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In vitro Evaluation of the Antiviral Activity and Cytotoxicity Effect of *Holothuria leucospilota* Sea Cucumber Extracts from the Persian Gulf

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Authors
Bahroodi S.[1] *MSc,*
Nematollahi M.A.*[1] *PhD,*
Aghasadeghi M.R.[2] *PhD,*
Nazemi M.[3] *PhD*

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ABSTRACT

Aims Antiviral activity and cytotoxicity effect of methanol and diethyl ether extracts from different parts of sea cucumber (*Holothuria leucospilota*) against HIV-1 were assessed on human oral epidermoid carcinoma cells (KB) and Human embryonic kidney 293T cells (HEK293T).

Materials & Methods Sea cucumber was collected at a depth of 10-30 m of Larak Island (Persian Gulf). Extracts were prepared by diethyl ether and methanol solvents. The antiviral activity of each extract was evaluated by inhibition of single-cycle replicable HIV-1 (SCR HIV-1) p24 Core antigen production in HeLa cells and cellular toxicity of different extracts were assessed, using a cell proliferation XTT kit.

Findings Antiviral activity of each extract showed that some concentrations were able to inhibit the replication of HIV-1. Diethyl ether extract of body wall with 2.79 TI index displayed the highest antiviral activity as well as less cytotoxicity effect.

Conclusion This study showed that crude extracts of *Holothuria leucospilota*, especially methanol and diethyl ether extracts of digestive organs and body wall had good cytotoxicity effect and antiviral activity, respectively.

Keywords Sea Cucumber; Antiviral; Cytotoxicity; Persian Gulf; *Holothuria Leucospilota*

[1]Fisheries Department, Natural Resources Faculty, University of Tehran, Karaj, Iran
[2]Hepatitis & AIDS Departments, Pasteur Institute of Iran, Tehran, Iran
[3]Persian Gulf & Oman Sea Ecological Research Institute, Bandar Abbas, Hormozgan, Iran

Correspondence
Address: Fisheries Department, Natural Resources Faculty, University of Tehran, Daneshkadeh Street, Karaj, Iran. Postal Code: 3158777871
Phone: +98 (26) 32223044
Fax: +98 (26) 32227765
mallahi@ut.ac.ir

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[1] Marine natural products [2] High-value components and bioactives from sea cucumbers for functional foods - a review [3] Structural and chemical defenses of echinoderms from the northern Gulf of Mexico [4] Antibacterial activity of extracts from the body wall of Parastichopus parvimensis (Echinodermata: Holothuroidea) [5] New bacterial species isolated from Malaysian sea cucumbers with optimized secreted antibacterial activity [6] a new sulfated saponin from sea cucumber, exhibits anti-angiogenic and anti-tumor activities in vitro and in vivo [7] Two new cytotoxic and virucidal trisulfated triterpene glycosides from the Antarctic sea cucumber Staurocucumis liouvillei [8] Global report: UNAIDS report on the global AIDS epidemic 2010 [9] Bioactivity of marine organisms: IV. Screening of some marine animals from the Indian coast [10] Development of single-cycle replicable human immunodeficiency virus 1 mutants [11] Antiviral effects of saikosaponins on human coronavirus 229E in vitro [12] The depolymerized fucosylated chondroitin sulfate from sea cucumber potently inhibits HIV replication via interfering with virus entry [13] Antiviral activity of Holothuria sp. a sea cucumber against herpes simplex virus type 1 (HSV-1) [14] Isolation of sphingoid bases of sea cucumber cerebrosides and their cytotoxicity against human colon cancer cells [15] Holothurinosides: New antitumour non sulphated triterpenoid glycosides from the sea cucumber Holothuria forskalii [16] Biologically active triterpene glycosides from sea cucumbers (Holothuroidea, Echinodermata) [17] Triterpene glycosides from sea cucumber Holothuria scabra with cytotoxic activity [18] Antioxidant and cytotoxic properties of two sea cucumbers, Holothuria edulis Lesson and Stichopus horrens Selenka [19] Inhibition of proliferation of PC3 cells by the branched-chain fatty acid, 12-methyltetradecanoic acid, is associated with inhibition of 5-lipoxygenase

