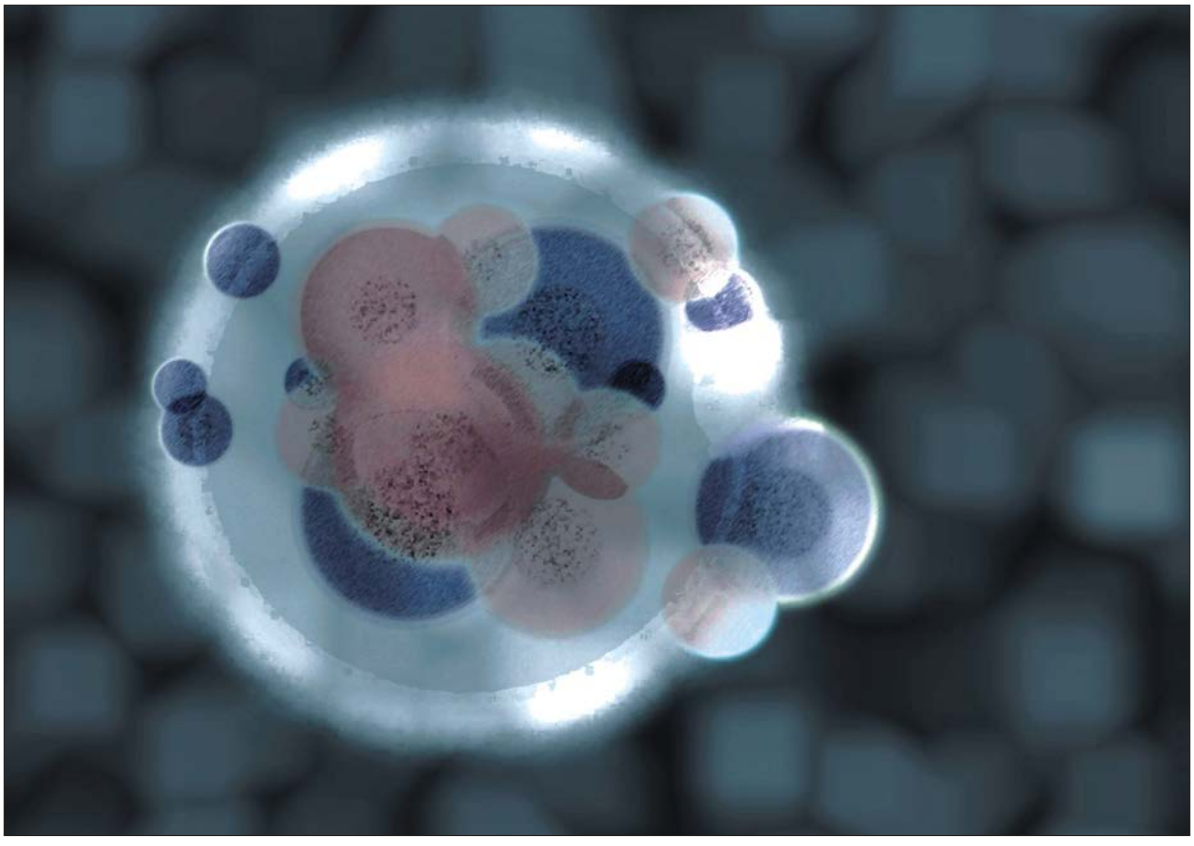

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In vitro Antiviral Activity of the Red Marine Alga from Persian Gulf, *Gracilaria salicornia*, Against Herpes Simplex Virus Type 2

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Abstract: This study was done to evaluate the anti-HSV-2 activity of the crude water of *Gracilaria salicornia* alga against HSV-2 in cell culture. The extract showed antiviral activity against HSV-2 not only before attachment and entry of virus to the Vero cells, but also on post attachment stages of virus replication. Regarding the calculated SI values of the extracts which were 44.4 and 38.5 for filtered and autoclaved extracts, respectively. It is concluded that the antiviral compound(s) in the water extract of this alga should be heat stable. Also the SI values for inhibition of the post attachment stages of HSV-2 replication were, 31.9 and 29.9 for filtered and autoclaved extracts, respectively. Therefore *Gracilaria salicornia* could be a good choice as a natural source for anti HSV-2 compound isolation.

Key words: Antiviral, *Gracilaria*, alga, herpes simplex virus

INTRODUCTION

Herpes simplex virus type 2 (HSV-2) is an enveloped virus which causes genital herpes and some other important complications such as encephalitis, meningitis, eye infections and cold sore. This virus can produce latent infection in the host for life and reactivated by stimulus to cause recurrent infections and lesions (Fields, 2001).

