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Evaluation Effect of Hydroalcoholic Extract of *Eucalyptus globulus* and *Artemisia draconculus* Compared with Acyclovir against Herpes Simplex Virus Type 1

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Abstract Herpes simplex virus 1 and 2 (HSV−1 and HSV−2), also known as Human herpes virus 1 and 2 are two members of the herpes virus family, Herpesviridae, that infect humans. Both HSV−1 (which produces most cold sores) and HSV−2 (which produces most genital herpes) are ubiquitous and contagious. They can be spread when an infected person is producing and shedding the virus. The present study was carried out to determine the effect of alcoholic extract of two herbs *Eucalyptus globulus* and *Artemisia draconculus* on herpes virus compare with acyclovir in Iran during 2011. After preliminary survey, two herbs *Eucalyptus globulus* and *Artemisia draconculus* were selected as a drug for the treatment of herpes virus. HSV−1 was isolated from patients and identified by specific monoclonal antibodies. Vero cells (African green monkey kidney cells) were cultured with Dulbecco's Modified Eagles' Medium (DMEM) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 100 IV/mL penicillin and l μg/mL streptomycin. Finally, the effect of these plants and Acyclovir compared with together on herpes virus. Results showed *Artemisia draconculus* could not reduce viral plaques significantly, however methanolic extracts of *Eucalyptus globulus* had a significant inhibitory effect against HSV−1 and concentration (200, 150, 50 μg/mL) has the best effect and (>200 μg/mL) Has lowest effect on HSV−1.The comparison of results exhibited that *Eucalyptus globulus* extract has more effects in different dilutions against HSV−1 in cell culture.

Keywords Herpes Virus; *Eucalyptus globulus*; *Artemisia draconculus*; Acyclovir

Background

The structure of herpes viruses consists of a relatively large double-stranded, linear DNA genome encased within an icosahedra protein cage called the capsid, which is wrapped in a lipid bilayer called the envelope (Roller et al., 2008). The envelope is joined to the capsid by means of a tegument. This complete particle is known as the virion. HSV−1 and HSV−2 each contain at least 74 genes (or open reading frames, ORFs) within their genomes, although speculation over gene crowding allows as many as 84 unique protein coding genes by 94 putative ORFs (Yang et al., 2012). These genes encode a variety of proteins involved in forming the capsid, tegument and envelope of the virus, as well as controlling the replication and infectivity of the virus (Xu et al., 1999). These genes and their functions are summarized in the table below. Cold sores, sometimes called fever blisters, are groups of small blisters on the lip and around the mouth. The skin around the blisters is often red, swollen, and sore. The blisters may break open, leak a clear fluid, and then scab over after a few days. They usually heal in several days to 2 weeks (Chuanasa et al., 2008). Cold sores are caused by the herpes simplex virus (HSV). There are two types of herpes simplex virus: HSV−1 and HSV−2. Both virus types can cause sores around the mouth (herpes labialis) and on the genitals (genital herpes) (Fatahzadeh and Schwartz, 2007).The herpes simplex virus usually enters the body through a break in the skin around or inside the mouth. It is usually spread when a person touches a cold sore or touches infected fluid-such as from sharing eating utensils or

