

The anti-proliferative and anti-angiogenic effect of the methanol extract from brittle star

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Abstract

Background: Anti-angiogenic therapy is a crucial step in cancer treatment. The discovery of new anti-angiogenic compounds from marine organisms has become an attractive concept in anti-cancer therapy. Because little data correlated to the pro- and anti-angiogenic efficacies of *Ophiuroidea*, which include brittle star, the current study was designed to explore the anti-angiogenic potential of brittle star methanol extract *in vitro* and *in vivo*.

Methods: The anti-proliferative effect of brittle star extract on A2780cp cells was examined by MTT assays, and transcriptional expression of VEGF and b-FGF was evaluated by RT-PCR. In an *in vivo* model, 40 fertilized Ross eggs were divided into control and three experimental groups. The experimental groups were incubated with brittle star extract at concentrations of 25, 50 and 100 µg/ml, and photographed by photo-stereomicroscopy. Ultimately, numbers and lengths of vessels were measured by Image J software. Data were analyzed with SPSS software ($p < 0.05$).

Results: Results illustrated that the brittle star extract exerted a dose- and time-dependent anti-proliferative effect on A2780cp cancer cells. In addition, VEGF and b-FGF expression decreased with brittle star methanol extract treatment. Macroscopic evaluations revealed significant changes in the second and third experimental group compared to controls ($p < 0.05$).

Conclusion: These finding revealed the anti-angiogenic effects of brittle star methanol extract *in vitro* and *in vivo* confer novel insight into the application of natural marine products in angiogenesis-related pathologies.

Key words: Angiogenesis, Anti-proliferative, Brittle star, Ovarian cancer, Marine organisms

Introduction

Angiogenesis, which is required for homeostasis, plays a crucial role in pathological events such as tumor proliferation, dysplasia progression, and metastasis (1). Increased angiogenesis contributes to the development of vascular-related diseases, especially cancer, and is a predictor of disease progression (2). Numerous angioregulatory peptides that control angiogenesis have been identified; however, an imbalance between pro- and anti-angiogenic factors can disrupt pathological angiogenesis (3). In cancer progression states, augmented expression of pro-angiogenic factors or reduction of angiogenic inhibitors can complicate cancer treatment strategies, requiring the need for further modalities (4).

Therapy that disrupts tumor vasculature is a fundamental strategy in cancer treatment. Hence, the use of novel anti-angiogenic compounds with vascular-disrupting capacity is a promising modality in tumor-targeted therapy (5).

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF, also known as FGF-2), as major endogenous pro-angiogenic cytokines that induce activation and migration of endothelial cells into tumors, are important pro-angiogenic factors involved in the regulation of tumor-related angiogenesis (6). Basic fibroblast growth factor promotes vascular sprouting via involvement in cell growth, migration, and differentiation (7). In tumor cells, increased VEGF expression, as a key regulator of

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angiogenesis, promotes invasion and metastasis. Therefore, VEGF suppression strategies are directed at tumor growth arrest, endothelial cell apoptosis, and extracellular matrix degradation and enhancement of other cancer therapeutic methods (3); therefore, targeting of VEGF and b-FGF pathways is pivotal in tumor metastasis prevention (8).

Ovarian cancer, a leading cause of cancer deaths in women worldwide, is spread primarily in the peritoneum through VEGF-mediated angiogenesis, which is associated with tumor aggression and poor survival (9). Growth, survival, and progression of tumors, such as in ovarian cancer, require enriched blood vessel networks; therefore, anti-angiogenic therapy has emerged as a good candidate for ovarian cancer treatment. The use of natural products with potent anti-angiogenic effects may help to reduce the side effects of synthetic chemotherapeutics on patients (10). A variety of terrestrial natural products have been proposed as potent angiogenic inhibitors with minimal toxicity, such as taxol, combretastatin, and camptothecin. Likewise, there is currently much enthusiasm for the identification of natural marine products as sources of angiogenic modulating compounds that may be promising in cancer and other related disease therapies (1). Brittle star (Ophiuroidea), the largest class of echinoderm, has sparked attention due to its arm regeneration capacity. Chlorinated biphenyls, triterpenoids, and polycyclic hydrocarbons have been identified as major bioactive metabolites in Ophiuroidea (11). Because there are few reports regarding anti-angiogenic compounds from the marine ecosystem, particularly echinoderms, and because no reported studies have examined the pro- or anti-angiogenic properties of Ophiuroidea, the present investigation aimed to explore the anti-angiogenic potential of brittle star methanol extract on ovarian cancer A2780cp cells and an *in vivo* chorioallantoic membrane (CAM) model.