Introduction

Every year, many bioactive compounds have been recognized and derived from various marine organisms. Search for discovering new metabolites has led to the isolation of 10,000 new combinations from marine animals such as seaweeds, Echinoderms, fishes, etc. In previous study, it was demonstrated that a remarkable number of these compounds have potential for use in medicine, pharmaceutical, cosmetic, and industrial sectors [1].

Sea cucumbers, informally named bêchedemer, or gamat, have been widely used in food or medicine purpose in Asia and Middle East communities [2]. Many bioactive compounds have been reported from different sea cucumber species. A number of these compounds possess biological activity [3-4]. Also, sea cucumbers have been well recognized as a bracing and traditional therapy in Chinese and Malaysian literature for their effectiveness in asthma, rheumatism, hypertension, constipation, impotence, cuts, and burns [5]. According to the literature survey, multiple pharmacological properties including antimicrobial, anticancer, anticoagulant, anti-hypertension, anti-inflammatory, antithrombotic, antioxidant, anti-angiogenic, antitumor, and wound healing have been attributed to chemical compositions, extracted from various sea cucumber species [2].

These pharmaceutical benefits and health functions of sea cucumbers can be ascribed to the attendance of significant amounts of bioactive compounds, especially chondroitin sulfates, triterpene glycosides (saponins), glycosaminoglycan, sulfated polysaccharides, sterols (glycosides and sulfates), phenolics, peptides, cerberosides, and lectins. Similarly, fatty acids of sea cucumber lipids fractions (diethyl ether fraction) are key components and liable for tissue repair and wound healing properties of this marine animal [2]. Also, the presence of Liouvillosides A and B (trisulfated triterpene glycosides) and fucosylated chondroitin sulfates in the extracts of these animals can exert antiviral activity against some strains [6-7].

The human immunodeficiency virus (HIV) is an RNA virus in the retrovirus subgroup that causes acquired immunodeficiency disease (AIDS) in humans. According to World Health Organization (WHO), 60 million people worldwide are infected with HIV and each day, 5,700 lose their lives because of this disease [8]. HIV tends to infect and kill T lymphocytes that leads to reduction and loss of host cellular immunity and will cause susceptibility to opportunistic infections.

The aim of this present study was to evaluate *in vitro* anticancer and antiviral activity of various parts of *Holothuria leucospilota* methanol and diethyl ether extracts against human oral epidermoid carcinoma cell line (KB) and HIV-1, respectively. Furthermore, we attempted to identify the potent extracts with particular emphasis on their cytotoxic activities.

Materials and Methods

Sample collection: All the samples were collected at a depth of 10-30 m around Larak Island in December 2012 and were frozen, using dry Ice immediately after transportation to the laboratory. They were maintained under a frozen state (-20°C) until extraction.

Extraction: Samples were defrosted with water and, then, solid particles on the body surface were removed with tap water. After cutting samples from both sides of the midline of the body, internal organs were separated from the body wall and washed before extraction.

Extracts of the samples were prepared following Naik *et al*. method [9]. Briefly, the cut sampled of fresh holothurians were transferred to an Erlenmeyer with 1000 ml diethyl ether solvent for the purpose of extracting semi-polar and non-poplar compounds. After 24h of soaking, the solution was filtered and evaporated to dryness with low pressure at 35-40°C, using Rotavap.

In order to isolate polar compounds, residues of the samples were soaked in methanol for 72 h. The concentrated methanol extracts were, then, dried to obtain crude semi-solid extracts. The crude extract was, then, weighed and the percentages of the sea cucumber extracts were calculated. Methanol extract evaporated to dryness with low pressure at 40-45°C, using Rotavap and, then, methanol extract was lyophilized.

