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## Short Communication

# Inhibitory Effect of Mentha Piperita Extracts against Herpes Simplex Virus Isolated from Eye Infection

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## Abstract

Herpes simplex virus (HSV) is one of the common pathogenic viruses of humans bing. This study aimed to determin anti-herpes virus activity of Mentha Piperita extracts in vitro. Mentha Piperita extracts can inhibit HSV infection when the cells treated before viral adsorbtion. HSV-1 were also inhibited by menthol after viral adsorption. HSV-1 viral particles were directly inhibited and viral yield was reduced when treated with Mentha Piperita extracts and menthol. Therefore, Mentha Piperita extracts and menthol showed anti-HSV activities at various stages of the viral replication cycle.

**Keywords:** Mentha Piperita, menthol, herpes simplex virus, virus replication, eye infection

## Introduction

Herpes simplex virus (HSV) is one of the most common viruses acquired by humans. HSV-1 belongs to the family Herpesviridae and sub-family Alphaherpesvirinae. All three viruses are neurotrophic and have the unique ability to remain latent in sensory and autonomic ganglia, innervating the site of primary infection for the lifetime of the host. This article emphasizes the most recent literature with respect to HSV-1 latency and reactivation, as well as treatment of herpes keratitis (HK) (1-3).

Ocular herpes simplex is usually caused by HSV-1 but also occasionally by the type 2 virus (HSV-2). Ocular manifestations of HSV are varied and include blepharitis, canalicular obstruction, conjunctivitis, corneal complications, iritis, and retinitis (4). Herpes simplex virus (HSV) ocular infection is the major cause of corneal blindness in developed countries. In developing countries it is becoming more prevalent, causing more blindness (5).

HERPES SIMPLEX virus (HSV) of the eye affects more than 400 000 people in the United States. Each additional ocular episode and its sequelae can have an increasing personal, social, and financial burden. A randomized, placebo-controlled clinical trial demonstrated that twice-daily oral acyclovir reduced the incidence of ocular HSV recurrences during 1 year in participants with a history of herpetic eye disease (6-8). Infections with HSV are usually acquired in early life. A US study found antibodies against HSV-1 in about 50% of people with high socioeconomic status and 80% of people with low socioeconomic status by age 30 years. It quoted a report which suggested overcrowding as a causal factor. However, only about 20–25% of people with HSV antibodies had any history of

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clinical manifestations of ocular or cutaneous herpetic disease. Ocular HSV is the most common cause of corneal blindness in high-income countries, and is the most common cause of unilateral corneal blindness worldwide (4).

Recurrent HSV is an epidemiologically important cause of infectious and inflammatory eye disease affecting children, adolescents, adults, and the elderly. The estimated prevalence of ocular HSV disease is 15 per 10,000 population. The incidence of first-episode ocular HSV at centres in Europe and North America is 4 to 13 per 100,000 person-years. The incidence of further episodes of HSV keratitis is 12 to 18 per 100,000 person-years. Worldwide, an estimated 10 million persons have had herpetic eye disease, with approximately two million individuals left with impaired vision of their affected eye (9, 10, 11).

The discovery of effective antiviral agents has been facilitated by advances in the fields of molecular biology and virology. In the pre-antiviral era, the widely held belief was that any therapeutically meaningful interference with viral replication would destroy the host cell upon which viral replication was dependent. A growing understanding of host cell–virus interactions and viral replication, however, has led to the development of safe and effective antivirals. These agents act by impeding entry of viruses into host cells; interfering with viral assembly, release, or de-aggregation; inhibiting transcription or replication of the viral genome; or interrupting viral protein synthesis (12).

Many clinical trials have been performed on the acute treatment of dendritic epithelial keratitis. Surveys of ocular antiviral pharmacology and of herpes simplex virus (HSV) eye disease have evaluated different interventions, but a systematic review of all comparative clinical studies has not previously been undertaken (13).

Mentha Piperita oil exhibited high levels of virucidal activity against HSV-1 and HSV-2 in viral suspension tests. At noncytotoxic concentrations of the oil, plaque formation was significantly reduced. Higher concentrations of Mentha Piperita oil reduced viral titers of herpesviruses (14).