Materials and Methods

Reagents

A2780cp human ovarian cancer cells were purchased from NCBI (National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). RPMI1640 Medium, FBS (fetal bovine serum), trypsin-EDTA, and antibiotic (penicillin-streptomycin) were obtained from Gibco-USA. The RNA isolation kit was from Roche (Germany). The cDNA synthesis and RT-PCR kits

were purchased from Pars Tous (Iran). Specimens of the brittle star (*Ophiocoma erinaceus*) were obtained from rocky intertidal flats of Persian Gulf waters. Methanol was purchased from Merck (Germany). HP-20 resin and MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) were obtained from Sigma (USA).

Preparation of brittle star methanol extract

After morphometric estimation of *Ophiocoma erinaceus* at the Research Center Applied Biology of Mashhad Islamic Azad University, specimens of brittle star were washed and stored at -80°C . For extraction, about 20 g samples of brittle star were dried, minced, mixed with 200 ml of methanol, and stirred for 72 h. The methanol solution was filtered through an 11 μm filter, concentrated in a vacuum evaporator, and stored at -20°C .

Cell culture

A2780cp cells were grown in RPMI 1640 cell culture medium containing 10% fetal bovine serum (FBS) (Gibco, USA) supplemented with L-glutamine (Sigma, USA) and 1% antibiotic at 37°C in an incubator containing 5% CO_2 . Treatment with brittle star extract was performed in the logarithmic phase of cell growth.

Cell proliferation assays

The effects of total brittle star extract on A2780cp cell growth were measured by MTT assays. The cells were plated at 10^4 cells/well in 96-well plates and incubated overnight. Then, medium was removed and the cells were incubated with the brittle star methanol extract at 0, 25, 50, or $100\mu\text{g/ml}$ for 24 or 48 h. After the treatment periods, cell viabilities were determined by MTT assays. In brief, 30 μl of a 1 mg/ml MTT in phosphate-buffered saline (PBS) solution were added to each well, incubated for 4 h at 37°C in the dark, and crystal formazan crystal which formed in viable cells dissolved with 100 μl of DMSO. Finally, the optical absorbance was measured at 570 nm on a spectrophotometer (Epoch, USA). All experiments were performed in triplicate.

RT-PCR

Changes in VEGF and b-FGF mRNA expression were analyzed by Revers transcription PCR (RT-PCR). Total cellular RNAs of A2780cp treated and

untreated cells were isolated by the High Pure RNA Isolation kit. Two μg of RNA were reverse transcribed to cDNA using an Easy cDNA Synthesis (ParsTous, Iran) Kit according to the manufacturer's protocol. Briefly, cDNA was synthesized in the presence of oligo dT, then incubated at 65 °C for 5 min followed by addition of the reverse-transcription (RT) premix and incubated at 25 °C for 10 min, 50 °C for 60 min, and 70 °C for 10 min, and then amplified according to manufacturer's protocol. Two μl of the synthesized cDNA were added to 10x buffer, MgCl_2 , 25 mM dNTP, Taq DNA polymerase, and the appropriate forward and reverse primers. Ultimately, RT-PCR was performed with 1 cycle at 95 °C for 4 min, 35 cycles at 94 °C for 30 sec for denaturation, 57 °C for 30 sec for annealing, 72 °C for 30 sec for extension, and 1 cycle for 5 min at 72 °C. The primers used were: b2m ($\beta 2$ microglobulin, used as a housekeeping gene) Forward 5' TGGTGCTTGGCTCACTGACC 3', Reverse 5' TATGTTCCGGCTTCCCATTCT 3'. Forward and reverse VEGF primers were 5' CTGCTGTCTTGGGTGCATTG 3' and 5' TTCACATTTGTTGTGCTGTAG 3'. Forward and reverse b-FGF primers were 5' CACCTATAATTGGTCAAAGTGG3' and 5' CAGAAATTCAGTAGATGTTTCCC 3'. Following amplification, the PCR products were electrophoresed in a 2% agarose gel and visualized by green viewer staining.

