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Original article

Antiviral Activity of Obtained Extracts from Different Parts of *Cupressus sempervirens* against Herpes Simplex Virus Type 1

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Abstract

Objective(s)
The aim of this study was to search for new antiviral agents from herbal medicines. Ethanol extracts of *C. semipervirens, C. semipervirens* var. *horizontalis* and *C. semipervirens* cv. Cereiformis were used in experiments to test their influence on herpes viruses (HSV-1).
Materials and Methods
HeLa cells monolayers were infected with herpes viruses (HSV-1). Antiviral activity of the plant extracts assessed using Hematoxylin & Eosin method and observed under a light microscope. All tests were compared with a positive control, acyclovir.
Results
Results showed that all three plants have antiviral activity against HSV-1 virus. The most active extract was the obtained extract from *C. semipervirens.* Among the different parts of this medicinal plant tested, the fruit's extract appeared to possess the strongest anti- HSV activity.
Conclusion
In conclusion, of the extracts tested in this survey all showed significant antiviral potency.

Keywords: Antiviral activity, *Cupressus sempervirens*, Cupressusaceae, HSV-1

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Introduction

A great variety of ethnomedicinal plants are being studied as a source of natural products useful in the development of novel drugs. It has been established that many of them inhibit several steps of the viral replication cycle of many DNA and/or RNA viruses (1). Herbal products have been used as folk remedies for different kinds of ailments including viral diseases (2).

There is a need to search for new compounds for treatment of viral infections since there is an increasing resistance to antiviral drugs (3).

The problems of viral resistance and viral latency leading to recurrent infections in immunocompromised patients have been documented earlier (4- 6).

A number of medicinal plant products have been shown to have antiviral activity (7, 8). Traditional plant extracts having anti-infective properties, have been screened for their antiviral activity (9).

The herpes simplex virus (HSV) pandemics continue to be unabated and pose a major public health threat. There are several *in vitro* and *in vivo* methods reported in the current literature to study the anti-herpetic activities of plant/herbal extracts or plant-derived molecules. Most commonly, researchers are using the cytopathic effect (CPE) on HSV-infected for preliminary studies and/or screening of large numbers of molecules/extracts (6). Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are agents of common infections with recurrent orofacial and genital lesions. HSV-1 predominantly causes epidermal lesions in and around the oral cavity. Herpes simplex virus type 1 is transmitted through contact with saliva and causes recurrent herpes labialis. (10).

Several plant-derived compounds warrant further evaluation as potential anti-HSV reagents (6).

Conifers are a small group of the flora of Iran (8 species from 8000 species) (11). Iranian conifers consist of two families: Taxaceae and Cupressaceae. The Taxaceae in Iran has only one species of *Taxus*. Iranian species of Cupressaceae consist of one species of *platycladus*, five species of *Juniperus* and one species of *Cupressus* namely: *C. sempervirns* L. This species is a monoecious and evergreen tree to 25 m, very variable in habit, bark thin, glabrous, grayish-brown, branches horizontally spreading, branchlets terete or slightly 4-angled, uniform rhombic leaves, obtus, dark green. Cones are usually large, hanging on short stalks, subglobose or ellipsoid, top rounded, usually 2-3 cm across, sometimes smaller, scales 8-14, back conex, multiple seeds on each scale ovate or narrowly oblong, wing nearly orbicular and narrow (12-14). This tree is distributed in Mediterranean regions of Europe, Russia, Turkey, Iran and Syria.

In Iran, this species have a variety, namely *C. sempervirens* var. *horizentalis* and a cultivar namely *C. sempervirens* cv. Cereiformis.

- *C. sempervirens* var. *horizentalis* (Mill) Gordon [Syn: *C. horizentalis* Mill; *C. sempervirens* f. *horizentalis* (Mill.) Voss.] has a broad and pyramidal growing horizontal branches. This is the wild form occurring in eastern Mediterrenean from Creta to Iran.

- *C. sempervirens* cv. Cereiformis (Carr.) Rehd. (Syn*: C. fastigiata* var. *cereiformis* Carr.) is a very narrow column with a very closely appressed branches (12- 17).

