Original Article

Antiviral Effects of *Aloe vera* (L.) Burm.f. and *Ruta Graveolens* L. Extract on Acyclovir-Resistant Herpes Simplex Virus Type 1

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Abstract

Herpes simplex virus type I (HSV-1) is a causative agent in a wide range of human diseases. With increasing drug resistance to anti-viral drugs, numerous studies are under way, particularly on medicinal plants. In this study, therefore, the effects of *Aloe vera* (L.) Burm. f. and *Ruta graveolens* L. (*R. graveolens*) extracts were evaluated on acyclovir-resistant HSV-1. The toxicity of extracts from *A. Vera* and *R. graveolens*, and also acyclovir was evaluated on HeLa cells by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay to obtain the highest non-toxic concentrations of the extracts and acyclovir on the cells. The effects of extracts and acyclovir HSV-1 were examined at different concentrations during a range of times. The virus titers were measured at different stages of the study using the TCID50 method. Minimum cytotoxic concentrations (MCC) of 12000 µg/ml and 125 µg/ml were determined for *A. vera* and *R. graveolens* extracts respectively. *R. graveolens* extract with a SI (Stimulation Index) of 15.33 had a higher antiviral effect than *A. vera*. extract with 3 log $TCID_{50}$ in 1 h and *R. graveolens* extract with 1.9 log TCID50 in 2 h after cell infection reduced the virus titer compared to the control. None of the tested concentrations of acyclovir had inhibitory effects on the virus replication. The extract of both plants had antiviral effects, but the extract of *R. graveolens* showed a higher antiviral impact on acyclovir-resistant HSV-1 compared to *A. vera* extract.

Keywords: Herpes simplex virus type 1, *Aloe vera*, *Ruta graveolens*, Antiviral effects

Introduction

The use of medicinal herbs in traditional medicine has a long history in many countries, including Iran. Many medicinal plants have antiviral properties, the extracts of which are used to treat various infectious diseases [1-3]. HSV-1 causes a wide range of mild and uncomplicated diseases, as well as life-threatening conditions such as encephalitis. The virus can cause eye infection followed by blindness in people with immunodeficiency. It also causes encephalitis, meningitis, and death through an attack on the central nervous system and the brain [4,5].

Several HSV-1 enzymes, such as polymerase and thymidine kinase, can be used as antiviral drug targets. Vidarabine, idoxuridine, and acyclovir are used for the treatment of HSV infections. The mechanism of action for these drugs is to inhibit the activity of viral enzymes, including DNA polymerase. Although acyclovir is still considered to be effective, its side effects, including its contraindication in breastfeeding period and the drug resistance, have limited the use of this drug [6,7] This, therefore, has further necessitated for access to new drugs (e.g., herbal medicines) that are effective with lower complications. It may be an effective step in achieving successful treatments of drug-resistant viral infections [8, 9].

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Aleo vera (Fig. 1) belongs to the Liliaceae family and contains a variety of minerals, vitamins, amino acids, anthraquinone, aloin, and emodin (with antiviral properties). The antiviral effects of *A. vera* on hepatitis, human immunodeficiency virus (HIV), HSV-2, and influenza virus have been shown in various research work [10-15].

R. graveolens - (Fig. 2) is a species of Rutaceae family that has many properties such as anticancer, anti-inflammatory, antihypertensive, antimicrobial, antifungal, and antiparasitic properties. A variety of glycoside, coumarins, lignins, quinolone alkaloids, and flavonoids have been extracted from this plant with potential anti-inflammatory, antioxidant, antibacterial, and antifungal activities [16-18]. In this study, the effects of the above extracts and acyclovir at different concentrations were comparatively investigated on the virus directly and also on different stages of HSV-1 replication inside the cell.

Fig. 1 Aloe vera

Fig. 2 *Ruta graveolens L.*

Material and Methods

Cells and HSV-1

The HeLa cells used in this study were purchased from the Pasteur Institute of Iran. Cells were proliferated using Dulbecco's Modified Eagle Medium (DMEM, Biosera, England) and Fetal Bovine Serum 10% (FBS Gibco, England). Penicillin (100 IU/ml) (penicillin, Biosera, England) and streptomycin (100 µg/ml) (streptomycin,

Biosera, England) were added to prevent bacterial contamination, and the media were then incubated at 37 °C with 5% CO2.