razors, kissing an infected person, or touching that person's saliva (Friedman, 2006). A parent who has a cold sore often spreads the infection to his or her child in this way. Cold sores can also be spread to other areas of the body. The first symptoms of cold sores may include pain around your mouth and on your lips, a fever, a sore throat, or swollen glands in your neck or other parts of the body. Small children sometimes drool before cold sores appear (Gershon et al., 2010). After the blisters appear, the cold sores usually break open, leak a clear fluid, and then crust over and disappear after several days to 2 weeks. For some people, cold sores can be very painful. Cold sores will usually start to heal on their own within a few days. But if they cause pain or make you feel embarrassed, they can be treated. Treatment may include skin creams, ointments, or sometimes pills (Carson et al., 2008). Treatment may get rid of the cold sores only 1 to 2 days faster, but it can also help ease painful blisters or other uncomfortable symptoms. The herpes simplex virus that causes cold sores cannot be cured. After you get infected, the virus stays in your body for the rest of your life (Chuanasa et al., 2008). Docosanol, a saturated fatty alcohol, is a safe and effective topical application that has been approved by the United States Food and Drug Administration for herpes labialis in adults with properly functioning immune systems. It is comparable in effectiveness to prescription topical antiviral agents. Due to its mechanism of action, there is little risk of drug resistance (El Sayed, 2000). The duration of symptoms can be reduced by a small amount if an antiviral, anaesthetic or non-treatment cream (such as zinc oxide or zinc sulfate) is applied promptly (Chattopadhyay and Khan, 2008). Effective antiviral medications include acyclovir and penciclovir, which can speed healing by as much as 10%. Famcyclovir or valacyclovir, taken in pill form, can be effective using a single day, high-dose application and is more cost effective and convenient than the traditional treatment of lower doses for 5~7 days (Garozzo et al., 2011). Lysine has been suggested as a treatment for herpes labialis based on *in vitro* studies, but the evidence is inconclusive in humans (Gebre-Mariam et al., 2006). Herpes simplex virus (HSV) infections are efficiently treated with antiviral drugs such as acyclovir (ACV). However, resistance

has been reported, mainly among immunocompromised patients (prevalence around 5%) and particularly allogeneic bone marrow transplant patients (prevalence reaching 30%) (Hammer et al., 2006). Resistance to ACV is associated with mutations on one of the two viral enzymes involved in the ACV mechanism of action: thymidine kinase (TK) and DNA polymerase. In 95% of the cases, ACV resistance is associated with a mutation in the TK gene as this enzyme is not essential for viral replication, unlike viral DNA polymerase, which is rarely involved in resistance (Hosono et al., 2008). Today, some of the herbal extracts used in the treatment of herpes sores. In this study the influence of alcoholic extract of the herb tarragon and eucalyptus compared with the drug acyclovir is reviewed.

1 Results

Trypan blue exclusion method showed that the *Eucalyptus globulus* and *Artemisia draconculus* extract had no serious effect on the proliferation of cells, up to concentration of 800 μg/mL (data not shown). Therefore, we could draw a conclusion that the CC50 (the concentration which causes 50% cytotoxicity effect) was more than 800 μg/mL.

Antiviral activity of the extract by TCID50 assay the inhibition of virus yield by *Eucalyptus globulus* and *Artemisia draconculus* extract was evaluated by TCID50 assay in Vero cells. *Eucalyptus globulus* extract showed strong antiviral activity against HSV−1 when added during the early stages of viral infection.

The degree of inhibition showed to be proportional to the concentration of the extract and when the concentration was higher than 200 μg/mL *Eucalyptus globulus* extract inhibited almost completely the virus yield. The EC50 value of the extract when treated during infection was 650 μg/mL and when treated after infection the EC50 decreased to 200 μg/mL, then the extract was more effective at low concentrations when present after viral infection. The results showed that compared to control wells with no *Artemisia draconculus*, different concentrations of aqueous and hydroalcoholic extracts of *Artemisia draconculus* could not reduce viral plaques significantly (data not shown), however methanolic extracts of *Eucalyptus*

globulus had a significant inhibitory effect against HSV−1. Results of cytotoxicity assays showed that the CC50 of aqueous methanolic extracts of *Eucalyptus globulus* were 200 μg/mL, 150 μg/mL, 50 μg/mL respectively. When applied one hour before Vero cell infection with HSV−1, methanolic Eucalyptus globulus at the concentration of 200 μg/mL, 150 μg/mL, 50 μg/mL caused a significant ($P<0.05$) reduction in viral plaques compared to the control samples (Figure 1; Figure 2).
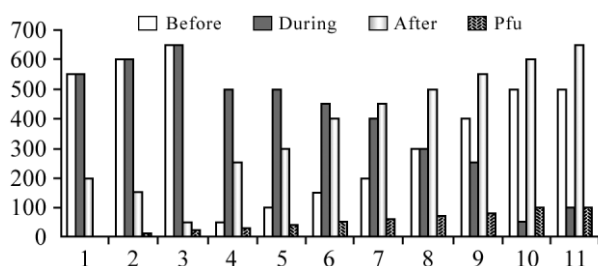

