

Evaluation of the Anti-proliferative Effects of *Ophiocoma erinaceus* Methanol Extract Against Human Cervical Cancer Cells

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Abstract

Background: Marine organisms provide appreciable source of novel bioactive compounds with pharmacological potential. There is little information in correlation with anti-cancer activities of brittle star. In the present study, anti-neoplastic efficacy of *Ophiocoma erinaceus* methanol extract against human cervical cancer cells was investigated.

Methods: The HeLa cells were cultured and exposed to brittle star methanol extract for 24 and 48 hr. The anti-proliferative properties were examined by MTT assay and the type of cell death induced was evaluated through morphological changes, flow cytometry, Annexin kit and caspase assay. To assess the anti-metastatic activity, wound healing assay was conducted and photographs were taken from the scratched areas. Further, to understand molecular mechanism of cell apoptosis, the expression of Bax was evaluated.

Results: The morphological analysis and MTT assay exhibited that the brittle star methanol extract can exert dose dependent inhibitory effect on cells viability (IC_{50} , 50 $\mu\text{g/ml}$). Flow cytometry and fluorescence microscopy demonstrated increment of sub-G1 peak, early and late apoptosis in HeLa treated cells. Wound healing migration assay showed that brittle star extract has anti-neoplastic efficacy by inhibiting cell migration. Caspase assay and RT-PCR analysis revealed that brittle star methanol extract induced caspase dependent apoptosis in HeLa cells through up-regulation of caspase-3 followed by up-regulation of Bax gene which is a hallmark of intrinsic pathway recruitment.

Conclusion: These results represented further insights into the chemopreventive potential of brittle star as a valuable source of unknown therapeutic agents against human cervical cancer.

Avicenna J Med Biotech 2016; 8(1): 29-35

Keywords: Apoptosis, Brittle star, Uterine cervical neoplasms

Introduction

Cancer is defined as a complex disease characterized by an uncontrolled growth, so that the loss of impressive natural or synthetic therapeutic agents has been the subject of various researches¹. Cervical carcinoma is a major health threat and one of the most common gynecological malignancies worldwide. Hence, discovery of novel potent natural agents is valuable to decrease the incidence and mortality of this disorder². The common modalities of cervical cancer treatment include chemotherapy, radiotherapy and surgery with low rates of complete response³. Therefore, there is an undeniable need to create innovative therapeutic strategies for cancer treatments, which act by modulating

cell proliferation, lowering the risk of malignancy, promoting cytostatic effects and blocking or delaying the progression of tumorigenesis⁴.

Apoptosis plays a key role in the elimination of premalignant cells which is distinguished by various morphological alterations, such as chromatin condensation and cell shrinkage⁵. There is the mutual correlation between apoptosis and cancer metastasis, so that alterations to the apoptotic pathway promote neoplastic metastasis. Therefore, the lack of efficacious chemotherapeutics against metastasis is considered the main obstacle in cancer therapeutics⁶.

It is shown that marine natural compounds can

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Received: 11 Jul 2015
Accepted: 15 Oct 2015

block tumor cell growth by apoptosis recruitment; in addition, high efficacy and low toxicity of these compounds have resulted in extensive researches for discovering novel drugs⁷. For instance, the natural anti-cancer compounds, such as Vinca alkaloids isolated from *Vinca rosea* and paclitaxel extracted from *Taxus brevifolia*, demonstrated the prominent role of natural resources in promoting conventional cancer therapy approaches⁸.

Meanwhile, marine environment represents a diverse array of organisms with unique biological properties which possess unidentified therapeutic compounds⁹. Despite the high biodiversity of the marine ecosystem, the investigation of pharmaceutical activity among marine natural products is relatively infrequent. Marine-based natural products are unidentified unlike terrestrial herbal agents; moreover, the quantification and detection of bioactive natural products from marine organisms with anti-cancer potential provide a unique clinical field¹⁰. Several surveys reported that lower marine organisms, particularly echinoderms, have proven to be a rich biomedical source of potential secondary metabolite¹¹.

Wijesinghe *et al* in 2013 evaluated the anti-cancer efficacy of sea cucumber (*Holothuria edulis*) extract on HL-60 leukemia cells and showed that this extract exerts pro-apoptotic effect against HL-60 cancer cells via up-regulation of Bax and caspase-3 protein¹².