Mint (mentha) is an herb which is well known for its antispasmodic, painkilling (1-3), anti-inflammatory, antispasmodic, decongestant, and antioxidant effects (4). Mentha Piperita is one of the mentha species (i.e., mentha piperita, Mentha Piperita oil, mentha arvensis, and cornmint oil) [5]. Menthol (29%) and menthone (20-30%) are the major components of the Mentha Piperita essential oil (15-17).

The extract and leaves are described as biological additives, but only the extract is reported to be used. Mentha Piperita Water is described as a flavoring agent or fragrance component, but is not currently in use. Mentha Piperita Oil is used at a concentration of < or = 3% in rinse-off formulations and < or = 0.2% in leave-on formulations. Mentha Piperita Oil is composed primarily of menthol and menthone (18).

Plants of the Lamiaceae family are used in traditional and complementary medicine, in particular in phytotherapy. A virucidal activity of extracts from lemon balm has been reported for herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), and recently been extended to other species of the Lamiaceae family (19-21).

Essential oils such as tea tree oil, lavender oil, thyme oil, Mentha Piperita oil and eugenol oil have been traditionally used by people for various purposes in different parts of the world (22).

In Iran, Mentha Piperita is used in traditional medicine as carminative, spasmolytic, anti-nausea and as a local treatment it can include cooling and mild analgesic effects. It has not been reported any data about toxic effects of using Mentha Piperita during pregnancy and breast feeding (23).

Mentha Piperita (Lamiaceae) is widely used in complementary medicine as constituents of medical products, cosmetic, food industry, flavouring, additives, beverage and pharmaceutical industries. The major constituents of Mentha Piperita from different sources were found such as menthone, menthofuran, menthol, pulegone, 1,8-cineole, isomenthone, limonene, menthyl acetate, terpenes, isomethone and carvone (23, 24). In

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this study Mentha Piperita extracts and menthol were selected for investigation of mode of action on various stages of HSV replication cycle.

## Materials and Methods

**Plant extracts and acyclovir.** Peppermint extract has been prepared from dried leaves from peppermint (Mentha×piperita L.) that were purchased from Caesar & Lorenz (Hilden, Germany). All plants were identified by plant specialist. Aqueous extracts were prepared briefly, by: boiling water (100 ml) was added to dried leaves (10g) and incubated for 15 min, subsequently filtered and cooled down. The resulting extracts were sterile filtered, aliquoted, and stored at -20°C.

The extracts were filtered, concentrated and lyophilized to form dried extracts. The dried extracts were reconstituted in dimethylsulfoxide (DMSO) and further diluted in medium for determination of cytotoxicity and anti-HSV activity. Pure menthol was purchased from Sigma-Aldrich and prepared as a stock solution of 160 mg/ml in DMSO Acyclovir (ACV), a commonly used as anti-HSV synthetic drug, was used as a positive control (Sigma Aldrich, USA). ACV was dissolved in sterile distilled water and was diluted with MEM before determination of anti-HSV activity. 50% inhibition concentration (IC50) of ACV was calculated.

**Cell lines and viruses.** Green monkey kidney (GMK) cells were grown in monolayers with Eagle's minimum essential medium (MEM) (Hyclone, UK) supplement with 10% heat inactivated fetal calf serum (Starrate, Australia) and 40 µg/ml gentamycin. Cells were incubated at 37°C in an atmosphere of 5% CO2 incubator. HSV-1 were propagated and grown on GMK cells. The virus stock was prepared from supernatants of infected cells and stored at -85°C until use.

**Cytotoxicity assay.** For the cytotoxicity assay, Mentha Piperita extracts and menthol (Sigma-Aldrich, USA) were prepared in DMSO and serial two-fold diluted with MEM. Nontoxic concentrations of Mentha Piperita extract used in the assay ranged from 0.009-0.080 mg/ml whereas methanol extract of Mentha Piperita ranged from 0.019-0.156 mg/ml. Each dilution of the plant preparations were added in quadruplicated wells of 96-well tissue culture plate. Then, the GMK cell suspensions containing 1x106 cells/ml were seeded into the culture plate. After 4 days of incubation, the cells were stained with 0.1% crystal violet in 1% ethanol for 15 minutes. The cytotoxicity was expressed as the 50% cytotoxic dose (CD50) and calculated according to modified protocol of Reed and Muench (25).

