Amirhossein S. Antiviral Activities of aerial subsets of Artemisia species against Herpes Simplex virus type 1 (HSV1) in vitro

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Background: Drug resistance to current anti-herpetic drugs has been increasingly reported. Therefore, there is a need for finding new antiviral agents, in particular from natural sources.

Objective: In the present study, antiviral activity of subset extracts obtained from aerial parts of Artemisia including A. incana, A. chamaemelifolia, A. campesteris, A. fragrans, A. annua, A. vulgaris, and A. persica were investigated against Herpes Simplex type I (HSV1).

Methods: Different concentrations of extracts (400, 200, 100, 50, 25, 12.5, 6.25, and 3.125 μg/mL) were obtained from subset of each plant separately, and used against KOS strain of HSV1 in HeLa cells. After 24 hours incubation, tetrazolium dye (MTT), was added. The dye absorption by viable cells was measured and compared to the positive control (extract-untreated cells) and acyclovir (as anti-viral agent).

Results: The extracts obtained from A. annua had the highest antiviral activity while those of A. chamaemelifolia showed the lowest activity.

Conclusion: Subset extracts of A. annua may be an appropriate candidate for further development of anti HSV1 infection.

Keywords: Antivirals, Artemisia, asteraceae, herpes simplex

Plants have been the target of research for a long period because of their unique properties. Due to the side effects that synthetic drugs might elicit, there is an increasing demand for traditional medicine as an alternative. Besides, bioactive components of plant extracts including different monoterpenoids, sesquiterpenoids, diterpenoid, flavonoids, fatty acids, and lignans have attracted the attention of scientists. [1-7]. HSV1, a member of Herpesviridae can cause disease ranging from Herpes Labialis to severe encephalitis. Herpes infection can also cause severe diseases in neonates, elderly, transplanted patients immuno suppressed by drugs and in patients with acquired immune deficiency syndrome. Resistance to current anti-herpetic drugs has been increasingly reported [8, 9]. This mandates the need to search for new therapeutic tools. This encouraged us to investigate anti-HSV1 effects of Iranian Artemisia. The genus Artemisia L is one of the largest and most widely distributed of the Asteraeae (Compositae) family. This is a large and heterogeneous genus, numbering over 400 species distributed mainly in the temperate zone of Europe, Asia, and North America [10-14]. The genus in Iran has 34 species, two of which are endemic to this country [14-16]. Different species of Artemisia have a vast range of biological effects including anti-malarial [17-20], anti-bacterial, anti-fungal [17-19, 21-23], and anti-oxidant [18-26]. Besides, there have been some reports on the inhibitory activity of Artemisia species against some types of viruses including HSV1 [27-31]. Therefore, the
The present study aimed to investigate the *in vitro* anti-HSV1 activities of extracts obtained from the aerial subsets of Iranian *Artemisia* species.

**Methods**

**Plant material**

Seven species of *Artemisia* were collected from different parts of Iran (Table 1). The Research Institute of Forest and Rangelands, Ministry of Jahad-E-Agriculture Iran identified these plants. Voucher specimens of the species have been deposited in the Herbarium of National Botanical Garden of Iran (TARI).

**Cell Culture**

HeLa cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, Gibco), penicillin G (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (25 μg/mL). The cells were incubated under a humid 5% CO₂ atmosphere at 37 °C.

**Extraction procedure**

The dried aerial parts of each species (100 g) were chopped in small pieces and then crushed into powder by a blender. Each sample was macerated in pure methanol for 24 hours. The samples were then extracted using a percolator (10 hours, 30 drops/min) [32]. The extracts were concentrated by a rotary evaporator and dried in an oven at 50 °C under reduced pressure to give 5-8 g. of solid residue. The solid residues (0.2 g) were dissolved in 100 mL of phosphate buffer containing 0.1% of ethanol, filtered, and sterilized using 0.22μm microbiological filters. Serial dilutions were prepared so that the concentrations of extracts were 400, 200, 100, 50, 25, 12.5, 6.25, and 3.125 μg/mL. The extracts were kept in the sterilized bottles in the fridge.

