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Original Article

Anti-viral Effect and Mechanism of Carvacrol on Herpes Simplex Virus Type 1

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Background and Aims: Herpes simplex virus type 1 (HSV-1) belonging to herpes viridae family is a normal human pathogen with benign lesions in immunocompromised patients which creates serious problems. Acyclovir is used to treat herpetic infections. Because of developing drug-resistant strains, the use of medicinal plants and their related compounds with fewer side effects is considered for the treatment of patients. In this study the antiviral effect and mechanism of carvacrol, a medicinal compound, on HSV1 virus was studied.

Materials and Methods: In this study, the maximum nontoxic concentration on vero cells was determined by MTT. The antiviral effect of the compound was determined by TCID50. The expression of early and late stages of viral replication (*UL52* and *UL27*) was evaluated by real time polymerase chain reaction method.

Results: The toxic concentration of carvacrol causing 50% cell death was 0.001%. The mechanism of action of the compound showed that pretreatment of HSV-1 with carvacrol prior to infection inhibits its ineffectivity approximately to 70%. The results also showed no reduction in the early and late gene expression of herpes virus replication.

Conclusions: Overall, the findings demonstrated that the carvacrol has inhibitory effect on HSV-1 by direct inhibition of free virus particles.

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Introduction

Herpes viruses are a large family of DNA viruses with an icosahedral capsid and genomes encoding 100-200 genes. These viruses have similar morphology and replication and can make a latent and recurrent infection. Herpes simplex viruses are normal human pathogens with benign lesions but sometimes cause lethal diseases. Herpes simplex virus type 1 (HSV-1) is a member of this family which has a short growing cycle and the latency in neurons [1]. After initial infection, these viruses tend to remain dormant in sensory ganglia, so that if the patient is placed under adverse conditions such as stress, sun exposure, radiation, ultraviolet, pressure, fever, hormonal factors and so on, or the immune system is suppressed or weakened, latent virus becomes active. Primary infection occurs due to skin, mucous membranes and eye contact with infected secretions. The virus further causes wounds around the mouth, herpes labialis, scorpion disease, corneal inflamm-ation and encephalitis [1, 2].

Viral replication begins by binding virus to specific receptors. After penetration and removing the coverage, the genome is released and the expression of Immediate early, early and late last is done. Finally virus is accumulated and released, which can be accompanied by cell lysis. Two of the most important genes involved in virus replication process are early and late genes (*UL52* and *UL27*, respectively) [3]. One of the drugs used in the treatment of injuries resulting from this virus is acyclovir. It is activated by viral thymidine which controls viral DNA polymerase and prevents viral replication in infected cells. Most chemical treatments against herpes simplex infections, which target viral proteins and involve in DNA synthesis, are almost successful in the treatment of infection caused by the virus. Nowadays, due to the mutant viruses without this enzyme, resistance to it especially in immuno-compromised people is increasing [2]. Therefore, the development of new drug compounds in order to treat diseases caused by herpes simplex virus is essential. Medicinal plants are appropriate choices for antiviral effect due to their low side effects on human. Carvacrol (2-methyl-5-1-methyl-ethyl phenol) is a phenol monoterpenoid that has shown by the previous studies to have a broad antimicrobial activity on pathogenic fungi, yeast, viruses, and bacteria [4]. Studies have indicated that monoterpenoid compounds such as carvacrol, in addition to savoring antimicrobial and anti-fungal activity are effective in anti-tumor and anti-cancer conditions [1, 5]. The antiviral effects on carvacrol has been tested on other viruses such as rotavirus and human respiratory syncytial virus [3]. In this study we investigated antiviral activity of this compound on HSV-1 and mechanism of the action of virus and its possible impact on the various stages of replication.

Materials and Methods

Carvacrol was prepared from department of physiology, in Shahid Sadoughi University of

Medical Science, in Yazd, Iran. Stoke concentration was 1 mg/ml which was dissolved in dimethyl sulfoxide (DMSO) and for all experiments final concentration of DMSO was below 1% with no effect on virus and cells. Acyclovir was prepared from Amin Pharmaceutical Company in Iran and was dissolved in distilled water to make a stock concentration of 100 µM [6]. This study was approved by Ethics Committee of **S**hahid Sadoughi University of Medical Sciences, Yazd, Iran.

