant in control group and duration treatment with enhancement dosage of sea cucumber extract, decreased formazan crystals

Detection of apoptosis by PI flow cytometry

Flow cytometry assay by propodium iodide conducted to indicate apoptosis by DNA fragmentation. The sub G1 peak determined cell apoptosis. Our observations demonstrated that 40 µg/ml of sea cucumber water extract enhanced sub-G1 peak as compared to untreated cells which is contributed with the correlation of cytotoxic effects of sea cucumber extract with apoptosis against CT26 cells (Figure 4).

Determination of apoptotic pathway induced by measurement of Caspase -3 and -9 activity

To determine the type of apoptotic pathway involved, the level of caspase activation was calculated colorimetrically (Figure 5). Our results elucidated intrinsic apoptotic pathway induced by sea cucumber extract is responsible of this cytotoxicity due to increase in caspase-3 and caspase-9 activity in treated cells as a concentration-dependent manner as compared with control.

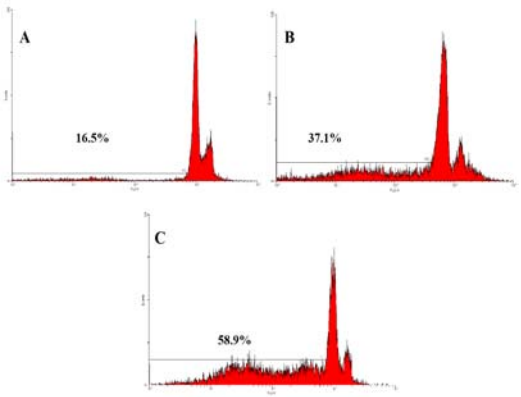

Figure 4. Flow cytometry analysis of untreated CT26 cells and with various concentrations of sea cucumber extract. A) CT26 cells without treatment, B) CT26 cells under treatment with 20µg/ml, C) CT26 cells exposed with 40µg/ml sea cucumber extract as IC_{50} value

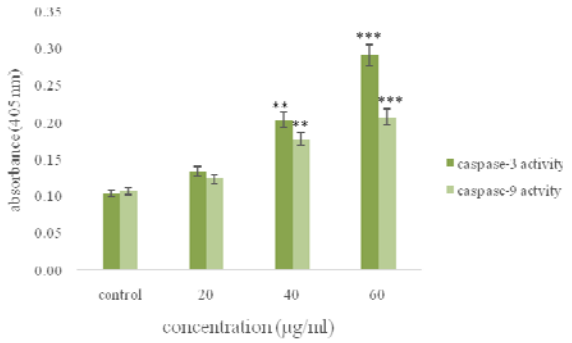

Figure 5. Effect of sea cucumber extract on caspase-3 and -9 activity in CT26 cells. Histogram represented caspase-3 and -9 activity in untreated cells and with 40, 60µg/ml sea cucumber extract

Reduction of CT26 implanted tumor growth

After elucidate *in vitro* cytotoxic effect of sea cucumber extract, we examined anti-tumor effect in colon cancer mouse model. As exhibited in the figure 6 no significant difference in body weight were visualized in treatment groups, but the oral administration of sea cucumber extract reduced tumor size and volume dose dependently as compared with control mice administrated with saline (Figure 6, 7). Further, as shown in Figure 6D, a reduced rate of mortality were exhibited in mice treated with100 and 400 mg/kg aqueous extract of *H. arenicola* which suggest that oral administration of sea cucumber extract correlates with prolonging the survival rate in the CT26 tumor-bearing mice. Beside, histological staining of tumor excised by H&E revealed apoptotic nuclei in colon carcinoma tissue in selective concentrations of sea cucumber extract (especially in 400 mg/ml). Since most chemotherapeutic drugs administrated orally, oral administration of sea cucumber extract introduced this natural product as a potent anti-tumor lead.

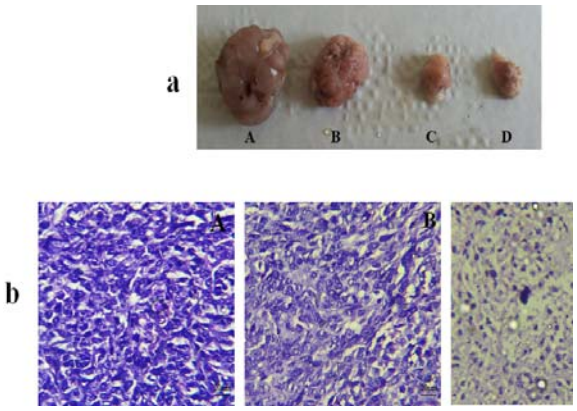

Figure 7. Effect of aqueous sea cucumber extract on CT26 tumor bearing mice. a) Morphological changes in tumor size A) control group and B, C, D) under treatment with sea cucumber extract (50, 100, 400 mg/kg body weight). b) Light microscopic observations on tumor structure (400×) of tumor cross sections in control group (A) and 100, 400 mg/kg experimental groups (B, C) respectively, indicating pyknotic nuclei in experimental group C