**Evaluation of viral efficacy to infect the cells**

To evaluate whether the preparation could infect cells, HeLa cells were cultured on the flat bottom of 96 well plates. Two hundred L of HeLa cells preparation containing 10⁴ cells was transferred into each well and incubated at 37°C for 24 hours. Then, 180 μL of the supernatant was removed and the cells were covered by 180 μL of viral preparation containing 5x10⁶ pfu/mL of HSV1 and incubated for 24 hours. Afterwards, 200 μL of culture media was removed and replaced by 200 μL of fresh culture media and 20 μL of MTT. The plates were covered by aluminum foil roundly and incubated for four hours at 37°C. Then, wells were emptied and 200 μL of dimethyl sulphoxide (DMSO) and 15 μL of glycin buffer were added. The dye absorption by viable cells was measured by ELISA reader and compared to the control wells that contained only Hela cells. The ratio of the infected cells to uninfected cells was an indicator for anti-viral effectiveness.

**Evaluation of anti-viral effect of extracts**

A cell preparation containing 10⁴ cells was passed into each 96 wells flat bottom plate. Twenty-four hours later, 100 L of a viral preparation containing 5x10⁶ pfu/mL of HSV1 (KOS strain) in fresh culture media was transferred into each well. KOS strain was kindly provided by the Virology laboratory, Faculty of Health, Tehran University of Medical Sciences. A serial dilution of *Artemisia* extracts belonging to Seriphidium section including *A. incana*, *A. chamaemelitolia*, *A. campesteris*, *A. fragrans*, *A. annua*, *A. vulgaris*, and *A. persica* at different concentrations including 400, 200, 100, 50, 25, 12.5, 6.25, and 3.125 μg/mL was prepared and transferred into the wells separately. Acyclovir (as the standard anti-viral agent) was also prepared at concentrations equal to the *Artemisia* extracts. After 24 hours, the supernatant was removed and the dye allowed to bind for 30 minutes and the plates were read by ELISA reader.

<table>
<thead>
<tr>
<th><em>Artemisia</em> species</th>
<th>Location</th>
<th>Collection time</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. annua</em></td>
<td>Islamabad near Maraveh tapeh-Shahrabad road (height, 940 m)</td>
<td>Sep 15, 2007</td>
</tr>
<tr>
<td><em>A. chamaemelitolia</em></td>
<td>Chovailly-Bajgiran road (height 1650 m)</td>
<td>Dec 24, 2007</td>
</tr>
<tr>
<td><em>A. campesteris</em></td>
<td>Maraveh tapeh-Shahrabad road (height 940 m)</td>
<td>Aug 8, 2007</td>
</tr>
<tr>
<td><em>A. fragrans</em></td>
<td>Maraveh tapeh-Shahrabad road (height 940 m)</td>
<td>Aug 8, 2007</td>
</tr>
<tr>
<td><em>A. incana</em></td>
<td>Khosph-Birjand (height 1290 m)</td>
<td>Dec 23, 2007</td>
</tr>
<tr>
<td><em>A. persica</em></td>
<td>Ghorogh Samieabad (height 909 m)</td>
<td>Aug 8, 2007</td>
</tr>
<tr>
<td><em>A. vulgaris</em></td>
<td>Ghorogh Samieabad (height 909 m)</td>
<td>Sep 19, 2007</td>
</tr>
</tbody>
</table>
and replaced by 200 μL of fresh media and 20 μL MTT, followed by incubation at 37°C for four hours. The supernatant was then replaced by 200 μL DMSO and 15 μL glycine buffer. Positive controls containing extract-untreated HSV1-infected cells were included in the experiments. The dye absorption by viable cells was measured by ELISA reader and compared to the control (extract-untreated cells) and acyclovir. Results were presented as mean of three independent experiments. Protection rate was determined by the ratio of viable cells to dead cells according to the following formula: [Total cells - infected cells] x 100/ Total.

**Results**

**Evaluation of the viral infectivity**

This experiment showed that the viral suspension was able to infect the HeLa cells and it can be used for the next experiments.

