



Anti-Vasculogenic Activity of a Polysaccharide Derived from Brittle Star via Inhibition of VEGF, Paxillin and MMP-9

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Background: Bioactive compounds such as terpenoids, chondroitin sulfate, and polysaccharides with added value can be found in pristine marine creatures. These compounds often do have highly valuable therapeutic applications such as being antioxidant, antitumorogenic, anti-inflammatory and anti-angiogenic. For the latter, varieties of angiogenesis factors can suppress this issue within the bodily tissues.

Objectives: The anti-angiogenic and anti-metastatic capacity of a polysaccharide derived from brittle star was investigated.

Material and Methods: The anti-proliferative effect of derived polysaccharide on umbilical vein endothelial cells (HUVEC) was measured using MTT (dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. The anti-angiogenic effect of the isolated polysaccharide was examined by Chorioallantoic membrane (CAM) assay. The transcriptional expression of VEGF (Vascular Endothelial Growth Factor) was evaluated by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). The anti-metastatic activity was investigated via scratch-wound healing assay. The levels of Paxillin and Matrix Metalloproteinase-9 (MMP-9) expression were analyzed by RT-PCR. Statistical analysis and mean comparisons ($p < 0.05$) were carried out by SPSS 16.

Results: Our results elucidated that the brittle star isolated polysaccharide exerted a dose dependent cytotoxic effect on the HUVEC endothelial cells. The CAM assay exhibited potent anti-angiogenic activity *in vivo*. The RT-PCR analysis showed that the extracted polysaccharide (40, 60 $\mu\text{g.mL}^{-1}$) down-regulated the VEGF expression. Further, the diminished attachment of endothelial cells demonstrated that the anti-invasiveness of the derived polysaccharide (25, 50 $\mu\text{g.mL}^{-1}$) was administrated via down-regulation of paxillin and MMP-9 mRNA expression.

Conclusions: Taken together, these results indicated that the polysaccharide extracted from brittle star was able to decrease the viability of the HUVEC cells, to suppress angiogenesis, and possibly act as a natural anti-angiogenic and anti-metastatic marine organic compound against angiogenesis related pathologies.

Keywords: Angiogenesis, Chick Chorioallantoic Membrane, Endothelial cells, MMP-9, VEGF.

1. Background

Angiogenesis, the process of new blood vessel multiplicity from pre-existing vessels, is considered imperative in malignant tumor growth. It is regulated by a balance of pro-angiogenic and angiostatic factors. Upon the switch of tumor cells to an angiogenic phenotype, angiogenesis contribute to the pathogenesis of numerous disorders, such as diabetic retinopathy, chronic inflammation, tumor growth and metastasis (1). Excess angiogenesis occurs through a series of steps, including stimulation of endothelial cells (ECs) by

autocrine and/or paracrine growth factors, proteolytic degradation of the basement membrane and surrounding extracellular matrix, EC migration, proliferation, and structural reorganization into a three-dimensionally tubular structure (2). Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were identified as positive regulators of angiogenesis (3). VEGF is a promising therapeutic target and a crucial angiogenesis factor, next to other previously reported angiogenesis inhibitors such as interferon, angiostatin, endostatin, TNP-470, bevacizumab, sunitinib and

erlotinib (5, 6). VEGF organizes tumor angiogenesis through binding to two tyrosine kinase receptors expressed on endothelial cells, namely, VEGFR1, VEGFR2 (4, 5).