Regarding ophiuroids (brittle stars), and their natural metabolite, an investigation has been conducted which compared them with other echinoderms. Nonetheless, polar steroids are proved to be an important natural product isolated from brittle stars which are responsible for their cytotoxicity, terpenes, naphthoquinones, phenylpropanoids, carotenoids and cerebrosides have been isolated from brittle stars and might have pharmacological activities¹³. The Persian Gulf has been recognized to be a rich source of unrevealed biological compounds and is considered as a habitat of numerous marine organisms¹⁴.

In 2007, Fatemi *et al* identified that species of *Ophiocoma erinaceus* (*O. erinaceus*) and *Ophiocoma scolopendrina* belonged to a single family of Ophiocomidae in Qeshm Island¹⁵. In a research performed by Keshavarz *et al* in 2012, it was found that a species of *O. erinaceus* were dominant in the sublittoral zone of the Persian Gulf¹⁶.

The main purpose of this study was to evaluate the cytotoxic and anti-metastatic efficacy of Persian Gulf brittle stars (*O. erinaceus*) methanol extract on human cervical cancer cells and whether this anti-tumor activity was mediated via an apoptotic mechanism.

Materials and Methods

Preparation of brittle star methanol extract

The species of *O. erinaceus* with a long body, thick spines on their arms, shorter arms compared to arm tips and diverse color compared to the common species

were used in our experiments. Specimens of the brittle star (*O. erinaceus*) were obtained from the rocky intertidal flats of the Persian Gulf waters. Firstly, morphometric estimation of *O. erinaceus* was conducted at the Research Center of Applied Biology at Mashhad Branch of the Islamic Azad University. Then, the specimens of brittle star were washed and stored at -80°C. For extract construction, brittle star samples (about 20 g) were dried, minced and mixed with 200 ml methanol (Merck, Germany). Then, the extract was constantly stirred (72 hr) at room temperature, filtered through an 11 µm Whatman filter and concentrated under a vacuum evaporator (Heidolph, Germany) before being stored. To prepare the stock solution (100 mg/ml), concentrated extract was dissolved in DMSO. For the experiments, the stock was diluted with serum-free cell culture medium to the final concentrations of DMSO which did not exceed 0.05%.

Cell culture

The HeLa cell (NCBI, Iran) was cultured in DMEM (Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA), 0.2 mM L-glutamine and 1% penicillin streptomycin (Gibco, USA). Cultures were maintained at 37°C and 5% CO₂ under humid atmosphere. Media were changed twice weekly and cultures were split 1:5 once a week.

In vitro cytotoxicity assessment of total extract of brittle star

The effects of total extract of brittle star on the proliferation of HeLa cells were measured by MTT assay. MTT assay provides an indication of mitochondrial integrity and activity, which is interpreted as a measurement of cell viability. Briefly, cells were seeded in 96-well tissue culture plates (10,000 cells/well) in complete DMEM medium, followed by incubation in 5% CO₂-95% atmosphere for 24 hr at 37°C. Cells were exposed to different concentrations of brittle star methanol extract (0, 12.5, 25, 50, 100, 200 µg/ml) for 24, 48, 72 hr followed by addition of MTT (Sigma, USA) (10 ml per well of 5 mg/ml stock solution) 5 hr prior to completion of incubation periods. Media were completely removed from each well and DMSO (200 µl) was added for solubilization of formazan crystals. After 10 min, absorbance was measured at 570 nm using a (Epoch, USA) spectrophotometer.

Cell morphological assessment

HeLa cells were seeded into a 24-well plate at a density of 1.0×10⁵ cells per well overnight, and different concentrations of brittle star methanol extract (0, 12.5, 25, 50, 100, 200 µg/ml) were added to the cultures. After 48 hr of incubation, the cells were washed in ice-cold Phosphate-Buffered Saline (PBS), and cell morphology was assessed using inverted microscope (Bio Photonic, Brazil).

Flow cytometry

HeLa cells were plated into a six well plate at a density of 5.0×10⁵ cells per well. After 48 hr of exposure to the brittle star methanol extract, the cells were col-

lected, fixed, permeabilized with 75% ice-cold ethanol, and stored at 20 °C. The cells were then suspended in 1 ml of lysis buffer (0.1% Triton X-100, 0.05 mg/ml propidium iodide, and 50 µl of 0.02 mg/ml RNase A), and after incubation for 30 min at 37 °C, the samples were analyzed using FACScan laser flow cytometer (FACS Calibur, Becton Dickinson, USA) ⁸.