In vivo angiogenesis assay using CAM model 40 fertilized Ross eggs were purchased from Toos Company (Iran) and randomly divided into a control group, which was stored using the normal conditions, and three experimental groups treated with concentrations of 25, 50, and 100 mg/ml of brittle star extract. The eggs were incubated at 38 °C and 55-65% humidity with automatic rotation. On day 2 of incubation a small hole was made in the shell under sterile conditions to reveal the air sac. Part of the shell was removed and the window was covered with sterile paraffin and coverslips (Fara, Iran). Then, the eggs were transferred to a sterile incubator and rotated manually twice a day to allow normal embryo development. On day 8 of incubation a 4×4 mm gelatin sponge containing agar in normal saline and albumin with 200 μl of antibiotic was placed on the CAM. The sponge contained 10 μl of brittle star extract. On day 12 of incubation, all samples were

photographed using a research photo stereo microscope (Ziess, Germany). The number and length of blood vessels around the gelatin sponge were recorded and measured using Image J software.

Statistical analysis

Quantitative data were obtained using SPSS software, version 16 (New York, USA) with a significance level of $p < 0.05$. T-test, ANOVA, and post hoc *Tukey* tests were performed.

Results

Effect of brittle star methanol extract on A2780cp cell growth

To assess the anti-proliferative effect of brittle star methanol extract on A2780cp cell viability, ovarian cancer cells were treated with elevating dosages of brittle star extract. As shown in Fig. 1, brittle star methanol extract inhibited cell viability in a dose /time-dependent manner.

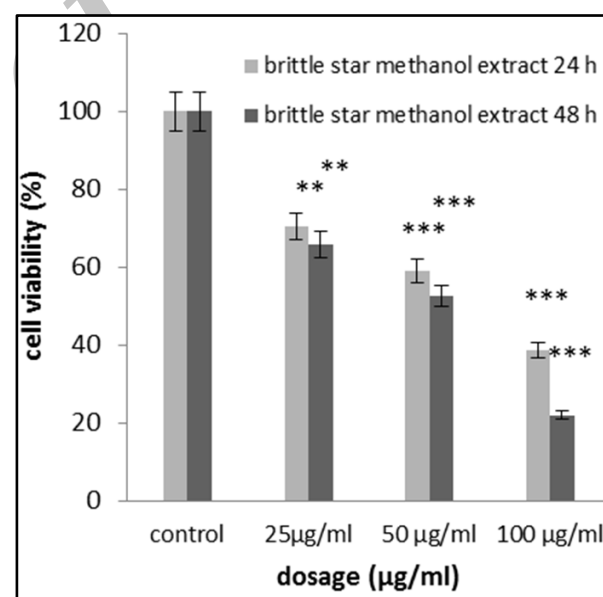


Fig. 1: Effect of brittle star methanol extract on viability of A2780cp ovarian cells after 24 and 48 h of treatment with brittle star methanol extract. Cell viability was determined by MTT assays. Data represent the mean \pm SD. ** = $p < 0.01$ and *** = $p < 0.001$.