**Plaque titration assay.** The cells were seeded into 24-well tissue culture plates and incubated at 37°C in a 5% CO2 incubator for 2 days. The GMK cells were grown to 70-80% confluence. Viral stocks were serially ten-fold diluted in MEM and each dilution was added to the cell monolayer. After 1 hour adsorption, the infected cells were then overlaid with overlay medium containing 1.5% carboxymethylcellulose and incubated at 37°C in a 5% CO2 incubator for 4 days before staining with 0.1% crystal violet in 1% ethanol for 15 minutes. Virus plaques were counted and expressed as plaque forming units per milliliter (PFU/ml).

**Plaque reduction assay.** Confluent cell monolayer in 24-well tissue culture plates were infected with 100-200 PFU/ 0.1 ml of HSV for 1 hour at room temperature. The infected cells were incubated with the various concentrations of crude Mentha Piperita extracts, menthol and ACV. The infected cells were then overlaid with medium, containing 1.5% carboxy-methylcellulose. After incubation for 3 days at 37°C in 5% CO2 incubator, the infected cells were stained with 0.1% crystal violet, in 1% ethanol, for 15 minutes. The percentage of viral inhibition after treatment with the extracts was calculated as percentage inhibition compared with the untreated viral infected cells control from triplicate experiments.

**Effect of plant extracts on pretreated cells.** Cell monolayers were treated with various nontoxic concentrations of crude Mentha Piperita extract and menthol for 1 hour. The extracts were removed before adding HSV inoculums. After incubation of the cells with HSV at room temperature for 1 hour, overlay

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medium was added. The infected cells were incubated at 37°C in a 5% CO2 incubator for 4 days. The number of plaques was counted and compared with controls.

**Effect of plant extracts on HSV during viral adsorption.** Confluent cell monolayers cultivated in 24-well tissue culture plate were infected with 200 PFU/ 0.1 ml of HSV. Then, nontoxic concentrations of crude Mentha Piperita extracts and menthol were added onto cell Monolayers and incubated for 1 hour at room temperature for virus adsorption. After that, the residual inoculum was removed and replaced by overlay medium containing 1.5% carboxymethylcellulose, and incubated at 37°C in a 5% CO2 incubator for 4 days.

After incubation, the virus plaques were stained with 0.1% crystal violet in 1% ethanol for 15 minutes. The number of plaques was counted and the 50% effective doses (ED50) were determined from dose-response curves.

**Effect of plant extracts on HSV after viral adsorption.** Confluent cell monolayers cultivated in 24-well tissue culture plates were infected with 200 PFU/ 0.1 ml of HSV and incubated for 1 hour at room temperature for virus adsorption. After viral adsorption, nontoxic concentrations of crude Mentha Piperita extracts and menthol were added onto the infected cells. Then, the cells were overlaid with overlay medium containing 1.5% carboxymethylcellulose and incubated for 4 days at 37°C in a 5% CO2 incubator. The number of plaques was counted and the 50% effective doses (ED50) were also determined.

**Effect of plant extracts on viral replication.** Cells were grown in 25 cm2 flasks until confluence, after which the cells were infected with 1x106 PFU/ml of HSV for 1 hour. After that, the infected cells were washed twice with PBS and treated with the highest nontoxic concentrations of crude Mentha Piperita extracts, menthol, and compared with ACV at IC50. Virus-infected cells in flasks containing medium with 2% fetal calf serum were also included as a drug negative control. The infected cells were further incubated at 37°C in a 5% CO2 incubator and the cells were collected at 18, 24 and 30 hours after viral infection. The infected cells were frozen and thawed twice before determination of virus titers using a plaque titration assay.

