Antiviral activity of Veronica persica Poir. on herpes virus infection

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Abstract: The lack of an effective anti-viral agent and the emergence of drug-resistant strains dictate a real need for discovery of novel therapies able to ameliorate viral infections. In this regards, medicinal plants and natural products offer safe and inexpensive platforms for discovery of efficient and novel anti-viral agents. We have investigated the potential anti-viral activities of Veronica persica Poir. as a medicinal plant against herpes simplex viruses (HSVs). In vitro screening of the ethanol plant extract against HSV-1 and HSV-2 infected Vero cells revealed the extract to show a dose-dependent inhibitory activity against both virus strains. After fractionation of the extract by a stepwise methanol gradient and evaluation of each fraction, the 80% methanol fraction displayed a pronounced inhibitory activity against the herpes viruses. The highest antiviral activity was observed when the Vero cells were treated with the extract both during and after infection by viruses. Moreover, the extract showed a prominent synergistic activity in combination with acyclovir anti-HSV therapy. Our findings revealed the potential of V. persica extract, especially its 80% methanol fraction, in inhibition of herpes simplex viral infections.

Key words: Medicinal plants; Bioactive phytochemicals; Herpes simplex virus; Natural products; Traditional herbal remedies; Botanicals.

Introduction

Herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) are responsible for cold sores, encephalitis, keratitis and genital herpes. The infections caused by these viruses (herpetic infections) are considered very common among adults and are dangerous especially in immunosuppressed patients (1-3). It is estimated that about 65-90% of people all around the world are infected by one of the herpes viruses (4). The approved drugs for treatment of HSV infections include acyclovir (ACV), penciclovir, valaciclovir, famciclovir, and ganciclovir (5). However, the prolonged applications of such anti-viral therapies have resulted in the emergence of the drug-resistant herpes virus strains (6, 7). Therefore, because of the appearance of drug-resistant strains as well as the absence of an efficient preventive vaccine, there exists a real need for the discovery of novel and efficient agents for treating these viral infections.

Medicinal plants with a safe and cost-effective profile, offer a promising option for the treatment of various diseases in humans (8-28). So far, many investigations have been carried out on screening of medicinal plant extracts intended for discovery of new therapeutic agents against viral infections (29-38). Such medicinal plants can offer alternative treatments with different mechanisms of action for managing HSV infections.

In this context, Veronica species are considered to be well-known traditional medicinal plants with a huge therapeutic potential. Until now, the anti-tumor, anti-rheumatic and wound healing activity of these species have been confirmed by different studies (39-42). Veronica persica Poir. or bird’s-eye speedwell is a widespread Veronica species in eastern Asia and Eurasia (43, 44). On account of different medical properties of Veronica species, investigation of V. persica extract as a lead for the discovery of a novel, natural and effective anti-viral agent could be of great interest. In this work, we have examined the potential anti-viral activity of V. persica extracts on HSV viruses. To our knowledge, this is the first report on the antiviral activity of this plant species.

Materials and Methods

Plant material and preparation of extract and fractions

Aerial parts (stems, leaves, and flowers) of Veronica persica Poir. were collected during the flowering stage in April 2016 from wild plants in the mountains of Meymand, Firuzabad County, Farz Province, Iran. The fresh parts of V. persica were used for the preparation of the ethanolic extract. The plant material (4000 g) was chopped and triturated with 85% ethanol solution, incubated at 4°C with shaking for 48 h, and then centrifuged at 2000 rpm for 10 min. The supernatant was collected into a clean flask and the pellet was discarded. The solvent was removed from the collected supernatant by evaporation. For preparation of a stock solution with a
concentration of 10 mg/mL, the extract was dissolved in 85% ethanol. The extract was diluted to the appropriate concentrations with medium containing 2% fetal calf serum. The ethanol extract was fractionated into different fractions by reverse phase chromatography (RP-C18 Sepack) with a stepwise methanol (MeOH) gradient: 0, 20, 40, 60, 80, and 100%.

