

## The pro apoptotic effect of brittle star dichloromethane extract on B16F10 melanoma cell line

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### ABSTRACT

Cancer is the second important reason of mortality in the world. In this regard melanoma was accounted as the most aggressive type of cutaneous cancer. Among drug extracted from natural products from marine organisms have been focused investigations related to chemotherapeutic agents derived from echinoderms such as sea cucumbers and starfish. In the present study, cytotoxic and apoptosis inducing potential of Persian Gulf brittle stars dichloromethane extract were evaluated against melanoma cancers. In this study, anti-proliferative effect of brittle stars dichloromethane extract on B16F10 melanoma cells examined by MTT assay and morphological characterization and death inducing effect of Annexin-PI and PI flow cytometry. The data analysis was performed by SPSS software and  $p < 0.05$  were considered significant. The dichloromethane extract of brittle star revealed significant cytotoxic effect on B16F10 melanoma cells with  $IC_{50} = 31 \mu\text{g/ml}$  which is stronger than inhibitory effect of methanol extract on melanoma cell growth. In addition, brittle star dichloromethane extract recruited apoptotic pathway in the response of  $31 \mu\text{g/ml}$  treatment. This study showed that certain concentrations of dichloromethane brittle stars possess cytotoxic activity that can be used as an anticancer agent used in clinical trial due to cell growth inhibition and apoptosis induction which offer therapeutic investigations of dichloromethane brittle star extract as complementary for melanoma treatment and prevention.

**Keywords:** Melanoma, marine, dichloromethane, brittle star, cytotoxic, apoptosis, cancer

### INTRODUCTION

Cancer is a threatened life human diseases and major leading cause of death after cardiovascular diseases in the world with highest mortality [1]. Reducing the incidence and morbidity of cancer has attracted oncological researches for discovering efficient ways to early diagnosis and treatment of cancer [2]. Melanoma is common skin cancer that account as sixth type of cancer with high metastatic capacity which have susceptibility to resistance against cytotoxic agents [3,4].

The origin of melanoma derived from environmental, molecular, biochemical and genetic factors [3]. The surgery, chemotherapy, radiation therapy, hormone therapy and immune therapy comprise effective strategy for melanoma treatment and prevention [1,5,6]. Despite recent progress related to melanoma chemotherapy drug resistance caused the relative failure that

encourage the need for new treatment options that leads to induction of apoptosis in cancer cells [4]. Since efficient anticancer agents are applied their cytotoxic effects through the induction of apoptosis in tumor cells, hence oncologist attempt to find innovative therapeutic compounds that destroy carcinoma lesions with low toxicity [8, 9, 10]. Natural products are bioactive compounds which have been used as chemotherapeutic agents in cancer treatment for controlling the cancer cell proliferation [11, 4]. Traditional medicine utilized medicinal extract with anti-cancer properties which have been isolated from terrestrial and marine resources and play an important role in cancer investigation field clinical trials [5]. In the field of marine organisms natural products, biologically active compounds have been identified from natural extract provide diverse agents with unique structural characteristics and varied biological and

pharmacology activities which isn't found in terrestrial natural products [12, 13]. Researchers follow multiple extraction methods to isolate novel efficacious secondary metabolites from sea natural such as sponges and other marine invertebrates because of natural product generation capacity that can be account as valuable source of new therapeutics in human diseases, particularly in cancer [14, 15]. The Echinodermata have been used in cell biology, molecular biology and immunology due to the presence of specialized cells such as colemocytes that create biological functions including phagocytosis, cytotoxicity and wound healing and various secreted compounds such as lectins and cytokines that reorganized their immunity responses [15, 16]. Among the echinoderms, many cancer compounds are extracted from sea cucumbers and starfish and the anticancer effects of brittle star is less studied.

Since, no one has tried to evaluate anticancer effect of dichloromethane brittle star extract on cancer cell lines, therefore the purpose of this paper was assessment the anti-tumor activity of dichloromethane brittle star *Ophiocoma erinaceus* (*O. erinaceus*) extracts on B<sub>16</sub>F<sub>10</sub> melanoma cells (6).

