

## Acyclovir and Propolis: Which have a Best Anti HSV-1 Effect?

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(Received: 21 April 2015; accepted: 20 July 2015)

Herpes simplex virus 1 (HSV-1), a neurotropic virus, commonly infects the skin and mucous membranes, and remains latent before erupting in response to different stimuli. HSV 1 is a pathogen of children and adults responsible for several disorders. Nucleoside analogues such as acyclovir and penciclovir are some drugs approved for the treatment of Herpes simplex virus. Nevertheless, these agents may cause a variety of toxic side effects, and viral strains resistant. Consequently, there is an increasing need for the discovery of more specific antiviral agents effective against the herpes simplex virus. In this laboratory experimental study, Various concentrations of propolis mouthwash (2/1; 4/1; 8/1) were added to cell monolayers. In order to investigate mouthwash antiviral effect and find out its inhibitory function, once before the entry of the virus into the cells (first method) and in the next step one hour after the addition of virus to cells (second method) during given times was added to the medium. Various concentrations of propolis at times 0/5, 1, 5 min (3, 5) with PFU/ml, 50 viruses were exposed and then were transferred to containers 96 cavities containing cell monolayer. In order to investigate the effect of drug type, one way ANOVA variance analysis statistical was used. In next step two-way ANOVA variance analysis statistical test was used to evaluate the effect of drug type with the study of the effect of concentration and time. (A) Before the entry of the virus into the cell: The greatest virus titer in the solution containing propolis is related to the concentration of 1.8 and for 1 minute, the solution containing acyclovir at times 0.5 and 1 min. (B) After the entry of the virus into the cell: The greatest virus titer in solutions containing propolis is related to the concentration of 1.4 and at 0.5 min, and in the solution containing acyclovir at 0.5 and 1 min. The role of propolis is prevention of virus entrance to intracellular, interaction with vero cell membrane, conversion of viral envelope, or direct on free virions of HSV.

**Key word:** Propolis, HSV-1, acyclovir.

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Herpes simplex virus 1 (HSV-1), a neurotropic virus, commonly infects the skin and mucous membranes, and remains latent before erupting in response to different stimuli<sup>1</sup>. Lesions are often painful, burning or pruritic and tend to recur in most patients<sup>2</sup>. HSV 1 is a pathogen of children and adults responsible for several

disorders. Including gingivo-stomatitis, pharyngitis, keratoconjunctivitis and encephalitis<sup>3</sup>.

Despite the symptoms caused by herpes infections are self limiting in healthy individuals, these can be extensive and prolonged in immunocompromised patients<sup>4</sup>. Nucleoside analogues such as acyclovir and penciclovir are some drugs approved for the treatment of Herpes simplex virus<sup>3</sup>.

Nevertheless, these agents may cause a variety of toxic side effects, and the emergence of viral strains resistant to these compounds could

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be considered as a growing problem, especially in immuno compromised patients<sup>5</sup>.

Consequently, there is an increasing need for the discovery of ,more specific antiviral agents effective against the herpes simplex virus. Natural products like plant extracts are very promising sources of compounds with antiviral activity. Due to the very low toxicity that the show for cells, and also due to the great variety of chemical constituents they have<sup>6</sup>.

Propolis has been known as a natural brownish – green resinous product collected by honey bees from part of trees and shrubs<sup>7</sup>. It is a combination of resin, essential oils waxes, and also includes amino acids, minerals, ethanol, vitamin A, B complex, E and flavonoids<sup>8</sup>.

The most important pharmacologically active constituents in propolis are flavonoids<sup>9</sup>. Flavonoids are well known compound that have antibacterial, antifungal, antiviral and anti-inflammatory properties<sup>9</sup>.

The flavonoids found in propolis caused a reduction of intracellular replication of herpesvirus strains<sup>10</sup>. The antiviral effect of ethanolic extract of propolis and selected constituents, e.g. caffeic acid, galangin, acacetin, kaempferol, chrysin and quercetin against HSV was analyzed in cell culture<sup>9</sup>.

A randomized single- blind comparative study concluded that the bee product propolis was more effective in the treatment of genital herpes than either placebo or acyclovir, a herpes drug marketed under the trade name Xouirax<sup>5</sup>.

Another study characterized the Experimental bases of Brazilian Propolis treatment using an HSV 1 infection model in mice. Together,

all results indicate that Brazilian propolis showed not only direct anti-HSV1 activity but also immunological activity against intradermal HSV-1 infection in mice. Especially, the immunological activity associated with IFN-g production – inducing Th-1 immunity in mice may contribute to the elucidation of various pharmacological actions of propolis in health and disease<sup>6</sup>.