Assessment of cell death by Annexin V/propidium iodide-staining

Annexin V/PI staining was carried out using Annexin V-FITC/PI staining kit provided by Abcam following manufacturer's instructions. Briefly, cells were plated into 48-well tissue culture plate and treated with different concentrations of brittle star methanol extract for 48 hr. Then, the cells were washed with PBS followed by incubation with prepared solution of Annexin V/PI in binding buffer for 15 min in the dark at room temperature. Finally, images were captured using fluorescence microscopy (Olympus, Japan).

Caspase assay

This assay was performed using quantification of caspase enzymatic activity (Abcam, UK) according to cleavage of p-nitroaniline and p-nitroanilide from the labeled substrate DEVD-pNA and LEHD-p-NA (4 mM), respectively. Briefly, 3×10⁶ cells were exposed to appropriate concentrations of brittle star extract for 48 hr. Then, the treated cells were trypsinized and lysed with 300 µl of chilled Cell Lysis Buffer, and centrifuged at 4 °C to obtain supernatant cytosolic extract rich in protein content. Then, cell lysates were minced with 5 µl of 2× reaction buffer and 5 µl of the conjugated substrate and incubated at 37 °C for 2 hr. Finally, the absorbance was read at 405 nm (Epoch, USA).

Wound migration assay

A wound migration assay was performed on 6-well plates coated with collagen (50 µg/ml). When the cells were grown to confluence, (approximately 80-90%), a scratch was made across the center of the plate to produce a wound area using a sterile 200 µl pipet tip. After 24 hr, cells were treated with IC₅₀ concentration of brittle star methanol extract for 48 hr, and were allowed to migrate into complete medium. Finally, photographs were taken at the edge of the wound areas.

Total RNA isolation and evaluation of the expression of Bax

RNA was isolated from untreated and brittle star methanol extract 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 s and 60 °C for 1 min. The sequences of primers used are as follows: B₂M Forward 5' TGGTGCTTGGCTCACTGACC 3', Reverse 5' TATGTTCCGGCTTCCATTCT 3' as the internal control gene. The forward

primer and reverse primer Bax were designed as 5' TTTGCTTCAGGGTTTCATCCA 3' and 5' CTCCATGTTACTGTCCAGTTCGT 3', respectively. Following amplification, the PCR products were subjected to electrophoresis in a 2% agarose gel and visualized by green viewer staining.

Data analysis

All the experiments were repeated at least in triplicates and the results were expressed as the mean±SEM. The statistical significance was evaluated using SPSS software by student's t-test. For all the comparisons, the level of p≤0.05 was considered significant.

Results

Cytotoxic effect of brittle star methanol extract on cell proliferation of cervical tumor cells

The cytotoxicity of brittle star methanol extract was evaluated using MTT assay. As shown in figure 1, brittle star methanol extract inhibited HeLa cell proliferation in a dose and time dependent manner at 24, 48, 72 hr. The IC₅₀ values were calculated as 81, 50.3 and 23 µg/ml, respectively.

The cell morphology was assessed using inverted microscope (Bio Photonic, Brazil) which clearly indicated apoptotic morphological changes, such as a reduction of cell volume, cell shrinkage, and cytoplasmic blebbing in treated cells (Figure 2).

Flow cytometry analysis

To detect apoptosis, flow cytometry analysis of DNA content using propidium iodide (PI) was carried out. The sub-diploid peak was considered as a valuable method for determination of cell apoptosis. The results revealed that after 48 hr of treatment with IC₅₀ concentration (50 µg/ml of brittle star methanol extract), there was a statistically significant increase in the sub-G1 peak compared with the control. The exposure to a higher concentration of brittle star methanol extract (100 µg/ml) induced a significant increase in the sub-

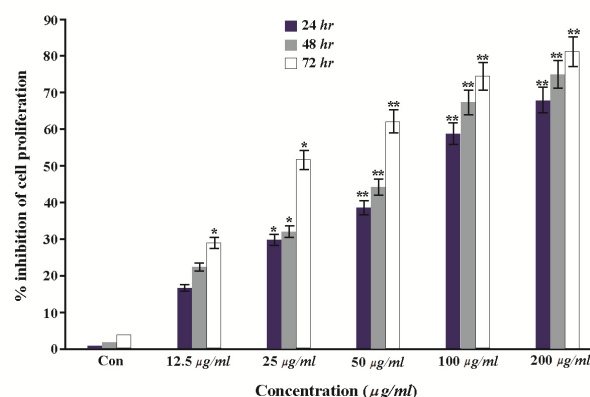


Figure 1. Cytotoxic activity of brittle star methanol extract in proliferation of HeLa cell line after 24, 48 and 72 hr using MTT assay at 570 nm. The data were represented as mean±SD and *p<0.05 and **p<0.005 were considered significant. The stars demonstrate significant difference between control (untreated) and brittle star methanol extract treated groups.