## MATERIALS AND METHODS

B16F10 cell line with code (C540) were obtained from NCBI (National Cell Bank of Iran). Medium (Roswell Park Memorial Institute) RPMI-1640, Fetal Bovine Serum (FBS), penicillin / streptomycin, trypsin and 3 [5, 4 - dimethylthiazol 2-5 - biphenyl tetrazolium bromide (MTT) were purchased from Sigma (USA). DAPI prepared from Appllichem (USA) and PI (propodium iodide), acridine orange obtained from Sigma (USA). Specimens of the brittle star (*O. erinaceus*) were obtained from rocky intertidal flats of Persian Gulf waters. Methanol, dichloromethane were purchased from Merck (Germany).

### Cell line and Cell culture environment

B16F10 cell line cultured in RPMI-1640 culture medium supplemented with 10 % FBS and 1 % penicillin / streptomycin in incubation temperature of 37°C under a humidified atmosphere containing 5% CO<sub>2</sub> and culture medium changed daily.

### Preparation of dichloromethane Brittle star extract

To prepare dichloromethane extract of brittle star, brittle stars (*O. erinaceus*) were collected from the Qeshm Island and immediately transferred to the Research Center Applied Biology of Mashhad Islamic Azad University and placed at -20. In the next step, collected brittle stars dried at room temperature then as equal of per gram of brittle stars was added 10 ml of methanol and 72 h placed on a shaker in the dark followed by filtration with whatman paper, and were exposed to dichloromethane and water for 6 hours . Finally the dichloromethane phase containing fat is concentrated under reduced pressure and then dried portion were placed at 4° C.

### Cell treatment, morphological observation and MTT assay

In order to treatment the cells harvested with trypsin-EDTA solution and were cultured in 96-well plates ( $2 \times 10^4$  cells/well). Then the cells were incubated overnight without treatment. After 24 h melanoma cells were exposed to various dosage of extracts (500, 250 and 125, 62.5, 31, 15 µg/ml).

After 24 and 48 h treatment period, morphological changes visualized and MTT assay were used for determination of cell viability on the basis of activity of succinate dehydrogenase mitochondrial enzyme in living cells that shift tetrazolium salt into insoluble formazan crystals. Therefore, after incubation with different concentration of dichloromethane extract 10 macro MTT (powder MTT 5 mg /ml PBS is provided) was added to each well directly for 4 h under 37°C and 5 % CO<sub>2</sub> incubation [10]. Finally, 80 µl DMSO (dimethylsulfoxide) was added in to each well to completely dissolve formazan crystals to each cell and the absorbance was measured by spectrophotometer (Epoch, USA).

### Apoptosis detection by DAPI staining

B16F10 cells cultured on coverslip, followed by treatment with desired concentration of brittle star dichloromethane extract for 24 h. Then cells were fixed with methanol for 5 min and loaded with 100  $\mu\text{g/ml}$  DAPI for 15 min at 37 °C in the dark. Eventually, stained cells observed under a fluorescence microscope (Olympus, Japanese).

### Apoptosis assay using flow cytometry images and histograms:

The morphology of treated cells visualized under inverted microscopy to evaluate cytotoxic effect of brittle star dichloromethane extract. To detect type of cell death induced by brittle star dichloromethane extract Annexin-PI staining and PI flow cytometry were used for determining the type of cell death induced. For performance Annexin-PI staining, briefly, cells were plated in 96 well plate and incubated with different concentration of brittle star dichloromethane extract for 24 h. Then, the cells rinsed with PBS and exposed with prepared solution of Annexin V/PI in Annexin V binding buffer for 15 min in dark at room temperature. Images were captured using fluorescence microscopy (Olympus, Japan). For accomplish PI flow cytometry analysis, B16F10 cells were plated into a sixwellplate at a density of  $4 \times 10^5$  cells overnight. After treatment period, the cells harvested and suspended in 1 mL of lysis buffer (0.1% Triton X-100, 0.05 mg/mL propidium iodide, and 1 mg/mL RNase A), for 30 min, then the samples were analyzed using FACScan laser flow cytometer (FACSCalibur, Becton Dickinson, USA).

