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Full Length Research Paper

Antiviral activity of *Avicennia marina* against herpes simplex virus type 1 and vaccine strain of poliovirus (An *in vitro* study)

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Avicenniaceae family is a member of true mangrove plants which has one genus, 11 species and several subspecies. *Avicennia marina* is the most current species among these plants in Iranian mangrove forest. Regarding to the presence of many active biological constituents in this plant and their applications in traditional and alternative medicine, the *in vitro* antiviral activity of its leaf extract on herpes simplex virus type-1 (HSV-1) and vaccine strain of polio virus (Sabin) in Vero cell line were determined. The CC50 of the extract was 5750.96 for Vero cells. The antiviral effect of the extract on HSV- 1 and vaccine strain of polio virus before and after the attachment of the virus particles to Vero cells were assessed. The IC50 values of the extract were 66 µg/ml and 137.24 µg/ml for before and after virus attachment stages of HSV-1 replication cycle respectively. The IC50 values of extract for vaccine strain of poliovirus were 145.7 and 314.3 µg/ml for before and after attachment stages of virus replication respectively. The SI values of the extract for the before and after virus attachment stages of viral replication cycle were 87.1 and 41.9 for HSV-1. The SI for the vaccine strain of poliovirus were calculated 39.5 and 18.3 for before and post attachment stages of this virus replication cycle ordinarily. The obtained SI values indicate that hot glycerin extract of *A. marina* leaves could be a good candidate for further studies in the area of antiviral compound developing.

Key words: HSV-1, vaccine strain of poliovirus, antiviral, *Avicennia marina*.

INTRODUCTION

Finding a new safe and efficient drug for treatment of viral respiratory disease, in particular retrovirus infections has been field of research for many scientist and significant attention has been paid to natural compounds. Knowledge of ethnopharmacology can lead to new bioactive plant compounds. Therefore, indigenous plants around the world and algae have been tested in many studies

(Goncalves et al., 2005; Iwashima et al., 2005; Lee et al., 2004; Nolkemper et al., 2006). Regarding these studies it has been shown that many plant species such as *Tridax procumbens, Carissa carandas, Mallotus philippensis, Streblus aspere, Terminatta alata, Macaranga pustulata, Sibbaldia micropetala, Hypericum cordifolium, Hypericum uralum* and *Maesa macrophylla* were active against the replication of poliovirus and herpes simplex-1 (HSV-1). Methanol extract of *Annona muricata* as well as aqueous extract of *Petunia myctaginiflora* indicated to inhibit HSV-1 cytopathic effect in Vero cells. By investigation which has done on some Indonesian plants in murine and human cell lines for HSV-1 and Polivirus, it has been found that *Piper aduncum* was active on poliovirus, while *Elytranthe tubaeflora* and *Melastoma malabathricum* inhibited HSV-1 (Felipe et al., 2006).

HSV-1 is widespread, enveloped and double stranded DNA agents which cause various infections in human and

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belongs to herpesviridae family. The virus causes recurrent infections of the nervous system located around the lips, in the eyes, in the mucous membrane of the oral cavity and genital as well (Fields, 2007). The drugs found to be clinically useful in the treatment of HSV- 1 are synthetic nucleosides such as acyclovir (ACV). The sever side effects and the emergence of drug-resistance mutants during long-term medication with these drugs have often limited their administration to patients (Bacon et al., 2003; Coen, 1996; Morfin and Thouvenot, 2003). Thus, the development of novel antiviral agents against this virus is still an important area of research. On the other hand, poliovirus is a non-enveloped virus which has single-stranded RNA. When the virus spreads to the central nervous system, it may develop paralytic poliomyelitis (Fields, 2007). The incidence of paralytic poliomyelitis has been reduced over the last decades, especially by the systematic use of a vaccine; however, the disease is still endemic in Asia and Africa (Felipe et al., 2006). However, despite of several different *in vitro* studies, we haven't been seen any report about clinical using of natural specific compound against poliovirus.

Avicennia marina (Forssk.) Vierh, has been traditionally used for treatment of rheumatism, small pox, ulcers and other ailments (Bandaranayake, 2002). This plant grows in the southwest parts of Iran and its antiviral activity on poliovirus and herpes simplex virus type-1 has not been reported yet from any other part of the world. Some studies were done about the anti parasitic, antifungal and antibacterial activity of *A. marina In vitro* antimalarial and anticandidal activities as well as cytotoxicity of *A. marina*. (Abeysinghe et al., 2006; Khafagi et al., 2003; Premanathan et al., 1999). In another study it was concluded that the leaves of *A. marina* have no effect on behavioral changes, morbidity or mortality in rats (Ali and Bashir, 1998). The present study was performed to evaluate *in vitro* antiviral activity of a crude extract of this plant's leaf against HSV-1 and polio virus.