The growth factors subsequently bind to their corresponding receptor tyrosine kinases (RTKs) within the endothelial cells in blood vessels (6, 7). This receptor binding results in the activation of the otherwise dormant endothelial cells, causing to divide and migrate towards the diseased tissues, or, in this case, the tumor cells (8). Adhesion molecules help the growing of new blood vessels to sprout forward. The sprouting of endothelial cells roll up to form new blood vessel tubes (9). Ultimately, these tubes form a network of new blood vessels that can circulate blood. With the new blood vessels now feeding it, the tumor can continually grow in size, invade other tissues and facilitate the spread of the cancer to other organs (10). The metastatic distribution of cancer is a leading cause of cancer related mortality. Matrix metalloproteinase are a family of zinc endopeptidases, which are pivotal components in the metastatic spread with the capacity for degradation of ECM proteins; these proteinases play a critical function in cancer cell invasion, metastasis and angiogenesis (11). MMP-2 and MMP-9 activities are attributed to aggressive, invasive or metastatic cancer stages that are considered principle targets for treatment and prevention of cancer (12).

The angiogenesis pathway is an appropriate attractive target for cancer treatment and prevention, which multiple modalities conducted to control this process. Therefore, prevention of the angiogenesis program can be promising in tumor suppression (13). Accordingly, different angiogenesis regulators have been discovered from natural sources in last 30 years (9). Amongst which, natural secondary metabolites with a considerable potential of being anti-tumorigenic through the suppression of angiogenesis and metastasis have found their place in the pharmaceutical industry (14).

The marine ecosystem is a unique storage of natural products with extraordinary structural and biological diversity, which provide various biomedicinal and therapeutic materials, and, hence, numerous investigations have been directed to the anti-cancer capacity of these constituents (15). Some marine invertebrates as well as echinoderms confer health benefits, and, thus, have been used in folk medicine (16). The pharmacological activity of marine organisms is associated with the presence of active metabolites, such as sterols, terpenoids, cerebrosides, saponins and polysaccharides, which give them unique biological properties (17).

Natural polysaccharides contain a variety of macromolecules that play essential roles in biological cascades, which make them efficient in biomedicine, regenerative medicine and cell therapy.

2. Objectives

Since the extraction of valuable natural polysaccharides is considered to be of great value (18), the aim of this research, for the first time, was to investigate the anti-angiogenesis/anti-invasiveness effects of brittle star extracted polysaccharide, both at *in vitro* and *in vivo* level.

3. Materials and Methods

3.1. Extraction of Brittle Star Polysaccharide

The preparation of polysaccharide from *Ophiocoma erinaceus* was performed for isolation of starfish crude polysaccharide with slight modifications as previously reported (19).

3.2. Cell Viability Test

The cytotoxic assay was evaluated, using the MTT assay. The HUVEC cells were cultured at a concentration of 10^4 cells/well in 96-well plates for 16 h, and incubated with various concentrations of 0, 6.25, 12.5, 25, 50, 100 $\mu\text{g} \cdot \text{mL}^{-1}$ extracted polysaccharide for 24, 48 h. The cytotoxicity of brittle star polysaccharide was investigated by the injection of 20 μL MTT solution and dissolving formazan crystals with 80 μL DMSO. The absorbance was measured at 570 nm by spectrophotometer (Epoch, USA).

3.3. Scratch-Wound Healing Assay

The effect of derived polysaccharide from brittle star on the migration of HUVEC cells was investigated via scratch healing assay. The cells were plated in 6-well culture plates. When the cells reached approximately 80% confluence, a wound track was created across the center of the plate with a sterile 200 μL pipette tip. The treatment was conducted at different concentrations of polysaccharide for 48 h. Photographs were taken at the edge of the wound areas under a light microscope (Bio Photonic, Brazil).

3.4. In vivo Chick Chorioallantoic Membrane (CAM) Assay

Ross fertilized eggs (40) were randomly divided into four groups, including a control group. The control eggs were stored in the normal conditions with no treatment, while experimental groups (1, 2 and 3) treated with 12.5, 25 and 50 $\mu\text{g} \cdot \text{mL}^{-1}$ brittle star polysaccharide. The fertilized eggs were incubated at 38 °C and 55-

Table 1. The sequences of primers and annealing temperatures.