Producing pseudotyped single-cycle replicable: Single-cycle replicable HIV-1 (SCR HIV-1) virions were constructed by deleting a 2-kb portion inside the Pol district of the HIV-1 genome from the pNL4-3 strain [10]. Pseudotyped SCR HIV-1 virions were created by the co-transfection of HEK293T cells with pmzNL4-3 (containing the mutated genome), psPAX2, and pMD2G plasmids from Addgene (www.addgene.org). The pmzNL4-3 plasmid encoded the HIV-1 full-length RNA with the bundling capacity containing the aforementioned deletion in the Pol district; the psPAX2 plasmid encoded HIV Gag and Gag-Pro-Pol polyproteins in addition to all the viral accessory proteins; and the pMD2G plasmid encoded the vesicular stomatitis virus surface glycoprotein (VSVG), which was vital for virion gathering and sprouting procedure. These pseudotyped virions could infect a wide range of cells, even without the CD4 receptor [10].

Following the co-transfection of the HEK293T cells with the aforementioned plasmids utilizing the Polyfect reagent (Qiagen, Germany), the supernatant containing virions were collected in 24, 48, and 72h. At the end, the viral stock was condensed 20 times by ultracentrifugation, p24 load was evaluated (HIV p24 ELISA, Biomerieux, France), and the stock was stored at -70°C.

Cell lines: The human immunodeficiency virus (HIV-1) infected HeLa, (HEK) 293T and KB cell lines, which were cultured at 37°C with 5% CO_2 in RPMI1640 medium (Biosera, England) and DMEM (Biosera, England), respectively. The media were supplemented with 10% fetal bovine serum (Biosera, England), 200 units/ml of penicillin G, and 80µg/ml of streptomycin (Sigma, USA).

XTT-based cytotoxicity assay: Cell proliferation XTT kit (Roche Diagnostics, Germany) was used to assess the cytotoxicity of methanol and diethyl ether extracts in HEK293T, HeLa and KB cells. Briefly, the cells were seeded in triplicate in 96-well plates in the presence or absence of various concentrations of methanol and diethyl ether extracts at 37°C with 5% CO2 for 3 days. Subsequently, 50µl of the prepared XTT mixture was added to each well and further incubated for 4h to allow for the formation of Formazan crystals. Absorbance was measured, using an ELISA plate reader (BioTek ELx800) at the wavelength of 450nm and reference wavelength of 690nm. Percent inhibition value was calculated, using the following formula:

$$Inhibition\ (\%) = [100 - (At/As)] \times 100$$

, where As is the absorbance of the solvent and At is that of the test sample. The cytotoxicity concentration of extracts that resulted in 50% reduction of the number of viable cells (CC50) was calculated from dose-response curves.

Inhibition of HIV p24 Core Antigen Production (HIV Replication): HeLa cells, which were used as target cells in this experiment, were grown at a seeding density of 6×10^4 cells per well in 24-well plates. Each well was infected with 400ng of 400ng P24 VSVG-SCR virions. After adsorption for 2h, cells were washed 3 times with pre-warmed DMEM to remove free virus particles. Cells were, then, incubated for 48h in a total volume of 500µl per well of fresh medium containing various concentrations of each extract (10,100 and 1000µg/ml). Nevirapine (HI-1/2 RT inhibitor) was used as positive control. After 48h, the p24 antigen (Ag) assay was performed on the supernatants by using a quantitative p24 ELISA method (HIV p24 ELISA, Biomerieux, France) according to the manufacturer's protocol.

Statistical analysis: Data are given as means ±SD. Statistical differences were calculated, using a one-way analysis of variance (ANOVA) followed by Duncan post Hoc. The $p<0.05$ was considered significant. The CC50 of extracts was calculated according to the method described by Cheng *et al.* [11]. The selectivity index (SI) was evaluated as the ratio of CC50 to IC50.