MATERIALS AND METHODS

Cell line and viruses

Monolayers of Vero cells (African green monkey kidney cells) were grown with Dulbeccos modified Eagle's Medium (DMEM; Gibco) supplemented with 5% fetal bovine serum (FBS; Gibco), 100 µg/ml penicillin and 100 µg/ml streptomycin. Cells (7 × 103/well) were seeded out into 24-well culture plates for cytotoxicity and antiviral assays, respectively, and incubated at 37°C in an atmosphere of 5% CO_2. Herpes simplex virus type 1 (HSV-1) strain KOS, received from the virology department of Tarbiat Modarres University and Polio virus (sabin; vaccine strain), purchased from health center of Bushehr University of Medical Science. The viruses were propagated in Vero cells and stored at -70°C until used. An aliquot of the viral stocks were tittered as TCID50/ml by using Reed and Muench method (Specter and Lancz, 1992).

Preparation of crude extracts

Leaves of *A. marina* were collected from the Naiband region beside the Persian Gulf (Southwest of Iran). They were washed, homogenized and then 40 gr of fresh leaves added to 100 mL of 10% glycerin-water solution (v/v). The mixture boiled for 20 min in 105°C. Then the extract was sterilized by using filter with 0.22 µm pore size.

Cytotoxicity assay

The cytotoxicity of the extract was achieved by culturing Vero cells for 72 h in contact with the amount of extract added to the cells . At the end of this time, viable cells were determined by trypan blue exclusion test as described elsewhere (Tolo et al., 2007). Whereby results were plotted as dose response curve and 50% cell growth inhibitory concentration (CC50) was obtained by using STATA statistical software.

Antiviral assay

The antiviral activity of extract against HSV- 1 and polio virus were measured by the CPE (Cytopathic Effect) inhibition assay test. Briefly, confluent monolayers of the cells were infected with 0.1 ml of the viruses suspension containing 10000 TCID50 and 0.1 ml of DMEM containing 2% heat-inactivated FBS. The inoculated cultures were incubated for 1 h. Then, the inoculums were removed and appropriate concentrations of the extract from minimal to maximal non-cytotoxic concentrations were added to each well based on serial dilution preparation. 0.1 ml of each virus suspension and 0.1 ml of culture medium without extract were used as virus control. For the cell control, 0.1 ml of culture medium with maximal non cytotoxic concentration of extract was added. Also for evaluation of probable antiviral effect of 10% glycerin solution, 0.1 ml of virus suspension and 0.1 ml of sterile 10%glycerin solution without extract was used. The plate was incubated at 37°C in a humidified atmosphere with 5% CO_2 for 72 h and the presentation of CPE was investigated daily. To determine the post attachment antiviral activity of the extract, the same protocol was done but the cells were pretreated with extract 2 h before viral infection. The degree of inhibition was expressed as percent yield of virus control (% virus control= CPE experimental group/ CPE virus control × 100) (Shuang-Cheng et al., 2002). The concentration reducing CPE by 50% in respect to virus control was estimated from graphic plots and was defined as 50% inhibited concentration (IC50) expressed in microgram per milliliter by using STATA modeling software. The selectivity index (SI) was calculated from the ratio CC50/IC50 (Kudi and Myint, 1999).

Statistical analysis

STATA statistical analysis software was used for the dose response curve drawing in order to IC50 and CC50 calculation (StataCorp., TX, LP; USA).

RESULTS

The cytotoxicity of the extract was determined by calculating CC50 which was 5750.96 µg/ml. The inhibitory effect of the extract on attachment stage for HSV-1 and poliovirus were measured as shown in Table 1 and 2. According to our data 10 µg/ml of the extract didn't show any antiviral effect, while 104 µg/ml of that extract lead to 100% inhibition of CPE formation due to HSV-1 replication in Vero cells. For poliovirus, 80 µg/ml

Table 1. Inhibition of HSV-1 replication by different concentration of the extract (Before virus inoculation).

