Holothurin B Isolated from Holothuria atra Inhibits Angiogenesis More Potent than Curcumin in Vitro

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Abstract

Background and objectives: Triterpene glycosides as the most bioactive components of sea cucumbers, have been considered for their various pharmacological properties especially anticancer and anti-metastasis activities. Due to the limited information on the biological properties of holothurin B as a marine triterpene glycoside, the present study aimed to examine its effect on angiogenesis and compare it with curcumin using human umbilical vein endothelial cells (HUVECs). Methods: Holothurin B was isolated from Holothuria atra and identified by NMR and Mass spectroscopic data. Cell survival was estimated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) technique and migration of cells was assessed by Transwell test. Angiogenesis was evaluated in vitro by tube formation assay. Results: Holothurin B reduced HUVECs survival with IC50 value of 8.16 µg/mL. At the concentrations of 5 and 7.5 µg/mL, it significantly decreased the number of migrated cells, the average length and size of tubules, and mean number of junctions; it was more potent than curcumin. Conclusion: Holothurin B could be considered as a potent antiangiogenic constituent through suppressing endothelial cell proliferation, migration and tubulogenesis in vitro, suggesting its potential for further animal and clinical investigations.

Keywords: angiogenesis inhibitors; cell migration inhibition; cell proliferation; holothurin B; human umbilical vein endothelial cells

Introduction

Angiogenesis involves the spreading out, movement, and differentiation of endothelial cells which lead to the development of new vasculature. This progress is organized by the balance between proangiogenic elements and inhibitors of angiogenesis. Stimulation or inhibition of angiogenesis may play a helpful role in the improvement of some pathologic situations [1]. Metastasis as the most dangerous event in cancer cells depends on angiogenesis. In tumors, angiogenesis helps them survive and multiply. Therefore, anti-angiogenic agents can inhibit metastasis by preventing from supplying of oxygen and nutrients to the cancer cells and eventually result in the tumor cells death [2]. Holothuria atra Jaeger or black cucumber which belongs to the Holothuroidea family, is a sea cucumber usually grown up in the Pacific and...
Indian Oceans. It has smooth, supple, black and often sandy skin with common size of 20 centimeters length [3]. Sea cucumbers are considered as plenteous origins of bioactive compounds for nutritional and medicinal applications [4]. These marine invertebrates have been used for treatment of dermatitis, wounds, hypertension, arthritis, and sexual dysfunction in Chinese folk medicine [5]. Various organic chemicals including flavonoids, alkaloids, glycosides, phenolic constituents, steroids and saponins have been identified in H. atra [6]. Holothurins are saponin or triterpene glycosides and are one of the main bioactive components isolated from this species and other holothurians. Holothurins are a group of toxins and can be toxic for humans if consumed in large quantities. They may have four sugars as holothurin A or two sugars as holothurin B in their sugar chain [4]. Significant cytotoxic, antitumor, anti-metastatic, anti-angiogenesis activities and moderate in vivo leishmanicidal effect have been described for holothurin A [6-9]. Limited investigations have been done on biological properties of holothurin B showing strong in vitro and moderate in vivo anti-leishmanial effects and also powerful cytotoxicity toward cancerous cells [9,10]. In the current investigation, holothurin B was isolated from Holothuria atra and its effect was evaluated on angiogenesis-related markers and compared with curcumin as a natural inhibitor of angiogenesis [11] in human umbilical vein endothelial cells (HUVECs). Many investigations have shown that curcumin has potent antiangiogenic effects in vitro and in vivo [11,12]. This curcuminoid compound with strong antitumor and anti-metastatic activities is currently being studied in several clinical trials for treatment of some cancers with reported beneficial effects on patients’ quality of life, increasing survival and reducing tumor indicators level [13].

Materials and Methods
Ethical considerations
The study was approved by the Institutional Research Ethics Committee of Isfahan University of Medical Sciences (ethical approval ID: IR.MUI.RESEARCH.REC.1398.579).