### Data analysis

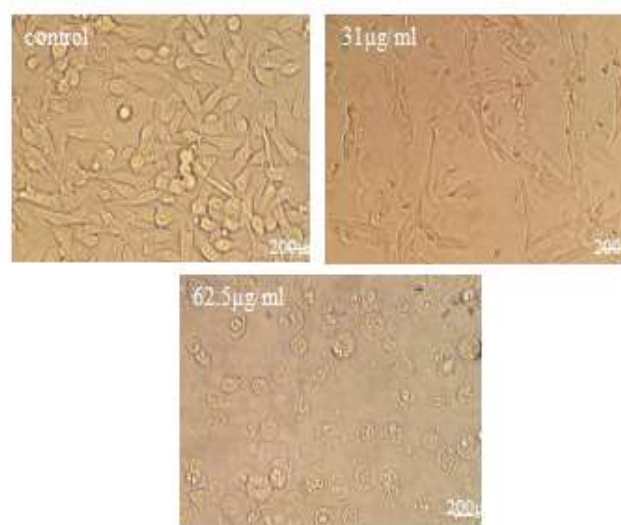
All the results were repeated at least triplicate and expressed as the mean  $\pm$  SEM. The statistical significance was evaluated using SPSS 16 software, one way ANOVA following by Duncan test. For all the comparisons, the level of  $p \leq 0.05$  was considered significant.

## RESULTS

### Cell morphological alteration

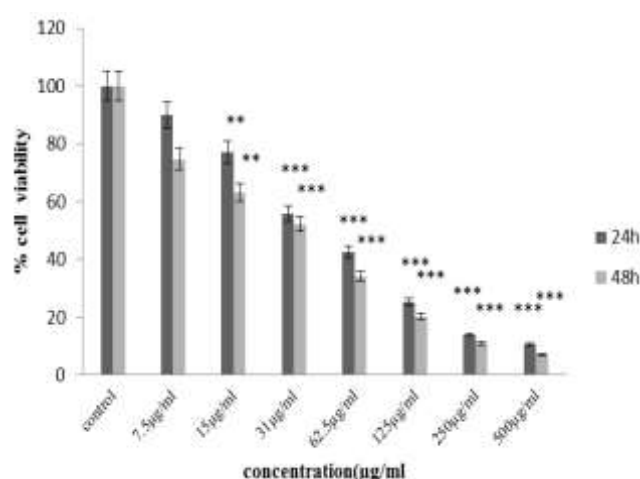
To determine cytotoxic effect of brittle star dichloromethane extract on B16F10 cells we assessed morphological changes of cells under

invert microscopy.



**Figure 1.** Effect of brittle star dichloromethane extract on cytomorphological changes of B16F10 cells. The cells exposed with indicated dosage of extract. As exhibited B16F10 Cells treated with  $\text{IC}_{50}$  value= 31  $\mu\text{g/ml}$  indicated clear alteration in cell morphology so that part of cell population showed round shape and others possess dendritic like shape while upper concentration indicated round shape as compare with untreated cells with fibroblast like morphology. Magnification= X 200.

As shown in figure1, the cell number reduced under treatment with increasing concentration of dichloromethane extract with obvious alteration in cell morphology and appearance of apoptotic bodies, so that in  $\text{IC}_{50}$  concentration (31  $\mu\text{g/ml}$ ) half of cells possess round shape and others have dendrite like shapes.



**Figure 2.** Brittle star dichloromethane extract significantly inhibited cell proliferation of B16F10 melanoma cells after 24 and 48h treatment as dose dependent manner. The data were represented as mean  $\pm$  SD and \* $P < 0.05$ , \*\* $P < 0.005$  and \*\*\* $P < 0.001$  were considered significant.