**Cell culture and herpes simplex virus type 1 and 2 (HSV-1 and HSV-2)**

Vero cell line CCL-81-ATCC (African green monkey kidney cells) was grown in monolayer culture in Dulbecco’s Modified Eagle Medium (DMEM) supplement with 5% (v/v) fetal calf serum and 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco), 2 mM l-glutamine (Gibco) and 1 mM sodium pyruvate (Gibco). Then, the cells were incubated at 37 °C in humidified air containing 5% carbon dioxide (CO₂). HSV-1 and HSV-2 were prepared with cooperation of the Institute Pasteur of Iran. Viruses were routinely grown on Vero cell and virus stock cultures were prepared from supernatants of infected cells and stored at -80 °C for further tests.

**Cytotoxicity assay**

The Vero cells were treated with different concentrations of the extract and the toxicity of the extract was assessed by morphological changes observed by optical inverted microscope daily in addition to the MTT assay, which was carried out as previously described (45). In brief, the cells were incubated with 50 µg/mL MTT solution at 37 °C for 5h. MTT is converted by mitochondrial succinate dehydrogenase enzyme into insoluble formazan. The cultures were washed with saline and then sodium dodecyl sulfate solution was added to dissolve the formazan. The absorbance was measured at 570 nm in a spectrophotometer (Jenway 6405 UV models) after 24h incubation at 37 °C, to determine the metabolic activity of the cells. The median cytotoxic concentrations of the samples (CC₅₀) were determined from dose-response curves.

**Viral infection assay**

The cells were seeded at 2 × 10⁴ cells/well in 96-well culture plates, in DMEM with 10% NBCS and antibiotics. After 24 h incubation, the medium was removed and cells were infected with 1 PFU (plaque forming unit)/cell for 2 hours at 37 °C. The infection development was appraised by plaque assay as previously described (45). In brief, the unabsorbed virus was removed after 2 h infection and cells were coated with a layer of 0.6% carboxymethylcellulose (CMC). After 48 h post-infection (p.i.), the CMC overlay was removed and cell monolayers were mixed with 10% formalin in saline. The cultures were stained with 1% crystal violet (Merck, Germany) and plaques were counted. The median inhibitory concentrations of test samples (IC₅₀) were determined from dose-response curves.

**Mechanism of antiviral activity**

The infected cells were treated with the most active MeOH fraction (80%-MeOH) at various times before, during or after infection as shown in Table 1.

To examine the direct effect of the tested extract on virus particle infectivity, 10⁴ PFU of the suitable virus particles were pre-incubated with 10 µg/mL of the extract at 25 °C for 35 min. Then these mixtures were diluted 10⁴ fold with fresh medium and cell monolayers were infected with the diluted mixture. In order to determine the effect of the extract on intracellular HSV-1 replication, cells were infected for 2 h by HSV-1 without extract treatment. Then, the medium was removed and replaced with fresh medium with or without the extract and the infected cells were removed from the wells by treatment with trypsin at 24 h p.i. The obtained cells were pelleted by centrifugation at 1500 rpm for 15 min, washed 3 times with saline solution. Each pellet was resuspended with 100 µL of physiological saline solution and the cells were broken by freezing and thawing them. The cell debris was removed by centrifugation at 1500 rpm for 10 min and the mixture containing the endogenous virus was next used for infecting cell monolayers (47).

**Synergistic effect of plant fraction and acyclovir**

The synergistic effect between the most active plant MeOH fraction and acyclovir (ACV) against herpes viruses was evaluated by treating the cells with different combinations of the plant fraction and ACV at the time and post-infection with the virus (47). Acyclovir (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in distilled water.

**Statistical analysis**

The extract was prepared in triplicate for all tests. Data were subjected to analysis of variance following a completely random design to determine the least significant difference (LSD) at $P < 0.05$ using SPSS v. 11.5 (IBM SPSS, New York, USA).

**Results**

After preparation of the *V. persica* ethanolic extract, to check its antiviral activity, HSV-1 and HSV-2-infected Vero cells were treated by different concentrations (0, 5, 10, 15, 30, 60 and 120, µg/mL) of the extract. The treatments were at the time and after infection of Vero cells by the herpes viruses. After incubation time, there

<table>
<thead>
<tr>
<th>Table 1. The infected cells were treated with 80%-MeOH at different times of infection.</th>
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<td>• During infection</td>
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<td>• Post infection</td>
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<td>• During and post infection</td>
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<td>• Post treatment</td>
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showed a significant selectivity and efficacy against both viruses ($P < 0.05$). Moreover, the obtained SI by this fraction was 451.6 for HSV-1 and 383.5 for HSV-2. The SI values of ACV for HSV-1 and HSV-2 were 340 and 300, respectively.