The current study investigated the antiviral activity of an aqueous and a special ethanol propolis extract named GH 2002 against herpes simplex virus type 1. Both extracts were prepared from propolis, well characterized in respect to its botanical and geographical origin as well as chemical composition<sup>8</sup>.

The aim of this study was the evaluation of effect of acyclovir and aqueous extract of propolis on HSV 1 in cell culture.

## MATERIALS AND METHODS

### The source of the virus and cell culture system

In this laboratory experimental study, HSV-1 virus was isolated from patient lip lesion with recurrent herpes labialis and was confirmed by neutralization test using an anti- HSV-1 pig antiserum (NIH, USA) .

Vero Cells (African green monkey kidney fibroblasts cell - cell bank of Pasteur Institute of Iran) that are appropriate cells for Cytopathic effect or CPE of Herpes simplex virus . according to following standard method were prepared. Vero Cell of cell culture medium, in cell culture flasks (Nunc, Denmark), which contains joined cell monolayer after washing by PBS (Phosphate-buffered saline), was trypsinized . After that, DMEM

**Table 1.** The virus titer (mean  $\pm$  SD), herpes type 1 in solutions containing propolis mouthwash and acyclovir at the stage before the entry of the virus in cell based on drug concentration and time

Drug type	Concentration	Time m		
		5/0	1	5
Propolis	2/1	13/0 $\pm$ 45/1	25/0 $\pm$ 73/2	11/0 $\pm$ 05/2
	4/1	67/0 $\pm$ 45/1	82/0 $\pm$ 70/2	6/0 $\pm$ 2
	8/1	51/0 $\pm$ 98/1	01/0 $\pm$ 75/2	87/0 $\pm$ 70/1
Acyclovir		62/0 $\pm$ 13/2	62/0 $\pm$ 13/2	24/0 $\pm$ 96/1
Control		70/4	70/4	70/4

Numbers in the table are the log of virus titer.

(Dibeccoo's modified Eagle's fresh growth medium) containing 5% FBS or Fetal bovine serum was added to the flask, and by frequently pipetting the liquid in the flask to suspend the cells in medium and cell aggregation breakage, homogenous cell suspension was prepared. The cell suspension is poured in new cell culture flasks to the cells attached to the bottom of the container and after proliferation and division; again cell complete monolayer is formed. For cells proliferation, cells every 3 days once in the same manner in passaged cell culture flasks were amplified.

#### Determination of cytotoxicity

Vero cells were cultured by above method in 24 holes sterile containers (Nunc, Denmark) in DMEM culture medium containing 8% fetal calf serum, 14% sodium bicarbonate,  $\mu$ /ml 100 penicillin,  $\mu$ /ml 100 streptomycin sulfate and 25/0 mg/m amphotericin B, after washing cell monolayers with PBS, increase serial concentrations (2/1; 4/1; 8/1) of propolis mouthwash were added to cavities with cell monolayers. After staining with Trypan blue dye exclusion method, during 5 min, 30 min, 1 h and 24 h, cells were counted. The mean and percentage of viable cells were calculated in cavities with mouthwash and 50% concentration of cytotoxicity (50 cc) was determined (regression).

#### Examining anti-HSV effect of propolis mouthwash

Various concentrations of propolis mouthwash (2/1; 4/1; 8/1) were prepared by holder medium (DMEM containing 2% fetal calf serum) and after washing with PBS, were added to cell monolayers. In order to investigate mouthwash antiviral effect and find out its inhibitory function, once before the entry of the virus into the cells

(first method) and in the next step one hour after the addition of virus to cells (second method) during given times was added to the medium. Various concentrations of propolis at times 0/5, 1, 5 min (3, 5) with PFU/ml, 50 viruses were exposed and then were transferred to containers 96 cavities containing cell monolayer. Controls for both methods include monolayers containing acyclovir (Merck, Germany) (positive control) and cells received no treatment (negative control). All containers for 4 days at 37 ° C and 5% Co<sub>2</sub> were placed in incubator. In order to investigate antiviral effect of the above solution, quant method (determination of infective dose 50% of Tissue culture or TCID<sub>50</sub>) were used. In this method the containers were investigated under inverted microscope, the cavities according to presence or absence of CPE were labeled as positive or negative. Finally, also virus titer (TCID<sub>50</sub>) was calculated by Karber method (12 and 15). All stages of the experiment were repeated twice, and the mean of calculated numbers were analyzed statistically as the final virus titer.