C. sempervirens is a medicinal plant. The dried leaves of this plant are used as an emmenagogue and for stomach pain (18) as well as for diabetes (19). The dried fruit of this plant is used for inflammation treatment (20), toothache, laryngitis (21), as a contraceptive (22), astringent, and antiphrastic (23). The dried seed of this tree has been used for wounds, ulcers, bruises, sores, pimples, pustules, skin eruptions, and erysipelas (24). The essential oil of the plant is used externally for headache, colds, cough, and bronchitis (25).

Virostatic activity of *C. sempervirens* with the help of the immune system by blocking virus entrance in host cells is previously reported (26). There seems to be an increasing possibility of finding biological activity among plants with recorded medicinal uses rather than plants randomly selected (27).

However, antiviral properties of three mentioned *Cupressus* species against herpes

virus type-1 have not been published. In the present study, the antiviral activity of ethanol extracts derived from leaves and fruits of *C. semipervirens, C. semipervirens* var. *horizontalis* and *C. semipervirens* cv. Cereiformis on HSV-1 in cultured HeLa cells were investigated.

Materials and Methods

Plant material

Plant specimens were collected from different parts of the country as follow:

- *C. semipervirens* var. *horizontalis* ("Zarbin" in Persian) from Sorkesh wood land, Aliabad Katool, Golestan province, north of Iran, height 950 m (2 Oct. 2002).
- *C. semipervirens* ("Sarve Shirazi" in Persian) from Ecological Garden of Nowshar, Mazandaran province, north of Iran, height 23 m (5 Oct. 2002).
- *C. semipervirens* cv. Cereiformis ("Sarve naz" in Persian) from campus of Ferdowsi University, Mashhad, Razavi Khorasan province, north east of Iran, height 920 m (6 Aug 2002).

Dr. M. Assadi, Research Institute of Forest and Rangelands, Ministry of Jahad Keshavarzi, Iran, identified these plants. Voucher specimens of the taxons have been deposited in the Herbarium of National Botanical Garden of Iran (TARI).

The collected materials were stored at –20 °C in order to avoid unfavorable changes in the chemical components (28).

Extraction and purification of compounds

All parts of plant (50 g) were crushed separately and soaked in 75 ml of ethanol 80% (V/V) for 24 hr and then percolated (10 hr, 30 drops/min) (29). The extracts were concentrated by a rotary evaporator and were dried in an oven at 40 °C to give 5-8 g of solid residue. These solid residues (0.2 g) were dissolved in 100 ml of phosphate buffer containing 0.1% of ethanol, filtered and sterilized using 0.22 µm microbiological filters. The final concentrations of extracts used in this research were 12.5, 25, 50, 100, 200 and 400 µg/ml.

Virus and cells

Human cervix carcinoma cell lines (HeLa), was used to provide target cells for virus infection in the Hematoxylin & Eosin (H&E) assay. Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin G, and 100 mg/l streptomycin and 0.25 mg/l amphotericin B In the antiviral assay, the medium was supplemented with 2% FCS and the above mentioned antibiotics.

The strain of HSV type 1 (HSV-1 strain KOS) used in this study was kindly provided by Dr R Hamkar, School of Public Health., Tehran University of Medical Sciences.

HSV-1 was propagated in HeLa cells. Virus titres were determined by cytopathic effects in HeLa cells and were expressed as 50% tissue culture infective dose ($TCID_{50}$) per ml. All viruses were stored at –70 °C until use.

Cytotoxicity

To evaluate cytotoxic effects of the plant extracts, 96 flat bottom well plates were covered by sterilized cellophane fragment to enable culturing HeLa cells on the cellophane. 200 µl of HeLa cells preparation containing 2.0×10^4 cells/ml was transferred into each well and incubated at 37 ºC for 24 hr The supernatant was removed and the cells were covered by different concentrations of plant extract at 12.5, 25, 50, 100, 200 and 400 µg/ml for 24 hr. Media was removed; cellophane fragments were dried and fixed by ethanol 70% (v/v). The cellophane was stained by H & E method (30) and observed under a light microscope.