Gene	Forward primers	Reverse primers	TM
GAPDH	5'GGCCAAGAT CAT CCA TGA CAA CT3'	5'ACCAGGACATGAGCTTGA CAA AGT3'	57
VEGF	5'CTGCTGTCTGGGTGCATTG3'	5'TTCACATTGTTGTGCTGTAG3'	59
MMP-9	5'GCCTGCACCACGGACGGTCGCTCC3'	5'GAGGTGCCGGATGCCATTACGTC3'	63
Paxillin	5'AGGGACTGGGTTCTGG3'	5'AAATCACAGGAATTGAAATGGG3'	56.5

65% humidity, and were rotated automatically. Two days after incubation, a small window in the shell concealing the air sac was opened in the eggs in sterile conditions. In which, part of the shell was removed and the window covered by sterile paraffin and lamellas. On day 8 of incubation in sterile conditions, a round gelatin sponge containing the albumen and agar in normal saline with 200 μ L of penicillin/streptomycin (to avoid contamination) was used, which was cut into 4 \times 4 mm and put on a Chorioallantoic membrane. The sponge was soaked using 10 μ L of the extracted polysaccharide. On the 12th day of incubation, all the cases were photographed using a research photo stereomicroscope (Ziess, Germany). The variables were included the number and length of the blood vessels, which for all samples was measured around gelatin sponge. The number and length of the vessels were measured using Image J electronic software.

3.5. Reverse Transcription-polymerase Chain Reaction of VEGF, MMP-9, Paxillin

To assess the anti-angiogenic and anti-metastatic action of isolated polysaccharide, the changes in the expression of VEGF, MMP-9 and Paxillin mRNA were examined by RT-PCR. Table 1 has indicated primers used for RT-PCR. HUVEC cell RNA was isolated using the High Pure RNA Isolation kit (Roche, Germany) according to the manufacturer's protocols and stored at -80 °C. Isolated RNA was reverse-transcribed to cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, US) according to the manufacturer's instruction. The produced cDNA (5 μ g) was added to 10 μ L *Taq* premix, 2 μ L forward primer, 2 μ L reverse primer, and distilled water.

3.6. Statistical Analysis

The results are presented as the mean \pm SD. 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 $p \leq 0.05$ was considered to be significant.

4. Results

4.1. Effect of Brittle Star Extracted Polysaccharide on HUVEC Cell Viability

The MTT assay and morphological analysis indicated that brittle star isolated polysaccharide exerted inhibitory effect on the viability of the HUVEC endothelial cells in a dose dependent manner. The IC_{50} value of isolated polysaccharide on HUVEC-7 cells was 25 μ g.mL⁻¹ after 24, 48 h treatment period (Fig. 1).

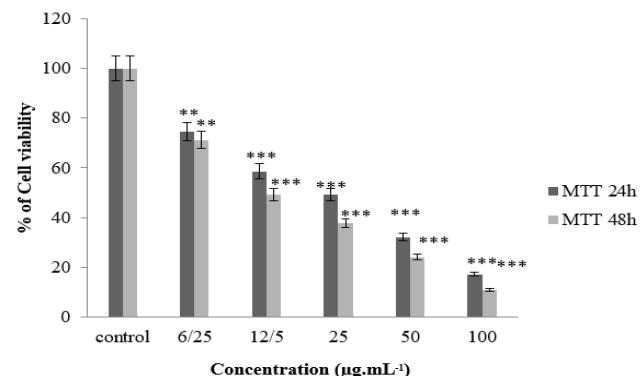


Figure 1. Inhibitory effect of brittle star extracted polysaccharide on cell viability of HUVEC endothelial cell line after 24, 48 h treatment by MTT assay. Data represented as Mean \pm SD and * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were considered significant.

The inhibitory concentrations of examined polysaccharide on HUVEC cells induced clear morphological changes, such as a cell shrinkage, cytoplasm blebbing and alteration of cell shape and size (Fig. 2). Thus, the brittle star polysaccharide was cytotoxic against HUVEC endothelial cells.

