Metastatic Inhibitory and Radical Scavenging Efficacies of Saponins Extracted from the Brittle Star (*Ophiocoma erinaceus*)

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Abstract

Echinodermata use saponins in chemical defense against pathogens and predators. The molecular mechanisms of antimetastatic effects of brittle star saponins are still unknown. The present study examined antioxidant capacity and invasive ability in HeLa carcinoma cells exposed to brittle star crude saponins. Discoloring methods with DPPH and ABTS and expression of SOD-2 with RT-PCR were used to estimate the antioxidant activity. The anti-invasive activity of extracted saponins was examined through adhesion of HeLa cells to extracellular matrix, wound healing and evaluation of the mRNA levels of MMP-2 and MMP-9 by real time-PCR. The results showed that extracted saponins had cytotoxicity against cervical cancer cells and ABTS and DPPH scavenging properties with IC₅₀ values of 604.5, 1012 µg/ml, respectively. Further, we found that, in wound healing assay, brittle star saponins could prevent invasion of HeLa cells in a concentration dependent manner. Furthermore, cell adhesion assay demonstrated blockage of cell attachment to extracellular matrix with an IC₅₀ concentration of 16.1 µg/ml. The significant dose dependent down regulation of MMP-2 and MMP-9 in treated cells demonstrated that isolated saponins can decline tumor metastasis *in vitro*. The brittle star saponins remarkably prevented cervical cancer invasion and migration associated with down regulation of matrix metalloproteinase expression. Therefore, saponins could be suggested as an anti-invasive candidate against cervical cancer and an antioxidant as well.

Keywords: Echinoderm - saponins - cervical cancer - attachment - aggression - antioxidant capacity

Introduction

Cervical malignancy is the second common carcinoma in women with high incidence and mortality rate in developed countries (Bai et al., 2014). Searching for natural products as the cure of cervix cancer is increasing due to the prevalence of non surgical approaches, however, finding various natural products with high curative properties is the main purpose of tremendous studies (Palasap et al., 2014). Many evidences proved that the peroxide and free radicals generated in the body are related with developing cancers and other degenerative pathologies, especially cervical carcinoma (Di Domenico et al., 2012).

The role of antioxidant substances in reducing malignancy effects has been improved by recent studies (Nourazarian et al., 2014). Searching for novel natural antioxidants seems necessary because of low endogenous antioxidant levels and side effects of synthetic antioxidants (e.g. butylated hydroxy anisole, BHA, and butylated hydroxy toluene, BHT, Pushparaj & Urooj 2014). Trend of extracting natural antioxidants from terrestrial biota is increasing. So, it is not surprising that marine realm being also fascinating based on its high biodiversity.

Marine ecosystem has been known as a rich realm comprising various antioxidant and anticaner natural compounds with low toxicity, high efficiency and none drug resistance (Guérard et al., 2010). One group of these components is including steroid, terpenoid or alkaloid saponins, which could be supposed as natural antioxidant and may apply as anticancer therapeutic agent (Huang and Zou, 2011). Most of plants and a few percentages of marine organisms can generate saponin compounds as a defense against pathogens and predators (Andersson et al., 1989). The saponin metabolites have cell permeabilizing effect, which is related to their biological activity and make then suitable to use as detergent and insecticides (Podolak et al., 2010).

However, surface activity of saponins affect cell membrane, animal growth, food intake, protein digestion,
animal reproduction and nutrient uptake via intestinal membrane (Cheok et al., 2014). On the other hand, saponins play an important role in biomedicine based on their pharmaceutical properties including hypcholesterolemic, anticaner, antifungal, antioxidant, anti-inflammatory and antitumoral activities (Thakur et al., 2011). Various mechanisms are involved in cytotoxicity of saponins, such as preventing drug efflux, inducing intrinsic and extrinsic apoptotic, evocating cell cycle arrestment, stimulating autophagy, suppressing angiogenesis, disintegrating cytoskeleton and reducing invasiveness (Podolak et al., 2010; Thakur et al., 2011).