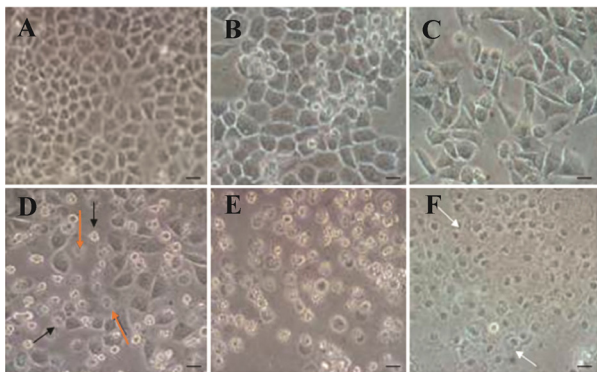


Figure 2. Cytomorphological alterations of HeLa cells treated with brittle star alcoholic extract. A) Control (without treatment) indicated accumulation of HeLa cells uniformly. (B, C, D, E, F) HeLa cells treated with 12.5, 25, 50, 100, 200 $\mu\text{g/ml}$ extract for 48 hr indicate apoptotic features such as cytoplasmic blebbing (orange arrows), round shape (black arrows) and apoptotic body formation (white arrows), respectively. Magnification= $\times 400$.

diploid peak, which is associated with the inhibitory effects of brittle star extract against HeLa cells (Figure 3).

Analysis of apoptosis by fluorescence microscopy

To confirm apoptotic alteration in HeLa cancer cells under treatment with brittle star methanol extract, Annexin V/PI staining was used. As reported in figure 4, the treated cells stained with PI (red nucleus) showed necrotic death and the cells stained with Annexin V-FITC (green cell surface), indicated apoptotic cell death. Under fluorescence microscopy, brittle star methanol extract treatment (100, 200 $\mu\text{g/ml}$) indicated a necrotic morphology and IC_{50} value showed apoptotic characteristics. These findings validated the morphological observation under the inverted microscope which showed morphological changes of apoptotic cell death in HeLa cells treated with brittle star methanol extract.

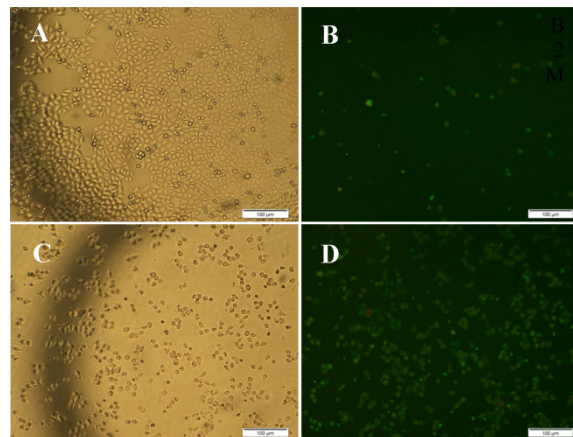


Figure 4. Apoptotic morphological alterations in HeLa treated cells with Annexin V-PI staining. (A, B) HeLa cells untreated observed by inverted fluorescence microscope, respectively. C, D) The HeLa treated cells with 50 $\mu\text{g/ml}$ brittle star methanol extract indicate externalization of phosphatidylserine to outer leaflet as one of the apoptosis main characteristic. Green color is indicating apoptotic cells (400 \times magnification).

Caspase assay

In order to assess the mechanism of induced apoptosis under exposure to brittle star extract, the enzymatic activity of caspase-3 and caspase-9 was evaluated. As shown in figure 5, the activity of caspase-3 and caspase-9 was remarkably increased in a dose dependent manner with IC_{50} value of 41.3 and 42.4 $\mu\text{g/ml}$ compared with the untreated cancer cells. Therefore, it elucidated the intrinsic pathways involved in apoptosis which were induced by brittle star extract in HeLa cancer cells.