Antiviral assay using H & E method

HeLa cells monolayers were grown in 96-well microtiter plates covered by sterilized cellophane fragment. Dilutions of the extracts, prepared as described above were added 1 hr before viral infection. Virus were added to each well and incubated at 37 °C in humidified 5% CO_2 atmosphere for 24 hr. Controls consisted of untreated infected, treated uninfected and untreated uninfected cells. Furthermore all tests were compared with a positive control, acyclovir (12.5, 25, 50, 100,

200 and 400 µg/ml). The 50% antiviral effective concentration, i.e. 50% inhibitory concentration of the viral effect (IC50) is expressed as the concentration that achieves 50% protection of treated infected cells from HSV-1 induced destruction.

The percent protection is calculated using the following formula: [Total cells- infected cells] ×100/Total. Data represented in Table 1.

Table 1. Doses inducing 50% growth inhibition (IC50) of extracts against herpes virus (HSV-1) compared with acyclovir.

Fraction		IC50 value (µg/ml)
C. Semipervirens	Leaf	6.76
	Fruit	4.12
C. Semipervirens	Leaf	23.53
Var. *Horizontalis*	Fruit	3.97
C. Semipervirens	Leaf	8.17
Cv. Cereiformis	Fruit	5.28
Acyclovir		10.01

Statistical analysis

The statistically different effects of tested compounds on the inhibition of HSV replication were compared with the control group or compared between different extracts using the Student's t-test. IC50 for each extract were obtained from dose-effect-curves.

Results

Assessment of anti-HSV activity

In the present study, the antiviral activity of ethanol extracts derived from leaf and fruit of *C. semipervirens*, *C. semipervirens* var. *horizontalis* and *C. semipervirens* cv. Cereiformis on HSV-1 in cultured HeLa cells were investigated.

The potential inhibitory effect of extracts against herpes virus was determined by treatment of viruses with the extract and subsequent infection of HeLa cells. In all experiments cells infected with untreated virus were used as control. Cytopathic effect (CPE) in HeLa cells infected by HSV-I showed in Figure 1. The percent reduction was calculated relative to the amount of virus produced in the absence of the extracts. In all antiviral plant extract assays different extract concentrations up to the maximum non cytotoxic concentration were used. All of the three extracts tested in this survey, showed antiviral activity against HSV-1 virus (Figure 2). Among the different parts of this plant tested, the fruit's extract appeared to possess the strongest anti- HSV activity ($P< 0.05$).

Assessment of anti-HSV activity of C. semipervirens

The most active extract was the extract of *C. semipervirens* which exhibited antiviral activity at concentrations ranging from 12.5 to 400 µg/ml (Figure 2a).