Acyclovir-resistant HSV-1 was obtained from the Department of Virology, Tarbiat Modarres University. The virus was titrated by 50% tissue culture infective dose (TCID50) method.

Preparation of Extracts and Medications

The plant species were collected from National Botanical Garden of Iran and verified by the experts by the Research Institute of Forest and Rangelands, Tehran, Iran with herbarium number MP.S. R 086 for *R. graveolens* and MP.S. A 098 for *A. vera* leaves were then cut, the contained gel was collected, and methanol (100 ml) was added to the collected gel (12.44 g). The extract was dried after complete gel dissolution in methanol. The dried extract (0.2 g) was dissolved in 10 ml of DMEM medium and passed through a 0.20 µm filter to be used for the next steps. The leaves of *R. graveolens* were dried, and ground and 100 ml of mixed water-methanol (80% alcohol + 20% water) were added to the powdered leaves (30 g). After 24 h, the extract was passed through a paper filter and adjusted to a volume of 100 ml with the same solvent. Next, 20 ml of the extract was dried at 37 °C, and 0.2 g of the dried extract was dissolved in 10 ml of DMEM medium, filtered by a 0.20 µm filter, and used for the next steps. Acyclovir powder was purchased from Sigma-Aldrich Inc., and 0.01 g of the powder was dissolved in 10 ml of DMEM medium and used for the next steps.

Cytotoxicity Method

The toxicity thresholds of *A. vera* and *R. graveolens* extracts were determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, diphenyltetrazolium bromide) assay. The 15,000 cells per 200 µl of DMEM medium containing 10% of the serum were cultured in 96-well plates. After 24 h of incubation and formation of cell monolayers in the wells, different plant extract concentrations, and acyclovir with a 2% serum medium were prepared and added individually to three wells. The same was applied for the control with an extract-free medium. After 72 h, the supernatants of the wells were removed and washed with phosphate buffered saline (PBS). Afterward, 20 µl of MTT (Merck) solution (0.005 g/ml PBS) and 80 µl of DMEM medium were added to each well. After 4 h of incubation at 37 °C and formation of formazan crystals, each well received 100 µl of DMSO (Merck) to dissolve the crystals. Three wells were also considered as blanks filled with 100 µl of serum-free medium and 100 µl of DMSO. The optical density (OD) of each well was measured at 570 nm by a Microplate Reader (Stat Fax 3200, USA). The cytotoxicity

percentage of the extracts was calculated by the following formula:

Cytotoxicity (%) = 1- (a-b)/(c-b) × 100, where:

 a = Average OD of test wells

 b = Average OD of blank wells

 c = Average OD of control wells

A concentration of the herbal extract leading to the survival of 50% of the cells was determined as 50% cytotoxic concentration (CC50). A cytotoxic concentration of the herbal extract that resulted in 90% survival of the cells was considered as effective minimal cytotoxic concentration (MCC). The above parameters were determined using a dose-response curve.

Direct Effects of Herbal Extracts and Acyclovir on HSV-1

An amount of 100TCID50/ml was prepared from the virus at an effective MCC of extracts. Since acyclovir has no direct antiviral effect, it was used as a control in comparison with plant extracts, therefore, the same was applied separately to the extract-free control and the acyclovir-containing medium. After 1, 2, 3, and 4 h, 200 µl of the above suspension was added separately to the wells of a 24-well plate containing monolayer cells for adsorption. After 1 h of virus absorption, the supernatants of the infected cells were removed followed by adding 1 ml of 1%-serum medium to the cells and incubating at 37 °C with 5% CO2 for 48 h. After incubation, the supernatant of each well was collected separately and virus titers were assessed by the TCID50 method.