Considerable amounts of saponin have been reported from starfish (ophiuroidea) and sea cucumber (echinoidae) (Prabhu and Bragadeeswaran, 2013). Brittle stars are closed related to starfish, comprise some components such as poly hydroxylated steroidal glycosides and disulfated sterols, which show various biological activities (e.g. hemolytic, antiproliferative and moderate cytotoxicity) (Wang et al., 2004). Arm regeneration is prominent feature of brittle star that make it popular as an experimental study (Czarkwiani et al., 2013). In the preliminary study we found cytotoxic, antioxidiant and hemolytic activity of brittle star (Baharara and Amini, 2015). However, it has been reported that saponins possess wound healing, cytoxic and hemolytic effect, we decided to extract saponin from brittle star and evaluate therapeutic effect of extracted saponin.

We have recently isolated the crude saponin from the Persian Gulf brittle star Ophiocoma erinaceus Muller & Troschel (family Ophiocomidae) using conventional extraction methods (Amini et al., 2014). This study has been carried out to evaluate antioxidant and anti-invasiveness efficacy of extracted brittle star saponin.

Materials and Methods

Reagents

Following list includes the materials used in the laboratory experiments: 1,1-Diphenyl-2-picryl hydrazyl (DPPH) and ABTS (2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) were purchased from Sigma (USA). All solvents (methanol, chloroform, n-butanol) and Diaion HP20 were purchased from Merck (Germany). HeLa was (cervical cancer cells) and NIH3T3 fibroblast normal cells purchased from NCBI (National Cell Bank of Iran). DMEM Medium, FBS (Fetal Bovine Serum), trypsin-EDTA and antibiotic (Penicillin-streptomycin) were obtained from Gibco (USA). MTT (3-[4,5- dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide was purchased from AppliChem (USA). The RNA isolation kit was purchased from Roche, Germany. The C-DNA synthesis kit and SYBR Green real-time PCR master mix were purchased from (Thermo Scientific, EU). The specimens of the brittle star Ophiocoma erinaceus (O. erinaceus) were collected by hand from a rocky intertidal flats of Qeshm island, Persian Gulf. The identification of samples was carried out using Clark & Rowe key.

Extraction of saponin fraction from brittle star

The saponin was isolated from the brittle star samples as described (Hu et al., 2010). First, the samples were washed using water, crushed into small pieces and immersed in ethanol at the room temperature for 3 days. Then, the extract was made by refluxing with ethanol for 3 h. In the next step, combined extract was evaporated under reduced pressure (Heidolph, Germany) and was defatted using chloroform/water solution for 6h. The water fraction was isolated using n-butanol. The organic layer was evaporated to yield n-butanol extract, which was dissolved in distilled water, applied using Diaion HP-20 resin column and washed using dionized water, 80% ethanol and 100% ethanol, respectively. Finally, the 80% ethanol fraction confirmed as the cure saponin with saponin detection methods. The saponin fraction was condensed; freeze dried and stored at -20°C.

DPPH radical scavenging assay

The color of DPPH and ABTS changes by reducing amount of hydrogen donating compounds, which is used for evaluating antioxidant potency. The comparison of scavenging activity of brittle star saponin and BHA against free-radical DPPH was evaluated as described (Tapondjou et al., 2011).

Briefly, the various concentrations of brittle star saponin fraction (0–2000μg/ml) were prepared in ethanol and 100 μl of each concentration of saponin fraction was added with 200μl of 1mM DPPH solution in ethanol. Then, the absorbance was measured at 517 nm after 30 min using a microplate reader(Epoch, USA). The bleaching activity was calculated using the following equation.

DPPH radical scavenging (%)=(control OD- sample OD/ control OD)×100.

ABTS decolorization assay

ABTS assay has been known as the in vitro antioxidant assay applying electron donating activity determination of brittle star saponin fraction. For this purpose, ABTS+ was dissolved by reaction between 7 mM ABTS stock solution and 2.45 mM K2S2O8. Then, the solution was maintained at room temperature for 12-16h. The reaction mixture (1 ml of standard, ascorbic acid or different concentrations of extracted saponin was mixed to 1ml of the ABTS stock solution) and incubated 30 min at room temperature. The optical density was taken at 734 nm. The decolorization activity of ABTS+ by saponin fraction was calculated using the equation:

ABTS scavenging effect %=((control absorbance – sample absorbance)/(control absorbance))×100.