**Anti-viral activities of plant extracts among Artemisia species**

We investigated anti-HSV1 activity of subset extracts obtained from aerial parts of *Artemisia* belonging to Seriphidium section including *A. incana*, *A. chamaemelifolia*, *A. campesteris*, *A. fragrans*, *A. annua*, *A. vulgaris*, and *A. persica*. Methanolic extracts were tested at various concentrations. Results showed that the extracts of aerial parts of *A. annua* had the highest anti-herpetic activity. Those of *A. chamaemelifolia* showed the lowest activity. The highest protection rates of *A. incana*, *A. chamaemelifolia*, *A. fragrans*, and *A. persica* methanolic extracts were observed at 12.5 μg/mL concentration being 50.69%, 46.53%, 60%, and 81.37%, respectively. The lowest protection rates of the aforementioned extracts were at 50 μg/mL concentration being 30.21%, 26.61%, 23%, and 25.69%, respectively. The protection rate decreased at higher concentrations in the aforementioned plant extracts particularly at concentration of 100, 200, and 400 μg/mL. The 100 μg/mL concentration showed very low activity against HSV1 and concentrations of 200 and 400 μg/mL showed cytotoxicity. The highest protection rates of *A. campesteris*, *A. annua*, and *A. vulgaris* methanolic extracts were observed at concentrations of 6.25, 12.5, and 25 μg/mL, which were 73.32%, 83%, and 63.94%, respectively. In these plants, the lowest protection rates were at concentrations of 50, 3.125, and 50 μg/mL being 24%, 69%, and 26%, respectively. Again, higher concentrations showed negligible protection rates (100 μg/mL) or cytotoxicity (200 and 400 μg/mL) (Figs 1 & 2). With respect to acyclovir, the highest protection rate was observed at 50 μg/mL being 81.43%, while the lowest protection rate was observed at 3.125 μg/mL being 38.67%. The same as plant extracts, higher concentrations showed negligible protection (100 μg/mL) or cytotoxicity (200 and 400 μg/mL).

**Comparison of anti-viral activities of plant extracts with positive controls**

HSV1-infected cells that were neither treated with extract nor with acyclovir served as positive control. The percentage of viable cells in the control well was 18%. Comparison between the protection rate of *A. chamaemelifolia* (the extract with weakest anti-HSV1 activity) and positive control indicated significant difference at all assessed concentrations (3.125, 6.25, 12.5, 25, and 50 μg/mL), with the viability being higher in the *A. chamaemelifolia* treated cells. Therefore, it may be deduced that other extracts are also associated with higher protection at these concentrations compared to positive control.

**Comparison of anti-viral activities of plant extracts to acyclovir**

In this research, anti-HSV1 activities of subset extracts obtained from aerial parts of Iranian *Artemisia* were investigated and compared with the anti-viral activity of acyclovir. *A. annua* extracts (as the strongest anti-viral extract) showed higher anti-HSV1 activities than acyclovir at 3.125, 6.25, 12.5, and 25 μg/mL concentrations. However, the anti-HSV1 activity of acyclovir was higher at 50 μg/mL than that of *A. annua*. A comparison of the anti-HSV1 activity of tested Artemisia extracts is shown in Table 2.

**Discussion**

We evaluated the anti-viral activities of seven species of *Artemisia* simultaneously. For this purpose, HSV1-infected HeLa cells were incubated with different concentrations of *Artemisia* extracts. The results showed that the extract obtained from the aerial parts of *A. annua* has the highest anti-herpetic activity but *A. chamaemelifolia* extract showed the lowest activity. The protection rate, which was determined by the ratio of the viable cells to the dead cells, decreased at higher concentrations of the extracts. A 100 μg/mL concentration showed very low activity against HSV1 and concentrations of 200 and 400 μg/mL showed cytotoxic effects as shown in Fig. 1.
There have been some previous reports on the antiviral activity of some Artemisia species. Saddi and colleagues reported the antiviral activity of essential oil obtained from the leaves of *A. aborescens* against HSV1 and HSV2 [27]. In another investigation, Chao-Mei et al. reported the inhibitory effect of *A. caruifolia* methanolic extract against HIV1 protease and demonstrated that this effect could be attributed to the presence of tri-p-coumaroylspermidine [28]. *A. verlotorum* aqueous extract has also been demonstrated to exert strong activity against feline immunodeficiency virus (FIV), which is a reliable model of HIV1 [29].