Comparing the Antiviral Effects of the Extracts and Acyclovir

In order to determine the effects of different extract concentrations on intracellular virus replication, confluent cells were first prepared in 24-well microplate. Thereafter, the cells were infected with 100 TCID50 of virus, and after 1 h of absorption, concentrations below effective MCCs were prepared separately from the extracts in 1 ml of DMEM containing 2% FBS and then added to the wells. One well received a medium containing acyclovir (30 µg/ml), and one well was supplied with an extract-free medium as a control. After 48 h, the supernatant fluids of the wells were collected, and virus titer was determined separately using the TCID50 method.

A concentration of each extract that resulted in 50% inhibition of virus replication compared to the control was considered as 50% inhibitory concentration (IC50) of the extracts. The selectivity index (SI) was calculated by dividing CC50 by IC50 of each herbal extract.

Effects of Herbal Extracts on Virus Replication

The length of the replication cycle of HSV-1 is about 18 to 24 hour and expression of the viral genome is performed sequentially in a cascade fashion in several stages at the specific times. Therefore, to determine the time of antiviral effect of the extract on the virus replication cycle were examined in three stages as follows:

1. Evaluation of the cells treated with plant extracts before virus adsorption

The cells cultured in two wells of 24-well plates were treated with a medium containing an MCC level of the extracts at 2 and 5 h before being infected with the virus. Following the above times, the supernatant was removed from each well. After washing the cells with PBS, 100 TCID50/ml virus was added to the cells. After 1 h of virus absorption, the cells were washed with PBS and were added 1-ml medium containing 2% FBS to each well. Following 48 h of incubation at 37 °C with 5% CO2, the supernatants of the cells were collected, and their virus levels were determined by TCID50 method.

2. Effects of extracts on the virus during virus adsorption

One well of a 24-well plate containing a cellular monolayer was incubated with 100 TCID50/ml of virus in a medium containing effective MCCs of the extracts at 37 °C for 1 h. The cells were washed subsequently, and a medium containing 2% FBS was added and incubated at 37 °C with 5% CO2 for 48 h. After incubation, the supernatants of the cells were collected, and the virus titer was determined by TCID50 method.

3. Effects of extracts on viral replication at different times after virus adsorption

Eight wells of the 24-well plate containing monolayer cells were washed and infected with 100 TCID50/ml of the virus and left 1 h for virus adsorption at 37 °C. The cells of one well were washed immediately after adsorption, and 1 ml of the extract-containing medium at effective MCC with 2% FBS was added to well. The other wells were washed after virus adsorption and received 1 ml of the culture medium. The supernatants of these wells were removed after 1, 2, 4, 8, 12, and 24 h of after adsorption and the extract-containing medium at effective MCC with 2% FBS was added to each well. An extract-free medium with 2% FBS was added to another well as a control. The 24- microplate was incubated at 37 °C with 5% CO2 for 48 h. The supernatants of the wells were collected after incubation, and their virus titers were measured separately by the TCID50 method.

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
Aleo vera	12000 ± 61	20000 ± 94	10000 ± 55	2.0
Ruta graveolens.L	125 ± 75	1150 ± 28	75 ± 3	15.33
Acyclovir	>10 ± 2.4	>100 ± 12.5	No activity	No activity

(a) Average concentrations of three independent experiments are shown in the table.

Statistical Analysis
The values of CC50, effective MCC, and IC50 were determined for the extracts using a dose-response curve (Prism, Graph Pad software). All three tests were repeated three times, and the results were analyzed by SPSS software at a significance level of P<0.05.

Results

Determining the Toxicity Thresholds of the Extracts on Cells
The effective MCC values were determined 12000 µg/ml for *A. vera* and 125 µg/ml for *R. graveolens* extracts. Concentrations of 20000 µg/ml for *A. vera* extract and 1150 µg/ml for *R. graveolens* extract were determined as CC50 of the extracts on HeLa cells by MTT method (Table 1).