Cell culture

The human cervical cancer cells, HeLa, were cultured in DMEM medium containing 10% FBS supplemented with 1% penicillin-streptomycin at 37°C, 5% CO2 incubator. The cells were maintained in a sub-confluent condition. All experiments were repeated for at least three times.

RNA extraction and RT-PCR analysis of SOD-2

In order to evaluate the extracted saponin on SOD-2 (Super Oxide Dismutase) mRNA levels, HeLa cells (2×10⁶) were treated using concentrations of saponin.
Then the total cellular RNA of treated and untreated HeLa cells was isolated using the High Pure RNA Isolation kit (Roche, Germany). Total RNA (2 µg) was reverse-transcribed to cDNA using random hexamer, or oligo(dT), and RT premix; and then amplified using RT-PCR Premix (Parstous, Iran). First, the produced cDNA (2 µL) was added to 10 µL Taq premix, 2 µL forward primer, 2 µL reverse primer and distilled water (Parstous, Iran). RT-PCR was performed 1 cycle reverse transcription at 95°C/4 min and 35 cycles as denaturation at 94°C for 30s, annealing at 59°C for 30 s, extension at 72°C for 30 s and 1 cycles 5 min at 72°C. The primers were: B2M Forward 5’ TGGTGTGTGGGTCTCAGTACC 3’, Reverse 5’ TATGGTTGGCTTCCCATTCT 3’ was used as the housekeeping gene. Forward 5’ AGCTATTTGGAATGTAATCAACTGG 3’ and Reverse 5’ TAAAGCAACATCAAGAATGCTACA 3’ for SOD-2. The PCR products were electrophoresed on a 2% agarose gel. The bands were became visible by green viewer staining and recorded using UV TEC gel documentation system (Cambridge, UK).

### Analysis of cell viability

The human cervical cancer cells and NIH3T3 fibroblast normal cells (10 4 cells/well) were cultured in DMEM medium to determine the effect of brittle star saponin on viability of cancer and normal cells. Then the cultured cells were incubated and treated using appropriate concentrations of saponin (0, 0.75, 3.1, 6.2, 12.5, 25, 50, 100 µg/ml). The viability of HeLa cancer cells and NIH3T3 normal cells was determined using MTT assay. First, after treatment period, 30 µl MTT solution (1 mg/ml) was dissolved in PBS and added to each well plate and incubated in the darkness for 4h. Then, the solution was formed crystal formazan in viable cells by dissolving with 100 µl DMSO. Finally, the absorbance was measured using a micro plate reader.

**MMP-2 and MMP-9 mRNA expression by Real time PCR**

Real-time RT-PCR was used to measure gene expression of MMPs (Matrix Metalloproteinase) in human cervical carcinoma cells. The total cellular RNA was extracted and quantified at OD between 260-280 nm. In the next step, according to the manufacturer’s protocol, total RNA reverse was transcribed to synthesize complementary DNA using a revert aid first strand cDNA synthesis kit (Thermo Scientific, EU), Real-time PCR was conducted on a Real-Time PCR Detection System (Bio-Rad CFX96) using SYBR Green real-time PCR master mix (Thermo Scientific, EU), 5 µl of reverse transcription product and primers (final volume of 20 µl). The sequences of the primers are listed in Table 1. The thermal cycle profile was 95°C for 20 s before the first cycle; 95°C for 20s, 59°C for 20sand 72°C for 30s, respectively, which were repeated 40 times and followed by 95°C for 1 min and 55°C for 1 min. The GAPDH was used as housekeeping control gene.

### Statistical analysis

The results are presented as the mean ± SEM. The experiments were carried out in triplicate. The significant differences among the means were analyzed by one-way ANOVA followed by the Tukey test. The level of p ≤0.05 was considered to be significant.