**Direct inactivation of viral particles.** For investigation of the effects of crude Mentha Piperita extracts on viral particles, the highest non-toxic concentrations of each extract and menthol were mixed with HSV and incubated for 1, 2, 3 and 4 hours at room temperature. After incubation, the inactivated viral particles were adsorbed onto cells for 1 hour at room temperature.

The infected cells were overlaid with overlay medium containing 1.5%carboxymethylcellulose and incubated for 4 days at 37°C in a 5% CO2 incubator. Then, infected cells were stained with 0.1% crystal violet.The percent of viral plaque inhibition by the extracts were calculated and compared with those of untreated controls.

**Statistical analysis.** Data were given as mean ± S.D. of three independent experiments. Statistical comparison between groups was analyzed by one way analysis of variance (ANOVA) and Post hoc Tukey's-b test. The p values less than 0.05 ($p< 0.05$) were considered significance.

## Results

Extracts of Mentha Piperita were tested for toxicity on GMK cells and CD50 values were calculated according to modified protocol of Reed and Muench [25]. The potential inhibitory effects of the Mentha Piperita extracts and menthol, which is pure compound found in Mentha Piperita, against HSV were investigated to clarify antiviral activity on pretreated cells, during viral adsorption and after viral adsorption period using plaque reduction assay. After pretreatment of the cells with the extracts, but before their infection with HSV, extract of peppermint were effective against HSV-1 with ED50 values of 62.70mg/ml and therapeutic indices (TI= CD50/ED50) of 1.79. Thus, higher TI reflected higher therapeutic potential of the extracts. However, the cells were not protected from HSV infection by menthol. During viral adsorption to the cells, ED50 values of the

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extract of Mentha Piperita on HSV-1 were 26.65 µg/ml. HSV1 was not inhibited by menthol during viral adsorption.

Anti-HSV activities of the Mentha Piperita extracts and menthol were also determined after viral adsorption. Direct inactivation of HSV-1 by the Mentha Piperita extracts was evaluated by plaque titration assay at 1, 2, 3 and 4 hours after viral inactivation and the results were compared with the untreated virus control. The extract of Mentha Piperita was also active against HSV-1 as the viral particles were completely inactivated within 2 hours.

The efficiency of extracts on viral growth kinetics was also observed at 18, 24 and 30 hours after viral infection, and compared with the inhibition of viral replication by ACV, and the virus control. ACV was used at ED50 concentrations, which were 1.5 µg/ml after treatment with HSV-1. After 30 hours of viral infection, HSV-1 yield was inhibited.

## Discussion

The emergence of drug resistant microbial pathogen is leading to the increased demand of potent antibiotics. The search of new biotic for screening large number of antimicrobial isolate cultured from nature.

Many aromatic plants used in phytotherapy are considered to be important sources for the production of raw materials or preparations containing phytochemicals that have significant activity against microorganisms. Thus, plant extracts and essential oils have been widely used in traditional medicine for treatment of many diseases. In this study, extracts of Mentha Piperita were able to prevent pretreated cells from HSV-1 infection.