Suppression of cell motility by brittle star methanol extract

To assess the inhibitory effect of brittle star methanol extract on the HeLa cell migration, the wound healing migration assay was performed. As shown in figure

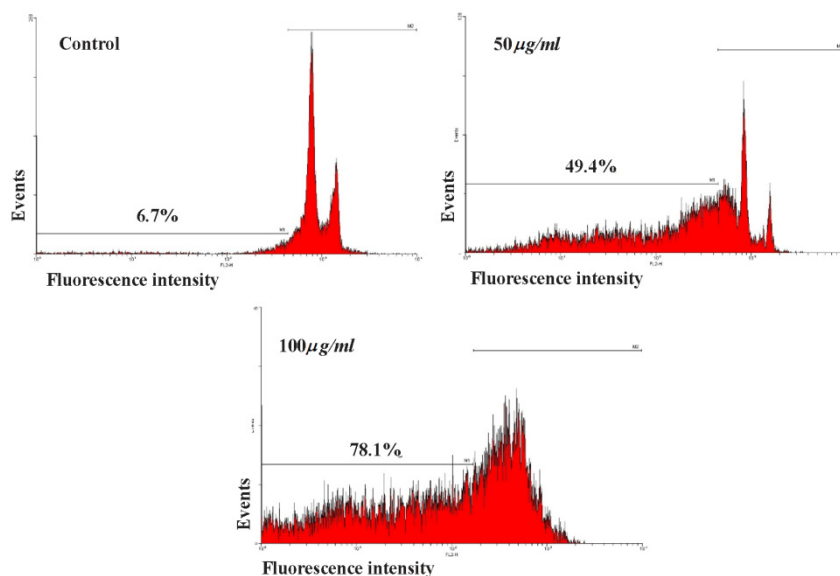


Figure 3. Detection of apoptosis using flow cytometry. Histogram of untreated and treated HeLa cells with 50, 100 $\mu\text{g/ml}$ brittle star extract indicated apoptosis induction in HeLa cells.

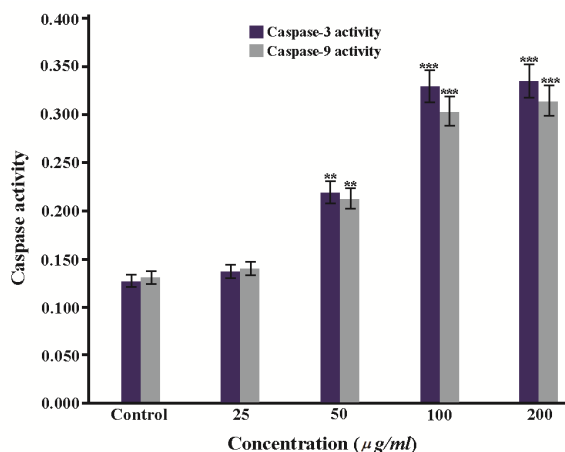


Figure 5. Elevation of the level of caspase-3,-9 activity in HeLa cells treated with brittle star methanol extract. Cells were treated with 25, 50, 100, 200 µg/ml of brittle star extract and were analyzed using a colorimetric caspase-3,-9 assay kit. **p<0.01, ***p<0.001 were considered significant. The stars demonstrate significant difference between control (untreated) and brittle star methanol extract treated groups.

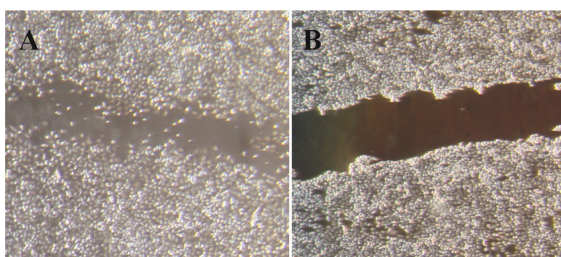


Figure 6. Inhibitory effect of brittle star alcoholic extract on cell migration of HeLa cells by wound healing assay. A) Control group, 48 hr after scratch formation B) Treatment group with 50 µg/ml of brittle star extract (IC₅₀ value), which exhibited the brittle star extract effectively suppressed cell motility 48 hr after scratch formation.