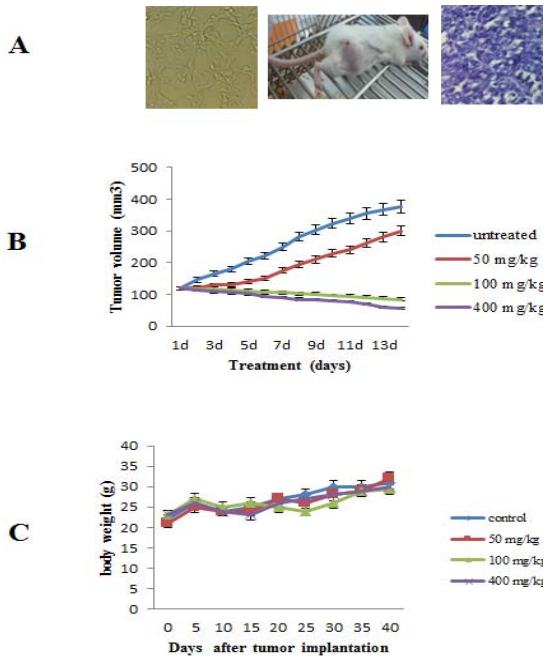

Figure 6. A) CT26 colon carcinoma cell line, mouse bearing colon cancer, histological section from tumor inoculated, (left to right, respectively). B) The alterations in tumor volumes in control and experimental groups during 14 days exposure with sea cucumber extract. All data are provided as mean ± SD. Sea cucumber extract suppress CT26 derived tumor volume. C) Evaluation of body weight in tumor-bearing mice treated with sea cucumber extract. There are no significant differences in body weight between experimental and control groups

Analysis of Bcl-2 expression

The mRNA expression levels of one apoptotic-related genes, Bcl-2, as anti-apoptotic member of Bcl-2 family, in transplanted colon tumor cells treated with inhibitory concentrations of sea cucumber extract were evaluated using the RT-PCR. The RT-PCR analysis indicated that the pretreatment of implanted colon cancer cells with sea cucumber water extract reduced the mRNA expression levels of Bcl-2 in a dose dependent manner compared to the untreated cells (Figure 8).

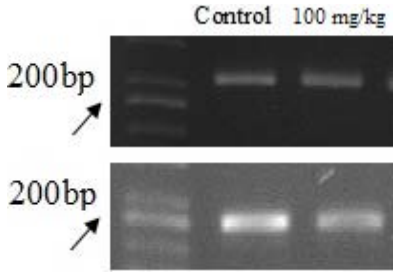

Figure 8. Effect of sea cucumber extract on expression of Bcl-2 mRNA in colon tumor cells. B2M mRNA was used as an internal control gene (230bp). Lane left to right: 50 bp molecular weight marker, control, 100 and 400 mg/kg extract, respectively. As shown in the figure, down regulation of Bcl-2 under treatment sea cucumber extract elucidated intrinsic apoptotic induction on colon tumor cells

Discussion

Extensive literature survey revealed that the presence of bioactive metabolites in sea cucumber species of and their biological efficacy on molecular targets and on cancer models have been suggested holothurians echinoderms a promising source of potent anti-cancer agents. In our study, anti-cancer effect of sea cucumber aqueous extract (*Holothuria arenicola*) in murine colorectal cancer CT26 cell line was evaluated. Morphological assessment exhibited that sea cucumber aqueous extract revealed a cytotoxic effect against CT26 cells as a dose dependent manner. Several research have exhibited that natural metabolites can attenuate colorectal carcinoma development (25). In 2013 Serra *et al* designed a study to investigate chemotherapeutic properties of hydro-alcoholic extract of Opuntia spp. fruits and reported that Opuntia spp. fruits possess an anti-proliferative activity against HT29 colon cancer that can be created due to existence phytochemical constituents such as betacyanins, flavonoids and phenolic acids (26). Nutritional phytochemicals such as dietary supplements, plant secondary metabolites, micro-organisms and medicinal herbs including *Cynaracar dunculus* and *Santalum album* have been proposed significant importance in prevention and treatment of colon carcinoma as one of the chronic diseases (25).

Ogushi *et al* investigated the cytostatic effect of sea cucumber aqueous extract and proved sea cucumber possess anti-tumor efficacy on human colon adenocarcinoma Caco-2 as dose dependent manner (27). Related to anti-cancer potential of sea cucumber Wijesinghe *et al* studied anti-tumor activity of aqueous edible sea cucumber (*Holothuria edulis*) extract on HL-60 leukemia cells and reported that this extract possess a pro-apoptotic activity against HL-60 cells via the augmentation of caspase-3 and reduction of Bcl-xl (28).