### Cytotoxic effect of brittle star dichloromethane extract on melanoma cells

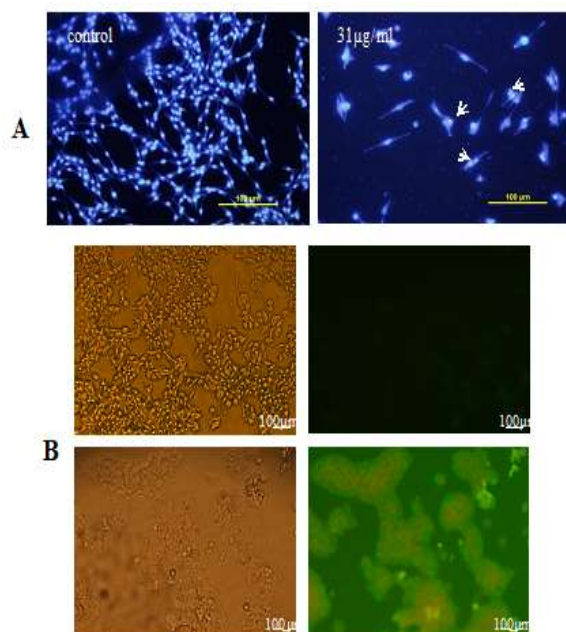
MTT assay was used to detect cytotoxicity of brittle star dichloromethane extract on melanoma cells. As revealed in figure 2, treatment with various concentration of dichloromethane fraction resulted in dose dependent reduction of cell viability.

### Brittle star dichloromethane fraction trigger apoptosis in B16F10 melanoma cells

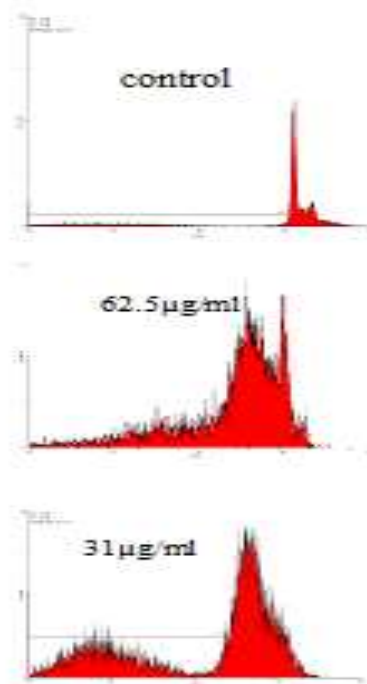
To determine the type of induced cell death under treatment with dichloromethane extract of brittle star DAPI and Annexin-FITC and propidium iodide (PI) staining and PI flow cytometry was performed according to kit instruction. Apoptotic cells characterized by membrane and nucleus changes such as lost membrane integrity, chromatin condensation and DNA fragmentation. To examine the nuclear alteration in response to dichloromethane fraction treatment, B16F10 cells were labeled with DAPI as cell permeable DNA dye. As shown in figure 3A, the IC<sub>50</sub> concentration of extract induced DNA fragmentation as crucial property of apoptosis that indicated by DAPI staining. Another characteristics of apoptosis is transfer of phosphatidylserine from inner leaflet to outer leaflet of cell membrane where AnnexinV bind to it and distinguish apoptotic cells. Figure 3B proved that dichloromethane fraction of brittle star can induce apoptosis in IC<sub>50</sub> concentration so that appearance of green color around the cell surface verify apoptotic features.

To validate apoptotic morphological features in response to dichloromethane extract treatment, accomplished flow cytometry based on propidium iodide staining. As shown in figure 4 treatment of B16F10 cells with brittle star dichloromethane extract resulted in a concentration dependent accumulation of cells in sub-G1 portion that sub-G1 increment is major indicator of apoptosis cell death.

These findings exhibited that brittle star dichloromethane fraction trigger apoptosis by sub-G1 cell cycle arrest in murine melanoma cells.



**Figure 3.** A) Images of B16F10 cells labeled with DAPI observed under fluorescence microscopy indicating DNA fragmentation under treatment with dichloromethane fraction of brittle star. B) Induction of apoptosis in B16F10 cells under incubation with IC<sub>50</sub> concentration of dichloromethane fraction of brittle star.



**Figure 4.** Effect of brittle star dichloromethane extract on DNA content and cell cycle of the melanoma cancer cells by flow cytometry histogram indicated sub-G1 cell arrest demonstrating involvement of an apoptotic cell death under treatment with dichloromethane brittle star extract.