To check if there is any correlation between time of treatment and the efficacy of the plant extract on viral infection, the cells were treated with 80%-MeOH fraction at different time points including before (incubation with cells, pre-incubation), during (at infection) and after cell infection (post infection, p.i.). In addition, the cells were treated at two times (at infection and p.i.). The optimum results were observed when the treatment was performed at two times: during (at infection) and post infection ($P < 0.05$). Sequentially, post infection, at infection and pre-incubation treatments were the other treatment time points in which the extract showed its inhibitory activity against the viruses.

Compared to was a dose-response effect: increasing concentrations of the plant extract, resulted in a decreasing trend in the percentage of plaque numbers (PFU) of both viruses. At the concentration of 120 µg/mL of the plant ethanol extract, the HSV-1 and HSV-2 viruses showed 35% and 25% significant reduction in PFU %, respectively ($P < 0.05$). In all concentrations, the antiviral activity of the plant extract was slightly higher for HSV-1 when compared to HSV-2. Such differences were observed especially at higher concentrations of the plant extract (60 and 120 µg/mL) (Figure 1).

In a comparative experiment, the selectivity of both plant ethanol extract and anti-viral drug acyclovir (ACV) against viruses was checked. It was observed that the selectivity index (SI, $CC_{50}/IC_{50}$) values of the ethanol extract are significantly lower than the ACV ones for both viruses ($P < 0.05$). In addition, the SI of ethanol extract was higher for HSV-1 compared with HSV-2 (Figure 2).

After fractionation of the ethanol extract using a stepwise methanol (MeOH) gradient, the antiviral activity of each MeOH fraction was evaluated. $CC_{50}$, $IC_{50}$ and SI for each fraction and ACV as the anti-viral drug positive control are summarized in Table 2. Among the fractions, 80%-MeOH exhibited the lowest $IC_{50}$ values (0.62 ± 11 for HSV-1 and 0.73 ± 21 for HSV-2), and

### Table 2. Antiviral activity of *V. persica* MeOH fractions and acyclovir against herpes viruses.

<table>
<thead>
<tr>
<th>Plant fractions</th>
<th>$CC_{50}$ (µg/mL)</th>
<th>$IC_{50}$ (µg/mL)</th>
<th>Selectivity index</th>
<th>$IC_{50}$ (µg/mL)</th>
<th>Selectivity index</th>
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<tr>
<td>0%-MeOH</td>
<td>450 ± 66</td>
<td>Inactive</td>
<td>-</td>
<td>Inactive</td>
<td>-</td>
</tr>
<tr>
<td>20%-MeOH</td>
<td>360 ± 34</td>
<td>155 ± 75</td>
<td>2.32</td>
<td>220 ± 34</td>
<td>1.63</td>
</tr>
<tr>
<td>40%-MeOH</td>
<td>350 ± 45</td>
<td>125 ± 33</td>
<td>2.8</td>
<td>200 ± 29</td>
<td>1.75</td>
</tr>
<tr>
<td>60%-MeOH</td>
<td>300 ± 59</td>
<td>85 ± 52</td>
<td>3.52</td>
<td>180 ± 35</td>
<td>1.66</td>
</tr>
<tr>
<td>80%-MeOH</td>
<td>280 ± 57</td>
<td>0.62 ± 11</td>
<td>451.6</td>
<td>0.73 ± 21</td>
<td>383.5</td>
</tr>
<tr>
<td>100%-MeOH</td>
<td>200 ± 77</td>
<td>60 ± 22</td>
<td>3.33</td>
<td>160 ± 44</td>
<td>1.26</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>150 ± 15</td>
<td>0.44 ± 0.1</td>
<td>340</td>
<td>0.5 ± 0.2</td>
<td>300</td>
</tr>
</tbody>
</table>

Note: Values are mean ± SD (n=3). Vero cell monolayers were treated with different doses of purified fractions of the plant extract at the time and after infection.