6B, HeLa cell migration was significantly controlled with IC₅₀ concentration of brittle star extract and the brittle star methanol extract in IC₅₀ concentration of 50 µg/ml could be efficient for inhibition of HeLa cell motility. These results revealed that brittle star methanol extract might possess anti-metastatic activity in cervical cancer cells.

Analysis of Bax expression

The mRNA expression levels of one apoptotic-related gene, Bax, as pro-apoptotic member of Bcl-2 family, in human cervical cancer HeLa cells treated with inhibitory concentrations of brittle star methanol extract were evaluated using the RT-PCR. The RT-PCR analysis using Image J software, indicated that the pretreatment of HeLa cells with brittle star alcoholic extract increased the mRNA expression levels of Bax in a dose dependent manner compared to the untreated cells (Figure 7). As shown in figure 7, up-regulation of Bax under treatment with brittle star methanol extract indicated the effect of intrinsic apoptotic pathway on

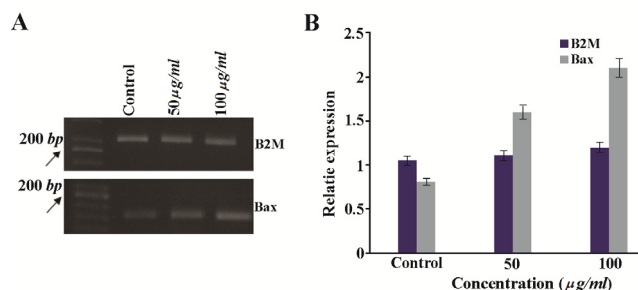


Figure 7. A) Qualitative assessment of the effect of brittle star methanol extract on expression of Bax mRNA (135 bp) in HeLa cells. B2M mRNA was used as an internal control gene (230 bp). B) The densitometry of gel quantitatively evaluated by image J indicates up-regulation of Bax pro-apoptotic gene in treated HeLa cells.

HeLa cell cytotoxicity under treatment with brittle star methanol extract.

Discussion

A unique characteristic of human cancers is the inability of tumor cells to undergo apoptosis. Therefore, the absence of effective treatment regimens and the resistance to conventional chemotherapeutic drugs has led to the introduction of innovative natural compounds for cancer prevention¹⁷.

In our investigation, the anti-neoplastic efficacy of brittle star methanol extract in human cervical cancer HeLa cell line was assessed. To evaluate the inhibitory concentrations of brittle star methanol extract, the HeLa cancer cells were treated with 0, 12.5, 25, 50, 100, 200 µg/ml of the extract for 24, 48 and 72 hr. Then, the growth inhibitory effect was measured by MTT assay. Figure 1 shows the HeLa cell growth was significantly inhibited in a dose dependent manner with inhibition rate of 38.6%, 58.7% (**p≤0.01), 44.2%, 67.3% (**p≤0.01) and, 62.2%, 74.5% (**p≤0.01) in treated groups of 50 and 100 µg/ml after 24, 48, 72 hr of treatment. The IC₅₀ concentration of brittle star extract for 48 hr of treatment was considered approximately 50 µg/ml which was used for following the assays. Cytomorphological observation revealed that the brittle star methanol extract indicated a potent anti-proliferative activity in HeLa cells in a dose-time dependent manner. In addition, the histogram of flow cytometry and fluorescence micrographs indicated that the IC₅₀ value of brittle star methanol extract induced apoptosis in HeLa cells and further, the increment of caspase-3 and -9 activity (**p≤0.001) and up-regulation of Bax gene showed that apoptosis was elicited through intrinsic apoptotic pathway in cervical cancer cells. This finding is in accordance with many studies that established the role of caspase-3,-9 and Bax in inducing programmed cell death¹². In addition, wound healing assay showed that the methanol extract of *O. erinaceus* exhibited anti-metastatic effects, which can be useful as an anti-invasive candidate for treatment of cervical metastatic carcinoma.

The high number of therapeutic drugs from terrestrial herbal extracts, structural diversity in marine organisms and existence of a little documentation about marine invertebrates have all stimulated more investigation for drug discovery from marine sources¹⁷.

The discovery of Dolastatins obtained from marine mollusk *Dolabella auricularia* or Halicondrins isolated from the Japanese sponge *Halichondria aokadai*, has encouraged extensive marine anti-cancer researches¹⁸.

According to Prado *et al*, the extracts from marine sponges and ascidians from the Brazilian coast are toxic against T47D cells. In addition, the IC₅₀ values of examined sponges extracts (approximately 30 µg/ml) strongly delayed the cell cycle progression which represented marine fauna as a valuable source of cytotoxic agents in oncological researches¹⁸.