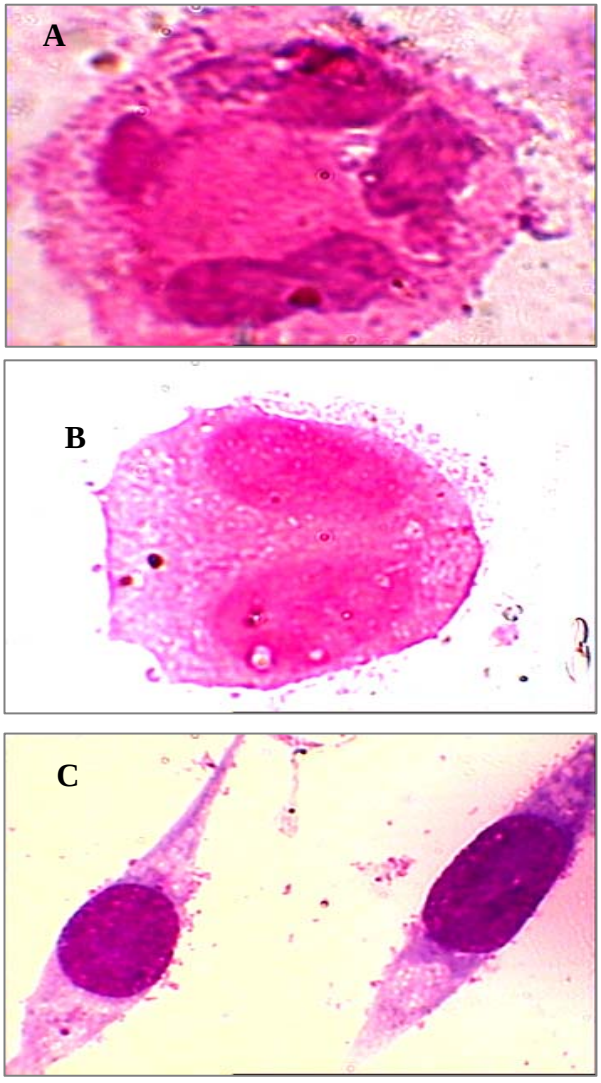

Figure 1. Cytopathic effect (CPE) in HeLa cells infected by HSV-I (A & B) (H&E stain).

Assessment of anti-HSV activity of C. semipervirens var. *horizontalis*

The ethanol extract of *C. semipervirens* var. *horizontalis* was also effective against HSV-1 at concentrations ranging from 12.5 to 400 µg/ml (Figure 2b).

Assessment of anti-HSV activity of C. semipervirens cv. Cereiformis

C. semipervirens cv. Cereiformis extract inhibited HSV-1 replication by 68.5% at the concentration of 12.5 µg/ml without showing cytotoxic effects, being more effective than the acyclovir as a positive control (Figure 2c).

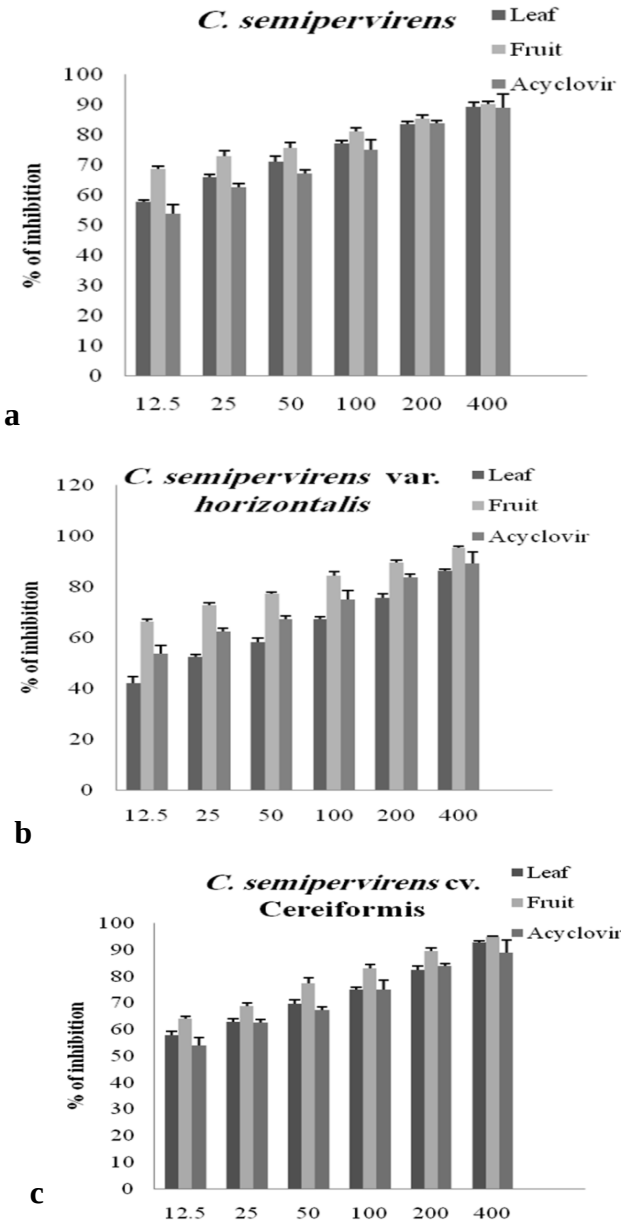

Figure 2. Dose-dependent effect of antiviral activity induced by extracts. a) *C. semipervirens* b) *C. semipervirens* var. *horizontalis* and c) *C. semipervirens* cv. Cereiformis Different concentrations of extract were added 1 hr after infection of herpes virus (HSV-1, white bars) to HeLa cells at 37 °C. After 3 days, inhibition was evaluated by Hematoxylin and Eosin (H&E) method and expressed as the inhibition rate. The x-axis indicates the concentration of extract (µg/ml). Each bar represents the mean±SEM of triplicate samples of three independent experiments.