Direct Effects of the Extracts and the Drug on the Virus
None of the plant extracts and acyclovir had direct virucidal effects on HSV-1 compared to the control. Virucidal activity values obtained for *A. vera* and *R. graveolens L.* extracts and acyclovir are showed in fig. 3.
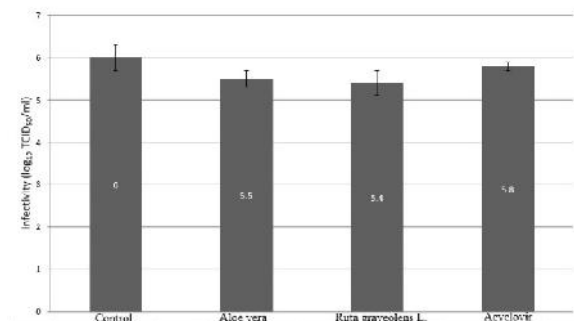
Fig. 3 Direct effects of *Aleo vera* (L.) Burm.f. and *Ruta graveolens* L. extracts at effective MCC on acyclovir-resistant HSV-1 at 1 hour. Acyclovir and both extracts had no significant changes in virus level compared to the control (P > 0.05). Error bars indicate standard deviations.

Effects of Herbal Extracts on Virus Replication
IC50 values of 10000 µg/ml and 75 µg/ml were obtained for *A. vera*, and *R. graveolens* extracts, respectively, against acyclovir-resistant HSV-1. As indicated in Table 1, SI values for *R. graveolens* and *A. vera* extracts are

15.33 and 2.0 respectively. The extract from *R. graveolens* showed a significantly higher antiviral effect than *A. vera* (P = 0.01).
The cells that were treated with both extracts prior and during virus adsorption did not show significant changes in virus level compared to the control.
A. vera extract at a concentration of 12000 µg/ml showed the highest antiviral effect at 1 and 2 h after adsorption compared to the control and to the other concentrations. In these hours, 3 logTCID50 and 2.4 logTCID50 led to a decrease in viral titers in comparison to the control. The extract of *R. graveolens* at a concentration of 125 µg/ml had the highest effect on the virus replication after 2 h of HSV-1 adsorption; at this time, it reduced 1.9 log TCID50 of the virus titer compared to the control (Fig. 4).
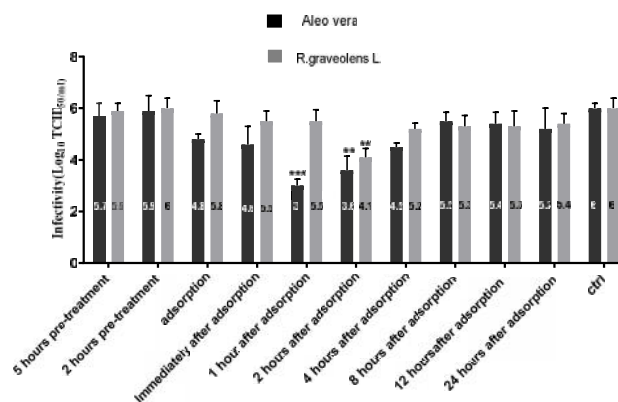
Fig. 4 Effects of *Aleo vera* (L.) Burm.f. and *Ruta graveolens* L. extracts on the virus replication at different times before and after adsorption with HSV-1. The extract of *A. vera* had a significant inhibitory effect (P= 0.003) *** on the virus replication 1 h after absorption. This extract concentration also had a significant inhibitory effect (P= 0.004) ** on virus replication after 2 h of virus adsorption. The extract of *R. graveolens* showed the highest inhibitory effect on virus replication (P= 0.005) ** compared to the control at 2 h after virus adsorption. Both extracts had no significant effects on virus replication at the other times (P > 0.05). Error bars represent standard deviations.

Discussion and Conclusion

The long history of successful medicinal plant usage due to their low side effects in the treatment of some viral

diseases has led to increasing research concerning the effects of medicinal plants on viruses to achieve alternative drugs [12].

Alipour *et al* [19], examined the effects of *Lilium* extract (from the same family as *A. vera*) on HeLa cells and showed that a concentration of 1250 ug/ml had a significant cytotoxic effect on HeLa cells. Our findings also revealed that *A. vera* extract at a concentration of 20000 µg/ml had a cytotoxic effect on 50% of HeLa cells after 72 h. Differences in the cytotoxic concentrations of *Lilium* extract on HeLa cells noted in the above study and that of ours can be attributed to differences in the type of materials used for the extraction. They used the leaves while the gel of the leaves were used for extraction in the present study, each of which has different compositions.