### Results

#### Antioxidant potential of brittle star extracted saponin

The antioxidant activity of the extracted saponin was tested using DPPH and ABTS assays. The free radical scavenging potential of brittle star saponin against DPPH, ABTS radicals were dose dependent in an IC50 concentration of 604.5 and 1012 µg/ml for ABTS and DPPH, respectively (Figure 1A and B), which were weaker than standard antioxidants such as BHA and ascorbic acid.

These results showed that brittle star extracted saponin can be introduced as novel natural antioxidant. Further, the expression of SOD-2, as one of the endogenous antioxidants enzymes, was evaluated and the results indicated that saponin can play its role by increasing superoxide dismutase levels (Figure 2). Hence, brittle star saponin fraction can interfere to oxidative damage induced by cervical cancer cells.
The cytotoxic effect of brittle star saponin

The cytotoxicity of brittle star saponin was examined by MTT assay. For this purpose, the human cervical cancer cells and NIH3T3 normal cells were incubated with various concentrations of isolated saponin (0, 0.75, 3.1, 6.25, 12.5, 25, 50, 100 µg/ml) for 24 and 48h. The viability of HeLa cells were inhibited by extracted saponin in a dose dependent manner (Figure 3). Although, the saponin with the concentration of 0.75 µg/ml showed no significant cytotoxicity compared to untreated cells. The IC₅₀ concentrations of extracted saponin for HeLa cancer cells determined approximately as 17.22 and 11.79 µg/ml for 24, 48 h, respectively which used in all subsequent experiments. However, NIH3T3 normal cells didn’t indicated remarkable cytotoxicity under exposure with brittle star saponin fraction. The morphological changes of HeLa cancer cells and NIH3T3 normal cells incubated with or without brittle star saponin were examined by inverted microscopy. As shown in figure 3, the cancer cells exposed to IC₅₀ concentrations of brittle star saponin revealed the obvious morphological alterations of apoptosis, such as, rounding, loss of adhesion, cell shrinkage and the appearance of apoptotic bodies as compared with untreated cells, while, brittle star saponin not induced significant morphological alteration on NIH3T3 normal cells suggesting a differential cytotoxic effect of brittle star saponin on cancer and normal cells.

Brittle star saponin suppressed HeLa cell adhesion and migration

Alteration of cell attachment to extracellular matrix

Figure 1. Antioxidant Capacity of Brittle Star Saponin. A) DPPH scavenging activity of extracted saponin as compared with BHA. B) ABTS radical-scavenging activities of extracted saponin as compared with ascorbic acid. Each value represents a mean±SD (n=10)

Figure 2. A) The Qualitative Changes in the Expression of SOD-2 mRNA was Analyzed by the Reverse Transcription-polymerase Chain Reaction Method. HeLa cells were treated with brittle star saponin and the mRNA expressions of SOD-2 were assessed by RT-PCR analysis that indicated antioxidant efficacy of isolated saponin. B) The densitometry of gel quantitatively evaluated by image J (n=3)

Figure 3. A) Analysis of Cytotoxicity of Brittle Star Saponin in HeLa Cervical Cancer Cells and NIH3T3 Normal Cells, A, B) Effects of extracted saponin on the viability of HeLa and NIH3T3 cells after 24 and 48 h treatment by MTT assay. Values represent as mean±SD (n=10) and the statistical significance of differences between untreated and treated cells was calculated (**p<0.01 and ***p<0.001). C, D) Morphological changes induced by brittle star saponin on HeLa and NIH3T3 cells. As shown, extracted saponin induced apoptotic morphological changes in HeLa cells, while it didn’t induce significant alteration on NIH3T3 normal cells. The images were captured by inverted microscope. a) untreated cells; b, c, d) Cells were treated with 12.5, 25, 50 µg/ml brittle star saponin for 24 h. (C) White arrows indicated cancer dead cells. C (Magnification × 200), D (Magnification × 400)
The effect of brittle star saponin on the adhesion of HeLa cells. Cells treated with 0, 12.5, 25, 50 µg/ml extracted saponin for 24 h and migration was assessed by wound healing assay. (x= scratch area, x1, x2, x3, x4= the closure of wound area which untreated and treated HeLa cells filled created area). B) Quantitative assessment of healing area on the basis of enclosed scratch area. C) The effect of brittle star saponin on the adhesion of HeLa cells. Cells treated with or without extracted saponin and were plated onto the surface of 96-well plates coated with Matrigel (2 µg/well). Then, cell attachment was measured at the indicated time points. (Data represent the mean±SD. ***p<.001, was considered significant, n=10)