Effect of brittle star methanol extract on VEGF and b-FGF mRNA expression

The mRNA levels of two angiogenic-related genes, VEGF and b-FGF in ovarian cancer cells treated with 50 and 100 $\mu\text{g/ml}$ brittle star methanol extract were evaluated using RT-PCR. The RT-PCR analysis indicated that the pretreatment of A2780cp cells with

brittle star extract decreased the mRNA levels of VEGF and b-FGF as compared with the untreated cells (Fig. 2).

Anti-angiogenic effect of brittle star methanol extract on CAM model

The anti-angiogenesis effect of brittle star alcoholic extract is shown in Fig. 3. Further, as shown in table 1, and Fig. 4, the examined extract has been affected number and length of blood vessel. The difference between the mean of length of blood branches in the control samples (34 ± 0.1 mm) and the first experimental group (32 ± 0.13 mm) was not statistically significant ($p > 0.05$); however, the differences in mean lengths between the control and the second (30.5 ± 0.09 mm) and third (24 ± 0.2 mm) experimental groups were significant ($p < 0.05$ and $p < 0.001$ respectively). The differences between the number of blood vessels in the controls (25.6 ± 1.1) and the first (22.4 ± 0.34), second (20.38 ± 0.75), and third (18.06 ± 0.78) experimental groups were all significant ($p < 0.05$ for the first and $p < 0.001$ for the second and third groups).

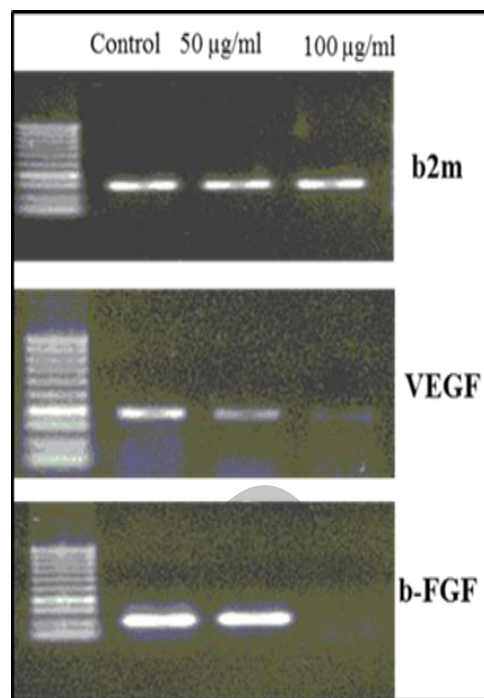


Fig. 2: A2780cp ovarian cancer cells were treated with brittle star alcoholic extract and the mRNA level of b2m, VEGF, and b-FGF was evaluated by RT-PCR and it showed that brittle star extract down-regulated the expression of VEGF, and b-FGF as compared with untreated group (control).

