Antiviral screening of four plant extracts against acyclovir resistant herpes simplex virus type-1

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Abstract: Herpes simplex virus type 1 (HSV-1) causes serious infections particularly in immunocompromised patients. Methanolic extract of four plants were evaluated for their anti-viral effects against acyclovir resistant HSV-1 in HeLa cell line. The 50% cytotoxic concentration (CC50) as well as the effective minimal cytotoxic concentration of each plant extract were evaluated by MTT assay. Antiviral effects of the plant extracts on HSV-1 were examined at different concentrations of the extract. The effective minimal cytotoxic concentration was evaluated at different times of virus replication after infection. Virus titration was assessed by tissue culture infectious dose 50 (TCID50) method. Among the 4 plant extracts evaluated only *Mentha pulegium* L. extract was shown to exert the highest antiviral activity, with selectivity index (SI) 10.25. Direct treatment of HSV-1 with *Mentha pulegium* L. extract resulted in 1.7 log10 TCID50 reduction in virus titers after one hour. The highest reduction in HSV-1 infectivity was obtained 1 hour after the infection of the cells with virus resulting in 2.1 log10 TCID50 reduction as compared to the control. The antiviral effects of *Mentha pulegium* L. extract on HSV-1 after virus infection was more remarkable than the virucidal activity.

Keywords: Antiviral, Plant extracts, Acyclovir resistant HSV-1.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) belongs to the family of Herpesviridae. Herpes simplex virus is extremely widespread throughout the world. It is responsible for many of the diseases such as herpes labialis, encephalitis, and infections of newborns (Kinchington *et al.*, 2012). Several antiviral drugs including acyclovir, penciclovir and valacyclovir which inhibit the virus-specific enzymes such as DNA polymerase have been used against HSV-1 infections (Burrel *et al*., 2012; Malvy *et al*., 2005). Some of these drugs might have harmful side-effects such as lethargy, confusion, hallucinations, tremors, headache, nausea, and diarrhea (Cunningham *et al*., 2012; Whitley, 2012). HSV-1 has, however, been reported to acquire resistance to some antiviral drugs such as acyclovir and penciclovir (Andrei and Snoeck, 2013; Vere Hodge and Field, 2013). The growing rate of virus resistance to these drugs is a common problem in immunocompromised hosts. It is therefore necessary to look for newer and more effective antiherpesvirus agents capable of killing this virus (Kokoska *et al.*, 2002; Visintini Jaime *et al.*, 2013; Müller *et al.*, 2007). In recent years, various reports on the effect of different plants extracts on viral infections have been published. Some extracts directly affect the host cells thereby preventing infection (Nolkemper *et al.*, 2010; Reichling *at al.*, 2008). Some of the phytochemicals compounds have antiviral effect by inhibiting the replication of viral nucleic acid or preventing the viral adsorption onto the cell (Zhang *et al.*, 2007). Due to the growing need for more effective antiviral agents, four methanolic crude extracts were screened in this study for in vitro antiviral activities against acyclovir resistant HSV-1 in HeLa cell line.

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MATERIALS AND METHODS

Compounds, cell line and virus

Human cervical HeLa cell line (Pasteur Institute, Tehran. Iran) was cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Biosera, England) supplemented with 10% fetal bovine serum (FBS; Gibco, Belgium), 100 IU/ml penicillin and 100 µg/ml streptomycin. The cells were maintained at 37ºC in 5% CO2 humidified atmosphere and were subcultured two times a week. Acyclovir was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acyclovir resistant HSV-1 was propagated on HeLa cells and tittered by 50% tissue culture infective dose (TCID50) method, and stored in small aliquots at -70ºC until use (Betancur-Galvis *et al*., 2002).

Preparation of the methanolic extract

The methanolic extracts of four plant leaves (*Mentha pulegium* L., *Salvia officinalis* L., *Hypericum perforatum*

L. and *Chelidonium majus* L.) were collected and prepared by the Research Institute of Forest and Rangelands, Tehran, Iran. Harvested plants were dried and grinded to fine powder, then 10 g of powdered dried leaves was added to 100 ml of methanol using a flask; the admixture was left at 25ºC for 72 h in order to macerate. The extract was then filtered using filter paper, and concentrated using a rotary evaporator at 40°C. The concentrates acquired were stored at -70°C until use for the tests.