![Figure 1. Antiviral activity of *V. persica* ethanolic extract on herpes viruses. Vero cells were infected with 1 moi (multiplicity of infection) of different herpes viruses, with or without treatment with various concentrations of plant extract. The cells were treated both at the time of infection and again post-infection with the extract up to the end of the assay. Plaque numbers (PFU) are presented as a percentage of the positive control (infected but untreated cell cultures). Values are means ± SD (n=3).](image1.png)

![Figure 2. Selectivity index (SI) of plant ethanol extract and acyclovir against HSV-1 and HSV-2. SI was determined as $CC_{50}/IC_{50}$. Values are means ± SD (n=3).](image2.png)

![Figure 3. Effect of the treatment time with the plant 80%-MeOH fraction on virus infection. Values are mean ± SD (n=3).](image3.png)
In another experiment, isolated glycosides (saikosaponins A, B2, C and D) from traditional medicinal plants showed higher selectivity and efficacy nowadays is of outmost attention. So far, different investigations have been carried out to assess the activity of medicinal plants and their products on different viral agents. For instance, in one examination, isolated glycosides (saikosaponins A, B2, C and D) from traditional medicinal plants showed a remarkable antiviral activity upon challenging with human coronavirus 22E9 (50). In another experiment, the ethanolic and aqueous extracts of Ocimum basilicum (sweet basil) were tested on coxsackievirus CVB1. It was found that some bioactive compounds derived from this plant are able to inhibit the replication of CVB1 (51). Hydrolysable tannins, isolated from the medicinal plant Terminalia chebula, were tested on dengue virus in another experiment (52). In this experiment, it was observed that punicalagin and chebulagic acid from the plant were able to inactivate the viral particles and inhibit their attachments to the host cells. Woodfordia fruticosa (53), Bupleurum spp. (54), Silybum marianum (55, 56), Corydalis saxicola (57) and Piper longum (58) are additional examples of investigated medicinal plants that have shown antiviral activities against different viruses such as enterovirus, hepatitis B, hepatitis C viruses, etc.

The inhibitory effects of different medicinal plants against HSV viruses were also investigated by several groups (59-61). In one interesting work, extracted natural products from the medicinal plant Cassia javanica showed potent inhibitory activity of HSV-2 replication (61). In another work, hydrolysable tannins extracted from some medicinal plants showed a great inhibitory action against HSV-1 by targeting the virus glycoproteins and finally reducing cellular attachment and host cell entrance (62). Melia azedarach (63), Houttuynia cordata (64), Rhododendron ferrugineum (65), Myrothamnus flabelifolia (66) and Digitalis lanata (67) are additional examples of medicinal plants containing various components with anti-HSV activities.

In this current work, we have investigated the potential antiviral activity of Veronica persica extract against the HSV viruses. V. persica is one of the famous medicinal plants of eastern Asia and Eurasia with a remarkable therapeutic potential. So far, the anti-cancer and anti-rheumatic activities of this plant have been confirmed by several researchers (39-42). However, as far as we know, there have been no report on evaluation of V. persica extract for any antiviral activities.

After obtaining an ethanol extract of the plant, its antiviral activity was checked using both HSV-1 and HSV-2 infected Vero cells. Our results revealed an antiviral activity of the ethanol extract in a concentration-dependant manner. However, the selectivity index values for the plant ethanol extract were low when compared to ACV as the antiviral medication. Subsequently, different MeOH fractions of the extract were obtained using reverse phase chromatography, and the antiviral activity of each fraction was checked on infected Vero cells. According to our results, with the exception of the 80% MeOH fraction, which showed a remarkable antiviral activity with a lower IC₅₀ and higher SI values for both HSV-1 and HSV-2, other MeOH fractions showed non-significant antiviral activities (P < 0.05). The 80% MeOH fraction also exhibited a much higher selectivity and efficiency against HSV viruses when compared to the antiviral medicine ACV.