Considering the complications of this virus some synthetic antiviral compounds such as acyclovir, penciclovir, vidarabine, etc 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 severe side effects and development of some resistant mutants of this virus especially during long term medication with antiviral drugs were reported (Malvy *et al*., 2005; Pottage and Kessler, 1995). Also, regarding the increasing prevalence of genital herpes, there is an urgent need to develop new anti-HSV-2 drugs.

In many studies to find 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). Some algae extracts which showed the antiviral activity *in vitro*, may be good antiviral compounds sources, especially for preventing transmission of some sexually transmitted viral infections (Cooper *et al*., 2002). In general marine algae contain some compounds such as sulfated polysaccharides and some other elements which have been found to be potent *in vitro* inhibitors for some viruses such as HSV-1 and HSV-2, but the amount and chemical composition of those compounds in different species of algae could be different (Cooper *et al*., 2002; Lee *et al*., 2004; Serkedjieva, 2004).

Up to now, no research has been done about the antiviral effects of Persian gulf algae, and the antiviral activity of *Gracilaria salicornia* has not been reported yet from any other part of the world. Therefore in the present study the anti-HSV-2 activity of the crude extract of this alga was evaluated in cell culture.

MATERIALS AND METHODS

This study was done at Persian Gulf Health Research Center of Bushehr University of Medical Sciences, Iran during 2005 to 2006.

Cell line and virus: In this study African green monkey kidney cell line (Vero) was used. Briefly, the cells were grown in 50 mL cell culture flasks (NUNC) or 24 wells cell culture microplates (NUNC) by using Dulbeccos Minimum Essential Medium (Gibco) containing 10% foetal bovine serum (Gibco).

Herpes simplex virus type 2 was isolated from clinical sample and confirmed by using anti HSV-2 type specific flourescent monoclonal antibody (DAKO).

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The virus was propagated in Vero cells and the titre of propagated viral stock was determined as TCID 50 mL^{-1} by using Karber method.

After titration, viral stock was dispensed in some sterile tubes which were stored at -70°C.

Cytotoxicity test: Cytotoxicity of alga extract was determined by culturing Vero cells for 72 h in the presence of increasing amounts of extract For each concentration of extract three wells were tested. Then viable cells were determined by the trypan blue exclusion test. By using STATA modeling software, results were plotted at dose response curve and 50% cell growth inhibitory concentration (CC50) was obtained.

Antivirial activity assay (CPE Inhibition assay): To begin with, Vero cells were grown in 24-well plastic plates (7×10^3 cells/well). Then the plates were incubated at 37°C 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 TCID50 and 0.1 mL of DMEM containing 2% FBS were mixed in each well of 24-well plates and appropriate concentrations of the extract from minimal to maximal non-cytotoxic concentrations were added to each well based on serial dilution preparation. For each concentration of extract three wells were tested.

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 37°C in a humidified CO_2 atmosphere (5% CO_2) and were investigated everyday for CPE presentation until 5 days post infection. For testing the probable post attachment antiviral effect of the extract, the same protocol was done but the extract was added 2 h 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% inhibited concentration (IC50) expressed in microgram per milliliter by using STATA modeling software. The Selectivity Index (SI) was measured from the ratio of CC50/IC50 (Kudi and Myrint, 1999; Kujumgier *et al*., 1999).

Preparation of alga extract: The alga *Gracilaria salicornia* was collected along the Bushehr coast of the Persian Gulf (South west of Iran) during summer of 2005. About 100 g of the fresh algae, 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 paper No. 1 filter paper. Then the crude extract was sterilized separately by two methods, filtering and autoclaving. These extracts were tested for their antiviral and cytotoxic activity.

Statistical analysis: STATA statistical analysis package was used for the curve plotting in order to IC50 and CC50 calculation.

RESULTS

The cytotoxicity of *Gracilaria salicornia* crude extracts which were sterilized by filtration and autoclaving methods on Vero cells were determined by calculation of CC50 which were 3643 and 3829 μg mL^{-1}, respectively.

We found antiviral activity of the extract against HSV-2 to be as follows:

Treatment of the Vero cells with different concentrations of crude extract concurrent with inoculation by HSV-2 as mentioned previously.

Filtered and autoclaved extracts at concentration 20 μg mL^{-1} did not show any antiviral effect while, 140 μg mL^{-1} of the filtered extract and 170 μg mL^{-1} of autoclaved extract could inhibit the performing of cytopathic effect completely due to HSV-2 replication inhibition in cell culture (Table 1 and 2).

Therefore the IC50 filtered extract was 82 μg mL^{-1} and that of autoclaved extract was calculated as 99.3 μg mL^{-1}.

Regarding the resulting IC50 and CC50 from each extract, the SI values were 44.4 and 38.5 for filtered and autoclaved extracts, respectively.