Figure 1 Incubation of cells with the *Eucalyptus globulus* extract before, during and after virus infection
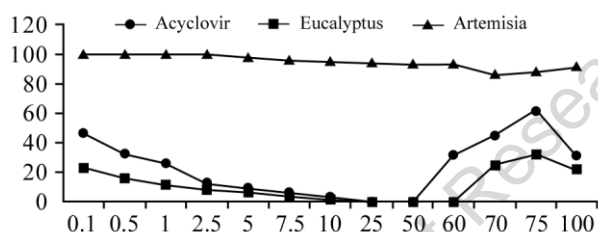

Figure 2 Effect of increasing concentration of *Eucalyptus globulus* and *Artemisia draconculus* extract and acyclovir on the titer of HSV−1

2 Discussion

Antiviral agents licensed currently for the treatment of herpes virus infections include acyclovir and derivatives, foscarnet and cidofovir, all of which inhibit herpes virus DNA polymerases (Khan et al., 2005). Some of these antiviral agents might produce toxic side-effects. In addition, the emergence of virus strains resistant to commonly used anti-herpes virus drugs is of importance, particularly in Immunocompromised patients (Ju et al., 2011). The development of viral resistance toward antiviral agents enhances the need for new effective compounds against viral infections. Thus, new antiviral agents exhibiting different mechanisms of action are urgently needed

(Knickelbein et al., 2009).

Medicinal plants produce a variety of chemical constituents with the potential to inhibit viral replication and compounds from natural sources are of interest as possible sources to control viral infection (Koch et al., 2008). These plants have been widely used to treat a variety of infectious and non-infectious diseases and represent an abundant source of new bioactive secondary metabolites. Thus plants continue to be a major source of new lead compounds (Batish et al., 2008). Besides small molecules from medicinal chemistry, natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases (Wei and Shibamoto, 2010).

In recent years, tendency of people for using traditional medication and medical plants have been arisen and even these remedies have been chosen as an alternative of industrialized medicine.

Herpes virus is one of common factors to produce different diseases in human body. Complications of herpes virus such as blister, skin rash, and meningitis and brain encephalitis can be pointed out.

According to acyclovir therapy against HSV virus, sometimes because of drug resistance or some restrictions in using industrialized drugs such as pregnancy, thinking about alternative therapies need to be felt.

Eucalyptus globulus and *Artemisia draconculus* are two species of native herbs in Iran and the history of their usage goes back to many years ago. In different parts of Iran they can be found and their frequency is high, so they can be used as an alternative treatment.

In this study the effects of two traditional herbs (*Eucalyptus Globulus* and *Artemisia Draconculus*) and acyclovir on herpes virus−1 were evaluated and their efficacy to suppress HSV−1 was reported. Based on results *Eucalyptus globulus* extract has more effects in different dilutions against HSV−1 in cell culture. According to our study, concentration (200 μg/mL, 150 μg/mL, 50 μg/mL) has the best effect and (>200 μg/mL) has lowest effect on HSV−1. About *Artemisia draconculus*, after varied examinations

in Vero cell line we could not find any inhibition effect against HSV−1 in different dilutions.

To conclude, because of the widespread *Eucalyptus globulus* consumption in different countries especially in Iran, the extraction of this plant can be used as an alternative treatment. For drug production, air spray or a tropical ointment instead of industrialized drugs such as acyclovir would be suggested in many cases and of course in all of these studies, dose of herbs and the best way of consumption should be tested before using them to increase their positive effects in patients.