## DISCUSSION

In previous study we evaluated cytotoxic effect of brittle star whole extract on A2780cp ovarian cancer cells and demonstrated dose and time dependent anti-proliferative effect of brittle star methanolic extract so that 50 µg/ml was considered IC<sub>50</sub> concentration [17]. In this study we investigated the anti-proliferative activity of brittle star dichloromethane extract on B16F10 cells and found that dichloromethane fraction have stronger cytotoxicity on B16F10 cancer cells. In addition, both extract induced apoptosis by treatment with IC<sub>50</sub> concentration that is desirable method to control cancer cell growth.

The anti-cancer activity of natural products to fight against cancer gain through inhibition of tumor cell proliferation, induced cytotoxicity, induction of apoptosis and suppression of metastasis and angiogenesis and eventually regulation of gene expression [7]. To date, a considerable quantity of marine natural resources have been shown as anticancer potential that more of them induced apoptosis to act [6]. Recently, many natural extract have been reported that possess therapeutic utility to treat melanoma [8]. The application of traditional medicine in prevention and treatment of melanoma as highly resistance skin cancer to chemotherapy can be considered as complementary strategy in treatment of melanoma [9]. There are several reports on the basis of utilization of natural terrestrial extract to combat melanoma [1].

Park *et al* in 2010 were examined the ethyl acetate fraction of *Phellinus linteus* and *Panax ginseng* on B16F10 melanoma cells and noted this extract suppressed melanoma cell growth via cellular differentiation and apoptosis induction that can be efficient in skin cancer treatment [1]. Hammerova in 2011 were evaluated the anti-neoplastic effect of Benzophenanthridine alkaloids and made obvious that alkaloids can be provide novel agents to treat malignant melanoma [18]. In traditional medicine *Lithospermum erythrorhizon* have been exerted anti-cancer activity and have been showed that possess melanoma therapeutic activity [10].

Zhang in 2013 assessed the anti-cancer efficacy of *Trametes robiniophila* aqueous extract on A875 melanoma cells and proved fungus water extract can be candidate in melanoma treatment via melanoma cell growth suppression and recruitment of apoptosis related genes as time and dose dependent manner [19]. A conducted

experiment by Cao *et al* in 2014 displayed quercetin played anti-melanoma effect indicating capability of melanoma in prevention and treatment of melanoma [20].

Correlated with secondary metabolites with anti-cancer activity from marine invertebrates Sima were provided a report. The echinoderms are rich from natural compounds and bioactive substances with biological activities that many of their effects related to starfish and sea cucumber have been studied but a few information is available from brittle stars [21].

Kumaran and coworkers were reported two species of starfish reveal remarkable antimicrobial and anti-cancer effect [22]. A study conducted by Mutee and colleagues exhibited that the starfish (*Acanthaster planci*) extract in comparison with conventional drugs induced apoptosis and inhibited human breast cancer cell proliferation higher compared with the chemotherapy drug tamoxifen which is similar to our results indicated anti-tumor capacity of marine echinoderm [23]. In 2004, Wang *et al* researched about bioactive metabolites of brittle star *Ophioplocus japonicas* and their investigation indicated the presence of naphthaquinone, phenylpropanoid, carotenoid, steroids and cerebrosides in Ophiuroids which attributed to cytotoxic and antibacterial effects [24].

In the regard of medical application of brittle star, Uzair and his colleagues at the same time studied the antiviral activity of natural extracts from marine organisms and discovered brittle star had a positive effect on HIV [25]. Prabhu and Bragadeeswaran in 2013 researched regard to hemolytic, antimicrobial and cytotoxic Features of *Ophiocnemis marmorata* (brittle star) crude extracts and showed that the ethanol extract had antimicrobial activity while methanol extracts had hemolytic activity and concluded the existence of steroidal related compounds in whole extract were responsible for their cytotoxic activity [26].

In summary since no reports is available related with anti-melanoma activity of star fish and brittle star, we highlighted for the first time that brittle star dichloromethane extract that is rich from fat inhibited melanoma cell growth and induced apoptosis. Although further studies are required to evaluate the anti-tumor effect of this extract in experimental model to apply for human melanoma treatment. Given that cytotoxic activity of brittle star dichloromethane extract notably

candidate marine ophiuroidea as an anti-carcinogenic agent used in clinical trial due to proliferation suppression and apoptosis elicitation and suggested brittle stars as rich source of natural Compounds with anticancer activity that can be depict their application in melanoma treatment complementary modality.

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