Chemicals
MTT kit (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Hakiman Shargh Research Co. (Iran). Fetal bovine serum (FBS) was purchased from Bioidea Co. (Iran). Geltrex reduced growth factor basement membrane matrix and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Gibco-BRL, Life Technologies Inc. (USA). Recombinant human vascular endothelial growth factor (VEGF) was provided from PeproTech, Inc. (USA). Calcein acetoxyethyl dye was purchased from Santa Cruz Biotechnology Inc. (Canada) and curcumin from Sigma-Aldrich Co., (USA). All other chemicals were obtained from Merck KGaA Co. (Germany).

Sea cucumber and isolation of holothurin B
Holothuria atra Jaeger was collected from Persian Gulf (Iran) in November 2019. The animal was identified by Dr Seyed Mohammad Bagher Nabavi, Khorramshahr Marine Science and Technology University, Khuzestan. The sea cucumbers were dried by freeze drier and extracted with ethyl acetate/methanol (1:1) by maceration method. The animal to solvent ratio was 1:5. The extract was partitioned by kupchan partitioning technique to hexane, dichloromethane, butanol, and water fractions. The sea cucumbers are rich in saponin compounds and butanol is the best solvent for saponin extraction. Therefore, butanol fraction was separated through medium pressure liquid chromatography using reverse silica gel and methanol/water solvent system. Holothurin B was isolated by several column chromatographic methods including medium pressure liquid chromatography (MPLC) and high-performance liquid chromatography (HPLC). The final purification of holothurin B was done with HPLC and its chemical structure was acknowledged by infrared (IR), different 1 dimensional and 2-dimensional nuclear magnetic resonance (NMR) and mass spectroscopy.

Cell culture
HUVEC cells were obtained from the National Cell bank of Iran (Pasteur Institute Tehran, Iran). They were grown in 75 cm² flasks using DMEM (Dulbecco's modified Eagle's medium) that improved with fetal bovine serum (FBS, 10%) and penicillin-streptomycin (1%) under controlled condition using an incubator. All experiments were done in 2 to 4 passages. Holothurin B and
curcumin were prepared in dimethyl sulfoxide (DMSO; 10%) to get various concentrations as per requirement and its final concentration was lower than 1% in HUVECs culture media.

**Cell survival assay**
The effect of holothurin B and curcumin on HUVECs survival was identified by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldtetrazolium bromide] kit based on a colorimetric method [14]. The cells were cultivated at 5×10⁴ cells in each well of a plate with 96 wells for 24 h. Next, HUVECs were exposed to holothurin B (1, 2.5, 5, 7.5 and 10 µg/mL) or curcumin (5, 10, 20, 40 and 80 µg/mL) and incubated during 24 h again [12,15]. Then the cells were treated with MTT reagent (4 h). After addition of DMSO, absorbance was measured at 570 nm using a spectrophotometer. Untreated cells were considered as negative control and three measurements was performed for assessment of cell survival during each treatment.

**Cell migration assay**
To examine cell migration, a 24-well Transwell insert membrane with (8.0 µm pores) was used (Costar, Corning Inc. US). About 5×10⁴ HUVECs/mL with culture medium (DMEM without serum) were added onto the upper portion of Transwell insert. The complete culture media was poured on the lower chamber and incubated for 30 min. Then vehicle or holothurin B (5 and 7.5 µg/mL) or curcumin (20 and 40 µg/mL) were added to inserts and the plate was preserved during night. After that, the cells that had not migrated were removed with a swab and the migrated cells were stabilized by ethanol solution (70%). Next, the crystal violet solution (1%) was used for staining of the migrated cells and the cells were counted by an inverted microscope [16].