Extract Concentration (µg/ml)	CPE inhibition (%)
10	0
26	15
40	30
64	50
72	55
80	60
88	65
96	70
100	90
104	100

Table 2. Inhibition of Poliovirus replication by different concentration of the extract (Before virus inoculation)

Extract concentration (µg/ml)	CPE inhibition (%)
80	0
112	30
144	50
176	70
208	90
240	100

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

Extract concentration (µg/ml)	CPE inhibition (%)
10	0
26	15
40	30
64	50
72	55
80	60
88	65
96	70
100	90
104	100

Table 4. Inhibition of Poliovirus replication by different concentration of the extract (after virus attachment to the cell)

Extract concentration (µg/ml)	CPE inhibition (%)
150	0
200	10
250	25
300	40
320	50
360	70
400	90
440	100

of the extract did not show any antiviral effect and 240 µg/ml of the extract inhibted CPE formation of poliovirus 100%. The IC50 values were determined 66 µg/ml and 145.7 µg/ml for HSV-1 and poliovirus respectively. Therefore the SI values for this stage of study were 87.1 and 39.5 for HSV-1 and poliovirus respectively.

As shown in Tables 3 and 4, we have found that 120 µg/ml and 150 µg/ml of the extract could not prevent the performing of cytopathic effect of HSV-1 and poliovirus, respectively after their attachment to the cells but 160 µg/ml and 440 µg/ml of that extract, showed 100% inhibitory effect on the viruses induced CPE. The IC50 values for the extract were determined 137.24 µg/ml and 314.3 µg/ml for HSV-1 and Poliovirus, respectively. The calculated SI values for this part of study were 41.9 and 18.3 for HSV-1 and poliovirus respectively.

DISCUSSION AND CONCLUSION

Plants are known as important sources of new chemical entities suitable for antiviral drug discovery and development (Akanitapichat et al., 2006). Many investigators have reported the inhibitory effects of algal extracts and their constituents on the replication of herpes simplex viruses (Park et al., 2005) . In previous studies, we reported that aqueous extracts of *Gracilaria salicornia* (a red alga) and *Cystoseirya myrica* (a brown alga) showed *in vitro* antiviral activity significantly (Zandi et al., 2007a, b). In other study antiherpetic activity of crude hot glycerin extract of Aloe Vera was established (Zandi et al., 2007c). Other reports also showed 70% aqueous ethanol extract of dried leaf and fruit of *A. marina* showed anti-encephalomyocarditis virus and anti-hepatitis B virus activities, respectively (Premanathan et al., 1999). In some other studies revealed that "Natural and synthetic flavonoids and interfere with picornavirus replication leading to preventing of decapsidation of viral particles and RNA release within cells or blocking viral RNA synthesis" (Conti et al., 1990; Genovese et al., 1995; Gonzalez et al., 1990; Salvati et al., 2004). Poliovirus is a nonenveloped virus and a member of picornaviridae family so investigation about the effect of the *A. marina* leaf extract on its replication cycle should be interesting. On the basis of previous studies the *A. marina* leaf extract have flavonoids and other effective compounds which shown moderate antiviral and antibacterial activity (Abeysinghe et al., 2006; Khafagi et al., 2003; Rui et al., 2004; Shraf, 2000).

Present study indicates that crude hot glycerin extract of fresh leaf *A. marina* showed antiviral activity against HSV- 1 and poliovirus *in vitro*, which could be lead to more studies for developing new antiviral drugs against these viruses.

According to our results, the extract affects both of viruses before adsorption more than after virus attachment to the cells. Furthermore, we found that this plant extract is more effective on HSV-1 comparing poliovirus. The extract may be assumed to block attachment of viruses to the cells through binding to the receptors of viruses or binding to the viruses ligands. However, it's important to consider not only the early stages of viruses replication such as adsorption and attachment of viruses to the cells could be inhibited by the extract but also the post attachment stages of replication of viruses could be affected by this extract. Regarding to the resulted IC50 for HSV-1 and Poliovirus in our study, we concluded that the used extract is more effective against HSV-1 comparing to poliovirus. This significant difference could be due to dissimilarity of viral structure between HSV-1 and poliovirus and/or differences in their replication cycles.

Elucidation of the antiviral mechanism(s) of this plant extract will be the subject of further research and should facilitate understanding of the complex interactions between viruses, compounds and cells and different susceptibility of viruses to the extract and its compounds. In another step of our research, cytotoxicity of hot glycerin extract of *A. marina* was tested. We have found that this extract showed less cytotoxicity than alcoholic one (Premanathan et al., 1999). This difference could be due to the type of solvent and/or the type of used cell line or some unknown factors. Because of a powerful antiviral activity and less cytotoxicity of the extract, identification of the most effective compounds and then quantitation of these elements are recommended for future studies. Also, further investigation on the antiviral activity of this plant on other viruses could be interesting.

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