In order to investigate the effect of drug type (regardless of its concentration and time) one way ANOVA variance analysis statistical test (one way ANOVA) was used. In next step two-way ANOVA variance analysis statistical test was used to evaluate the effect of drug type with the study of the effect of concentration and time. Then, if there is difference in different levels of drug type, concentration and time, in order to reveal the exact differences contrast method was used ( $0/05 > \hat{\alpha}$ ), all statistical analyzes were performed using the software SPSS16.

**Table 2.** The virus titer (mean  $\pm$  SD), herpes type 1 in cells containing propolis mouthwash and acyclovir at stage after entry of the virus into the cell based on drug concentration and time

Drug type	Concentration	Time m		
		5/0	1	5
Propolis	2/1	25/0 $\pm$ 12/1	64/0 $\pm$ 95/0	42/0 $\pm$ 82/0
	4/1	16/0 $\pm$ 87/4	11/0 $\pm$ 82/3	0 $\pm$ 04/2
	8/1	61/0 $\pm$ 12/4	18/0 $\pm$ 09/3	71/0 $\pm$ 67/3
Acyclovir		0 $\pm$ 36/0	0 $\pm$ 36/0	0 $\pm$ 18/0
Control		14/5	14/5	14/5

Numbers in the table are the log of virus titer

### Findings

- The results of cell toxicity of acyclovir and propolis investigation with different concentrations showed that cytotoxic concentration which led to destroy 50% of monolayer cells (CC50), was not available in studied concentrations.
- To assess the antiviral effects of propolis mouthwash before and after the entry of the virus in the cell, virus concentration was measured and analyzed.

#### Before the entry of the virus into the cell

The greatest virus titer in the solution containing propolis is related to the concentration of 1.8 and for 1 minute, the solution containing acyclovir at times 0.5 and 1 min (Table 1).

In next step in two-way variance analysis it was revealed that in each of the concentrations of 1/2, 1/4, 1/8 drug type had a statistically significant effect on virus titer (p value <0/001); so that virus titer was the lowest amount in acyclovir solution. It was also found that the "time" had a statistically significant effect on virus concentration (p value <0/001) so that the highest concentration of virus was in 0.5 minutes. Later it was found that interactions (interactions) factors "drug type" and "time" also had a statistically significant effect on virus titer (p value <001/0).

#### After the entry of the virus into the cell

The greatest virus titer in solutions containing propolis is related to the concentration of 1.4 and at 0.5 min, and in the solution containing acyclovir at 0.5 and 1 min (Table 2).

In the next step in two-way ANOVA analysis it is revealed that in concentration of 1.2, "drug type" had a statistically significant effect on virus titer (p value=0/011) so that virus concentration in the solution of acyclovir was the lowest. Also "time" and factors interaction "drug type" and "time" had statistically significant effect on the virus concentration (0/05> p value).

At concentrations of 1.4 and 1.8 "drug type", "time" and factors mutual effect "drug type" and "time" had no statistically significant effect on the virus titer (0/05> p value).

At concentration of 1.8, "drug type" had a significant effect on virus titer (0/06 <p value) so that the virus titer in the solution containing acyclovir was the lowest but "time" "time" mutual effect and "drug type" had statistically no significant effect on the virus titer (05/0 <p value).

### DISCUSSION

Use of oral rinse in treatment or prevention of several oral Diseases enumerate as a simple, In expensive and acceptable method for patient's of common oral diseases, recurrent intraoral herpetic infections, unexplained standard topical dray for its treatment yet. Now routine treatment of oral herpetic infection is the use of topical or oral acyclovir. In as much as possibility of drug resistance in some patients, for exp. Immuno suppressive patients, therefore attain the oral rinse with antiviral activity and minimum adverse effects, can improve considerably remedy of these lesions.(2,6)

In this study we were investigating the antiviral activity and mode of action of propolis extract in comparison with acyclovir.

Several concentrations of propolis extract (1/2, 1/4 , 1/8) were serially added to the DMEM (Dul becco's modified minimal essential medium) in different periods of time (0.5 , 1 , 5 minutes), one phase prior to virus inoculation to DMEM (First way), and next phase 1 hour after virus inoculation to DMEM (Second way), to examine the anti-HSV activity and determination of cyto toxicity.

Control groups for two phases were cell mono layers containing Acyclovir (Positive control), and cells without treatment (Negative control). Cytotoxic concentration, that results in 50% destruction of mono layers cells (cc50), was not in involved concentrations, for both Acyclovir and propolis.

