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Full Length Research Paper

Evaluation of *in vitro* antiviral activity of a brown alga (*Cystoseira myrica*) from the Persian Gulf against herpes simplex virus type 1

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The hot water extract of a brown marine alga, *Cystoseira myrica,* from the Persian Gulf was evaluated as an antiviral compound against KOS strain of HSV-1 in cell culture. The extract exhibited antiviral activity against herpes simplex virus type 1 (HSV-1) not only during absorption of virus to the cells, but also on post attachment stages of virus replication. The water extract of *C. myrica* was sterilized by filtration and autoclaving, respectively. The IC_{50} for filtered extract was 99 µg/ml and the IC_{50} for autoclaved extract was 125 µg/ml. Based on resulted selectivity index (SI) values of the extracts, which were 33.4 and 28.2 for filtered and autoclaved extracts, respectively, we found that the antiviral compound(s) in the water extract *of C. myrica* to be heat stable. Also, the SI values for inhibition of the post attachment stages of HSV-1 replication were 23.1 and 21.7 for filtered and autoclaved extracts, respectively. The IC_{50} in this phase of study were 143 and 162 µg/ml for filtered and autoclaved extracts, respectively. Therefore, *C. myrica* could be a good candidate as a natural source for anti-HSV-1 compound(s) isolation.

Key words: *Cystoseira myrica*, HSV-1, antiviral, Persian Gulf.

INTRODUCTION

The herpes simplex virus type 1 (HSV-1) is the primary cause of oral-facial and pharyngeal infections and may cause herpetic whitlow, as well as severe and sometimes dangerous infections of the eyes and brain. HSV-1 also accounts for 10 to 15% of all genital herpetic infections. This virus can produce latent infection in the host for life and is reactivated by stimulus to cause recurrent infections and lesions (Fields, 2001). Considering the complications of this virus, some synthetic antiviral compounds were developed for treatment of active herpetic infections, but they are not effective for the treatment of latent infections (Naesens and De Clercq, 2001). On the other hand, the severe side effects and development of some resistant mutations of this virus, especially during long term medication with antiviral drugs, were reported (Malvey, 2005; Pottage and Kessler, 1995).

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Therefore, for many researchers, finding new natural antiviral compounds is very interesting. In many studies looking for novel antiviral agents, some plants and algae extracts were tested on different viruses including the herpes viruses (Yoosook et al., 1999; Lopez et al., 2001; Lee et al., 2004; Serkedjieva, 2004). In some of these experiments different species of brown algae were tested for their antiviral activity.

However, until now, no research has been done with respect to the antiviral effects of brown algae from the Persian Gulf and the antiviral activity of *Cystoseira myrica* has not yet been reported from any other part of the world. Therefore, in the present study, the anti-HSV-1 activity of the crude water extract of this alga was evaluated in cell culture.

MATERIALS AND METHODS

Cell line and virus

African green monkey kidney cell line (Vero) was used as a proper cell line for HSV-1 replication. Briefly, the cells were grown in 50 ml

Table 1. Inhibition of HSV-1 replication by different concentrations of the filtered extract (at the same time of virus inoculation).

Extract concentration (µg/ml)	CPE inhibition (%)
20	0
40	10
60	25
80	40
10	50
130	75
130	75
150	85
170	100

Table 2. Inhibition of HSV-1 replication by different concentrations of the autoclaved extract (at the same time of virus inoculation).

Extract concentration (µg/ml)	CPE inhibition (%)
20	0
50	5
80	15
100	30
120	45
130	55
170	80
200	100

cell culture flasks (NUNC) or 24 well cell culture microplates (NUNC) by using Dulbeccos Minimum Essential Medium (Gibco) containing 10% foetal bovine serum (Gibco). Herpes simplex virus type 1, KOS strain, was received as a gift from the virology department of Tarbiat Modarres University. The virus was propagated in Vero cells and the titre of propagated viral stock was determined as $TCID_{50}$/ml by using Karber method.