4.2. Inhibitory Effect of Brittle Star Polysaccharide on Migration of HUVEC Cells

The scratch wound healing assay was conducted to understand the effects of the extracted polysaccharide on the migration of HUVEC endothelial cells. As exhibited in Figure 3, HUVEC cell migration was significantly decreased with inhibitory concentrations

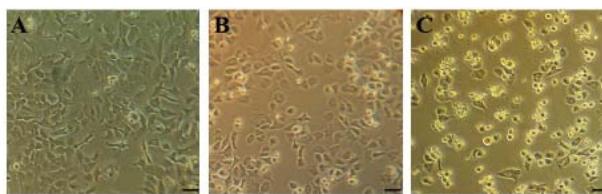


Figure 2. Cytomorphological alterations of HUVEC cells treated with brittle star isolated polysaccharide. A) Control (without treatment). B, C) HUVEC Cells treated with 25, 50 $\mu\text{g.mL}^{-1}$ for 48 h. $\times 400$

of brittle star polysaccharide extracted (25, 50 μmL^{-1}) in a concentration dependent manner. These results confirmed that brittle star isolated polysaccharide might be considered efficient for suppressing the migration of endothelial cells.

4.3. Inhibitory Effect of Brittle Star Polysaccharide on CAM Model

Comparing the mean length of the blood branches (34 ± 0.10 mm) in the control samples with the length (31 ± 0.04 mm) in the first experimental group was not remarkable ($p > 0.05$). However, changes of length in the second (30 ± 0.11 mm) and third (25.9 ± 0.14) experimental group compared with the control were

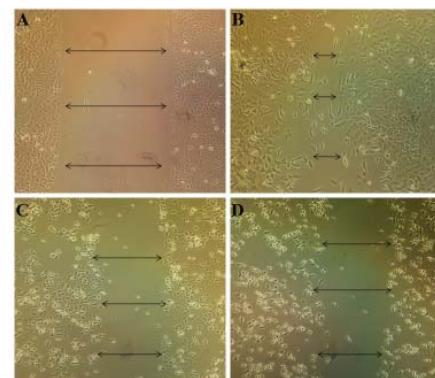


Figure 3. Inhibitory effect of brittle star isolated polysaccharide on cell migration of HUVEC cells by scratch healing assay. A) Untreated cells at the time of scratch assay. B) Control groups 24 h after scratch assay. C, D) Experimental groups with 25, 50 $\mu\text{g.mL}^{-1}$ extracted polysaccharide, which indicated that this treatment effectively inhibited cell migration of HUVEC endothelial cells. Magnification $\times 200$.

statistically significant. Moreover, the average number of blood vessels in the first experimental group (22.6 ± 1.11) compared to the control revealed significant reduction ($p < 0.05$). On the other hand, the number of blood vessels in the second (19.29 ± 0.47) and third