The effect of *Nepeta pungens* (Bunge) Benth. hydroalcoholic extract was investigated on the measles virus, and no virucidal effect was detected on this virus. In other words, the extract of *N. pungenus* had no virucidal effect in extracellular conditions [20]. Likewise, the direct effects of *A. vera*, *R. graveolens*, and acyclovir concentrations (virucidal effect) were investigated on HSV-1 in extracellular media in part of this study, and the results showed no significant reductions in the virus titer [21].

The effects of *A. vera* extract at different concentrations were investigated on HSV-1 in part of this study. The results showed a concentration of 12000 g/ml as an effective MCC of *A. vera* extract, with an inhibitory effect on the virus replication. Sydiskis *et al* studied the effect of *A. vera* extract on HSV-2 in Vero cell line and found that the extract at a concentration of 15500 µg/ml reduced the amount of HSV-2 by 90% [21]. The difference between the above and our studies can be attributed to a higher resistance of Vero cells than Hela cells against the cytotoxic effects of *A. vera* extract.

The antiviral effects of *Dracaena* extract from the Liliaceae family was examined on HSV-1 and HSV-2 in Vero cell line [22]. The results showed inhibitory effects of *Dracaena* extract on both HSV-1 and 2 at the time of viral adsorption and, to a weaker level, after viral adsorption.

Yarmolinshy *et al* [23], also evaluated the antiviral properties of *Lilium* (Liliaceae family) and found the highest inhibitory effect of *Lilium* extract on HSV replication in Vero cell line immediately after viral infection of the cells. The extract also inhibited the virus replication within hours after viral adsorption. Also, Hayashi *et al* [24], investigated the antiviral properties of Yuuca (Liliaceae family) on HSV-1 and 2 in HeLa cell line and showed that Yuuca extract could inhibit viral replication at 0 and 3 h after virus adsorption through inhibition of protein synthesis in the virus. Zandi *et al* [25], investigated the antiviral effect of *A. vera* on HSV-2

in Vero cell line and showed that the extract exhibited an inhibitory effect at the attachment, entry, and replication stages of the virus. Their results are consistent with part of our results on Hela cells, in that *A. vera* extract with 12000 g/ml had a significant inhibitory effect on HSV-1 at 1 and 2 h after virus adsorption.

Wei Wang *et al*[26], studied the antioxidant and antimicrobial activity of *Phellodendron amurense* Rupr (Rutaceae family) and indicated that acridone content of the plant had a strong activity at low concentrations against HSV-1 and HSV-2 leading to a reduction in plaque measurement. The present study determined that the hydroalcoholic extract of *R. graveolens* could decrease the virus 1.9log TCID50 titer at 2 h after virus adsorption into HeLa cells compared to the control.

Alpha-phase genes are expressed based on the time of HSV replication cycle [5], Since the alpha-phase genes in HSV are expressed at the first 4 h of virus replication, it seems that the extracts of *A. vera* and *R. graveolens* exert their antiviral effects through interference with the expression of alpha genes, or via the function of proteins present in this phase. Yet, a more comprehensive examination is needed to determine the relationship between the extract effects on the alpha-phase proteins.

Our observations demonstrate that both *A. vera* and *R. graveolens* extracts have antiviral effects against acyclovir-resistant HSV-1. Nevertheless, a higher SI for the extract of *R. graveolens*, compared to that of *A. vera*, suggests that the former has a greater antiviral effect than the latter. There is a high difference between the CC50 value and the effective MCC in *R. graveolens* extract. Moreover, the effective MCC of this extract could decrease the virus 1.9log TCID50 titer after 2 h of adsorption. Accordingly, it can be concluded that the extract of *R. graveolens* contains important anti-viral compounds, and effective antiviral compounds with more purity can be achieved to examine their effects on viral replication by using other extraction methods along with further examination of the extracts. The results of the above study showed the antiviral effect of the studied extracts, but due to the limited time of the project and also the lack of financial resources, was not possible to accurately investigate the cause of interference of these extracts in different stages of virus replication, including mRNA synthesis or protein expression.

Conflict of Interests

There were no conflicts of interest associated with this study.

Acknowledgment

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