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 (IC50, 50 μg/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.

**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 researches 1. 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 disorder 2. The common modalities of cervical cancer treatment include chemotherapy, radiotherapy and surgery with low rates of complete response 3. 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 tumorigenesis 4.

Apoptosis plays a key role in the elimination of premalignant cells which is distinguished by various morphological alterations, such as chromatin condensation and cell shrinkage 5. 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 therapeutics 6.

It is shown that marine natural compounds can...
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 drugs. 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 approaches.

Meanwhile, marine environment represents a diverse array of organisms with unique biological properties which possess unidentified therapeutic compounds. 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 field. Several surveys reported that lower marine organisms, particularly echinoderms, have proven to be a rich biomedical source of potential secondary metabolite.

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 protein. 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 activities. The Persian Gulf has been recognized to be a rich source of unrevealed biological compounds and is considered as a habitat of numerous marine organisms.

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 Island. 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 Gulf.

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 inter-tidal 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 μl 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 5.0×10⁴ 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 FACS 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, 3x10⁶ 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 2x 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 Forward5’TGGTGCTCTTGG CTCACTGACC 3’, Reverse 5’TATGTGCGCTTCC CATTCT 3’ as the internal control gene. The forward primer and reverse primer Bax were designed as 5’TGGCTTCAGGGTTCCATCCA 3’ and 5’CTCCAT GTTACTGTCAGTTGCTGT 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-G1 peak.
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) show-
ed 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 µg/ml) indicated a necrotic
morphology and IC50 value showed apoptotic charac-
teristics. These findings validated the morphological
observation under the inverted microscope which show-
ed morphological changes of apoptotic cell death in
HeLa cells treated with brittle star methanol extract.

Caspase assay
In order to assess the mechanism of induced apopto-
sis 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 cas-
pase-9 was remarkably increased in a dose dependent
manner with IC50 value of 41.3 and 42.4 µg/ml com-
pared with the untreated cancer cells. Therefore, it elu-
cidated the intrinsic pathways involved in apoptosis
which were induced by brittle star extract in HeLa can-
cer cells.

Suppression of cell motility by brittle star methanol extract
To assess the inhibitory effect of brittle star metha-
nol extract on the HeLa cell migration, the wound heal-
ing 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 µg/ml brittle star extract indicated apoptosis induction in HeLa cells.

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 µg/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×magnification).
6B, HeLa cell migration was significantly controlled with IC\textsubscript{50} concentration of brittle star extract and the brittle star methanol extract in IC\textsubscript{50} 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 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 prevention 17.

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 IC\textsubscript{50} 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 IC\textsubscript{50} 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 death 12. In addition, wound healing assay showed that the methanol extract of \textit{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 sources 17. The discovery of Dolastatins obtained from marine mollusk Dolabella auricularia or Halicordinis isolated from the Japanese sponge Halichondria aokadai, has encouraged extensive marine anti-cancer researches 18.

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 \( \mu \text{g/ml} \)) strongly delayed the cell cycle progression which represented marine fauna as a valuable source of cytotoxic agents in oncological researches 18.

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 \( \mu \text{g/ml} \) 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 extract 19.

Regarding marine fauna, several studies reported the cytotoxic effects of marine sponges 20. 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 manner 21.

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 spongiaserata with cell growth inhibition, loss of membrane integrity and mitochondrial membrane potential reduction induced more apoptotic morphology 22, 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 cells 23. 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 agents 11.

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 \( \mu \text{g/ml} \)) was more efficient in the early arrest of cell growth and recruitment of the apoptosis process 25. In contrast, the IC50 of brittle star methanol extract (50 \( \mu \text{g/ml} \)) demonstrated that Acanthaster planci had more cytotoxicity against cancer cells. A few studies have been reported about the Persian Gulf ophiuroidae 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 cells 26. 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.

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