Cellular cytotoxicity test

The cytotoxicity effect of plant extract was determined in HeLa cells by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) test. Briefly, cells were cultured in 96-well plates at a number of 2×10^4 per well in 200µl of medium in triplicates. After culturing, the cells were grew for 24 h and then treated with different plant extract concentrations. After 72 h of incubation, the medium was removed and 20µl of MTT (5 mg/ml in PBS) was added per well, and incubated for 4 h at 37ºC. The insoluble formazan crystals were dissolved by adding 100 µl of dimethylsulfoxide (DMSO; Sigma-Aldrich Co.) to each well. After 10 min incubation, absorbance was recorded at OD570 using Microplate Reader (Stat Fax 3200 Micro plate Reader, USA). Cytotoxicity percentage was calculated as follows:

$$\text{Cytotoxicity (\%)} = \frac{(a-b)}{(c-b)} \times 100$$

Where: a is mean OD570 value from a well added with medium containing various concentrations of extract, b is mean OD570 value derived from Blank wells (wells which contain only 100 µl DMSO), c is mean OD570 value derived from control wells added with culture medium without extract (Shafiee *et al.*, 2012). The 50% cytotoxic concentration (CC50) and the effective minimal cytotoxic concentration was assessed from dose-response curve.

Virucidal assay

The 100 TCID50/ml of HSV-1 were incubated at 25°C for 1 h with effective minimal cytotoxic concentration of plant extracts and with DMEM medium without plant extract for control. After incubation, HeLa cells monolayer, grown in 24-well plates, were infected with 200 µl of each viral suspensions. For virus adsorption, infected cells were incubated at 37°C for one hour. After that, infected cells were washed and medium without plant extract was added to them, and incubated at 37°C for 48 hours**.** Then, supernatants of each infected cell were collected and virus titers were assessed by the end point dilution method and expressed as TCID50/ml by comparing with the control (Koch *et al.*, 2008).

Screening for antiviral effect of plant extracts

HeLa cells, cultivated in 24 wells culture plates, was infected with 100 TCID50/ml of HSV-1 and incubated at 37°C for 1 hour. After incubation, the residual medium on the infected cells was removed and different concentrations which were lower than effective minimal cytotoxic concentration of plant extracts were added to them and incubated at 37°C for 48 hours. Medium without plant extract was added to cells in another well for control. After that, the supernatants of each well were collected and HSV-1 titers were assessed by TCID50 assay. The 50% inhibitory concentration (IC50) of each plant extract was calculated as the concentration that inhibited 50% of tissue culture infective dose when compared to controls. The selectivity index (SI) was calculated by the ratio of CC50 to IC50 for each assayed plant extract (Civitelli *et al.*, 2014).

Antiviral assay

Evaluation of the plant extract was performed in three distinct stages, as described below:

a) Effects of the plant extract on pretreated cells

Cell monolayers, cultivated in 24-well plate were treated with the effective minimal cytotoxic concentration of plant extract for 5 and 2 h in individual wells. Medium without plant extract was added to control cells. After incubation, the supernatant was removed and the cells were washed with PBS. After that, the cell monolayers infected with 100 TCID50/ml of HSV-1 and incubated at 37°C for 1 hour. After virus adsorption, medium with 2% FBS was added to the infected cells in individual wells; then incubated at 37°C for 48 hours. After that, the supernatants of each well were collected and titers of the virus were determined.

b) Effects of the plant extract on HSV during viral adsorption

The confluent cells, grown in 24-well plate, were infected with 100 TCID50/ml of HSV-1 in medium with plant extract at effective minimal cytotoxic concentration and incubated at 37°C for 1 hour. After virus adsorption, supernatant was removed and the cells were washed with PBS, and the medium with 2% FBS was added to the wells; the cells were then incubated at 37°C for 48 hours. After incubation, the supernatants of wells were collected and titers of the virus were determined.

c) Effects of plant extract on HSV-1 replication at different times after viral adsorption

The cultivated cells in 24-well plate were infected with 100 TCID50/ml of HSV and incubated at 37°C for one hour. Immediately after virus adsorption, the supernatant was removed and the infected cells were washed with PBS. After that, medium with plant extract at effective minimal cytotoxic concentration was added to the infected cells and incubated at 37°C for 48 hour. In other wells, infected cells were washed after adsorption and medium was added to them. The medium was then removed 1, 2, 4, 8, 12 and 24 h after viral adsorption and

the medium with plant extract at effective minimal cytotoxic concentration was added into the wells; the cells were maintained at 37°C for 48 hour. The supernatants of the infected cells in each well were collected and the virus titers were assessed (Civitelli *et al.*, 2014; Hung *et al.*, 2015).

