

# Antiviral activity of *Holothuria sp.* a sea cucumber against herpes simplex virus type 1 (HSV-1)

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**Abstract. – BACKGROUND:** Finding the new bioactive compounds with antiviral activity from the natural resources are in interest of many drug discovery scientists. Sea cucumber is among the marine organisms a traditional food item in Asia with different applications in traditional medicine.

**METHODS:** In current study, a cold water extract of the *Holothuria sp.*, one type Persian Gulf's sea cucumber was evaluated for its antiviral effects against KOS strain of Herpes Simplex Virus type 1 (HSV-1) in cell culture. The half maximal inhibitory concentration (IC<sub>50</sub>) values were calculated for anti-adsorption activity and intracellular antiviral activity of the crude extract separately.

**RESULTS:** The extract exhibited antiviral activity not only against the virus adsorption to the cells, but also on virus intracellular replication. The CC<sub>50</sub> for sea cucumber extract was 32.57 mg/ml. The IC<sub>50</sub> values for the inhibition of the virus adsorption to the cells and virus intracellular replication were 120.2 and 189.9 µg/ml respectively. Selectivity index (SI) value for anti-adsorption activity was 189 while that value for the extract's intracellular antiviral activity was 172.

**CONCLUSIONS:** The results showed that *Holothuria sp.*, water extract has remarkable antiviral effect against HSV-1 in cell culture and it is crucial to investigate the mechanism(s) of action of extract. Moreover, identification of the effective compound(s) within the extract would be necessary for future studies towards developing the new natural antiviral agent against HSV-1.

**Key Words:**

Antiviral activity, Herpes simplex virus type 1 (HSV-1), Sea cucumber, *Holothuria sp.*, Marine organism.

## Introduction

Viral diseases have been always a major health problem and human beings always have been tried to find the new antiviral drugs. Cold sore or fever blister is one of the prevalent viral diseases and its main causative agent is herpes simplex

virus type 1 (HSV-1) an enveloped DNA virus belongs to the *Herpesviridae* family<sup>1</sup>.

Establishment of the latent infections due to HSV infection in sensory ganglia is the main barrier for its treatment<sup>2</sup>. Regarding the prevalence and importance of HSV-1 complications around the world several attempts have been done in the field of drug discovery for this virus but most of the antiviral agents for herpes viruses especially nucleoside analogues possess severe side effects and are not able to cure HSV-1 infections completely<sup>3</sup>. Indeed, following a long period of use of nucleoside analogues, drug resistant virus mutants have been emerged<sup>4</sup>. Therefore, finding the effective and novel anti HSV-1 agents especially within the natural resources seems crucial. Several studies have been conducted to find the good candidate as anti-HSV-1 compound among various types of natural resources such as plants and marine organisms<sup>5-7</sup>. Such natural compounds need to be isolated and screened for possible antiviral activity, and it can be a new hope for the synthesis of new anti HSV-1 agents<sup>8</sup>.

Marine organisms were studied for their bioactive compounds with different applications<sup>9-11</sup>. Sea cucumber is among the marine organisms a traditional food item in Asia due to its delicate flavor and texture<sup>12</sup> and also a traditional medicine in Japan and China<sup>13</sup>. Moreover, the antifungal, antitumor and antioxidant activities of the sea cucumber's extract were demonstrated beside the other biological activities<sup>14-16</sup>.

There is no report about the anti-HSV-1 activity of *Holothuria sp.* a sea cucumber until now. Therefore, in present study we investigate the effect of a crude water extract of this sea cucumber against HSV-1 intracellular replication as well as its activity against virus adsorption to the host cells as an *in vitro* study.

## Materials and Methods

This study was done at Persian Gulf Marine Biotechnology Research Center (Bushehr University of Medical Sciences, Bushehr, Iran).

### *Sea Cucumber Harvesting*

Sea cucumbers were harvested freshly from Bushehr port, in the north part of Persian Gulf. They were sent to the Persian Gulf Marine Biotechnology Research Center in dark plastic bags filled with fresh seawater for extraction and further processing.