Findings

The results of this study indicated that the methanol extract of sea cucumber digestive organ had cytotoxic properties and inhibited the growth of KB cells. As results showed (Table 1), this extract with 2.49 TI index had the best anti-HIV activities among all of the extracts. Accordingly, digestive organ methanol extract with this kind of cytotoxic profile is recommended for further investigation, including bioassay-guided fractionation of the bioactive compounds. Generally, methanol digestive organs extract with 2.46 TI index (Table 1) demonstrated better cytotoxic effect than other extracts obtained from *H.leucospilota*.

The effects of various sea cucumber extracts in viability of treated cancer cells compared to the controls is illustrated in Diagram. 1A to D. As shown in Diagram. 1B, methanol digestive organs exhibited anti-proliferative effects and caused to significant diminish cancer cells after treatment ($p<0.05$). Noteworthy is that this extract had less cytotoxicity against HEK293T cells compared other extracts.

Result for antiviral activity of each extract is summarized in Table 2. Our results showed that all the extracts had a considerable cytotoxic effect on the host cell line. The anti-HIV effects of different concentration extracts of sea cucumber are shown in Diagrams. 2A to F. The finding demonstrated that none of extracts could inhibit HIV-1 replication significantly ($p<0.05$); however, the methanol digestive organs extract in 100µg/ml concentration was more potent against HIV-1 replication as well as less cytotoxicity effect compared to other extracts (Diagram. 2A). In addition, body wall extract with 2.79 TI index had a relatively better antiviral activity than other extracts (Table 2).

Discussion

Marine organisms are currently being considered as suitable natural sources, from which therapeutic agents can be derived. So far, a lot of natural products have been examined for finding antiviral compounds from marine animals [1]. In recent years, marine invertebrates such as sea cucumbers, achieved popularity among researchers. This reputation is not only for their nutritive compound, but also because of their high potential therapeutic uses in the medical sciences [2].

Results of this investigation showed that sea cucumber methanol extracts of different parts had marked inhibitory activity on HIV-1 replication and the IC50 rate ranged from 35.89 to 337.60. Huang *et al*. in 2013 [12] investigated inhibitory effects of fucosylated chondroitin sulfates (FuCS-1) derived from the sea cucumber on HIV viral replication. FuCS-1 is a non-toxic compound that is soluble in polar solvents. FuCS-1 HIV-1 replication inhibitory activity results from binding of FuCS-1 to HIV-1 gp120 protein and inhibition of the virus infection by preventing from entering virus to cells and not affecting on virus transcriptase enzyme activity [12]. The present findings seem to be consistent with other studies, indicating that these extracts perform a vital role by disrupting virus penetration into host

cells.

Also, in 2014 Farshidpour *et al*. investigated the antiviral activity of *Holothuria sp*. against herpes simplex virus type 1 (HSV-1). The results of another investigation exhibited the antiviral effect polar extract of *Holothuria sp*. against HSV-1 in cell culture [13]. Moreover, some compounds such as Liouvillosides A and B, which are trisulfated triterpene glycosides, were isolated from Antarctic sea cucumber, and examined by Maier *et al*. In the present study, the latter compounds may cause strong cytotoxic effects, as well [7].

The current study demonstrated that diethyl ether extracts obtained from different parts of the *H.leucospilota* also had good inhibitory effects against HIV-1 replication. Their IC50 was variable from 5.03 to 37.01µg/ml (Table 2). Among all different experimental extracts, diethyl ether body wall extract with 2.79 TI index showed the best antiviral effects. This effect might be caused by the presence of bioactive substances such as cerebroside and some glycolipids, which were extracted from other marine animals. Less cytotoxicity effect of diethyl ether extracts of *H.leucospilota* compared with methanol extracts on host cells seem to derive from the absence of triterpene glycosides and saponins in these extracts [14].