The anti-HSV1 properties of extracts from the medicinal plants used in this research could be due to multiple different components in these plants. The phytochemical characterization of extracts and the identification of bioactive compounds are now needed.

### Table 2. Relative comparison of the Anti-HSV1 activity of different Artemisia extracts.

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>Order of observed anti-HSV1 activity</th>
</tr>
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<tbody>
<tr>
<td>3.125</td>
<td><em>A. annua</em> &gt; <em>A. campesteris</em> &gt; <em>A. persica</em> &gt; <em>A. incana</em> &gt; <em>A. fragrans</em> &gt; Acyclovir &gt; <em>A. vulgaris</em> &gt; <em>A. chamaemelifolia</em></td>
</tr>
<tr>
<td>6.25</td>
<td><em>A. annua</em> &gt; <em>A. campesteris</em> &gt; <em>A. persica</em> &gt; Acyclovir &gt; <em>A. fragrans</em> &gt; <em>A. incana</em> &gt; <em>A. vulgaris</em> &gt; <em>A. chamaemelifolia</em></td>
</tr>
<tr>
<td>12.5</td>
<td><em>A. annua</em> &gt; <em>A. persica</em> &gt; <em>A. campesteris</em> &gt; Acyclovir &gt; <em>A. fragrans</em> &gt; <em>A. incana</em> &gt; <em>A. vulgaris</em> &gt; <em>A. chamaemelifolia</em></td>
</tr>
<tr>
<td>25</td>
<td><em>A. annua</em> &gt; <em>A. persica</em> &gt; Acyclovir &gt; <em>A. vulgaris</em> &gt; <em>A. campesteris</em> &gt; <em>A. fragrans</em> &gt; <em>A. incana</em> &gt; <em>A. chamaemelifolia</em></td>
</tr>
<tr>
<td>50</td>
<td>Acyclovir &gt; <em>A. annua</em> &gt; <em>A. incana</em> &gt; <em>A. vulgaris</em> &gt; <em>A. campesteris</em> &gt; <em>A. chamaemelifolia</em> &gt; <em>A. persica</em> &gt; <em>A. fragrans</em></td>
</tr>
</tbody>
</table>

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**Fig. 1** Picture of *Artemisia annua* [36]
Unknown is also the method of action of active components of subset from our extract extracts. The presence of flavones such as 4', 6, 7-trihydroxy-3', 5'-dimethoxyflavone and 5', 5-dihydroxy-3', 4', 8-trimethoxyflavone [20], exiguaflavone A and B [33], artemetin, bonanzin, eupalitin, and chrysosplenetin [34] in the extracts of Artemisia have been previously reported and anti-viral activities of flavones have been broadly demonstrated [35]. Some of these phytochemicals might be responsible for the anti-viral activity of Artemisia species used in this research. As for the A. annua, artemisinin (a safe and commonly used anti-malarial medication) is among the most important candidates accounting for the observed antiviral effects. In previous studies, artemisinin and its derivatives such as artesunate, have been shown to possess antiviral activities against a range of viruses including HSV1, human cytomegalovirus, Epstein-Barr virus, hepatitis B virus, hepatitis C virus, HIV1, and bovine viral diarrhea virus [30]. In addition, sterols including sitosterol and stigmasterol have been isolated from A. annua, as virus inhibitory agents [31]. Further studies have to be conducted to determine if there is a substantial difference or even any synergistic or antagonistic effect among different components existing in these medicinal plants, which can affect anti-viral activities. This work also needs to be expanded to other viruses and extended to studies in animal models.

In conclusion, extracts of A. annua and related species may be appropriate candidate for further therapeutic studies against herpes viruses.

Acknowledgments

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References