One of the important questions in our experiment was the probable effects of pre- and post-infection treatments, as well as to examine the effectiveness of the plant extract at different infection time points. To answer this question, the Vero cells were treated by the 80% MeOH plant extract fraction before, during and after infection with HSV viruses. After the experiment, it

**Discussion**

Currently, combination therapy is one of the highly recommended choices for treatment of viral infections. The newly identified genetic diversity among isolated human HSV viruses is just one of the reasons that push experts toward combination therapies. On the other hand, discovery of new therapies is essential not only for enhancing the effectiveness of treatment, but also for treatment of infections caused by drug-resistant HSV strains (48). Currently, one of the main concerns about the HSV infections is their progression to a severe form and becoming resistant to the common therapies such as ACV. In such circumstances, current therapies are not able to suppress the viral replication adequately and may fail to reduce the cellular viral load patients (6, 49). Low bioavailability and potency, low efficacy and having several side effects by the current HSV infection therapies, compel a need for discovery or designing novel treatments without the above-mentioned drawbacks.

In this context, medicinal plants are one of the safe and efficient options in searching for novel antiviral therapies. Screening traditional herbal medicines to obtain natural-based antiviral agents with lower side effects, higher selectivity and efficacy nowadays is of outmost attention. So far, different investigations have been carried out to assess the activity of medicinal plants and their products on different viral agents. For instance, in one examination, isolated glycosides (saikosaponins A, B2, C and D) from traditional medicinal plants showed a remarkable antiviral activity upon challenging with human coronavirus 22E9 (50). In another experiment,

**Figure 4.** Synergistic effects between plant 80%-MeOH fraction and acyclovir. Vero cells were treated independently with 0.1 μg/mL of plant fraction or 0.01μg/mL acyclovir at and post infection with the different viruses, or with a mixture both of those (0.1 μg/mL of plant fraction and 0.01μg/mL acyclovir). Plaque number (PFU) of the treated cultures are presented as a percentage of the positive control. Values are mean ± SD (n=3).
was observed that treatment of cells at the time of infection and again at post infection led to a higher efficacy of the extract against viruses. Such findings revealed the inhibitory action of the extract on both before and after the viral infections and suggest the interfering action of the extract on cellular entrance of the viruses. On the other hand, the post infection treatment of cells merely showed better results in reducing viral plaque formation when compared to treatment at infection. The 80% MeOH fraction also showed a reasonable inhibitory effect on endogenous viruses indicating the inhibitory activity of the extract on intracellular virus replication.

Another interesting finding was the higher sensitivity of HSV-1 to the V. persica extract when compared to HSV-2. In almost all anti-viral evaluations using ethanol extract and 80% MeOH fraction, HSV-1 showed a higher sensitivity to V. persica. This phenomenon also was observed in all treatment time points indicating a higher effectiveness of the plant extract against HSV-1. In order to check the synergistic effects of the extract when applied along side the antiviral drug ACV, in a separate experiment, specific amounts of 80% MeOH fraction of plant extract and ACV were used for treatment at both during and after viral infection of cells. The cells which received both ACV and the plant extract fraction showed a higher reduction in plaque formation by both viruses. Similarly, HSV-1 again showed a higher sensitivity especially to the plant extract. Meanwhile, the group receiving only the ACV resulted in a similar reduction in PFU% of both viruses. These findings exhibited the notable potential of the plant extract in combination therapies against HSV viruses.

Since most anti-viral therapies still are not ideal to completely prevent or inhibit the viral infections, development and/or discovering novel and efficient anti-viral agents is of utmost importance at this time. As far as we know, there is no vaccine for prevention of HSV infections. On the other hand, researchers still are in search for more efficient and safe anti-HSV agents. Based on the literature, there are currently no available drugs that are able to target the latent HSV-based infections. In this regard, medicinal plants offer novel potential and currently are of center of attention as efficient sources of anti-microbial and anti-viral agents. Here, we have demonstrated the remarkable anti-HSV activity of V. persica plant on both HSV-1 and HSV-2 viruses. Our results also revealed that the 80% MeOH fraction of the V. persica extract is able to significantly reduce the viral plaque of both viruses. Considering the importance of combination therapy in eradication of viral infections, the V. persica extract showed a superior performance in inhibition of HSV viruses when applied along with ACV. However, more experiments are necessary to investigate the applicability and efficiency of combination therapies using both standard therapeutics and natural agents. In conclusion, we introduced V. persica as a medicinal plant with anti-HSV activity. However, more experiments are recommended to find the specific mechanisms of activity of this plant on viruses. We are certain that such plants and their products will continue to play key roles in drug discovery investigations, especially for anti-viral drug development.

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Conflicts of Interest
The authors declare no conflict of interest.

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