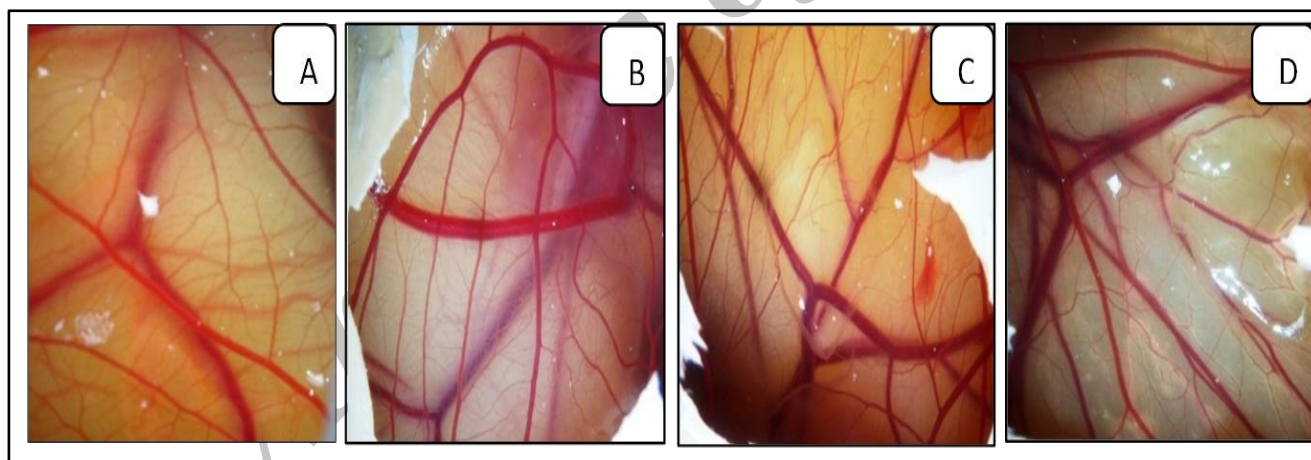


Fig. 3: Photomicrographs of CAM in the control and treated samples. Control (A) and experimental samples treated with brittle star extract at concentrations of 25 (B), 50 (C), or 100 (D) µg/ml.

Table 1. The vessel number and length in control and experimental groups. All data are presented as mean \pm SD.

Groups	Mean of length of blood vessels (mm) mean \pm SD	p-Value	Mean of number of blood vessel mean \pm SD	p-Value
Control	34 \pm 0.1	-	25.6 \pm 1.1	-
25 µg/ml	32 \pm 0.13	$p > 0.05$	22.4 \pm 0.34	$p < 0.05$
50 µg/ml	30.5 \pm 0.09	$p < 0.05$	20.38 \pm 0.75	$p < 0.001$
100 µg/ml	24 \pm 0.2	$p < 0.001$	18.06 \pm 0.78	$p < 0.001$

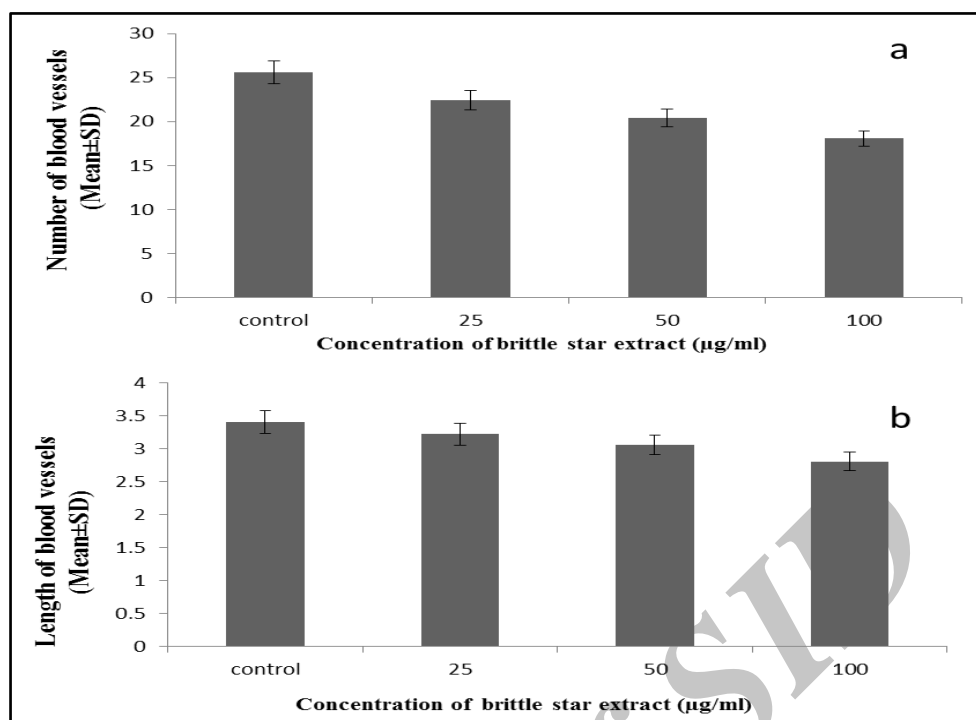


Fig. 4: Average of number (a) and length (b) of blood vessels in control and experimental samples treated with brittle star extract (* $p < 0.05$, ** $p < 0.001$). The extract has been affected number and length of blood vessels.