**Capillary tube formation assay**
For assessment of the effect of holothurin B or curcumin on angiogenesis in vitro, tube formation assay was done using Geltrex. Briefly, a 24-well plate under ice-cold condition was covered with Geltrex and preserved for 30 min in an incubator. About 1.2×10⁵ cells were seeded in each well and treated with holothurin B (5 and 7.5 µg/mL), curcumin (20 and 40 µg/mL) or 2 ng/mL of VEGF (as the reference). Then, the plate was placed in an incubator for 24 h. After that, Hanks balanced salt solution was used for washing the cells and they were stained with calcein acetoxymethyl dye. Photographs of each well were obtained with a camera attached to an inverted fluorescent microscope (10×). The average tubules size, length and junctions were estimated in each well (at least 5 fields) using AngioQuant software [17].

**Statistical analysis**
Records were stated as mean ± standard error of mean (SEM). Evaluation was statistically performed using one-way analysis of variance (ANOVA) and Tukey post-hoc test by SPSS software (version 23.0). The p value below 0.05 reflected the statistically significant difference.

**Results and Discussion**
Antiangiogenic approaches have been considered as the potential strategies to hamper tumor progression and cancer metastasis as well as other disorders associated with pathological angiogenesis such as atherosclerosis, fibrogenesis, systemic lupus erythematosus and diabetic retinopathy [2,18]. Recent attempts for developing new therapeutic agents that regulate angiogenesis have led to the identification of some natural compounds derived from herbal or animal resources with antiangiogenic effects.

In this study, we isolated holothurin B from *Holothuria atra* and identified its structure by different NMR and Mass spectroscopic data (supplementary file). The IR spectrum displayed an absorption band correlated with a γ-lactone moiety at 1736 cm⁻¹, strong broad absorption bands at 3424 and 1073 cm⁻¹ characterizing glycosidic structure, and also, two further absorption bands at 1258 and 1210 cm⁻¹ due to sulfate group. The ¹H-NMR, ¹³C-NMR, and HMBC spectra exhibited resonances linked to seven CH₃ groups at δH 0.8, 0.84, 0.86, 0.99, 1.02, 1.20, 1.41 (δC 16.1, 22.4, 22.4, 27.4, 21.9, 19.3, 22.5), one alkenyl group (δH 5.23; δC 114.5, 152.6) and one lactone carbonyl group (δC 174.0). The spectra displayed a broad singlet resonance due to a -CH proton linked to a carbon with an -OH group at δH 4.42 (δC 70.1) and a C- bearing a hydroxyl at δ 87.9, proposing the existence of the holostane structure with 9 (11) en-3, 12, and 17 triol group. The assignments of the NMR signals fitting to aglycone moiety obtained from the H- and C-NMR, resulted in the recognition of aglycone moiety as 9 (11)-holostene-3β, 12α, and 17α triol. The ¹H- and ¹³C-NMR spectra demonstrated signals for two anomic protons.
and their connected C, δH 4.72 (δC 105.1), δH 4.81 (δC 105.6). The sugar chain assignment and other linkages were confirmed using HMBC spectrum. As presented in Figure 1, final elucidation showed the structure of compound as 3-O-[D-quinovopyranosyl-(1→2)-β(4-O-sulfo)-β-D-xylpyranosyl]-9(11)-holostene-22-ketone-3β,12α,17α-triol.

The survival rate of HUVECs was evaluated after 24 h exposure to holothurin B or curcumin using MTT method. As shown in Figure 2, treatment with 7.5 and 10 µg/mL of holothurin B significantly reduced HUVECs viability to % 66.7±6.9 (p<0.01) and % 8.5±1.9 (p<0.001), respectively compared to the untreated cells and its IC<sub>50</sub> value was 8.16 µg/mL (95% CI = 7.18-9.14). Because of notable inhibitory effect from 10 µg/mL, holothurin B was used with 5 and 7.5 µg/mL in further studies.