Brittle star saponin modulates the down-regulation of MMP-2 and MMP-9 expression

MMPs (Matrix Metalloproteinases) are zinc-binding enzymes that their expression levels are attributed with tumor metastasis. To identify the molecular basis of anti-metastatic activity of brittle star saponin, the cervical cancer cells were exposed using different concentrations of isolated saponin and then performed using real time-PCR assay. Figure 5 shows the transcriptional expression of MMP-2 and MMP-9 in HeLa cells. The brittle star saponin dose dependently decreased MMP-2 and MMP-9 mRNA expression in a dose dependent manner (Figure 5). Therefore, the marked down-regulation of MMP-2 and MMP-9 demonstrated anti-invasiveness properties of the brittle star saponin via suppressing enzymatic degradation processes in cervical cancer.

**Discussion**

Dual Interaction between cancer cells and normal cells may produce reactive oxygen species, which can affect cancer metastasis (Gupta et al., 2014). A previous study showed that any damage in endogenous antioxidant system, as a consequence of antioxidant metabolic rate...
abnormality, may enhance the accumulation of reactive oxygen species. Therefore, the proliferation of cancer cells in metastatic state may become faster as a result of free-radical-mediated injuries (Nishikawa and Hashida, 2006).

Our previous observations exhibited that brittle star methanol extract has cytotoxic and antioxidant ability (Baharara and Amini, 2015). In this study, the antioxidant capacity of isolated saponins has been confirmed by DPPH and ABTS methods. Therefore, the brittle star’s radical scavenging activity of methanol extract may be a result of saponin compounds. The antioxidant activity of saponin may be attributed to its ability of terminating the radical chain reaction by electron or hydrogen donating to the DPPH and ABTS radicals. There are many reports related to antioxidant and cytotoxic properties of saponin metabolites. The cytotoxic and radical scavenging capacity of triterpenoid saponins from *Terminalia vorensis*, which widely used in African traditional medicine, were established by Ponou (2010).

Tapondjou (2011) were examined the dimeric cytotoxic and antioxidant properties of triterpenoid saponins extracted from *Butyrospermum parkii* Kotschy (Sapotaceae) and found that the isolated saponin has a significant inhibitory effect on the proliferation of A375 and T98G cells. The study also showed that the isolated saponin can play an antioxidant role against DPPH, ABTS and nitric oxide free radicals. Arslan & Çelik (2013) have succeeded to extract crude saponin from *Gypsophila arrostii* Guss, *G pilulifera* Boiss, & Heldr and *G simonii* Hub. They evaluated antiradical potency using DPPH and ABTS assays and found strong antioxidant and low hemolytic activities for the saponin extract. Weng et al (2014) found antioxidant efficacy of the saponins extracted from root bark of *Aralia taibaiensis* Z.Z.Wang & H.C. Zhengas, which has been known as one of the Chinese medicine herbs.

All studies mentioned above, same our study, confirmed antioxidant efficacy of natural saponin metabolites, which can be used for prevention and suppression of carcinogenesis. Additionally, declining the intrinsic antioxidant defense mechanisms can exacerbate promotion of malignancy metastatic state. Hence, controlling levels of antioxidant enzymes can suppress tumor metastasis (Badjatia et al., 2010).