### *Preparation of Sea Cucumber Extract*

The sea cucumbers were washed by tap water carefully at the laboratory to eliminate all sea water residues from their body. The sea cucumber body was cut bilaterally at dorsal midline, after taking out all the internal organs and residues the body was washed by distilled water properly. The body was cut into small pieces and kept at 55°C overnight for drying. The dried body pieces were blended into powder form for extraction. A cold water extract was prepared from dried body powder of the organism by mixing and homogenizing in cold double distilled water. The residues of the prepared extract were separated by filtration method using Whatman paper No.1 filter paper. The clarified extract was sterilized by a syringe filter with 0.2 micron pore size (Millipore, Billerica, MA, USA). The aliquots of the sterile extract were stored at -20° C till the time of experiments.

### *Cell Line and Virus*

In this study HEp-2 cell line was used as an appropriate human cell line for HSV-1 propagation and antiviral experiments. Cells were grown and propagated using Dulbeccos' Minimum Essential Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, NY, USA) and incubated at 37°C in the presence of 5% CO<sub>2</sub>. At the time of virus propagation and antiviral assays serum concentration was reduced to 2%. Herpes simplex virus type 1 (KOS strain) was used in this study as virus of interest.

Titration of the propagated viral stock was performed by TCID<sub>50</sub> (Tissue Culture Infectious Dose) method using Karber formula. After titration, viral stock was aliquoted and stored at -70°C.

### *Cytotoxicity Assay*

Cytotoxicity of the sea cucumber extract against HEp-2 cells was measured using trypan blue exclu-

sion test as described previously<sup>6</sup>. Briefly, a monolayer confluent HEp-2 (human epithelial-2) cell line was prepared in a 24 wells cell culture microplate (NUNC: Nunlon products). Then, the cells were treated by different concentrations of the extract in triplicate. After 72 h viability of the treated cells were determined by the trypan blue exclusion test. Results were plotted using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA) to determine the half maximal cytotoxic concentration (CC<sub>50</sub>) of the tested extract.

### *Antiviral Activity Assay (CPE Inhibition Assay)*

We have used CPE (Cytopathic effect) inhibition assay method to evaluate the antiviral activity of the tested extract<sup>17</sup>. Briefly, monolayers from HEp-2 cells were prepared in 96-wells cell culture microplate (2×10<sup>3</sup> cells/well). Thereafter, the culture medium was removed from each well and each well was inoculated by 100 TCID<sub>50</sub> of HSV-1 triplicately. The microplate was kept at 37°C for 1 hour for virus adsorption. After washing the cells by sterile PBS (phosphate buffered saline) to remove the un-absorbed viruses, the cells were overlaid by DMEM (Dulbecco's Modified Eagle's Medium) with 2% FBS (fetal bovine serum) containing increasing concentrations of the extract and incubated at 37°C for 5 days and investigated for CPE presentation daily.

For the virus control, virus suspension containing 100 TCID<sub>50</sub> was added to the 3 wells of microplate. Also, 3 wells of each row were chosen as test control for the maximum non-toxic concentration dose of the extract without virus.

The value of virus replication inhibition was expressed as percent yield of virus control (% virus control = CPE experimental group/CPE virus control × 100)<sup>17</sup>. The concentration of the extract that reduced 50% of CPE presentation compared to virus control wells, was estimated from graphic plots defined as 50% inhibited concentration (IC<sub>50</sub>) expressed in microgram per milliliter using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA).

### *Anti-adsorption Activity Assay*

To evaluate the effect of sea cucumber extract on virus adsorption to the cells a confluent monolayer of HEp-2 cell line was infected with 100 TCID<sub>50</sub> of HSV-1 in the presence or absence of different concentrations of the extract. The microplate was incubated at 37° C for 1 hour for virus adsorption. After washing the cells by sterile PBS for two times, the cells were overlaid by DMEM with 2%

FBS and kept at 37°C in a humidified chamber in the presence of 5% CO<sub>2</sub> for five days. Inhibition of the CPE presentation was used to measure the effect of the extract as described earlier.

### Statistical Analysis

Graph Pad Prism for Windows, version 5 (Graph Pad Software Inc., San Diego, CA, USA, 2005) was used to determine the half maximal cytotoxic concentration (CC<sub>50</sub>) and half maximal inhibitory concentration (IC<sub>50</sub>) values of the extract<sup>18</sup>. Selectivity Index value (SI) was defined as the ratio of CC<sub>50</sub> to IC<sub>50</sub> value.

## Results

### Cytotoxicity of the *Holothuria sp.* Crude Extract

The cytotoxicity of the water extract of sea cucumber crude extract was determined by treatment of the HEp-2 cells with increasing concentrations of the extract. The CC<sub>50</sub> for the used extract was 32.57 mg/ml (Figure 1).