Discussion

Angiogenesis is a complex physiological process crucial to pathogenesis in all stages of cancer progression. The development of new blood vessels is essential for adequate blood supply in malignancy expansion. Hypoxia is considered to be a major stimulus that triggers the release of cytokines, chemokines, and pro-angiogenic factors to sprout new blood vessels and degrade basement membrane that lead to metastasis (12). Thus, angiogenesis suppression is a promising approach in current anti-cancer therapy and efforts to find natural angiogenesis inhibitors has attracted much attention (5). Herein we demonstrated that brittle star methanol extract significantly inhibited A2780cp cell growth at 50-100 µg/ml. This result was consistent with our previous experiments in which brittle star methanol extract demonstrated similar toxicity against HeLa cervical cancer cells. However, the CAM assay demonstrated that the formation of new blood vessels was significantly less in the experimental groups in a dose-dependent manner than in the control group. VEGF and b-FGF are pro-angiogenic factors released in response to inadequate oxygen or nutrients, or mutations in oncogenes or tumor suppressor genes in tumor beds (6). Accordingly, the effect of the brittle star methanol extract on VEGF and b-FGF

expression was assessed. RT-PCR revealed dose-dependent reductions in VEGF and b-FGF mRNA expression.

There are many reports related to anti-angiogenicity in natural products. Several phytochemicals, such as polyphenols, flavonoids, and chalcones, have been shown to influenced angiogenesis (13). For example, Cao et al, demonstrated that resveratrol, a bioactive compound obtained from grape, had anti-angiogenic properties (14).

He, et al. (2009) reported anti-angiogenic activity in a zebrafish model in rhubarb root, an ingredient in traditional Chinese compounds for treatment of inflammation, and anthraquinone derivatives (15). In 2010, He et al. reported that n-hexane and ethyl acetate fractions from *Alpinia oxyphylla*, in *in vivo* and *in vitro* assays, exhibited anti-angiogenic effects in on zebrafish embryos, endothelial cells, and MCF-7 and Hep-G2 tumor cells (16).

In 2012 He et al. isolated bioactive metabolites from *Alpinia caerulea* and investigated anti-angiogenic function of *Alpinia* on umbilical endothelial cells. Their results showed that fruit extract is rich in diterpenoids identified as zerumin A and (E)-8(17),12-labdadiene-15,16-dia. At 20µg/ml

the fruit extract inhibited vessel formation by 25% in a zebrafish model (17).

In addition, He et al. (2011) examined rhein, a bioactive substrate from rhizome of *Rheum palmatum* and showed suppression of angiogenesis via influence on angpt2 and tie2 mRNA expression (18).

The anti-angiogenic activity of julibroside J8 was examined by Hua in 2009. Julibroside J8 is a triterpenoid saponin extracted from *Albizia julibrissin* that inhibited microvessel formation in a CAM model at 10-50 µg/egg and reduced tumor vessel density in transplanted colon carcinoma cells in Balb/C nude mice at 0.5-3 mg/kg. This result demonstrated promising inhibitory effects on cancer progression (19). Varinska and his colleagues studied *in vitro* and *in vivo* anti-angiogenic effects of the flavonoid precursor 4-hydroxychalcone (Q797) and proposed Q797 as a new angiogenesis inhibitor due to its selective effect on endothelial and human epithelial cancer cells via VEGF and b-FGF modulation and expression (20).

Previous investigations established that VEGF plays an important role in epithelial and endothelial cell proliferation (7). Perk et al. (2009) reported 1-furan-2-yl-3-pyridin-2-yl-propenone, a selective cyclooxygenase (COX) inhibitor, significantly inhibited VEGF production in HT1080 human fibrosarcoma cells, and breast cancer cell angiogenesis in CAM assays (21). Jiang et al. (2013) examined the anti-angiogenic activity of deoxypodophyllotoxin as a natural microtubule destabilizer and indicated suppression of angiogenesis *in vitro*, *ex vivo*, *in vivo*, and vascular disrupting effects (5).