In an investigation by Althunabit *et al.*, they assessed the cytotoxic effect of several sea cucumber species (*Holothuria scabra, Holothuria leucospilota* and *Stichopus chloronotus)* and so reported that all organic extract of sea cucumber displayed anti-proliferative properties against (A549) non-small cell lung cancer cells, in addition, they revealed that the water and organic extracts of *Holothuria edulis* Lesson (Holothuriidae) and *Stichopus horrens* Selenka (Stichopodidae) elicited cytotoxic effects against A549 and TE1 cancer cells and proposed that *H. edulis* and *S. horrens* can be considered as an anti-cancer agent (29).

The cytotoxic mechanisms underlying the anti-colon cancer activity of sea cucumber in this research has been investigated. DAPI and AO-PI assay demonstrated that IC_{50} value elicited apoptosis cell death via intrinsic pathway through up-regulation of caspase-3 and caspase-9 activity. This result is in agreement with many investigations proved that the caspase-3 and caspase-9 activation are recruitment proteases of mitochondrial programmed cell death.

Previously, anti-cancer properties of sea cucumber extracts have been demonstrated and have been shown that bioactive agents extracted from sea cucumbers such as saponins, sulfated polysaccharides, 12-methyltetradecanoic acid (12-MTA), philinopside E, triterpene glycoside compounds, glycosaminoglycan, and chondroitin sulfates are considered as main components responsible for anti-tumoral properties of this marine echinoderms. Therefore, the pro-apoptotic effect of *H. arenicola* on CT-26 cancer cells probably can be due to the existence of these secondary metabolites in the aqueous extract.

A similar research in 2009 have been demonstrated that an ethanol extract from *Vitexagnus-castus* fruits is rich in flavonoids compounds that inhibited human colon cancer cells via apoptosis induction and can be used as chemotherapeutic source against colon cancer (30).

Investigation about the anti-proliferative and anti-radical activities of an enzymatic extract from brown algae of *Ecklonia cava* together with its crude polysaccharide and crude poly phenolic fractions was conducted by Athukorala *et al.* They demonstrated the strong selective cell proliferation inhibition on all cancer cell lines tested especially at a concentration of 5.1 µg/ml of crude poly phenolic fractions extract, containing high poly phenol amount. It showed IC_{50} value on murine colon cancer (CT-26) cell line associated with programmed cell death (31). Hajiaghaalipour *et al*. accomplished interesting research related to anti-cancer properties of tea extract that is widely consumed on the colorectal cancer cell line, HT-29 and concluded that this extract exhibited cytotoxic effect on HT-29 cells with an IC_{50} value of 87 µg/ml with increment of caspase-3, -8, and -9 activity in colorectal cancer cells that determined the apoptosis dependent to caspase pathway (32).

In another study, the effects of fucoidan as one of brown algae bioactive metabolite were examined on HT-29 and HCT116 human colon cancer cells by Kim *et al.* and their findings confirmed that fucoidan could cause significant anti-proliferative activity and pro- apoptotic effect of HT-29 and HCT-116 cells in a dose-dependent manner mediated via the death receptor-mediated and mitochondria-mediated apoptotic pathways (33).

In addition, anti-tumor effects of *H. arenicola* aqueous extract were examined *in vivo* whether elucidated sea cucumber can be consider as potent candidate for treatment and prevention of colorectal carcinoma. In 2010, Silva-Stenico *et al* verified anti-tumor activities of intra- and extracellular cyanobacteria extracts against murine cancer cell

line, and colon carcinoma CT-26 and their observations revealed that methanol intracellular cyanobacteria extract has inhibitory activity against colon cancer cell line (34). Arghiani *et al.* evaluated anti-tumor efficacy of sesquiterpene ferutinin against colon carcinoma. In this study natural product extracted from Ferula exhibited cytotoxic and pro-apoptotic effect *in vitro* and *in vivo* against colon cancer without damaging the spleen and liver (35).

Our *in vivo* results confirmed that aqueous extracts of sea cucumber regressed mouse CT26 colon carcinoma and improved reduced colon cancer in mice which in according with other investigations verifying anti-tumor potential of natural products.

Conclusion

The in vitro and *in vivo* findings in this study revealed that the Persian Gulf sea cucumber species (*H. arenicola*) elicited anti-tumor activity via apoptosis induction that may consider as an alternative in pre-clinical treatment of colorectal carcinoma in future. Thus, detection of its chemical constituents is highly recommended.

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Conflict of interest

The authors declare that they have no conflict of interests.

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