Cytotoxicity test

The cytotoxicity of alga extract was determined by culturing Vero cells for five days in the presence of increasing amounts of extract. Viable cells were determined by the trypan blue exclusion test. The results were plotted, at dose response curve and by using STATA statistical software, the 50% cell growth inhibitory concentration (CC_{50}) was obtained.

Antiviral activity assay (CPE Inhibition assay)

Vero cells were grown in 24-well plastic plates (7×10^3 cells /well). Then the plates were incubated at 37ºC in the presence of 5% CO_2 until the cells became confluent. Thereafter, the culture medium was removed from each well 0.1 ml of virus suspension containing 10000 $TCID_{50}$ and 0.1 ml of DMEM containing 2% FBS were mixed in each well of the 24-well plates and appropriate concentrations of the extract from minimal to maximal non-cytotoxic concentration were added to each well based on serial dilution preparation. For the virus control, 0.1 ml of virus suspension and 0.1 ml of culture medium without extract were used. For the cell control, 0.1 ml of culture medium with maximal non cytotoxic concentration of extract was added.

The plates were incubated at 37ºC in a humidified CO_2 atmosphere (5% CO_2) and were investigated every day for CPE presentation until day 5 post infection. For testing the probable post attachment antiviral effect of the extract, the same protocol mentioned above was performed, but the addition of the extract was two hours post inoculation of cells with virus. The degree of inhibition was expressed as percent yield of virus control (% virus control = CPE experimental group/ CPE virus control ×100). The concentration of extract which reduced CPE 50% with respect to virus control was estimated from graphic plots defined as 50% inhibitory concentration (IC_{50}) expressed in microgram per milliliter by using STATA modeling software. The selectivity index (SI) was measured from the ratio of CC_{50}/IC_{50} (Kudi and Myrint, 1999; Kujumgier et al., 1999).

Preparation of alga extract

C. myrica was collected along the Bushehr coast of the Persian Gulf (Southwest of Iran). About 100 g of the fresh alga, corresponding to 10 – 12 g of dry alga material was homogenized in 500 ml cold double distilled water. The mixture was clarified by filtration using Whatman No.1 filter paper and the light brown extract resulted. The water extract of *C. myrica* were sterilized by filtration and autoclaving, respectively. These extracts were tested for their antiviral and cytotoxic activity.

Statistical analysis

STATA statistical analysis package was used for drawing the dose response curve in order to IC_{50} and CC_{50} calculation.

RESULTS

The cytotoxicity of *C. myrica* extracts which were sterilized by filtration and autoclaving methods on Vero cells were determined by calculation of CC_{50}. The resulted CC_{50} for filtered and autoclaved extracts were 3310 and 3528 µg/ml respectively. Treatment of the Vero cells with different concentrations of the cold water extract at the same time of inoculation by HSV-1 was done. Based on data shown in Table 1, 20 µg/ml of the filtered extract did not show any antiviral effect, while the application of 170 µg/ml of that extract lead to 100% inhibition of CPE formation due to HSV-1 replication in Vero cells. Therefore, the IC_{50} of this extract by using STATA software is 99 µg/ml. The data for autoclaved crude extract of *C. myrica* is shown in Table 2. We found that 20 µg/ml of the autoclaved extract didn't show any anti-HSV-1 effect; although 200 µg/ml of that extract lead to 100% inhibition of HSV -1 related CPE formation. The IC_{50} for the autoclaved extract in this part of the study was calculated to be 125 µg/ml. The SI values were calculated at 33.4 and 28.2 for filtered and autoclaved extracts, respectively, based on the IC_{50} and CC_{50} from each extract.

Also, antiviral activity of the crude extracts was evaluated on post attachment stages of the virus replication cycle. In this phase of research, based on the data in Table 3, we have found that 20 µg/ml of the filtered ex-

Table 3. Inhibition of HSV-1 replication by different concentrations of the filtered extract (after virus attachment to the cell).