Provision and maintenance of cell lines

Vero cells (National Center of Genetic and Biological Resources, Iran) that were prepared under the supervision of Academic Center for Education, Culture and Research (ACECR) University, were grown in monolayer culture with Dulbecco´s Modified Eagle´s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (all Gibco, Karlsruhe, Germany) and was kept in incubator with 5% CO_2 at 37°C [6, 7].

Preparation and virus replication of HSV-1

HSV-1 strain KOS (Tarbiat Modares University, Iran) was used for experiments. HSV-1 stock cultures were prepared from supernat-ants of infected cells and were stored at -70°C. Infectivity titers were determined by a tissue culture infectivity dose ($TCID_{50}$) method [8].

Cytotoxicity test

In order to achieve a concentration of drug which was nontoxic on vero cells, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) test was used. In this test, cells were seeded into the 96 well plates. Plates were kept in incubator at 37°C with 5% CO_2 for 24 hrs. Then, the cells were washed with preheated phosphate buffered saline (PBS), and serial dilution of carvacrol from 0.1% to 0.00001% was added to vero cells. After 48 hrs of incubation at 37°C, MTT dye uptake was determined by measuring the optical density at 570 nm in a spectro-photometer (Biotek Instrument Model: Box 998, United States). Wells containing medium with 1% DMSO were used as control [9].

Determine the viral titer by $TCID_{50}$ method

To determine the appropriate concentration of the virus that makes pathological change in 50% of the cells, $TCID_{50}$ method was used. Virus infectivity was quantified by estimating the 50% $TCID_{50}$ using standard cell culture procedures. Briefly, when cells reached 80% confluency 96-well microtitre plates, six replicates were infected with 1.8 ml DMEM combination without serum as a diluents and 0.2 mol virus, and then were incubated for 1 hr at 37°C in a humidified atmosphere of 5% CO_2 until viruses were absorbed into the cell. Afterwards, the media was removed and DMEM containing 2% FBS was added into 96-well plates and incubated for 48 hrs at 37°C. Cytophatic effect was checked by an inverted microscope (ACCU-SCOPE, United States) and infectivity titers were expressed as $TCID_{50}$/ml based on the Karber formula. The virus infectivity was then compared and analyzed to determine the optimal sample formulation under the different conditions of preparation and storage [10, 11].

Antiviral activity

The antiviral activity of carvacrol was evaluated with $TCID_{50}$ assay. Different concentration of the drug was incubated with virus for 1 hr at 25°C. Then the resulting compound was inoculated to the cells for 1 hr at 37°C, and then the cells were subjected to $TCID_{50}$ assay. The 50% inhibitory concentration (IC_{50}) of the compound was evaluated from dose response curves [12-14].

HSV-1 incubation with carvacrol prior to infect vero cells with virus

In this experiment, the ability of drugs to bind to the virus and change their ability to infect cells was tested. For this purpose, the desired concentration of HSV-1 virus with non-toxic concentrations of carvacrol was incubated for 1 hr at room temperature and then was added to the cells and removed from the cells and media containing 2% FBS was added to the cells. The results were recorded after 24 to 48 hrs by $TCID_{50}$ assay [15]. Beta pinene was used as a monoterpene compound control which had been prepared from Carl Roth (Karlsruhe- Germany). This compound is solved in ethanol; during testing we noted that final ethanol concentration should not exceed 1%.

Treatment of vero cell before infecting cells with HSV-1

Drugs were examined for their ability to bind to cell receptors and to prevent the virus. For this purpose cells and drug were incubated for 1 hr at 37°C. Then the cells were washed and HSV-1 with defined concentration of carvacrol were added to the cells and were incubated for 1 hr at 37°C. Afterwards, the medium were removed, and DMEM medium containing 2% FBS were added and the cells were checked after two days.