Honey is cheap and readily available as compared to acyclovir. It would be interesting to proceed with studies that would include the effects of honey on viral shedding. (4)

One of the most common oral and extraoral lesions caused by viruses is RHL. Although, RHL is self-limiting, the use of antiviral medications can reduce shedding, infectivity, Pain, size and duration of lesions. (1) For many years, significant efforts have been made to identify the antiviral agent with different mechanism of action.

Propolis Posses different mechanism of action and it might be a good agent against HSV. The antiviral effect of flavonoids and selected constituents were induced by cytotoxicity on HSV-1 infected cells.(5,6)

Chrysine and keampferol caused a

concentration-dependent reduction on cell growth and viability, whereas quercetin reduced infectivity and intracellular replication (2).

Antiviral activity of an aqueous extract of propolis has been previously shown in vitro and in an animal model of herpes simplex virus (HSV) infection by Hweihel and Isanu. The same authors observed that addition of the propolis extract to a cell culture before or at the time of viral infection completely blocked development of infection, thus concluding that propolis could block the cell membrane receptor for HSV or induce internal changes in the host cells, which in turn affect the virus replication cycle. (11)

Amoros et al investigated the in vitro antiviral activity of resin balsam against HSV and could detect a virucidal effect when herpes virus was pretreated with propolis, but pretreatment of cells with propolis did not inhibit viral replication (7)

Schnitzler suggest propolis extracts interfere with virion envelope structures or are masking viral compounds which are necessary for absorb or entry into host cells. Apparently, free herpes virus is very sensitive to propolis extracts and the inhibition of HSV-1 appears to occur before entering the cell but not after penetration of the virus (9).

Though acyclovir is a safe drug for the treatment of primary and recurrent herpes, it may induce nephro toxicity and neurotoxicity. Cases of allergic dermatitis have been encountered with the use of acyclovir. (1)

For evaluating of anti-HSV activity of propolis in comparison with Acyclovir in two ways explained, total virus titre evaluated in Karber method.

When HSV-1 was incubated with the propolis extract prior to virus inoculation of the cells, characterized that after 0.5 min of incubation in all concentrations of propolis, herpes virus titre was strongly reduced in comparison with positive control group and after 1 min of incubation in all concentrations, herpes virus titre was increased. 5 min of incubation, herpes virus titre was reduced a bit, but for acyclovir the values 0.5 and 1 min after incubation were equal and reduced some what 5 min after incubation, whereas the values for Negative control group were the most. There for

the effect kind of drug in virus titre is significant and Acyclovir had maximum titre of virus.

Also, the effect of time in virus titre is significant (time- dependent activity), and maximum virus titre was in 0.5 and 1 min. In continuous, the effects of interaction of kind of drug and time in virus titre are significant, In other wise, propolis with 1/2 , 1/4 concentrations and 0.5 min of incubation has more efficacy in comparison with Acyclovir. (Prior to virus inoculation to cell)

After virus inoculation of the cells, the use of acyclovir demonstrated strongly reduced titer of herpes virus in comparison with all concentrations of propolis, and among of all concentrations of propolis, concentration of 1/2 propolis was more significant in comparison it can be said that antiviral effect of propolis at the First stage (Prior to virus inoculation to cell), is more prominent than acyclovir but later (after entrance of viruses in to the cells), inhibitory effect of acyclovir is more significant than propolis. ( $P < 0.05$ )

These differences, represent manner of antiviral action of propolis and acyclovir. Inasmuch as acyclovir acts as inhibition of viral DNA Polymerase, which occurs after activation by viral thymidin kinase, there for the most effective is on intracellular virus, reciprocally, propolis, acts as inhibitory effect prior to the entrance of virus into the cell.

Consequently, may be attribute the role of propolis to prevention of virus entrance to intracellular, interaction with vero cell membrane, conversion of viral envelope, or direct on free virions of HSV. Regarding to the findings of this study, and usefull effect of propolis oral rinse on free viruses, can be used for reduction of viral contamination in mouth fluids (after 30 sec of gargling) and consequently reduction of cross-contamination risk of oral fluids in personal contact, and produced Aero cells within Dental Procedures, in Asymptomatic Shedding of virus, also reduction of infectivity of herpetic oral lesions. More over, propolis acts as inhibitory effect without any need for viral thymidin kinase, there for it can be considered as new found option in topical treatment of HSV lesions, that resistant to acyclovir, especially in patient's with immune system defects, such as HIV+ patient's.

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