Virucidal effect of peppermint oil on the enveloped viruses herpes simplex virus type 1 and type 2 in vitro

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Summary

The virucidal effect of peppermint oil, the essential oil of Mentha piperita, against herpes simplex virus was examined. The inhibitory activity against herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) was tested in vitro on RC-37 cells using a plaque reduction assay. The 50% inhibitory concentration (IC₅₀) of peppermint oil for herpes simplex virus plaque formation was determined at 0.002% and 0.0008% for HSV-1 and HSV-2, respectively. Peppermint 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 by 82% and 92% for HSV-1 and HSV-2, respectively. Higher concentrations of peppermint oil reduced viral titers of both herpesviruses by more than 90%. A clearly time-dependent activity could be demonstrated, after 3 h of incubation of herpes simplex virus with peppermint oil an antiviral activity of about 99% could be demonstrated. In order to determine the mode of antiviral action of the essential oil, peppermint oil was added at different times to the cells or viruses during infection. Both herpesviruses were significantly inhibited when herpes simplex virus was pretreated with the essential oil prior to adsorption. These results indicate that peppermint oil affected the virus before adsorption, but not after penetration into the host cell. Thus this essential oil is capable to exert a direct virucidal effect on HSV. Peppermint oil is also active against an acyclovir resistant strain of HSV-1 (HSV-1-ACV⁵⁰), plaque formation was significantly reduced by 99%. Considering the lipophilic nature of the oil which enables it to penetrate the skin, peppermint oil might be suitable for topical therapeutic use as virucidal agent in recurrent herpes infection.

Key words: Mentha piperita, peppermint oil, virucidal activity, herpes simplex virus, acyclovir-resistant HSV-1

Introduction

Herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are common human pathogens which cause epidermal lesions in and around the oral cavity, the eye, in the pharynx, and the oesophagus as well as in the mucous membrane of the genitals. Infections in immunocompromised patients are usually more severe than in immunocompetent hosts. Both viruses, which can be distinguished by serological and molecular methods, are able to establish a latent infection in the nervous system that can be reactivated. Several drugs are currently available for the management of HSV infections such as acyclovir or penciclovir. Acyclovir and related synthetic nucleosides interfere with viral DNA replication through activation by viral thymidine kinase. The incidence and severity of disease produced by herpes simplex virus...
have been increasing in recent years (Cassady and Whitley, 1997), especially in the immunocompromised host where viral resistance to acyclovir represents a particular problem. The prevalence of resistance in acyclovir-treated immunocompromised individuals is approximately 6% (Christophers et al. 1998). This trend has led to search for alternative antitherpetic agents that have a wide range of efficacy without serious adverse effects, and which are effective for viral strains resistant to current antiviral agents.

For the past decades, a large number of antitherpetic screening experiments on medicinal plant extracts and plant derived secondary metabolites (e.g. flavonoids, anthraquinones, naphthodianthrones, polyphenolics) have been reported (Reichling, 1999; De Logu et al. 2000). Essential oils obtained from fruits, leaves, seeds, stem bark, and roots of many plants have been widely used in traditional medicine. Among others, antibacterial, antifungal, immunomodulatory, antiinflammatory, and antirheumatic activities have been described (Saller et al. 1995; Reichling, 2001; Saller and Reichling, 2001). Hitherto, there are only little informations on the effects of essential oils on viruses or viral infections. Recently, the antitherpetic activity of several essential oils of different plant sources as well as of various constituents of essential oils was demonstrated (Bourne et al. 1999; Hayashi et al. 1995; Sivropoulou et al. 1997; Benencio and Courreges, 1999). A fungicidal activity of tea tree oil the essential oil of Melaleuca alternifolia, against dermatophytes and filamentous fungi could be demonstrated recently (Hammer et al. 2002) and the antifungal activity of tea tree oil against Candida albicans has been analysed (D’Auria et al. 2001). Furthermore, we reported the antitherpetic activity of Australian tee tree oil and eucalyptus oil (Schnitzler et al. 2001).

Menthae piperitae aetheroleum (peppermint oil) is an essential oil derived from leaves of Mentha piperita L. Peppermint oil is widely used in traditional as well as in complementary medicine, especially in phytotherapy, for the external treatment of different human diseases e.g. of various pain conditions, including headache syndromes (Göbel et al. 1995), postherpetic neuralgia (Davies et al. 2002) or mild bacterial or fungal infections of the skin. Recently, its antibacterial activity against gram-positive and gram-negative bacteria was clearly demonstrated (Reichling et al. 1999). But until now the antiviral properties of peppermint oil against HSV-1 and HSV-2 have not been described.

