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Original article

Antiviral activities of aerial subsets of *Artemisia* species against Herpes Simplex virus type 1 (HSV1) *in vitro*

Mehrangiz Khajeh Karamoddini[a], Seyed Ahmad Emami[b], Masoud Sabouri Ghannad[c], Esmaeel Alizadeh Sani[b], Amirhossein Sahebkar[d]

[a]Department of Microbiology, Qhaem Medical Center, [b]Department of Pharmacognosy, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad 91775-1365; [c]Department of Microbiology, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan 65178-3-8736; [d]Biotechnology Research Center and School of Pharmacy, Mashhad University of Medical Sciences, Mashhad 91775-1365, Iran

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, flavonioids, 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 Astraceae (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

***Correspondence to*:** Dr. Masoud Sabouri Ghannad, Department of Microbiology, Medical school, Hamadan University of Medical Sciences, Hamadan 65178-3-8736, Iran. E-mail: sabouri39@yahoo.com

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 amphotricin B (25 µg/mL). The cells were incubated under a humid 5% CO_2 atmosphere at 37 ^{O}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 0.22µ 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 10^4 cells was transferred into each well and incubated at 37^{O}C for 24 hours. Then, 180 □l of the supernatant was removed and the cells were covered by 180 µL of viral preparation containing $5x10^6$ 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^{O}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 10^4 cells was passed into each 96 wells flat bottom plate. Twenty-four hours later, 100 □L of a viral preparation containing $5x10^6$ 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. chamaemelifolia, 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

Table 1. List of seven species of *Artemisia* plants screened for antiviral activity.

Artemisia species	Location	Collection time
A. annua	Islamabad near Maraveh tapeh-Shahrabad road (height, 940 m)	Sep 15, 2007
A. chamaemelifolia	Chovailly-Bajgiran road (height 1650 m)	Dec 24, 2007
A. campestris	Maraveh tapeh-Shahrabad road (height 940 m)	Aug 8, 2007
A. fragrans	Maraveh tapeh-Shahrabad road (height 940 m)	Aug 8, 2007
A. incana	Khosph-Birjand (height 1290 m)	Dec23, 2007
A. persica	Ghorogh Samieabad (height 909 m)	Aug 8, 2007
A. vulgaris	Ghorogh Samieabad (height 909 m)	Sep 19, 2007

and replaced by 200 µL of fresh media and 20 µL MTT, followed by incubation at 37^{O}C for four hours. The supernatant was then replaced by 200 µL DMSO and 15 µL glycin 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 .fragrens* 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 □g/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 cytotoxicty (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 □g/mL concentration showed very low activity against HSV1 and concentrations of 200 and 400 □g/mL showed cytotoxic effects as shown in **Fig. 1**.

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

Concentration (µg/mL)	Order of observed anti-HSV1 activity
3.125	*A. annua* > *A. campesteris* > *A. persica* > *A. incana* > *A. fragrans* > *Acyclovir* > *A. vulgaris* > *A. chamaemelifolia*
6.25	*A. annua* > *A. campesteris* > *A. persica* > *Acyclovir* > *A. fragrans* > *A. incana* > *A. vulgaris* > *A. chamaemelifolia*
12.5	*A. annua* > *A. persica* > *A. campesteris* > *Acyclovir* > *A. fragrans* > *A. incana* > *A. vulgaris* > *A. chamaemelifolia*
25	*A. annua* > *A. persica* > *Acyclovir* > *A. vulgaris* > *A. campesteris* > *A. fragrans* > *A. incana* > *A. chamaemelifolia*
50	*Acyclovir* > *A. annua* > *A. incana* > *A. vulgaris* > *A. campesteris* > *A. chamaemelifolia* > *A. persica* > *A. fragrans*

Fig. 1 Picture of *Artemisia annua* [36]

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* aquous 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.

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.

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