Discussion

Since a long time, medicinal plants have been used to treat viral infections. The chemical diversity, structural complexity, lack of substantial toxic effects, and broad spectrum of antiviral activity of natural products, make them ideal candidates for new therapeutics. In fact, terpenoids isolated from medicinal plants have attracted attention because many of them exhibit specific antiviral effect against HSV-1 and 2, and the coronavirus Sars-CoV, *in vitro*. Triterpenoids and limonoids isolated from Meliaceae species proved to inhibit HSV-1, HIV-1 and RSV multiplication (31).

Viruses are classified as important pathogens among different kinds of microorganisms which cause infections. Viral rapid transmission, high infectivity and multiple viral mutations are some different aspects of research which have attracted the attention of scientists. Infected HeLa cells with HSV-I were incubated with different concentrations of the Iranian medicinal plants extracts. H & E staining method was performed and the results were evaluated by CPE effect in comparison with uninfected cells.

It is notable that based on the data obtained in this research, all ethanol extracts show major antiviral effects in comparison with acyclovir used as a control. In all three taxons which were investigated in this research, fruit extracts demonstrated stronger anti HSV-I effect than subsets. Increased concentration of subset extracts showed antiviral effect as well. The strongest anti HSV-I effect was shown by *C. sempervirens* var. *horizentalis* fruit extract with less effect presented by *C. sempervirens* cv. Cereformis, subsets. The properties of plant extracts obtained were probably due to the presence of similar components in the extracts including flavonoids, tannins, lignans, monoterpenes, sesquiterpenes and diterpens. The anti HSV-I effect of the extract may be resulted from each separate component or synergistic effect of entire components. Synergy effects of the mixture of bioactive constituents and their byproducts contained in plant extracts are claimed to be responsible for the improved effectiveness of many extracts, because the plant extracts consist of complex mixtures of major compounds, minor concomitant agents and fibres, which can all

be involved in the synergy effects (32). Plants use complex mixtures of secondary compounds of different structural classes to protect themselves against herbivores, bacteria, fungi and viruses. These complex mixtures may contain secondary metabolites, which are specific for a single target (monotarget secondary metabolites). A majority of secondary metabolites, however, can interfere with several targets (multitarget secondary metabolites) in a pleiotropic fashion. The composition of such extracts appears to be optimized, since the components are not only additive but apparently synergistic in their bioactivity (33). Antiviral effects of lignans and sesquiterpene constituents from the essential oil of the phytoalexines have been confirmed (34-37). The oil extracted from *C. sempervirens* contains terpinen-4-ol. The anti HSV-I effect of this component has been reported by Lipipun *et al* (38). Moreover, antiviral activities of lignans have been also reported by San Feliciano *et al* 1993 (39). Podophylotoxine is a component belong to lignan so that it exists as a general components in fruits which acts as anti HSV-I. Anti HSV-I activities of extracts may be due to the property of galic acid, one of the tannins component exists in fruits *Cupressus* spp. in high concentrations. San Feliciano *et al* (39) reported anti HSV-I activity of apigenine as a component of flavonoides exist in Cupresseace family. Antiviral activities of *C. sempervirens* proanthocyanidins against retroviruses such as HIV and HTLV have been reported (40).

Conclusion

Of the extracts tested in this survey, all showed significant antiviral potency. After the successful detection of active plant extracts, the substances responsible for the bioactivity must be isolated and chemically characterized. Further analysis, including additional purification of the extracts, along with further antiviral testing are currently being conducted.

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