Here we report studies on peppermint oil for its inhibitory activity on plaque formation of HSV-1 and HSV-2 in cultured cells. Furthermore, we investigated for the first time the antitherpetic activity of Menthae piperitae aetheroleum against an acyclovir-resistant strain of HSV-1.

Materials and Methods

Peppermint oil

The essential oil tested was purchased from Caesar & Lorenz, Hilden, Germany. Peppermint oil was analysed as 1% solution in n-hexane. Gas chromatography was performed using a Carlo Erba MFC 500 chromatograph equipped with a Spectra Physics Integrator SP 4290 as described previously (Schnitzler et al. 2001). A gas chromatograph Carlo Erba HRGC 4160 was coupled via an open, split interface to a Finnigan MAT 4500 mass spectrometer (Schnitzler et al. 2001). Peppermint oil was dissolved in ethanol and added to the cell culture medium.

Acyclovir

Acyclovir (ACV) was purchased from Glaxo SmithKline Beecham and dissolved in sterile water.

Cell cultures

RC-37 cells (African green monkey kidney cells) were grown in monolayer culture with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (FCS), 100 mg/ml penicillin and 100 mg/ml streptomycin. The monolayers were removed from their plastic surfaces and serially passaged whenever they became confluent. Cells were plated out onto 24-well and 6-well culture plates for cytotoxicity and antiviral assays, respectively, and propagated at 37 °C in an atmosphere of 5% CO₂.

Viruses

Herpes simplex virus type 1 (HSV-1) strain KOS (Paris et al. 1980), HSV-2 strain HG52 (Dolan et al. 1998) and HSV-1-ACVres, an acyclovir-resistant HSV-1 strain (Knopf, 1987) were used for experiments. HSV-1-ACVres was a kind gift from Dr. C. Knopf, German Cancer Research Center, University of Heidelberg. HSV-1-ACVres exhibits a single-point mutation at the DNA polymerase gene (C → G nucleotide conversion in position 2496). As a result of the mutation the amino acid alanine was replaced by valine in position 719 of the DNA polymerase. Virus was routinely grown on RC-37 cells as described previously (Rösen-Wolff et al. 1988). Herpes simplex virus stock cultures were prepared from supernatants of infected cells and stored at −80 °C. Infectivity titers were determined by a standard plaque assay on confluent RC-37 cells.

Cytotoxicity assay

For cytotoxicity assays, cells were seeded into 24-well plates at a density of 5 × 10⁴ cells per well and incubated for 24 h at 37 °C. The medium was removed and fresh DMEM containing the appropriate dilution of the peppermint oil was added onto subconfluent RC-37
cells in ten replicates for each concentration of the drug. Wells containing 1 ml medium with 1% ethanol but no drug were also included on each plate as controls. After 4 days of incubation, the growth medium was removed and viability of the drug treated cells was determined in a standard neutral red assay (Söderberg et al. 1996). This assay quantifies the number of viable cells after their exposure to toxicants by measuring the amount of neutral red dye taken up by the cells. Medium was replaced by 1 ml DMEM supplemented with neutral red at 40 mg/ml and incubated for 3 h at 37 °C. The medium was removed and cells were rinsed with 1 ml of 0.5% formaline in 1% CaCl2 for 1 min. Finally the cells were dissolved in 50% ethanol with 1% acetic acid, incubated for 5 min on a shaker and neutral red dye incorporated by the viable cells was eluted. The neutral red dye uptake was determined by measuring the optical density (OD) of the eluted neutral red at 540 nm in a spectrophotometer. The mean OD of the cell-well controls was assigned a value of 100%. Uptake of the supravital dye neutral red has been shown to be linear with cell numbers. The cytotoxicity of the drug which reduced viable cell number by 50% (TC50) was determined from dose-response curves.

Direct plaque assay
Inhibition of virus replication was measured by plaque reduction assay. Usually $2 \times 10^3$ plaque forming units (pfu) were incubated with different concentrations of peppermint oil for 1 h at room temperature. Serial dilutions of the treated virus were adsorbed to the cells for 1 h at 37 °C. The residual inoculum was discarded and infected cells were overlayed with medium containing 0.5% methylcellulose. Each assay was performed in six replicates. After incubation for 4 days at 37 °C, monolayers were fixed with 10% formalin. The cultures were stained with 1% crystal violet and subsequently plaques were counted. By reference to the number of plaques observed in virus control monolayers (untreated cultures), the concentration of test compound which inhibited plaque numbers by 50% (IC50) was determined from dose-response curves.