### Antiviral Activity

#### Anti-Adsorption Activity

Treatment of the HEp-2 cells with different concentrations of the cold water extract at the same time of virus inoculation to the cells was performed. The

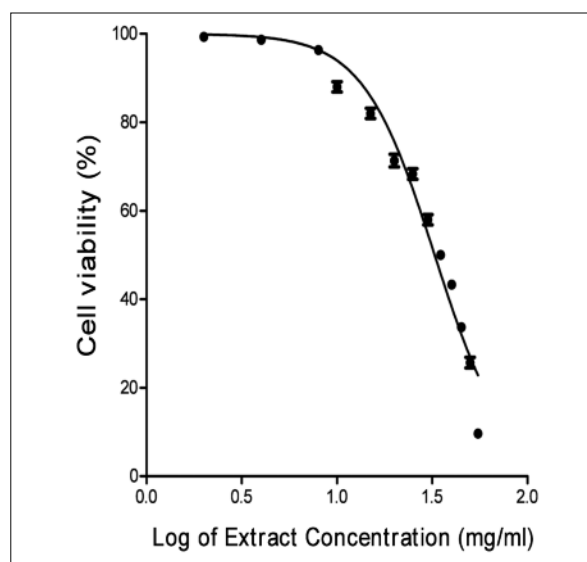
results showed that 80 µg/ml of the extract did not exhibit significant anti-adsorption activity and there was no inhibition on virus CPE presentation. However, 160 µg/ml of the extract inhibited virus CPE presentation by 96.6% ± 2.8 and and IC<sub>50</sub> value for the extract was 120.2 µg/ml (Figure 2).

### Intracellular Antiviral Activity

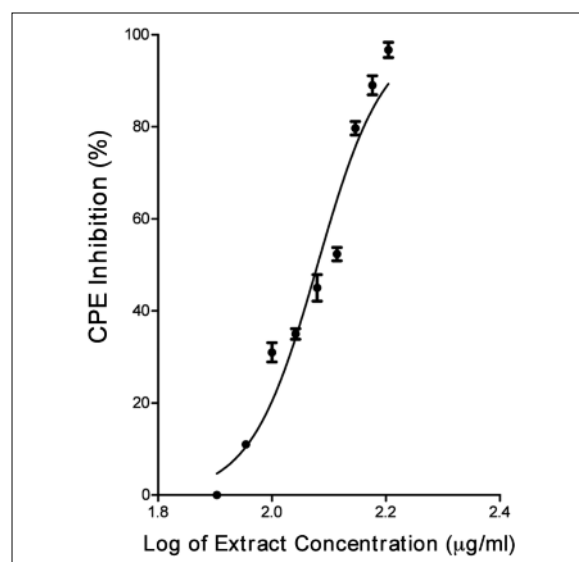
Antiviral activity of the sea cucumber extract was evaluated against intracellular replication of HSV-1 in HEp-2 cells by adding the different concentrations of the crude extract to the virus inoculated cells. It was found that 50 µg/ml of the extract did not inhibit the virus replication within the host cells significantly and the presentation of the HSV-1 related CPE was decreased by 5.6% ± 2. However, 400 µg/ml of the extract could prevent the virus CPE presentation 98.3% ± 2.8 compared to the non-treated infected cells (Figure 3). The IC<sub>50</sub> value for intracellular antiviral activity of the extract was 189.9 µg/ml.

## Discussion

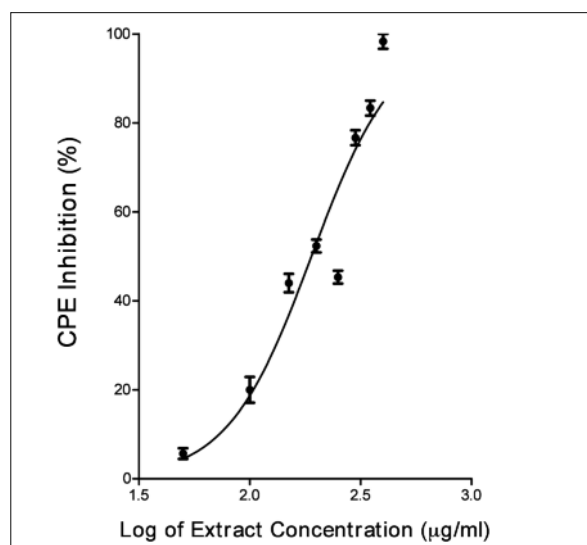
Traditional medicines have been used to prevent or treat HSV-1 infections for a long time. Accordingly, a large number of natural products have been examined for their antiviral effects on HSV-1<sup>2,3,6,7</sup>. A crude water extract of *Holothuria sp.* a sea cucumber from Persian gulf as a marine organism of