There was significant reduction in the HUVECs survival to % 82 ± 3.5 and % 32 ± 3.1 after exposure to the concentration of 40 and 80 µg/mL of curcumin, respectively (p<0.05) with IC<sub>50</sub> value of 70.39 µg/mL (95% CI = 60.59-80.19) (Figure 3). Curcumin was used with 20 and 40 µg/mL in further experiments. In the current study, holothurin B caused a major decrease in the HUVECs survival. Previous investigations have presented holothurin A as an anticancer constituent which prevents proliferation, migration and invasion of cancerous cells [8]. In the study of Zhao and co-workers, holothurin A<sub>1</sub> isolated from *Pearsonothuria graeffei* repressed survival of HepG-2 cell with IC<sub>50</sub> value of 3.40 μM, though its IC<sub>50</sub> value was 5.60 μM in human endothelial cells (ECV-304) after 24 h treatment [7].

Cytotoxic properties of holothurin A and B isolated from *Holothuria moebii* were estimated toward rat and human glioma cells by Yu et al. [19]. Their findings revealed potent anti-proliferative activities with IC<sub>50</sub> range of 0.99 to 4.03 μM (1.17 - 4.79 μg/mL) for holothurin A and IC range of 1.39 to 8.64 μM (1.23 - 7.63 μg/mL) for holothurin B in different glioma cells [19]. Wang et al. also assessed cytotoxicity of holothurin A and B derived from *Holothuria scabra* toward cancer and normal cell lines [14]. In their study, holothurin A showed IC<sub>50</sub> values of 3.76, 8.94 and 3.46 μg/mL against human cervical cancer (HeLa), leukemia (K562) and hepatoma (HePG2) cells, respectively and IC<sub>50</sub> value of 3.84 in normal hepatocyte HL-7702 cells. While holothurin B showed more potent suppressing effects on the proliferation of all mentioned cell
Holothurin B inhibits angiogenesis

lines with IC_{50} values as 2.05, 3.64, 1.79 and 2.69 µg/mL, respectively [14]. Similarly, Yan et al. described the stronger cytotoxic effects of holothurin B compared to holothurin A in human gastric cancer, leukemia and hepatoma cells [20]. In fact, the aglycon part of holothurin B is similar to holothurin A and also both compounds contain n-quinovose and D-xylose and sulfuric acid as the sodium salt, but holothurin B does not have two other monosaccharide residues of holothurin A in the sugar chain of aglycone [4]. It seems that more potent cytotoxic action of holothurin B is related to the fewer monosaccharide residues at the sugar group [14]. However, it should be noted that both compounds have shown toxic effects on normal cells and must be used in non-cytotoxic concentrations during pharmacological studies.

It has been proposed that some proteins such as BCL2 family proteins, histone deacetylase 1 and tyrosine-protein phosphatase non-receptor type 2 which have crucial roles in regulating the cell cycle and apoptosis may be the targets of holothurin A and B in their anticancer activities [18]. The potential of holothurin A for interaction with topoisomerase II alpha as an important enzyme in replication of DNA has also been suggested for its cytotoxic effect toward cancerous cells [21].

Our finding also exhibited the impact of holothurin B on declining HUVECs migration as a critical step in angiogenesis and suppressing other angiogenesis-related markers including average length, size and junctions of tubules during tube formation. As shown in Figure 4, all treatments significantly reduced HUVECs migration using Transwell method in a concentration dependent manner (p<0.001). Holothurin B at 7.5 µg/mL concentration decreased cell migration to % 6.4 ± 2.3 and curcumin at its higher concentration (40 µg/mL) decreased the migration to % 28.8 ± 5.1. However, it should be taken into account that the potent suppressive effect of holothurin B at its higher concentration on cell migration may be partly due to its cytotoxic effect.

The impact of holothurin B and curcumin on angiogenesis was estimated using tube formation assay on Geltrex basement membrane matrix. Figure 5 represents the photomicrographs of HUVECs during tube formation in which tube-like constructions was seen in normal control cells (Figure 5A). VEGF induced stimulatory effect on differentiation of HUVECs morphology into a tube-like shape (Figure 5B). Exposure to holothurin B and curcumin resulted in a remarkable declining in formation of capillary tubules compared to the normal HUVECs (Figures 5C-5F).