Mode of antiviral activity
In order to determine the mode of antiviral action, cells were pretreated with peppermint oil before viral infection, viruses were incubated with peppermint oil before infection and cells and viruses were incubated together during adsorption or after penetration of the virus into the host cells. Peppermint oil was always used at the nontoxic concentration of 0.01%. Cell monolayers were pretreated with the oil prior to inoculation with virus by adding the essential oil 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 inoculum was added. For pretreatment of herpes simplex virus about $2 \times 10^3$ pfu of HSV-1 and HSV-2 were incubated in medium containing 0.01% peppermint oil for 1 h at room temperature prior to infection of RC-37 cells. For analysing the antiviral inhibition during the adsorption period, the same amount of HSV-1 or HSV-2 was mixed with the drug and added to the cells immediately. After 1 h of adsorption at 37 °C, the inoculum was removed and cells were overlaid with medium containing 0.5% methylcellulose. The effect of peppermint oil against HSV was also tested during the replication period by adding peppermint oil after adsorption to the overlay medium, as typical performed in antiviral susceptibility studies. All experiments mentioned above were performed in parallel with acyclovir to test the suitability of the assay. Each assay was run in six replicates. Plaque reduction assays were carried out as mentioned above and number of plaques of drug-treated cells and viruses were compared to untreated controls.

Results

Chemical characterisation of peppermint oil
Since the chemical composition of peppermint oil is important for its antiviral activity, it was chemically characterized before using it in the bioassay. The major components of peppermint oil were identified by comparing its mass spectral data with those of authentic terpene standard, literature data, and mass spectral data stored on the spectrometer database as well as by coinjection with authentic substances. The major constituents of the peppermint oil used consisted of menthol (42.8%), menthone (14.6%), isomenthone (5.9%), menthylacetate (4.4%), cineole (3.8%), limonene (1.2%) and carvone (0.6%). Cineole and limonene were found in a ratio of 3 to 1. A ratio greater than 2 is always considered to be a characteristic feature of a native peppermint oil.

Cytotoxicity
Peppermint oil was dissolved in ethanol and added to the medium at a final concentration of 1% ethanol. Ethanol by itself did not exhibit any toxic effect on RC 37 cells. The effect of peppermint oil on the growth of eucaryotic cells was examined. Monolayer cultures of RC-37 cells were grown in 0.001–0.1% drug-containing medium and after 4 days of incubation, cell viability was determined in the neutral red assay. Peppermint oil concentrations up to 0.01% did not show any visible changes in cell morphology and cell density, whereas complete cell death was observed at a concentration of
0.03% peppermint oil. Cytotoxicity is expressed as the toxic concentration, which is required to reduce cell growth by 50%. The toxic concentration (TC50) of peppermint oil for RC-37 cells is 0.014%. Acyclovir was not cytotoxic at a concentration of 22.5 µg/ml.

Virucidal activity

The virucidal action of peppermint oil against herpes simplex viruses was evaluated. The viruses were exposed for 1 hour to various concentrations ranging from 0.0001–0.06% of the compound in suspension assays. Since the initial dilution of the essential oil was always performed in ethanol and all assays contained 1% ethanol final concentration, additional tubes containing virus and 1% ethanol were used as controls. Briefly, the samples were diluted in medium and aliquots of each dilution were adsorbed on cells for 1 h at 37 °C. Ethanol at a final concentration of 1% had no effect on virus titers. The 50% inhibitory concentration (IC50) of peppermint oil was determined at 0.002% and 0.0008% for HSV-1 and HSV-2, respectively (Fig. 1). The results are presented as a percentage of virus control and are the mean values from three independent experiments. Peppermint oil inhibited plaque formation of HSV-1 and HSV-2 in a dose-dependent manner. At a concentration of 0.01% peppermint oil, which is still not cytotoxic, the titres of HSV-1 and HSV-2 are reduced by 82% and 92%, respectively. Higher concentrations of peppermint oil reduced viral titers by 94% for HSV-1 and by 92% for HSV-2 (Fig. 1). Prior to application to the cell monolayer the higher concentration of the essential oil was diluted to reach nontoxic levels.