In the second part of this study, antiviral activity of the crude extracts were tested on post attachment stages of the virus replication cycle. We found that 190 μg mL^{-1} of the filtered and autoclaved extracts could prevent the CPE performing of HSV-2 in Vero cells completely.

Meanwhile both extracts did not exhibit any antiviral activity at concentration 20 μg mL^{-1} against HSV-2. Based on antiviral activity of different concentration of the both extracts the IC50 value for filtered and autoclaved extracts were 114 and 128 μg mL^{-1}, respectively (Table 3 and 4).

Table 1: Inhibition of HSV-2 related cytopathic effect (CPE) by using different concentrations of the filtered extract (At the same time of virus inoculation)

Extract concentration (μg mL^{-1})	CPE inhibition (%)
20	0
30	5
50	20
70	40
90	55
110	80
130	90
140	100

Table 2: Inhibition of HSV-2 related cytopathic effect (CPE) by using different concentrations of the autoclaved extract (At the same time of virus inoculation)

Extract concentration (μg mL^{-1})	CPE inhibition (%)
20	0
40	10
60	20
80	30
110	50
130	85
140	90
170	100

Table 3: Inhibition of HSV-2 related cytopathic effect (CPE) by using different concentrations of the filtered extract (After virus attachment to the cell)

Extract concentration (μg mL^{-1})	CPE inhibition (%)
20	0
40	5
50	10
70	20
100	40
120	50
140	70
190	100

Table 4: Inhibition of HSV-2 related cytopathic effect (CPE) by using different concentrations of the autoclaved extract (After virus attachment to the cell)

Extract concentration (μg mL^{-1})	CPE inhibition (%)
20	0
60	10
80	25
120	45
140	50
160	70
170	80
190	100

The SI values for filtered and autoclaved extracts in this part of research were 31.9 and 29.9.

DISCUSSION

For many years researchers have been trying to find new antiviral compounds from natural materials such as plants and algae because of their minimal side effects (Richards *et al*., 1978; Kudi and Myrint, 1999; Lopez *et al*., 2001). In some studies the antiviral properties of the extracts of some marine algae were established (Serkedjieva, 2004; Preeprame *et al*., 2001; Ghosh *et al*., 2004; Lee *et al*., 2004; Hayashi *et al*., 2006; Pujol *et al*., 2006). In most of the studies done in this area the viruses which were tested, belonged to the herpesviridae family especially, HSV-1 and HSV-2.

In the present study we chose HSV-2 due to its ability to cause different clinical complications and its increasing prevalence in communities (Rosen, 2006). On the other hand HSV-2 is a good example of enveloped viruses. Therefore discovery of natural antiviral compounds against this virus should be interesting. In most studies, some effective chemical compounds have been identified (Lee *et al*., 2004; Damonte *et al*., 1996), but the antiviral effect of *G. salicornia* crude extract has been tested for the first time in this study. Some works on aqueous extracts of algae, on specific carbohydrates in them (carrageenans) and on other sulfated polysaccharides like dextran sulfate and heparin suggest that these molecules may inhibit both DNA- and RNA-viruses infections and may operate both outside of and within infected cells (Baba *et al*., 1988; Ghosh *et al*., 2004; Mitsuya *et al*., 1988).

Since we prepared crude water extract of *G. salicornia*, it is obvious that the resulting IC50 and CC50 values are not comparable with their counterpart studies in which the purified fractions were tested.

For sterilization of the extract we used autoclave beside filtration and we have found that the autoclaved extract showed acceptable IC50 and based on SI values of this extract, it could be a good choice for anti- HSV-2 natural compound, although in most studies the filtering method has been used for extract sterilization.

The resulting IC50 values for filtered and autoclaved extracts which were tested at the same time as that of virus inoculation were 82 and 99.3 μg mL^{-1}, respectively and the related SI values were 44.4 for the filtered extract and 38.5 for the autoclaved extract. In one study the SI for the extract of Ceramium rubrum a red marine alga was 4.9 for HSV-2 (Serkedjieva, 2004). Therefore, according to our study both extracts were good for inhibiting the start point of virus replication.

In another step of our research, we tested the autoclaved and filtered extract for their probable post attachment inhibition effects on HSV-2 replication. Based on IC50 and CC50 values of each extract the SI was 31.9 for the filtered extract and 29.9 for the autoclaved extract. These data showed that the water crude extract of *G. salicornia* could prevent the initial stages of HSV-2 replication such as adsorption and attachment of the virus to the host cell. It also exhibits a reasonable effect on inhibiton of virus replication after attachment of HSV-2 to the Vero cells.

Based on these data, it could be concluded that water soluble extract of *G. salicornia* could be a good choice for natural antiherpetic compound development. Further investigations such as purification of water crude extract and *in vivo* studies are recommended for future researches.

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