The investigations conducted by Zandi *et al* showed that the aqueous extract of red algae *Gracilaria corticata* collected from Bushehr coast in IC₅₀ concentrations of 9.336 and 9.726 µg/ml exhibited anti-tumor activity against Jurkat and molt-4 tumor cells that validated the therapeutic potential of marine flora which showed more cytotoxicity as compared with brittle star methanol extract¹⁹.

Regarding marine fauna, several studies reported the cytotoxic effects of marine sponges²⁰. The cytotoxicity of Brazilian marine sponges crude extracts, *Aplysina caissara*, against a hepatoma cell line, in agreement with our results indicated that sponge extracts like brittle star extract trigger the inhibition of cancer cell proliferation in a dose dependent manner²¹.

The evaluation of anti-neoplastic potential of six species of marine sponges collected from the Spanish coast on BE(2)-M17 neuroblastoma cells documented the water extract of *Halichondria panicea*, *Pachymatis majohnstonia*, *Ophlita spongiaseriata* with cell growth inhibition, loss of membrane integrity and mitochondrial membrane potential reduction induced more apoptotic morphology²², which, consistent with our findings, demonstrated the involvement of intrinsic apoptotic pathways under exposure to marine fauna extracts.

The anti-proliferative activity of marine sponge organic extracts (hexane, ethyl acetate and *n*-butanol) in human cancer cell lines demonstrated the high cytotoxicity of extracts in HL-60 cells. In addition, their findings showed that the ethyl acetate extract of *Jaspis* sp. with a significant increase of cells in the sub-G1 phase, induced apoptosis in KB cells²³. In contrast, our results exhibited that methanol extract of brittle star exerted pro-apoptotic effect on HeLa cells via translocation of phosphatidylserine to extrinsic leaflet, sub-G1 increment and caspase activation.

Related to anti-tumor activity of marine invertebrates, echinoderms with presentation of novel bioactive natural products and broad biological activities have provided a novelty in the development of new therapeutic anti-cancer agents¹¹.

Althunibat *et al* evaluated the anti-proliferative activity of the Malaysian sea cucumber species, *Holothuria scabra*, *Holothuria leucospilota* (*H. leucospilota*) and *Stichopus chloronotus*. Their research revealed that due to the high level of total phenols, C33A (human cervical cancer) and A549 (human non-small lung carcinoma) were much more sensitive to the water extract of *H. leucospilota*²⁴. In accordance with these results, our study revealed that methanol extract of *O. erinaceus* conducted cytotoxic effect on HeLa (cervical cancer) growth cells, demonstrating anti-cancer potential of echinoderms.

Investigation of the anti-cancer potential of *Acanthaster planci* starfish extract along with Tamoxifen (a non-steroidal selective estrogen receptor modulator) in human breast cancer cells indicated that the sea star extract compared with Tamoxifen (IC₅₀=15.6 µg/ml) was more efficient in the early arrest of cell growth and recruitment of the apoptosis process²⁵. In contrast, the IC₅₀ of brittle star methanol extract (50 µg/ml) demonstrated that *Acanthaster planci* had more cytotoxicity against cancer cells. A few studies have been reported about the Persian Gulf ophiuroidea and their biological effects. For example, Andersson *et al* reported the antibacterial and cytotoxic effect of saponin is like compounds extracted from brittle star on leukemia cells²⁶. Prabhu *et al*. indicated hemolytic and cytotoxic properties of crude extracts from *Ophiocnemis marmorata*²⁷ which consistent with pro-apoptotic and anti-metastatic properties of *O. erinaceus* extract in this study, proposed the anti-cancer potential of brittle star methanol extract.

Conclusion

Taken together, our results indicated apoptosis inducing effect of *O. erinaceus* methanol extract. Further, it suggests the presence of active metabolites with appreciable pharmacological potential in *O. erinaceus* as one of marine fauna, which are a valuable source of anti-cancer and anti-metastatic agents that can be used against cervical cancer in anti-cancer experiments.

Acknowledgement

This work was performed in the Research Center for Animal Development Applied Biology and was supported by the Vice Chancellor for Scientific Research of Mashhad Branch, Islamic Azad University. The authors give special thanks to the Central Lab of Mashhad University of Medical Sciences.

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