3 Material and Methods

3.1 Cell culture and Virus

Vero cells (African green monkey kidney cells) were cultured with Dulbecco's Modified Eagles' Medium (DMEM) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 100 IV/mL penicillin and l µg/mL streptomycin. The cells were maintained at 37℃ in a humidified atmosphere with 5% CO_2 and were subculture two or three times a week. HSV−1 was isolated from patients and identified by specific monoclonal antibodies. Viruses were quantified in terms of the 50% tissue culture infective dose (TCID50) by endpoint dilution, with the infectious titer determined by the method of Reed and Munch (14), and stored in small aliquots at −70℃ until use.

3.2 Virus stock

HSV−1 was isolated from the lip lesions of a patient and its genus was confirmed by neutralization test using guinea pig anti-HSV−1 serum and monoclonal anti-HSV−1 antibodies against HSV glycoprotein's D and G.

3.3 Plant materials

*Eucalyptus globulu*s and *Artemisia draconculus* were collected from a farmland in Kerman and identified at the department of Virology of Kerman university of Medical Sciences. The dried leaves were pulverized and 200 gr of pulverized sample was extracted with 500 mL of 80% methanol by maceration for 72 h. The methanol extract was concentrated in a rotary evaporator, lyophilized and thereafter preserved for further use. *Eucalyptus globulus* and *Artemisia draconculus*

stocks were prepared by dissolving 10 mg of each extract in one mL distilled water. It was then sterilized by filtration.

For cytotoxicity and antiviral assays, the stock solutions of *Eucalyptus globulus* and *Artemisia draconculus* were diluted in the maintenance medium of Dulbecco's modified Eagle's growth medium (DMEM, Sigma) supplemented with 2% fetal bovine serum (Gibco, Germany), 0.14% (v/v) sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin sulphate, and 0.25 µg/mL amphotericin B.

3.4 Cytotoxicity assay

In order to test the effect of the Eucalyptus globulus and Artemisia draconculus extract on vero cells, 5×10^4 cells, (in l mL DMEM, supplemented with 10% FBS) were seeded in to each well of micro plates, cultured for 6 hr at 37℃, cells were allowed to grow for additional 48 h in the presence of increasing amounts of extract (10 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL, 600 µg/mL, 800 µg/mL and 1 000 µg/mL), The cytotoxicity of the extract was determined on a conventional hemocytometer using the trypan blue exclusion method. The 50% cytotoxic concentration (CC50) was defined. As the concentration, which caused a 50% reduction in the number of viable cells.

3.5 Incubation of cells with the extract before, during and after virus infection

Eucalyptus Globulus and *Artemisia draconculus* hydroalcoholic extract was dissolved in serum free DMEM and incubated with semi-confluent cell in 24 well tissue culture plates in increasing concentration from 10 to 1 000 µg/mL for 2 h at 37℃. After removal of the extract, the cells were washed with phosphate buffered saline (PBS) and then infected with HSV−1 at multiplicity of infection (MOI) 1. After l h incubation, the unabsorbed virus was removed, the cell monolayer was washed with PBS and further incubated in DMEM with 2% FBS. Controls consisted of Vero cells untreated and Vero cells infected with HSV1.

For determination of antiviral activity of the extract during and post virus infection the assay was performed as described above, with the exception that the extract was added together with the virus and after

adsorption, correspondingly. After 48 h incubation at 37℃, virus titer was determined by the endpoint dilution method and expressed as TCID50 /mL.

EC50, the concentration needed to restrain 50% virus infection compared to untreated infected cells, were determined directly from the curve obtained by plotting the inhibition of the virus yield against the concentration of the samples.

3.6 Statistical analysis

The mean number of viral plaques of two different experiments was compared with oneway analysis of variance (ANOVA) using SPSS v11.5. Dunnett test was used as the post hoc test. A $P<0.05$ was considered statistically significant.

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