Lee (2010) investigated *Phellinus linteus* methanol and aqueous extracts on proliferation and migration of HUVEC cells and found that only the methanol extract significantly inhibited angiogenesis *in vitro* and *in vivo* (22).

Huang (2013) reported anti-metastatic and anti-angiogenic activity of carotenoid lycopene against human peripheral blood mononuclear cells, and CAM assays elucidated that this effect was associated with elevated cytokine levels, especially IFN-γ and IL-12, along with down-regulation of MMP-2 (23).

Yoo and his coworkers (2008) evaluated the action of *Saururus chinensis* aerial parts extract on

angiogenesis and inflammation and observed strong CAM angiogenesis suppression, along with inducible nitrous oxide synthase (iNOS) and COX-2 attenuation and *in vivo* anti-inflammatory effects (24).

In a similar investigation, Jung et al. examined anti-inflammatory and anti-angiogenic activities in ethanol extracts of dried parts of *Salvia plebeian*, which is commonly used as a palliative for inflammatory lesions in traditional medicine, and found significant inhibition of chick CAM angiogenesis and anti-inflammatory effects in an air pouch model. The inhibition of angiogenesis was associated with iNOS and reactive oxygen species (ROS) reduction in stimulated macrophage cells, indicating the antioxidant capacity of *Salvia plebeian* (25).

Yi et al. (2012) showed that *Gleditsia sinensis* ethanol extract has anti-angiogenic and anti-metastatic activity via down-regulation of endothelin-1 and matrix metalloproteinase 2 and may have anti-cancer potential (26).

Recent research has shed light on the potential value of chemotherapeutic drugs to improve survival rates in ovarian cancer patients (27). Several natural metabolites and dietary compounds, such as curcumin and resveratrol, are being studied in oncological research in combination with chemotherapy. Failures in common therapeutic strategies, such as surgery and chemotherapy, have highlighted alternative modalities for ovarian cancer treatment (28).

Vascular endothelial growth factor plays an important role in ovarian carcinogenesis (6). Bevacizumab (Avastin), a prominent anti-VEGF treatment, in combination with chemotherapy, inhibits the binding of VEGF isoforms to the VEGF receptor (VEGFR). This is considered to be the most efficient modality against ovarian cancer cell line insensitive to chemotherapy. Other VEGFR inhibitors in clinical development include aflibercept, ramircumab, and semaxanib (9).

Down-regulation of VEGF is a chief strategy that contributes to increased survival in ovarian cancer patients. Therefore, bevacizumab and other VEGF inhibiting compounds offer promise in ovarian cancer treatment (29). For example, Rhode et al. (2007) demonstrated that ginger significantly inhibited ovarian cancer cell proliferation and suppressed

VEGF and IL-6 secretion, resulting in NF- κ B inhibition (10).

Recently, interest has increased in the biological potential of natural marine products on human health. Namvar et al. (2013) demonstrated that a poly-phenolic component of seaweed *Sargassum muticum* methanol extract (SMME) exerted antioxidant and anti-proliferative activity on breast cancer cells. Also, SMME induced apoptosis and inhibited *in vivo* angiogenesis in MCF-7 and MDA-MB-231 cells, which suggests that seaweed may be a potent source of bioactive compounds (30).

These reports demonstrate the anti-angiogenic properties of natural products and provide novel insight into the marine ecosystem as an immense

source of active metabolites with therapeutic applications.

Conclusion

Brittle star methanol extract inhibited angiogenesis in the CAM model and suppressed cell proliferation and VEGF and b-FGF mRNA expression in A2780cp ovarian cancer cells. These data provide additional therapeutic information about Persian Gulf brittle star, which may have therapeutic potential in the treatment of angiogenesis-related pathologies.

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