STATISTICAL ANALYSIS

The CC50 as well as the effective minimal cytotoxic concentration and IC50 of each plant extract were assessed from dose-response curve (Prism, Graph Pad). Data were compared using Student's t-test. The level of statistical significance was set at a P-values < 0.05.

RESULTS

Cellular cytotoxicity

The effective minimal cytotoxic concentration of plant extracts were assessed at 900 µg/ml for *Mentha pulegium* L., at 2400 µg/ml for *Salvia officinalis* L., at 370 µg/ml for *Hypericum perforatum* L. and at 1600 µg/ml for *Chelidonium majus* L. and the CC50 values of the plant extracts on HeLa cell line were obtained by MTT assay (table 1).

Direct effects of the plant extract on HSV-1

Among the 4 plant extracts tested, only *Mentha pulegium* L. extract presented virucidal activity. The virus titers decreased after 1 h treatment with the *Mentha pulegium* L. extract at effective minimal cytotoxic concentration compared to controls, so that the virus titer significantly decreased from $10^{6.3}$ TCID50/ml in control to $10^{4.6}$ TCID50/ml in treated viruses ($p<0.05$). Virucidal activity values obtained for each extract and acyclovir are showed in fig. 1.

Antiviral effect of plant extracts

The dose-response activity of the four plant extracts against HSV-1 determined by TCID50 method. The IC50 for acyclovir resistant HSV-1 were revealed in a broad range between 700 µg/ml for *Salvia officinalis* L. extract and 100 µg/ml for *Mentha pulegium L.* extract. The selectivity indices for the examined plant extracts against HSV-1 are shown in table 1. *Mentha pulegium* L. extract was found to have the highest SI value against acyclovir resistant HSV-1, hence it was chosen for the subsequent antiviral assay.

Antiviral activity of Mentha pulegium L. *extract on viral replication*

The cells that were treated with the *Mentha pulegium* L. extract prior and during viral infection did not show significant changes in virus titer compared to the control. Treatment of the infected cells with the *Mentha pulegium* L. extract immediately after virus adsorption and also 1, 2, and 4 h after the infection resulted in a higher antiviral activity compared to other treatment times and also the control (fig. 2). Treatment of infected cells with *Mentha pulegium* L. extract after 1 h of virus infection exerted the highest inhibitory effect on virus replication, so that the virus titer significantly decreased from $10^{6.3}$ TCID50/ml of control to $10^{4.2}$ TCID50/ml in the infected cells at this time ($p<0.05$).

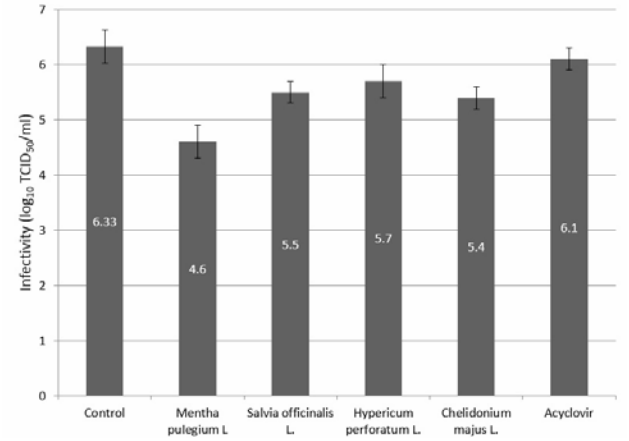

Fig. 1: Direct effect of acyclovir and plant extracts at effective minimal cytotoxic concentration on acyclovir resistant HSV-1 at 1 hour. Average infectivities of three independent experiments are shown in the graph. Error bars indicate standard deviations. TCID50, Tissue culture infectious dose 50.

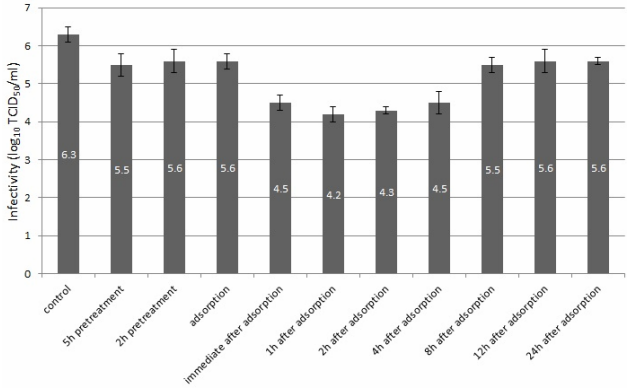

Fig. 2: Inhibitory effects of methanolic extract of *Mentha pulegium L.* at effective minimal cytotoxic concentration on pretreated cells, during and after the viral infection at different times. Average infectivities of three independent experiments are shown in the graph. Error bars indicate standard deviations. TCID50, Tissue culture infectious dose 50.