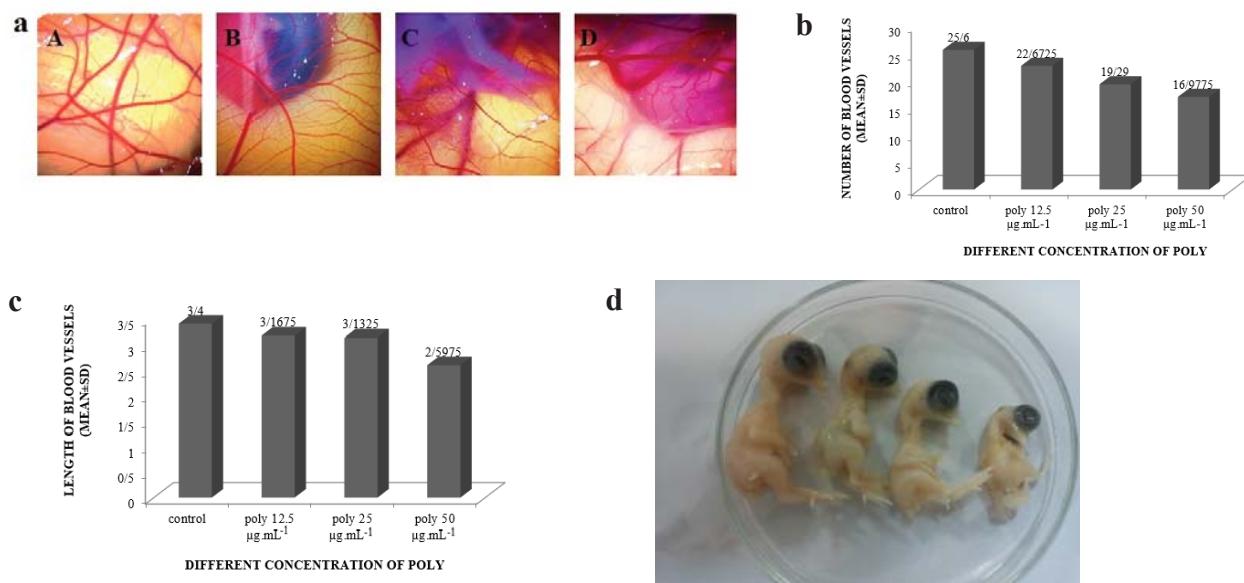


Figure 4. a) Screen shots of CAM in the control and treated samples. Reduction of angiogenesis in the samples treated with different concentrations of brittle star isolated polysaccharide compared with control (A) CAM treated with isolated polysaccharide at $12.5 \mu\text{g.mL}^{-1}$, (B) CAM treated with isolated polysaccharide at $25 \mu\text{g.mL}^{-1}$, (C) CAM treated with isolated polysaccharide at $50 \mu\text{g.mL}^{-1}$ (D). b, c) Average number and length of blood vessels treated with extracted polysaccharide compared with untreated group (* $p < 0.05$, ** $p < 0.001$), d) The reduction size of chick fetus that indicate insufficient blood supplies under treatment with extracted polysaccharide, left to right: control, treated groups at 12.5 , 25 , $50 \mu\text{g.mL}^{-1}$.

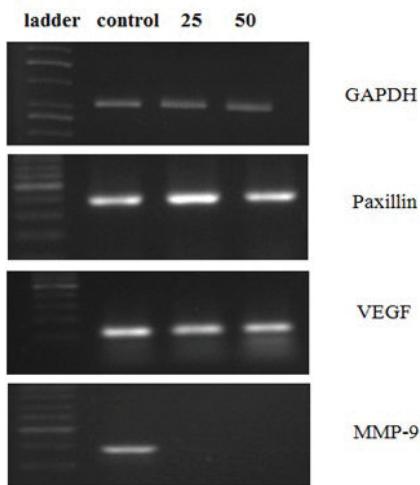


Figure 5. HUVEC endothelial cells were treated with brittle star polysaccharide extract. The mRNA expressions of VEGF, MMP-9 and Paxillin were evaluated by RT-PCR analysis that displayed suppression of isolated polysaccharide on mRNA expressions of pro-angiogenic and metastatic factors.

(16.97 ± 0.56) experimental groups declined reasonably at ($p < 0.001$) compared to the control group (Fig. 4).

4.4. Expression of VEGF, MMP-9 and Paxillin in HUVEC Cells

RT-PCR data showed a reduction ($p < 0.05$) in the gene expression levels of VEGF, MMP-9 and Paxillin under treatment with concentrations of 25 and 50 $\mu\text{g.mL}^{-1}$ isolated polysaccharide, dose dependently, in comparison with the control group (Fig. 5).

5. Discussion

Tumor angiogenesis is a critical aspect of cancer therapeutic methods that include endothelial cell growth, differentiation and migration with involvement of pro-angiogenic signals (20). These signals promote tumor angiogenesis and therefore have been topic of interest (21). In addition, the blockade strategies of tumor vessel proliferation that may decrease the metastasis process have been considered for elaborating ectopic angiogenesis during tumor invasiveness (22). However, one crucial aim in cancer research is the development of therapeutic strategies with minimum side effects and higher efficacy. One of such approaches would be the use of traditional medicine (23), which was also confirmed herein.