**Figure 1.** Cytotoxicity of sea cucumber crude extract against HEp-2 cells. trypan blue exclusion test was performed on HEp-2 cells after 72 hours treatment with increasing concentrations of the extract. Results are presented as percentage of cell viability from triplicate assays.



**Figure 2.** Effects of sea cucumber crude extract against HSV-1 adsorption to the HEp-2 cells. CPE inhibition assay was used to evaluate the antiviral activities. All experiments were performed in triplicates. Data were plotted using Graph Pad Prism Version 5.



**Figure 3.** Antiviral activity of sea cucumber crude extract against HSV-1 intracellular replication. CPE inhibition assay was used to evaluate the antiviral activities. All experiments were performed in triplicates. Data were plotted using Graph Pad Prism Version 5.

interest was evaluated for its possible antiviral activity against HSV-1 in cell culture system. The half maximal cytotoxic concentration of the that the water extract of *Holothuria Sp.* does not exhibit significant cytotoxicity against HEp-2 cell line which is a good advantage for using this type of extract for any future medicinal use.

The selectivity index (SI) for the anti-adsorption activity of the extract is 189. Therefore, the extract showed potent antiviral activity during the adsorption of virus to the host cells. The mechanism(s) conferring the antiviral properties of the tested extract against HSV-1 adsorption and attachment is unknown. One of the possible mechanisms for the sea cucumber crude extract anti-adsorption activity against HSV-1 can be due to inactivation of the viral particles. It is demonstrated that some derivatives such as sulfated polysaccharides are present in sea cucumber<sup>19</sup>. Moreover, antiviral activity of some sulfated polysaccharides from various marine organisms have been reported<sup>20,21</sup>. Therefore, inactivation of HSV-1 particles through saturation of virus ligands by sulphated polysaccharide derivatives those are present in *Holothuria sp.* water extract could be a possible mechanism for anti-adsorption effect of the extract. The other possible mechanism of action for this part of study includes interaction of different constituents that present in the *Holothuria sp.* crude extract with host cells surface molecules including HSV-1 receptors that might be lead to the receptor masking. However,

as one of the limitation of our study we would suggest to investigate about the exact mechanism(s) of anti-adsorption activity of *Holothuria sp.* extract for future studies. Indeed, investigation about the probable direct extracellular activity of that extract against virus particles seems to be addressed in the future.

Based on SI value of the extract for its intracellular antiviral activity against HSV-1 (SI = 172) it could be concluded that the extract of interest exhibits significant *in vitro* anti-HSV-1 activity.

There are various possibilities for the observed intracellular anti-HSV activity from sea cucumber water extract including, prevention of virus uncoating, inhibition of viral DNA synthesis, virus transcription blocking for some certain genes, viral protein production and processing and even maturation of the viral particles. However, further investigations it would be recommended to investigate about the molecular and cellular mechanism(s) of action of sea cucumber extract. Also, identification of the active constituent(s) of the extract would be crucial as one aspect of the neighbor researches.

It has been reported that acidic mucopolysaccharide from *Stichopus japonicas*, one species of sea cucumbers has multiple pharmacologic actions, such as antitumor, immunologic regulation, anticoagulated blood and antiviral activity<sup>22</sup>. Two types of trisulfated triterpene glycosides from the Antarctic sea cucumber *Staurocucumis liouvillei* belonging to the family *Cucumariidae* were found with virucidal activity against HSV-1<sup>23</sup> that could be supportive for our hypothesis about one of the possible mechanisms of *Holothuria sp.* extract against HSV-1 adsorption that must be investigated in future studies.

There are also some other identified constituents in sea cucumbers such as triterpenoids, saponins and chondroitin sulfate<sup>24-26</sup> that their antiviral activity but from different natural resources against different viruses have been reported<sup>27-29</sup>.

## Conclusions

The presence of those above mentioned bioactive compounds together with the other constituents in the body of *Holothuria sp.* would justify our results.

## Conflict of Interest

The Authors declare that there are no conflicts of interest.

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