Vero cell incubation with carvacrol after cell infection with HSV1

The ability of combination in affecting virus infection in intercellular were tested. After the penetration of the virus to infected cells, drug was added to cells. Wells containing medium with 1% DMSO but no compound were also used as control. After two days, cells were subjected to $TCID_{50}$ assay [16].

Attachment assay

To determine antiviral effect of carvcrol on attachment of virus, vero cell monolayers were grown in 24-well culture plates and then prechilled at 4°C for 1 hr. After aspirating the medium, cells were infected with virus in the absence of the maximum noncytotoxic carvacrol concentrations. Then, cells were kept at 4°C for another 3 hrs. After removing the medium, cells were washed with PBS [17-19]. Finally, the effect of carvacrol on attachement was checked with $TCID_{50}$ assay. *Melissa officinalis* with a stock concentration of 1.43 mg/ml was used as a positive control [16].

The evaluation of HSV-1 penetration to cells

To determine the effect of carvacrol on viral penetration, cells were prechilled at 4°C for 1 hr followed by infection with virus for 2 hrs at 4°C. Carvacrol was added for another 30 min. at 4°C. To allow viral penetration, the temperature of incubator was changed to 37°C. After 30 min. at 37°C, cells were treated with citrate buffer (135 mM NaCl, 10 nM KCl, 40 mM sodium citrate, pH 3) to stop penetration and to inactivate attached,

unpenetrated virions. After removing the buffer, the cell was overlaid with medium and then the effect of carvacrol on penetration was checked with $TCID_{50}$ assay [18].

Real Time polymerase chain reaction (RT-PCR)

The level of gene expression of late and early genes were determined by RT-PCR. For this purpose, vero cells were infected with HSV-1 in 24 well plates at 37°C for 1 hr, then cells were overlaid with medium and carvarol. Acyclovir was used as a control. After 48 hrs, the cellular RNA was extracted using a kit from RIBO-PREP Company (Moscow, Russia). Then, reverse transcription of RNA was performed by cDNA synthesis kit (Fermantas, USA), yielding cDNA. In the presence of specific primers, the cDNA was used for RT-PCR using SYBR green. The primer pairs for *UL52* were forward (5′GACCGACGGGTGCGTTATT3′) and reverse (5′GAAGGAGTCGCCATTTAGCC3′); for *UL27* were forward (5′GCCTTCTT CGCCTTTCGC3′) and reverse (5′CGCTCGTGCCCTTCTTCTT3′) [20]. Glyceral-dehyde 3-phosphate dehydro-genase (*GAPDH*) was used as the reference gene which primer pairs were *GAPDH* forward (5′CCCACTCCTCCACCTTTGAC3′) and *GAPDH* reverse (5′TCTTCCTCTT GTGCTCTTGC3′). Comparison of gene expressions was performed by the delta delta Ct method.

Results

The results of the toxicity of carvacrol on vero cells using MTT

The effect of different concentrations of the drug on vero cells MTT assay processed for 48 hrs led to, a desired result (TC_{50} was 0.001%).

Determination of 50% inhibitory concentra-tion of carvacrol against virus

In order to determine inhibitory concentration of carvacrol against virus, non-toxic drug with different concentrations of carvacrol was exposed to the virus and then was inoculated into the cells. The concentration range tested for carvacrol was up 0.00001% - 0.00075%, which was obtained by MTT assay. 50% of inhibitory concentration of carvacrol against virus was 0.0002% (Table 1).

Results of carvacrol antiviral mechanism against HSV-1

The results of virus titer by $TCID_{50}$/ml was $10^{4.5}$. Results show that carvacrol decreases herpes up to 70%. There was no reduction when drug enters the cell before entering the virus. The result showed that Carvacrol has no effect on replication. Carvacrol has no effect in attachment. Based upon data, carvacrol has no effect on penetration (Fig. 1).

The results of early and late virus gene expression of HSV-1

RT-PCR result revealed that this drug had no role in the early and late gene expression (Fig. 2).