In this study, the transcriptional level of superoxide dismutase under exposure with brittle star saponin fraction was investigated. We found that extracted saponin fraction can exhibit antioxidant capacity via augmentation the level of superoxide dismutase, which is one of involved endogenous antioxidant enzymes. Kumaraguruparan (2002) found a negative correlation between cancerous and normal individuals. Kumaraguruparan found that in the cancerous state, the level of glutathione peroxidase was significantly lower and superoxide dismutase activities was higher compared to the control group.

Further, in this study, the levels of metastatic associated molecules were evaluated to identify the mechanisms that inhibited cervical cancer metastasis by brittle star saponin. MMP-2 (Matrix Metalloproteinase-2 or gelatinase-A) and MMP-9 (Matrix Metalloproteinase-9 or gelatinase-B), which are generated by metastatic cancer cells, play a major function in progressing tumor invasion and migration (Li et al., 2014). Furthermore, it has been demonstrated that MMP expression is associated with cervical carcinoma promotion (Roomi et al., 2010). Therefore, It is not surprising that finding novel components to suppress tumor metastasis is the main purpose of oncological studies (Byambaragchaa et al., 2013). The results of this study showed that brittle star extract comprises crude saponin with hemolytic and cytotoxic properties. Additionally, our results elucidated that crude saponin extracted from brittle star can significantly blocked cancer migration of HeLa cells through suppression of MMP-2 and MMP-9 expression.

Previous studies revealed that other saponin metabolites have also antimetastatic effects. For example, Platycodon D a type of saponin extracted from the *Platycodononis Radix*, which has some anti-tumor and anti-invasiveness properties as it can inhibit growth, adhesion and migration of hepatocellular carcinoma cells along with suppression the adhesion of HepG2 cells to Matrigel (Li et al., 2014).

In another study, Man et al (2011) examined antitumor activity of diosgenyl and penogenylsaponins extracted from *Rhizoma Paradis*. They found that these saponin substances possess anti aggressive efficacy against B16F10 melanoma cells through suppression of MMP-2 and MMP-9 activity in translational level. The role of saponin isolated from *Platycodon grandiflorum* on invading and migrating HT1080 cells were estimated and proved that this metabolite has inhibitory effect on invasion and migration of the cancer cells by decreasing activity of MMP-9 and MMP-2. Therefore, the metabolite has been introduced as an anti-invasive drug for treatment of colon cancer (Lee et al., 2008).

Ganoderic acid is an active terpenoid extracted from *Ganoderma Lucidum*, which can inhibit adhering cells to extracellular matrix by suppressing MMP-2 and MMP-9. Therefore, ganoderic acid could be suggested as a useful clinical agent against high metastatic lung carcinoma (Xu et al., 2010). Regarding to cervical cancer, it has been determined that polyphenol compounds such as Curcumin, resveratrol and ferulic acid have antioxidant capacity, which can interfere with each stage of cervical carcinogenesis. It is exhibited that arsine trioxide as a natural substance, can be consider as an inhibitor for cervical cancer, which can block attach to Fibronectin and Matrigel, reduce the MMP-2 expression and induce the E-cadherin expression (Yu et al., 2007). There is few reports about the ability of starfish and brittle stars in gelatinase expression. Suh et al (2011) explained that the ethyl acetate fraction extracted from *Asterias amurensis* has anti-atherosclerotic effect, which is a result of suppressing MMP-9 and MMP-2 activities in human aortic smooth muscle cells.

In contrast, we found an anti-metastatic activity of brittle star saponin, which seems a result of reducing the attachment of cervical cancer cell to extracellular matrix and down regulation of MMP-2 and MMP-9. Therefore, we suggested that brittle star crude saponin has an anti-migration effect through the down-regulation of MMP-2 and MMP-9. In addition, extracted saponin exerted preferential cytotoxic effect against cancer cells.
with minimum cytotoxicity on fibroblast normal cells. Therefore, these results introduced brittle star saponins as a novel anti-metastatic lead for the control and avoid of progression metastatic cervical cancer related to oxidative stress in future. Nevertheless, further studies on the metastasis pathways genes regulated by brittle star crude saponins seem necessary.

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Elaheh Amini et al


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