DISCUSSION

HSV-1 can cause critical infections such as encephalitis and disseminated infections in neonates. Immunocompromised hosts are at high risk of severe HSV infections (Rozenberg *et al.*, 2011; Meyding-Lamadé and Strank, 2012). Several drugs such as acyclovir, vidarabine and penciclovir are currently

Table 1: Selectivity indices of acyclovir and plant extracts against acyclovir resistant HSV-1(a).

Extract	Effective Min. cytotoxic conc. (µg/ml) ± SD	CC50 (µg/ml)± SD	IC50 (µg/ml) ± SD	SI
Mentha pulegium L.	900 ± 32.1	4100 ± 96.09	400 ± 26.09	10.25
Salvia officinalis L.	2400 ± 76.3	2900 ± 70.94	2000 ± 110	1.45
Hypericum perforatum L.	370 ± 12.58	750 ± 29.09	300 ± 18.02	2.5
Chelidonium majus L.	1600 ± 80.2	3100 ± 90.73	600 ±36.85	5.16
Acyclovir	>10 ± 2.4	>100 ± 12.5	NA	NA

(a) Average concentrations of three independent experiments are shown in the table. NA: no activity

available for treatment of HSV-1 infections. Acyclovir resistant HSV can also cause persistent and disseminated diseases in immunocompromised patients (Piret and Boivin, 2011).

There are various reports on the antiviral activity of plant extracts that can result in the development of novel drugs. Some ethanolic plant extracts which are most effective against free HSV particles affect the adsorption of the virus to host cells and some extracts directly affect the virus infected cells thereby preventing the infection (Nolkemper *et al.*, 2010; Reichling *at al.*, 2008). Reichling and colleagues (2008) have determined the effects of *P. sidoides* extract on HSV-1 and HSV-2 before infection, during adsorption and after penetration into the host cells. They reported that treatment of these viruses with *P. sidoides* extract before and during the adsorption stage did reduce the plaques and they suggested that *P. sidoides* extract inhibits virus adsorption or penetration due to covering of the viral proteins that are necessary for attachment or entry into the host cells (Schnitzler *et al.*, 2008).

Amoros and colleagues evaluated the virucidal activity of propolis against HSV-1 and showed that pretreatment of virus with propolis had virucidal effect but treatment of host cells with propolis did not interfere with virus adsorption (Amoros *et al.*, 1992). These data are in accordance with our findings related to *Mentha pulegium* L. extract which showed that virus titer decreased after one hour of treatment of HSV-1 with ethanolic *Mentha pulegium* L. extract, compared to controls, but pre-treatment of HeLa cells with *Mentha pulegium* L. extract did not prevent virus adsorption or viral replication.

Some plant extracts exhibited significant antiviral effect during the intracellular replication steps.

Several studies have been undertaken for evaluation of antiviral action of plant extracts at different phases in the HSV-1 replication cycle (Yoosook *et al.*, 2000). Our results also show that treating the cells with the *Mentha pulegium* L. *extract* 1 to 4 hours after virus infection can significantly reduce virus titer compared to other post-infection time schedule and the control. Since, the alpha or immediate early genes of HSV are expressed 2 to 4 hours after the infection it can be hypothesized that, the inhibitory effect of the *Mentha pulegium* L. extract 1 to 4 h after the infection could have prevented the synthesis of alpha or immediate early proteins or could have repressed the function of these proteins after their expression in the cell.

CONCLUSIONS

It was observed in this study that in vitro pretreatment of the virus with *Mentha pulegium* L. extract, was found to have a virucidal effect, and also the treatment of the acyclovir resistant HSV-1 infected cells with the *Mentha pulegium* L. extract interfered with the viral replication. However, more investigations are needed to better understand the mechanism of *Mentha pulegium* L. extract effect on acyclovir resistant HSV-1. More research should also be conducted on the constituents of this extract to identify the major antiviral component in the extract.

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