Table 1. The results of the drug selectivity index

Drug	Max noncytotoxic concentration	TC_{50}	IC_{50}	SI (TC_{50}/IC_{50})[a]
Carvacrol	0.00075%	0.001%	0.0002%	5
Acyclovir	100 µm	≥ 100 µm	1.8 µm	≥ 56

[a] Selectivity index (SI) is the ratio of TC_{50} and IC_{50}.
TC_{50}= 50% toxic concentration; IC_{50}= 50% inhibitory concentration

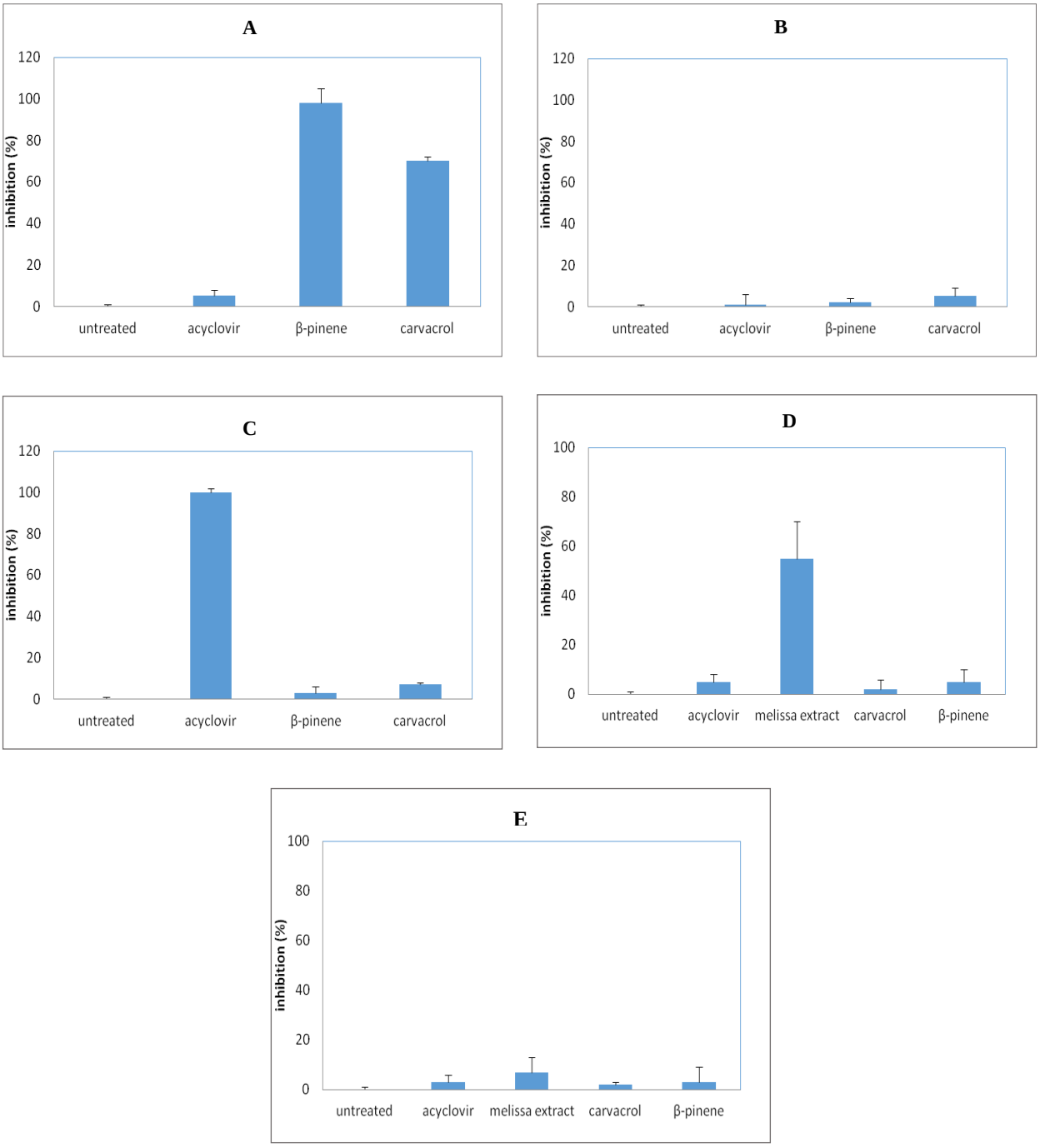

Fig. 1. Results of pretreatment of virus (A); pretreatment of cell (B); replication results (C); attachment results (D); penetration results (E)

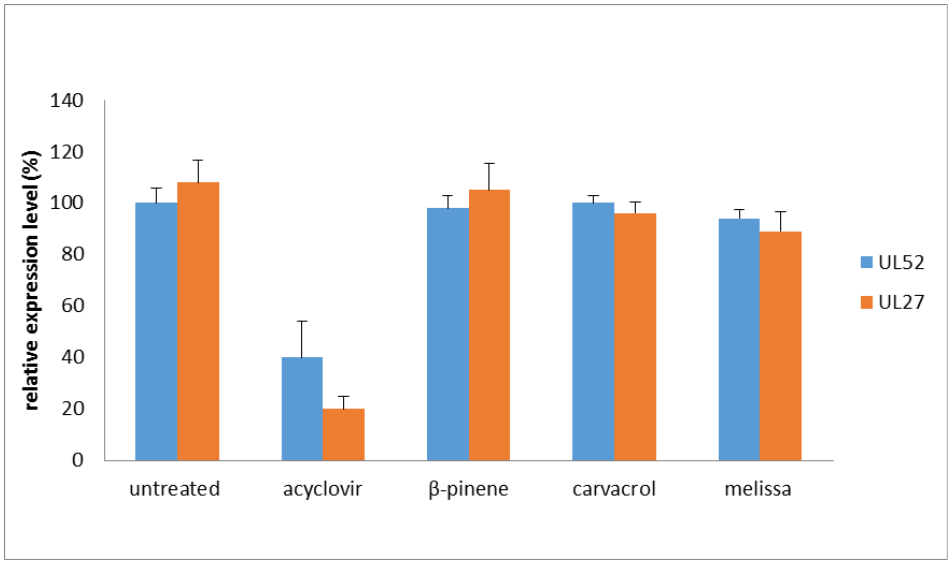

Fig. 2. Results of early and late gene expression of HSV-1

Discussion

Herpes virus can cause lifelong infection. This infection is latent and is associated with reactivation. Many of the known human herpes viruses infect most of the world's population. HSV-1 leads to different clinical symptoms from hepetic labialis to an acute encephalitis in humans [1, 2]. Drug for the treatment of diseases caused by this virus is acyclovir. However, due to prolonged use of the drug, resistant strains of the drug have been identified. Acyclovir resistant strains can cause severe clinical consequences in patients [2].

The use of medicinal plants to treat and prevent various diseases has been widely growing. Recently, due to drug resistance and its side effects, use of drugs of plant origin for the treatment of diseases has increased. In this study, we tried to investigate the effect of carvacrol combination on HSV-1. Many other studies have been conducted to determine the effects of plant extracts on HSV-1. A study by Khanavi and colleagues in 2009 presented the chemical components of essential oils of thyme and oregano. It was found that essential oil of thyme twigs air contains 24 compounds including: thymol (38%), carvacrol (34.96%), *p-cymene* (7.17%) and beta-caryophyllene (2.71%) [21]. In another study by Cinati and colleagues it was shown that one of the constituents of licorice is carvacrol [22].

Based on this study and other studies by Mardani et al. [23], Farahani et al. [24], Sabouri Ghanad et al. [25] and Monouri et al. [26] on plant extract, our study was determined to check antiviral activity of carvacrol on vero cells. Carvacrol is a compound with the scientific name Methyl ethyl 2-methyl phenol 5-1 which has an anti-bacteria and anti-fungi effects. Magi et al. in 2015 showed the antimicrobial effect of carvacrol on *Streptococcus pyogenes* [27]. Maximum nontoxic concentration of this compound determined with MTT assay was 0.0001.

The antiviral effect of the drug was tested and IC_{50} was 0.0002. With TC_{50}/IC_{50}, selectivity index was calculated which was 5. According to the research that was conducted by Amoros et al. in 1992, the drugs with selectivity index higher than 4 are considered preferable [28]. Astani et al. worked on some essential oils and monoterpenoids and some of their selectivity indexes were as follows: Thyme oil: 6.4, Thymol: 2.8, Citral: 12.9 [29].

According to this study, carvacrol which belonged to monoterpenoids and one of the compounds of plants essential oils had similar selectivity index. In a study conducted by Mardani et al. in Iran in 2012, the antiviral effect of Shirazi thyme essential oils, was determined. Carvacrol was one of the compounds that its antiviral effect against HSV-1 was considered. In this pilot study, cytotoxicity of essential oils in different concentrations was performed on vero cells. The result showed that the concentration which destroyed 50% of vero cells was 0.0676, and the concentration which inhibited detection of viral plaque was 0.0059. Also tested essential oils in 0.01, 0.02 could prevent the virus completely [23]. Based on the previous studies, thyme essential oils can prevent the virus completely. In this study, carvacrol, as a part of thyme essential oils, reduced the virus up to 70%, so it showed that another compound of thyme essential oils has antiviral effect. The concentration used was 0.01 and 0.02 %. However, in this study the concentration of 0.00075% was inhibited by 70% that is a good result for carvacrol compound.

In another study which was performed by Farahani in 2012, the antiviral effect of thyme on HSV-1 was tested *in vitro*. The antiviral effect of this plant was considered by preventive effects of cytopatic virus. Thyme essential oils prevent herpes virus proliferation and in non-toxic concentration shows antiviral effect on HSV-1 [24].

Another study by Pilao and his colleagues on *Mentha pulegium* essential oils showed that carvacrol is the main compound thus its antiviral effect on human and animals was investigated. They concluded that these essential oils are able to inhibit different human and animal virus such as Rota virus, bovine diarrhea virus and respiratory syncytial virus *in vitro* [15]. In the next phase, drug mechanism has been considered. This experiment was performed in three methods: incubation of HSV-1 with carvacrol before infection with vero cells with the virus (Pretreatment of virus), treatment of vero cells with carvacrol before contaminating cells with HSV-1 (Pretreatment of cell), and incubation of vero cells with carvacrol after infecting the cells with HSV-1 (replication). Carvacrol shows 70% decrease in pretreatment of virus. In another study carried out by Monavari and colleagues on thyme essential oils in 2013, the antiviral effect of this extract against HSV-1 before infection cells with the virus was probed. In this article, Melissa and B-pinene was used as positive control which both showed 100% decrease [26].

In this study, in pretreatment of the cell, only *Mellissa* showed 60% decrease. In replication,

carvacrol has no decrease because it cannot enter the cell while acyclovir, as a positive control, has 100% decrease which is due to its mechanism that affects on replication. After this stage, antiviral effect of carvacrol on penetration and attachment modes was checked which showed no inhibitory effect on virus. In final stage, primary and delay gene was checked by RT-PCR method, and it resulted in that carvacrol shows no change in none of them although acyclovir showed decrease for *UL52* and *UL27* 40% and 20%, respectively. It means that it plays important role for gene expression decrease. We resulted that the use of plant drugs is more effective and has no side effects; carvacrol compound showed ant-viral effects in pretreatment process.

This study suggests that compounds such as Carvacrol and beta-pinene are monoterpenes and the synergism between them can be considered as a next step. We reached a good coclusion on low toxicity and anti-herpes virus by *in vitro* study. *In vivo* studies can be studied on animal laboratory.

Conflict of Interest

The authors declare that they have no competing interests.

Acknowledgment

There is no acknowledgement to declare.

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