The extracted polysaccharide from brittle star suppressed the viability of the HUVEC endothelial cells in a dose dependent manner and exerted an anti-angiogenic effect via down regulation of VEGF transcription. Furthermore, the brittle star isolated

polysaccharide significantly ($p < 0.05$) reduced the length and the number of the vessel branches in the experimental groups relative to the control and proved to be anti-angiogenic using the CAM model. It is characterized that angiogenesis suppression is a prominent strategy to prevent tumor migration and metastasis (24). In the previous study, we demonstrated that brittle star extract interferes with the proliferation of ovarian cancer cells with valuable capacity in the inhibition of angiogenesis of tumor cells via down-regulation of FGF and VEGF (7).

MMP-2 and paxillin, the two pivotal inhibitors in the development of a therapeutic strategy for metastasis suppression (25, 26), were blocked as represented by decreased migration action in treated HUVEC. This may be illustrative of the anti-metastatic activity of the extracted polysaccharide.

Augmented levels of pro-angiogenic signals have been reported as an essential element in patients with metastatic cancers (27). RT-PCR analysis confirmed that the relative levels of VEGF transcript in HUVEC cells were decreased by brittle star extracted polysaccharide after 48 h. The anti-angiogenic potential of brittle star polysaccharide was also examined *in vivo* with CAM model and showed the dose dependent reduction of blood vessel numbers and branching patterns. There are many surveys relating to the anti-angiogenic efficacy of certain natural polysaccharides (28).

Previous studies reported that polysaccharides possess anti-angiogenic potential that is efficient in cancer therapeutic researches. The anti-angiogenesis capability of polysaccharides from the mycelia of *Antrodia cinnamomea* elucidated that this compound exhibited an anti-angiogenic effect by reducing VEGF emission along with attenuation of neo-vascularization in the CAM model and repression of tube organization in HUVEC cells (29).

Huang et al. (2012) studied the anti-angiogenic activity of polysaccharides derived from *Lycium barbarum* and proved that extracted polysaccharide noticeably inhibited the angiogenesis in a dose dependent manner by the suppression of PI3K function, HIF-1a protein disaggregation and attenuation of VEGF mRNA scale, thus prevented neovascularization by modulation of PI3K/HIF-1a/VEGF pathways (30).

Related to anti-angiogenic inhibitors from marine sources, Liu et al. (2012) examined the anti-angiogenic efficacy of sulfated polysaccharides in brown algae on the HUVEC based cell culture model and concluded that fucoidan ($100 \mu\text{g.mL}^{-1}$) reduced micro-vessel outgrowth via suppression of VEGF-A expression (31). The examination of sulfated polysaccharide of *Sepiella maindroni* ink on metastasis and angiogenesis

demonstrated that the extracted polysaccharide prohibited the invasion and motility of SKOV3 (ovarian) and B16F10 (melanoma) cancer cells through mitigation of MMP-2 transcription (21). Lu and his coworkers (2013) reported that polysaccharide derived from the brown seaweed indicated an anti-angiogenic effect by modulation of bFGF activity (32). In addition, Senthilkumar (2013) confirmed the anti-vasculogenic potential of marine sources, particularly sponge derived compounds (33).

Here and for the first time, it was established that brittle star isolated polysaccharide has a significant anti-angiogenic potential mediated via down-regulation of VEGF and the anti-metastatic activity associated with inhibition of MMP-9 and paxillin expression. The data proved the curative efficacy of brittle star extracted polysaccharide for the prevention of angiogenesis-related disorders.

Conflict of Interest

The authors declare that they have no conflict of interest to report.

Authors' Contribution

All authors have participated equally in the present study.

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