There have been numerous studies that investigated new neutral cytotoxic and antitumor compounds from marine animals [7]. To our knowledge, there is no investigation to show the cytotoxicity effect of crude extracts or bioactive compounds obtained from sea cucumber with comparison between normal and carcinoma cell lines. In 2005, Tian *et al*. demonstrated antitumor and antiangiogenic properties of *triterpene glycoside* extracted from sea cucumber [6]. The *H.leucospilota* body wall methanol extract inhibited proliferation of KB cells, while this component exhibited a slight cytotoxic effect on normal cells. Identification and purification of active components of the extracts can lead to an increase in the therapeutic index and inhibitory effects on cancer cells. A study by Rodriguez *et al*. in 1991 reported antiviral activity and cytotoxicity effect of 5 types of *triterpene glycoside*, which were isolated from sea cucumber (*Holothuria forskalii*). Cytotoxic properties of sea cucumber methanol extracts on cell lines might be caused by saponins in these extracts [15]. Cytotoxicity effect of terpene glycosides results from the ability of this compound in integrating with the cell membrane and ion channels, which then creates pores in cells. This will eventually cause cellular osmoregulation dysfunction and ultimately cell death [16].

Han *et al*. investigated the cytotoxicity effect of triterpene glycosides extracted from *Holothuria scabra* and showed that these compounds had a strong cytotoxic effect on 5 types of the cell lines [17]. These findings indicated the potent cytotoxicity effect of methanol extracts on normal cells. These results were consistent with those of other studies and demonstrated that diethyl ether extracts obtained from *H. leucospilota* had a great cytotoxicity effect on both cell lines. Diethyl ether extracts of sea cucumber contains rich alkaloid sphingoid compounds [18]. Alkaline sphingoid leads to morphological changes in chromatin pressed parts and increases *caspase 3* activities, which can lead to reduced viability of the cells by apoptosis. Sphingolipids derived from sea cucumber can be used as anti-colon cancer agents [14]. Yang *et al*. [19] proved that 12-methyltetradecanoic acid chain, extracted from sea cucumber, inhibited proliferation of prostate cancer cells (PC3). Moreover, wound healing properties are ascribed to long chain fatty acid in sea cucumber lipid fractions [2].

Conclusion

Among all the investigated extracts, diethyl ether extract obtained from body wall demonstrated the best TI index for anti-viral properties. However, this component has cytotoxicity effect on the host cell. *H. leucospilota* digestive organs methanol extract, which had less cytotoxicity, affect inhibitedHIV-1 replication more significantly compared to other extracts (Diagram 2A). Also, this extract showed the best cytotoxicity effect. Thus, we expect to find more bioactive compounds in this extract. The evidence form this study suggests that methanol extract of digestive organs and diethyl ether extract of body wall of *H. leucospilota* are a good candidate for isolation and purification of bioactive components and effective materials with anticancer and antiviral activities.

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Ethical Permissions: All procedures performed in this study were in accordance with the guidelines approved by the Ethics Committee of Pasteur Institute of Iran.

Authors' Contribution: Bahroodi, S (First author), Introduction author/ Original researcher/ Discussion author (25%); Nematollahi, M A. (Second author), Assistant (25%); Aghasadeghi, M R. (Third author), Methodologist/ Statistical analyst (25%); Nazemi, M. (Fourth author), Methodologist (25%).

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References

1- Faulkner DJ. Marine natural products. Nat Prod Rep. 1996;13(2):75-125.