Quantitative analysis revealed the proangiogenic activities of VEGF through enhancement of average tubules length, size, and mean number of junctions (p<0.001). Conversely, significant inhibitory effects were observed after exposure to holothurin B and curcumin in tube formation in comparison with normal untreated HUVECs. All tested concentrations were associated with a notable decrease in the quantitative markers of capillary tube formation. The less average length, size and junctions of tubules were detected in cells treated with 7.5 µg/mL of holothurin B (p<0.001) (Figures 6A-6C).

In previous investigations Holothurin A has displayed antiangiogenic activities [7,8]. It was able to persuade apoptosis in HUVECs and to hamper in vitro angiogenesis in tube formation and moreover, in vivo angiogenesis in chicken chorioallantoic membrane [8].

In the study of Zhao and co-workers, holothurin A_{1} showed anti-metastasis activities through reduction of cell adhesion in HepG-2 cells and also adhesion of tumor cells to endothelial cells, decreasing of migration rates in HepG-2 cells during a scratch wound assay and inhibition of angiogenesis by disruption of tube structure in matrigel in vitro test.
Figure 5. Representative fluorescence photomicrographs of tube formation in HUVECs (10× magnification); the cells were cultured on Geltrex and incubated without (A) or with VEGF 2 ng/mL (B), curcumin 20 µg/mL (C), curcumin 40 µg/mL (D), holothurin B, 5 µg/mL (E) or holothurin B 7.5 µg/mL (F) for 24 h.

Figure 6. Effect of holothurin B or curcumin on capillary tube formation on Geltrex; HUVECs were cultured on Geltrex and incubated without or with holothurin B (5 and 7.5 µg/mL) or curcumin (20 and 40 µg/mL) or VEGF (2 ng/mL) for 24 h. Data were quantified by determining the average tubules length (A), tubules size (B) and mean number of junctions (C) and shown as means ± SEM from triplicate tests. *p<0.05, **p<0.01 and ***p<0.001 versus control (untreated cells).
They also found the potential ability of holothurin A₁ in suppressing the expression of VEGF, matrix metallo-proteinase-9 (MMP-9), and nuclear factor- kappa B (NF-κB) and elevating the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1), as a controller for MMP-9 activity which may offer some mechanisms in its antiangiogenic activities [7]. Based on the findings of the present study, holothurin B showed inhibitory effect on angiogenesis-related markers stronger than curcumin as a well-known inhibitor of angiogenesis. However, regarding the cytotoxic effect of holothurin B, selecting non-cytotoxic concentrations should be considered in future studies. Curcumin is a natural phenol diarylheptanoid and the most important substance of turmeric [22]. Various investigations have confirmed the chemopreventive, anticancer and antiangiogenic activities of this curcuminoid compounds [11,23]. Curcumin directly inhibits angiogenesis through several mechanisms including suppression of many proangiogenic proteins such as epidermal growth factor, VEGF, transforming growth factor, cell adhesion molecules and cytokines such as tumor necrosis factor-α. Moreover, it inhibits some signaling pathways involving NF-κB, matrix metalloproteinases, protein kinase C, urokinase plasminogen activator system and cyclooxygenase-2 [11].

**Conclusion**

Holothurin B, a marine-derived triterpene glycoside could be considered as a potent antiangiogenic agent through suppressing endothelial cell proliferation, migration and tubulogenesis in HUVECs. Moreover, its inhibitory effects on angiogenesis were stronger than curcumin as a natural well-known inhibitor of angiogenesis. Additional studies should be developed for clarifying the molecular and cellular mechanisms of the antiangiogenic activities of holothurin B and its clinical impact for pathological angiogenesis.

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**Author contributions**

Afshan Yegdaneh designed the pharmacognostical related studies; Leila Safaeian contributed to the concept, design, definition of intellectual content, manuscript preparation and editing; Mina Mirian, Nasim Dana and Mohadeshe Taheri participated in cellular procedures, data acquisition and statistical analysis.

**Declaration of interest**

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

**References**


**Abbreviations**

DMSO: dimethyl sulfoxide; HUVECs: human umbilical vein endothelial cells; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide