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Original Research

# Susceptibility of herpes simplex virus type 1 to monoterpenes thymol, carvacrol, *p*-cymene and essential oils of *Sinapis arvensis* L., *Lallemantia royleana* Benth. and *Pulicaria vulgaris* Gaertn

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**Abstract:** In recent years, with increased the prevalence of viral infections and having no specific for their treatment and also the continuous appearance of resistant viral strains, the finding of novel antiviral agents is necessary. In this study, monoterpenes of thymol, carvacrol, *p*-cymene and essential oils from *Sinapis arvensis* L., *Lallemantia royleana* Benth. and *Pulicaria vulgaris* Gaertn. were screened for their inhibitory effect against herpes simplex virus type 1 (HSV-1) *in vitro* on Vero cell line CCL-81-ATCC using a plaque reduction assay. The antiviral activity of three monoterpenes (thymol, carvacrol and p-cymene) and three essential oils were evaluated by cytotoxicity assay, direct plaque test. In addition, the modes of antiviral action of these compounds were investigated during the viral infection cycle. Results showed that the inhibitory concentrations ($IC_{50}$) were determined at 0.002%, 0.037%, >0.1%, 0.035%, 0.018% and 0.001% for thymol, carvacrol, *p*-cymene, *S. arvensis* oil, *L. royleana* oil and *P. vulgaris* oil, respectively. A manifestly dose-dependent virucidal activity against HSV-1 could be exhibited for compounds tested. In order to determine the mode of the inhibitory effect, compounds were added at different stages during the viral infection cycle. At maximum noncytotoxic concentrations of the compounds, plaque formation was significantly reduced by more than 80% when HSV-1 was preincubated with *p*-cymene. However, no inhibitory effect could be observed when the compounds were added to the cells prior to infection with HSV-1 or after the adsorption period. *Conclusion:* These results indicate that compounds affected HSV-1 mostly before adsorption and might interact with the viral envelope. Thymol exhibited a high selectivity index and seems to be a promising candidate for topical therapeutic application as antiviral agent for treatment of herpetic infections.

***Key words:*** HSV-1; Antiviral activity; Viral infection cycle; Antiviral agents.

## Introduction

Medicinal and aromatic plants have been widely used to treat many infectious diseases (1-7). Essential oils (EOs) are complex mixtures of volatile, lipophilic and aromatic plant secondary metabolites (8-11). The EOs including monoterpenes has a wide application in traditional medicine (12). The main constitutes of EOs include mono- and sesquiterpenes, arising from the isoprenoids pathway, and their oxygenated derivatives such as alcohols, ketones, esters, aldehydes, phenols and oxides (13-15). Previous studies have reported the biocide activity of EOs against many different clinically pathogens (16-18). Thymol (2-isopropyl-5-methyl phenol) and it's isomer, carvacrol (2-methyl-5-(1-methylethyl) phenol), are phenolic components which they and their various ether and ester derivatives have many biological activities such as antibacterial, antifungal, antioxidant, free radical scavenging and anti-inflammatory properties (19-21).

Thymol is naturally found in the oils of thyme (*Thymus vulgaris* L., oregano (*Origanum vulgare*), *Satureja thymbra*, *Thymbra capitata* and *Monarda fistulosa* L. (22-24). These phenolic components may affect cell activities by interacting with membrane proteins and cause proton imbalance across the membrane. The *p*-cymene is a naturally occurring aromatic organic compound. It is classified as an alkylbenzene related to a monoterpene. It is a constituent of a number of essential oils, most commonly the oil of cumin and thyme. Significant amounts are formed in sulfite pulping process from the wood terpenes.

Rad et al. (25) have evaluated *Sinapis arvensis* L. EO; they reported an antibacterial activity against five pathogenic multi-drug resistant bacteria and antioxidant activity. They also investigated *Lallemantia royleana* Benth. EO; the results showed antifungal and antibacterial activities of this plant (26). In addition, they studied *Pulicaria vulgaris* Gaertn. EO and illustrated that potent antimicrobial and cytotoxic activities of this EO may be attributed to its high contents of thymol, carvotanacetone and thymol isobutyrate (1). So far no studies have

been performed on susceptibility of herpes simplex virus type 1 to these three oils.

The herpes simplex virus (HSV) has a 186 nm diameter, and a linear double-stranded DNA of 152 kbp. It belongs to the family Herpesviridae, subfamily Alphaherpesvirinae, and genus Simplex-virus. It has two serotypes: herpes simplex virus type 1 (HSV-1, causing orofacial, mucocutaneous infections and encephalitis) and type 2 (HSV-2, causing anogenital infections) (27, 28). Some of antiviral drugs such as acyclovir, famciclovir and valacyclovir can be used to decreasing the clinical signs of infection and may suppress the virus, but might cause toxic side-effects and drug resistance (by decreasing the thymidine kinase activity of HSV and alteration of the viral DNA polymerase functionality) (29-32). Therefore, obtaining new effective compounds against HSV infections is an important matter.

The present study focused on the investigation of the antiviral activity of three monoterpenes (thymol, carvacrol and *p*-cymene) and three essential oils including *Sinapis arvensis* L., *Lallemantia royleana* Benth. and *Pulicaria vulgaris* Gaertn. against herpes simplex virus type 1 (HSV-1) and the mode of antiviral action of these compounds during the viral infection cycle.

## Materials and Methods

### Monoterpene and essential oils preparation

Thymol, carvacrol and *p*-cymene were purchased from Roth (Karlsruhe, Germany). These monoterpenes met high purity standards. Structural formulas of the monoterpene compounds are shown in Figure 1. Based on previous studies the essential oils of *Sinapis arvensis* L. (25), *Lallemantia royleana* Benth. (26) and *Pulicaria vulgaris* Gaertn. (1) were prepared. Briefly, dried aerial parts (leaves, stems and flowers) (200 g) of each plant were subjected to hydrodistillation for 3 hours using a Clevenger-type apparatus in accordance with the method outlined by the British Pharmacopeia (33). The obtained essential oils was dried over anhydrous sodium sulfate (Sigma-Aldrich, USA) and kept at 4 °C until analysis and further assays. Major identified components of these essential oils are shown in Table 1.

The monoterpenes and essential oils were dissolved in ethanol (0.5%, Merck) and further diluted in medium for cell culture tests. Because of the ethanol at concentration below 1% has no effect on cells and viruses (34).

### Acyclovir

Acyclovir (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in distilled water and used as positive control.

### Cell culture and herpes simplex virus type 1 (HSV-1)

Vero cell line CCL-81-ATCC (African green monkey kidney cells) was grown in monolayer culture in Eagle minimum essential medium (MEM) supplemented with 5% (V/V) fetal calf serum (FCS) (Gibco), 100 U/mL penicillin (Gibco), 100 µg/mL-1 streptomycin (Gibco), 2 mM l-glutamine (Gibco) and 1mM sodium pyruvate (Gibco). Monolayers were removed from surfaces and serially passaged until they became confluent. The cells were plated out onto 6-well for antiviral assays and 96-well culture plates for cytotoxicity test, and cultivated at 37 ºC in an atmosphere of 5% carbon dioxide ($CO_2$). For all assays, herpes simplex virus type 1 (HSV-1) strain KOS was used. Viruses were routinely grown on Vero cell and virus stock cultures were prepared from supernatants of infected cells and stored at -80 ºC for further

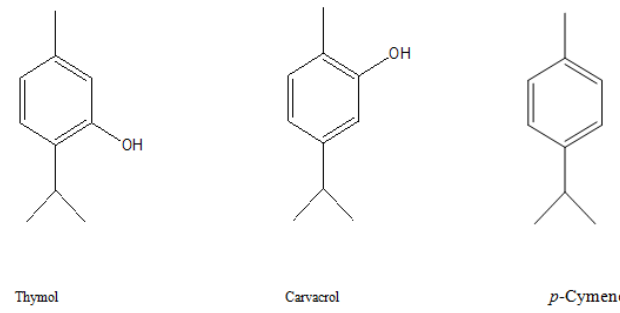


**Figure 1.** Structural formulas of monoterpenic compounds used in this study.

**Table 1.** Major components of essential oils of medicinal plants.

| Plant Name | Collection area | Major Components | References |
|---|---|---|---|
| *Sinapis arvensis* L. | Hamun Lake, Zabol, Iran | **Stem:** 1-butenyl isothiocyanate (18.4%), benzyl isothiocyanate (15.15%), Cubenol (15.12%), dimethyl trisulfide (6.12%), octadecane (4.14%), Hexadecane (4.09%), 6,10,14 trimethylpentadecane-2-one (3.85%), thymol (3.44%), Spathulenol (2.64%), dimethyl tetrasulfide (2.22%), indole (1.91%), 1-epi–and Octadecanal (1.14%).<br>**Flower:** cubenol(14.32%),2-phyenylisothiocyanate(7.45%),dimethyltrisulfide (5.24%), thymol (4.62%), δ-cadinene (3.40%), 6,10,14-trimethylpentadecane-2-one(3.25%),9 methylthiononanonitrile(3.21%), hexadecane (3.02%), 1-epi-cubenol (3.01%), %), octadecane (2.41%), spathulenol (1.58%), octadecanal (1.42%). Indole (1.41%) and nanodecane (1.01%). | (25) |
| *Lallemantia royleana* Benth. | Meymand mountains, Firuzabad, Iran | *trans*-pinocarvyl acetate (26.0%), pinocarvone (20.0%), verbenone (7.1%), (*E*)-β-ocimene (4.1%), *trans*-carveol (5.3%), 3-thujen-2-one (5.1%), pulegone (4.4%), *cis*-carveol (3.5%), linalool (3.4%), dihydrocarvyl acetate (2.5%), β-cubebene (2.1%), carvacrol (1.6%), *trans* pinocarveol (1.6%), β-pinene (1.5%), myrtenal (1.5% and terpinolene (1.1%). | (26) |
| *Pulicaria vulgaris* Gaertn. | Hamun Lake, Zabol, Iran | thymol (50.22%), *p*-menth-6-en-2-one (carvotanacetone, 20.2%), thymol isobutyrate (16.88%), menthan-2-one (4.31%), 1-methyl-1,2 propanedione (4.13%), 2,5-dimethoxy-*p*-cymene (4.01%), myrtenol (1.22%), linalool (1.1%) and β-myrcene (1.9%). | (1) |

assays.

### Cytotoxicity assay

The cells were seeded into 96-well plates and incubated at 37 °C for 24 h. The medium was removed and fresh MEM containing a dilution of the essential oils or monoterpenes was added onto subconfluent cells in three replicates for each of the drug concentrations. In this assay, wells containing medium with ethanol (1%) but no drug were also comprised on each plate as controls. After 72 h of incubation, the growth medium was removed and viability of the drug treated cells was determined in a standard neutral red test (35). The neutral red dye uptake was determined by measuring the optical density (OD) of the eluted neutral red at 540 nm in a spectrophotometer (Jenway 6405 UV models). The OD mean of the cell-control wells was assigned a value of 100%. The cytotoxic concentration of the drug which diminished viable cell number by 50% ($TC_{50}$) was detected from dose-response curves.

### Direct plaque test

The plaque reduction assay was used for analysis of inhibition of virus replication. All compounds were dissolved in ethanol and added to the medium at a final concentration of 1% ethanol, wells containing 1% ethanol but without drug were also included on each plate as controls. Serial dilutions of the monoterpenes-treated or oil-treated virus were adsorbed to Vero cell for 1 h at 37 °C. The residual inoculum was removed and infected cells were overlaid with medium containing 0.5% methylcellulose. Monolayers were fixed with 10% formalin after incubation for 4 days at 37 °C. The cultures were stained with 1% crystal violet (Merck, Germany) and then plaques were counted. The concentration of test compounds which inhibited plaque numbers by 50% ($IC_{50}$) was determined from dose-response curves.

### Mode of antiviral activity

In order to determine the mode of antiviral action the cells and viruses were incubated with acyclovir or compounds at different stages during the viral infection cycle. Cells were pretreated with acyclovir or compounds before viral infection, viruses were incubated with acyclovir or compounds before infection and cells and viruses were incubated together with acyclovir or compounds after penetration of the virus into the host cells. All compounds were dissolved in ethanol and added to the medium at a final concentration of 1% ethanol, wells containing 1% ethanol but without drug were also included on each plate as controls. The compounds or acyclovir were always used at the maximum noncytotoxic concentration. Cell monolayers were pretreated with the compounds prior to inoculation with virus by adding the compounds or acyclovir to the culture medium and by incubation for 1 h at 37 °C. The compound was aspirated and cells were washed immediately before the HSV-1 inoculum was added. For pretreatment of herpes simplex virus about $2\times10^3$ pfu of HSV-1 were incubated in medium containing the maximum noncytotoxic concentration of the compounds for 1 h at 25 °C prior to infection of Vero cells. After 1 h of adsorption at 37 °C, the inoculum was removed and cells were overlaid with medium containing 0.5% methylcellulose. The

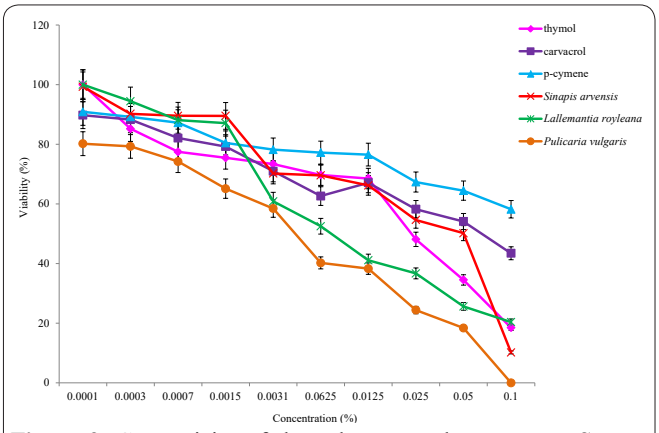


**Figure 2.** Cytotoxicity of thymol, carvacrol, *p*-cymene, *Sinapis arvensis* L., *Lallemantia royleana* and *Pulicaria vulgaris* essential oils on Vero cells. Assays were repeated independently three times and data presented are the mean of three experiments.

effect of compounds against HSV was also tested during the replication period by adding the compounds after adsorption to the overlay medium, as typically performed in antiviral susceptibility assays.

## Results

### Cytotoxicity

Monolayer cultures of Vero cells were grown in 0.00001-0.1% drug-containing medium and after 4 days of incubation, cell viability was determined in the neutral red assay. The toxic concentration ($TC_{50}$) of the compounds for Vero cells was 0.014% for thymol, 0.051% for carvacrol, > 0.1% for *p*-cymene, 0.054% for *S. arvensis* oil, 0.071% for *L. royleana* oil and 0.001% for *P. vulgaris* oil (Figure 2).

### Antiviral activity of monoterpenes and essential oils against HSV-1

The potential inhibitory effect against HSV-1 of different compounds was determined by pretreatment of the virus with the corresponding compounds for 1 h at room temperature and subsequent infection of Vero cells. The dose response curves are shown in Figure 3 demonstrating a dose-dependent activity of the tested

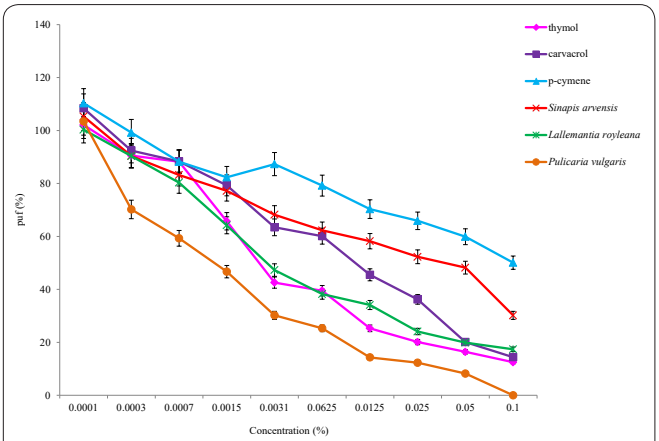


**Figure 3.** Determination of the $IC_{50}$ of thymol, carvacrol, p-cymene, *Sinapis arvensis*, *Lallemantia royleana* and *Pulicaria vulgaris* essential oils against HSV-1. Viruses were incubated for 1 h at room temperature with increasing concentrations of the compounds and immediately tested in a plaque reduction assay. Assays were repeated independently three times and data presented are the mean of three experiments.

**Table 2.** Selectivity indices (SI) of monoterpenes and essential oils for HSV-1.

| Compounds | $TC_{50}$ (%) | $IC_{50}$ (%) | Selectivity index (SI) |
|---|---|---|---|
| Thymol | 0.014 | 0.002 | 7 |
| Carvacrol | 0.051 | 0.037 | 1.37 |
| *p*-cymene | > 0.1 | > 0.1 | ND* |
| *Sinapis arvensis* L. | 0.054 | 0.035 | 1.54 |
| *Lallemantia royleana* Benth. | 0.071 | 0.011 | 6.45 |
| *Pulicaria vulgaris* Gaertn. | 0.001 | 0.001 | 1 |

*ND: not determined.

compounds. Selectivity indices for different essential oils and monoterpenes were calculated as the ratio $TC_{50}$/$IC_{50}$ and are given in Table 2. A high selectivity index was found for thymol.

## Mode of antiviral activity

To identify the step at which replication might be inhibited, cells were infected with HSV-1 after preincubation of the cells with essential oils and monoterpenes, pretreatment of the virus with the compounds prior to infection, addition of the compounds after adsorption during the intracellular replication period. The cells infected with untreated virus as well as acyclovir were used as control during all assays. The percent reduction was calculated relative to the amount of virus produced in the absence of the compounds. In all experiments maximum noncytotoxic concentrations of monoterpenes and essential oils were used and the results are shown in Figure 4. Pretreatment of cells with the compounds and addition of all the compounds during the replication phase did not reduce virus production. However pretreatment of HSV with the analyzed compounds prior to infection caused a significant reduction of infectivity ranging from about 50% for *P. vulgaris* oil to >80% for *p*-cymene. These results show that the antiviral effect of the examined compounds were mainly exerted prior to adsorption of HSV to the host cells. The results showed that acyclovir was effective against HSV-1 with the highest antiviral activity when added during the replication period (Figure 4).

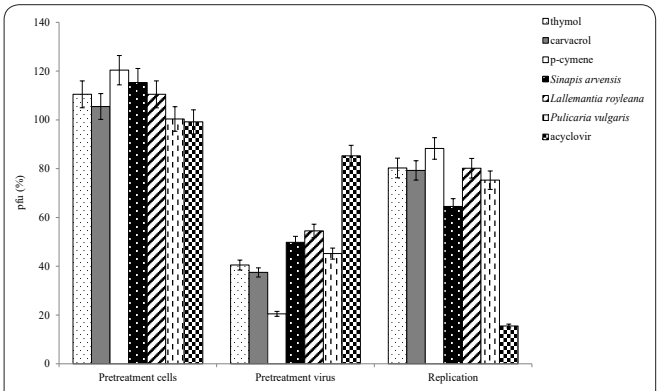


**Figure 4.** Mode of inhibitory effect of thymol, carvacrol, p-cymene, *Sinapis arvensis*, *Lallemantia royleana* and *Pulicaria vulgaris* essential oils against HSV-1 during different periods of the viral replication cycle. Virus or cells were treated with the maximum noncytotoxic concentration of the compounds. Acyclovir was used as a control. Assays were repeated independently three times and data presented are the mean of three experiments.

## Discussion

In this study we evaluated the antiviral activity of monoterpenes of thymol, carvacrol, *p*-cymene and essential oils of *S. arvensis* L., *L. royleana* Benth. and *P. vulgaris* Gaertn. against HSV-1 *in vitro*. The monoterpene *p*-cymene exhibited the highest level of antiviral activity against HSV-1 in viral suspension tests. At maximum noncytotoxic concentration plaque formation was reduced by 80%. Among the essential oils, *P. vulgaris* essential oil reduced virus plaque formation by about half. In order to determine the mode of antiviral action, either cells were pretreated before viral infection or viruses were incubated with noncytotoxic concentrations of drugs before infection, or after penetration into the host cells. Pre-treatment of the cells with these drugs had no or only minor effect on the production of infectious virus and plaque formation. The same results were found when the monoterpenic compounds or essential oils were added during the replication period of the infection cycle. However antiviral activity was observed for monoterpenes and essential oils when herpesvirus was incubated with these drugs prior to host cell infection. These results suggest that the investigated drugs directly inhibit herpes virus infection and might interfere with virion envelope structures or mask viral structures which are necessary for adsorption or entry into host cells. The inhibition of HSV by the tested drugs appears to occur before adsorption but not after penetration of the virus into the cell. It remains to be determined whether the inhibitory effect of compounds is due to binding of the compounds to viral proteins involved in host cell adsorption and penetration. De Logu et al. (36) reported an inactivation of herpesviruses and prevention of cell to cell spread by *Santolina insularis* essential oil. However, no antiviral effect was observed during the intracellular replication phase, which is in accordance to our results. Isoborneol, a monoterpene and a component of several plant essential oils, showed virucidal activity against HSV-1 and specifically inhibited glycosylation of viral proteins (37). The application of the monoterpene cineole protected mice against infection with HSV-2 (38). Since essential oils are able to inhibit acyclovir-resistant HSV-1 isolates (34), the mechanism of interaction between monoterpenes, essential oils and acyclovir with HSV must be different. Acyclovir inhibits virus replication by interference with the DNA polymerase inside the cell, whereas monoterpenes and essential oils probably inactivate HSV before it enters the cell. Astani et al. (32, 39) also showed high antiviral activity for essential oils and isolated monoterpenes when herpesvirus was incubated with these drugs

prior to host cell infection. Viral resistance to acyclovir represents a particular problem; the prevalence of resistance in acyclovir-treated immunocompromised individuals is approximately 4 to 7% (40). Therefore other antiherpetic agents which are effective for viral mutants resistant to current antiviral agents are of great interest for topical treatment.

In our study, the tested monoterpenes showed a higher antiviral activity compared to the tested essential oils. These findings are in contrast to results in antiviral activity, where the complex mixture of the essential oil revealed a higher antiviral activity (39) than single monoterpenes. However the antiviral activity of single monoterpenes does not contribute equally to the antiviral activity of the essential oil. An important predictive value for future application of these drugs is their selectivity index; Amoros et al. (40) recommended a selectivity index of at least 4 as appropriate. According to this suggestion, the monoterpene thymol is a suitable antiherpetic agent and might be applied in recurrent herpes labialis.

## Conflicts of interest

The authors declare no financial or other conflicts of interest.

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