Extract concentration (µg/ml)	CPE inhibition (%)
20	0
40	5
60	10
110	25
130	40
150	50
170	70
210	100

Table 4. Inhibition of HSV-1 replication by using different concentrations of the autoclaved extract (after virus attachment to the cell).

Extract concentration (µg/ml)	CPE inhibition (%)
20	0
60	5
100	20
130	35
160	45
170	50
220	80
240	100

tract could not prevent the performing of cytopathic effect of HSV-1 in Vero cells but 210 µg/ml of that extract inhibited the HSV-1 related CPE formation 100% in cell culture. The IC_{50} value for filtered extract is 143 µg/ml. The data in Table 4 shows the CPE inhibition of the different concentration of autoclaved extract on post attachment stages of HSV-1 replication cycle. As shown in Table 4, 240 µg/ml of autoclaved extract could prevent the CPE exhibition of HSV-1 in cell culture 100%, whereas a concentration of 20 µg/ml of that extract didn't show any anti-HSV-1 effect. Therefore, the IC_{50} of that extract in this phase of study was 162 µg/ml. The SI values for filtered and autoclaved extracts in this part of the research were 23.1 and 21.7, respectively.

DISCUSSION

Interest in employing antiviral compounds from natural sources like plants or algae has been enhanced by researchers and the consumers' preference for natural medicines and concerns about the toxic effects of synthetic antiviral materials. There are some publications about antiviral properties of different species of marine algae such as brown, green and red algae (Damonte et al., 1996; Lopez et al., 2001; Lee et al., 2004; Serkedjieva, 2004; Richards et al., 1978; Preeprame et al., 2001; Ghosh et al., 2004; Hayashi et al., 2006; Pujol et al., 2006). In most studies done in this area, the viruses tested belonged to the herpesviridae family, especially herpes simplex viruses. In the present study we chose HSV-1 for our research because of its ability to perform different clinical complications and its increasing prevalence in communities (Rosen, 2006). Also, HSV-1 is a good example of enveloped viruses. Therefore, the discovery of natural antiviral compounds should be interesting. In some studies in this direction, some brown algae were tested. However, until now there has been no other study on the antiviral effect of *C. myrica*. This was valuable for us especially because we had easy access to this alga on the Bushehr coast.

We have prepared water extract and for the sterilization of the extract we have used a filtration method besides autoclaving. We have found that the autoclaved extract showed the acceptable IC_{50} and based on SI values of this extract, it could be a good choice for anti- HSV-1 natural compound, although in most studies the filtering method was used for extract sterilization. The IC_{50} values for filtered and autoclaved extracts tested at the same time as that of virus inoculation were 99 and 125 µg/ml, respectively. Also, the SI values were 33.4 and 28.2 for the filtered and autoclaved extracts. This data showed that the crude extract of *C. myrica* could prevent the initial stages of HSV-1 replication such as absorption, attachment and/or entry to the host cell.

In another step of our research, we tested the autoclaved and filtered extract for their probable post attachment inhibition effects on HSV-1 replication. Based on IC_{50} value of each extract, the SI for filtered extract was 23.1 and for the autoclaved extract was 21.7. Therefore, the water extracts of *C. myrica* also exhibit antiviral effect on post attachment stages of HSV-1 to the Vero cells, probably on internalization of virus to the cells because we have done this phase of study at 37ºC. Meanwhile, in one study which evaluated the antiviral activity of sulphated polysaccharides from *Sargassum patens*, a brown alga, against HSV-2, suggested that the antiviral mode of action of purified sulphated polysaccharide of that alga could be ascribed to the inhibition of virus adsorption (Zhu et al., 2004). Also, in another study the antiviral effect of diterpens from *Dictyota pfaffi* a Brazilian brown alga, on HSV-1 was evaluated and the results indicated that the diterpenes affected an early step of the replicative cycle (Barbosa et al., 2004). These results suggested that *C. myrica* water extract might be a candidate for natural antiherpetic compound development. Further investigations such as purification of water crude extract and in vivo studies are recommended for future studies.

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