2- Bordbar S, Anwar F, Saari N. High-value components and bioactives from sea cucumbers for functional foods - a review. Mar Drugs. 2011;9(10):1761-805.
3- Bryan PJ, McClintock JB, Hopkins TS. Structural and chemical defenses of echinoderms from the northern Gulf of Mexico. J Exp Mar Biol Ecol. 1997;210(2):173-86.
4- Villasin J, Pomory CM. Antibacterial activity of extracts from the body wall of Parastichopus parvimensis (Echinodermata: Holothuroidea). Fish Shellfish Immunol. 2000;10(5):465-7.
5- Farouk AEA, Hamid Ghouse FA, Ridzwan BH. New bacterial species isolated from Malaysian sea cucumbers with optimized secreted antibacterial activity. Am J Biochem Biotechnol. 2007;3(2):60-5.
6- Tian F, Zhang X, Tong Y, Yi Y, Zhang S, Li L, et al. PE, a new sulfated saponin from sea cucumber, exhibits anti-angiogenic and anti-tumor activities in vitro and in vivo. Cancer Biol Ther. 2005;4(8):874-82.
7- Maier MS, Roccatagliata AJ, Kuriss A, Chludil H, Seldes AM, Pujol CA, et al. Two new cytotoxic and virucidal trisulfated triterpene glycosides from the Antarctic sea cucumber Staurocucumis liouvillei. J Nat Prod. 2001;64(6):732-6.
8- UNAIDS. Global report: UNAIDS report on the global AIDS epidemic 2010 [Internet]. Geneva: UNAIDS; 2010 [cited 2015 Jul 2]. Available from: http://www.unaids.org/globalreport/
9- Naik CG, Kamat SY, Parameswaran PS, Das B, Bhattacharya J, Ramani P, et al. Bioactivity of marine organisms: IV. Screening of some marine animals from the Indian coast. Mahasagar. 1989;22(2):99-104.
10- Zabihollahi R, Sadat SM, Vahabpour R, Aghasadeghi MR, Memarnejadian A, Ghazanfari T, et al. Development of single-cycle replicable human immunodeficiency virus 1 mutants. Acta Virol. 2011;55(1):15-22.
11- Cheng PW, Ng LT, Chiang LC, Lin CC. Antiviral effects of saikosaponins on human coronavirus 229E in vitro. Clin Exp Pharmacol Physiol. 2006;33(7):612-6.
12- Huang N, Wu MY, Zheng CB, Zhu L, Zhao JH, Zheng YT. The depolymerized fucosylated chondroitin sulfate from sea cucumber potently inhibits HIV replication via interfering with virus entry. Carbohydr Res. 2013;380:64-9.
13- Farshidpour F, Gharibi S, Taherzadeh M, Amirinejad R, Taherkhani R, Habibian A, et al. Antiviral activity of Holothuria sp. a sea cucumber against herpes simplex virus type 1 (HSV-1). Eur Rev Med Pharmacol Sci. 2014;18(3):333-7.
14- Sugawara T, Zaima N, Yamamoto A, Sakai S, Noguchi R, Hirata T. Isolation of sphingoid bases of sea cucumber cerebrosides and their cytotoxicity against human colon cancer cells. Biosci Biotechnol Biochem. 2006;70(12):2906-12.
15- Rodriguez J, Castro R, Riguera R. Holothurinosides: New antitumour non sulphated triterpenoid glycosides from the sea cucumber Holothuria forskalii. Tetrahedron. 1991;47(26):4753-62.
16- Chludil HD, Murray AP, Seldes AM, Maier MS. Biologically active triterpene glycosides from sea cucumbers (Holothuroidea, Echinodermata). In: Rahman Au, editor. Studies in natural products chemistry: Bioactive natural products, part 1. 28th Volume. Amsterdam: Elsevier Science; 2003. pp. 587-615.
17- Han H, Li L, Yi Y, Wang X, Pan M. Triterpene glycosides from sea cucumber Holothuria scabra with cytotoxic activity. Chin Herb Med. 2012;4(3):183-8.
18- Althunibat O, Ridzwan B, Taher M, Daud JM, Jauhari Arief Ichwan S, Qaralleh H. Antioxidant and cytotoxic properties of two sea cucumbers, Holothuria edulis Lesson and Stichopus horrens Selenka. Acta Biologica Hungarica 2013;64(1):10-20.
19- Yang P, Collin P, Madden T, Chan D, Sweeney-Gotsch B, McConkey D, et al. Inhibition of proliferation of PC3 cells by the branched-chain fatty acid, 12-methyltetradecanoic acid, is associated with inhibition of 5-lipoxygenase. Prostate. 2003;55(4):281-91.