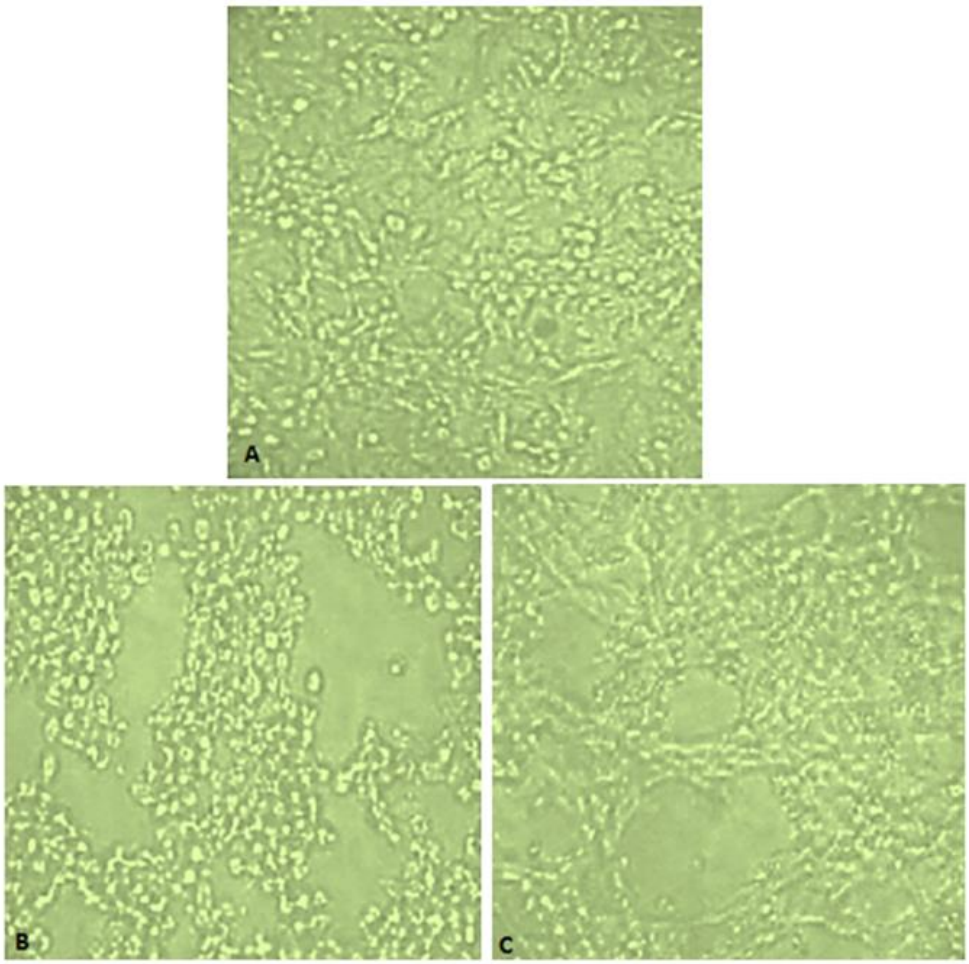


**Fig. 1.** The HSV-1 cytopathic effect and its inhibition by the Echinacea purpurea extracts, A: control VERO cell/B: Herpes virus injected VERO cell with CPE/C:VERO cell treated by Echinacea purpurea extract with reduced CPE

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Small plaque sizes were determined by measurement the diameter of plaques comparing with controls. The small plaque might result from reduction of viral infectivity to the neighboring cells.

However, inhibitory effect of menthol on HSV was observed only when menthol was applied after HSV-1 adsorption.

Time dependent virucidal effects of the Mentha Piperita extracts on HSV-1 particles were shown by reduction of amount of viral plaque by 100%. The virucidal activity of the crude Mentha Piperita extracts was better than menthol. The similar results were observed since essential oils of Mentha Piperita showed high level of virucidal activity against HSV-1 and were effective against HSV before viral adsorption (25).

The cardinal antiviral activity of aqueous Lamiaceae extracts appears to be virucidal and through this mechanism affecting the interaction of the virion with the cell. The activity is rapid since even simultaneous addition of virus and extract to cells allowed for full antiviral potency (26).

Our findings demonstrated that the activity of methanol and dichloromethane extracts of Mentha Piperita showed higher anti-HSV activity during and after viral adsorption. Thus, the extracts were able to interfere during the attachment stage of the viruses by directly inhibition of viral particles and also during the viral replication and viral protein expression as observed from the reduction of viral yield and viral proteins. Menthol, which is the main compound in the Mentha Piperita extracts showed anti-HSV activity after viral adsorption. Thus, activity of menthol against HSV occurred during viral replication. The results from this study demonstrated the antiviral activity of the Mentha Piperita extracts and indicated the potential for development of an alternative therapeutic antiherpetic agent.

## Conclusion

The current study exhibits inhibitory activity of plant extract against HSV. Further research on the isolation of active pure compound from plant help us to carry out studies on drug resistant viral strains. This will also encourage doing the research on the plant bioactive compounds against the viral strains. The identified compounds will be reducing the frequency of recurrences of acute HSV infections.

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