The antiviral activity of acyclovir was determined by adding increasing concentrations of the drug after the adsorption of the virus. At a concentration of 0.0225 µg/ml ACV plaque formation was reduced by 37.9%, whereas at a concentration of 22.5 µg/ml plaques were reduced by 90.2% (data not shown). A clearly dose-dependent effect could be observed.

In order to analyse a possible time-dependent virucidal effect, HSV-1 was incubated with nontoxic concentrations of peppermint oil for different amounts of time, ranging from 1 min to 4 h. After 1, 10, 20, 30, 60 min and 2, 3, and 4 h, an aliquot was removed and assayed for remaining infectivity on confluent monolayers of RC-37 cells in 6 well plates by plaque assay. A clearly time-dependent activity could be demonstrated (Fig. 2), after 2 h of incubation the peppermint oil exhibited an antiviral activity of about 98% and more than 99% after an incubation period of 3 h.

![Fig. 1. Determination of the 50% inhibitory concentration (IC50) of peppermint oil against HSV-1 and HSV-2. Viruses were incubated for 1 hour at room temperature with increasing concentrations of peppermint oil and immediately tested in a plaque reduction assay. These experiments were repeated independently two times and data presented are the mean of three experiments.](image1)

![Fig. 2. Time-dependent activity of peppermint oil against HSV-1. HSV-1 was incubated with nontoxic concentrations of peppermint oil for different amounts of time.](image2)
Mode of antiviral action

Herpesvirus replication is characterized by a cascade of coordinately regulated events. The inhibitory effect of peppermint oil was determined following addition at different times during viral infection. To identify the step at which replication might be inhibited, cells were infected with HSV after preincubation of the cells with peppermint oil, pretreatment of the virus with the essential oil prior to infection, addition of the essential oil during adsorption or after the adsorption period. In all experiments untreated virus infected cells were used as control. The percent reduction was calculated relative to the amount of virus produced in the absence of the compound. In all assays the nontoxic concentration of the essential oil was used. Pretreatment of cells with the essential oil did not reduce virus production (Fig. 3). However pretreatment of HSV-1 and HSV-2 with peppermint oil prior to infection caused a significant reduction in the amount of plaques. Nontoxic concentrations of peppermint oil caused a reduction of infectivity of 82% and 92% for HSV-1 and HSV-2, respectively. When peppermint oil was added only during the adsorption period, virus titres were reduced by 18% for HSV-1 and by 25% for HSV-2 (Fig. 3). In contrast, no significant effect on viral growth could be observed when the essential oil was added only to the overlay medium after the adsorption period immediately following the removal of the unadsorbed virus inoculum. These results suggest that the virucidal effect of peppermint oil is exerted prior to viral infection of host cells. All experiments were performed in parallel with acyclovir to test the suitability of the assay. Acyclovir achieved the highest antiviral effect when applied during the replication period (data not shown), plaques were reduced by 90.2%. When this drug was added to the cells prior to infection, no antiviral effect could be observed. Pretreatment of the virus or addition of the drug during the adsorption reduced plaque formation by 79.8% and 72.9%, respectively.

Virucidal activity against HSV-1-ACV<sub>res</sub>

The virucidal activity of peppermint oil was also analysed against HSV-1-ACV<sub>res</sub>, an acyclovir resistant HSV-1 strain. HSV-1-ACV<sub>res</sub> exhibits a single-point mutation in the DNA polymerase gene which leads to an amino acid exchange. Therefore, viral replication cannot be blocked anymore by the drug acyclovir. HSV-1, HSV-2 and HSV-1-ACV<sub>res</sub> were incubated with 0.01% peppermint oil and the virucidal activity was measured by plaque reduction assay. All virus strains were significantly inhibited by the essential oil, the titre of HSV-1ACV<sub>res</sub> was reduced by 99% (Fig. 4).

Discussion

HSV-1 is transmitted through contact with saliva and causes recurrent herpes labialis, whereas HSV-2 is transmitted primarily by sexual contact and associated with urogenital and neonatal infections. There is only little information on the effects of essential oils against viral infections. Antiviral activity of tea tree oil against tobacco mosaic virus was reported previously (Bishop, 1995). All reports on the inhibitory activity of pepper-
Peppermint oil against HSV are anecdotal descriptions. Therefore we analysed the possible inhibitory effect of peppermint oil against herpes simplex virus infection in vitro.

Experiments to assess the toxicity of peppermint oil indicate a moderate toxic behaviour in cell cultures. The toxicity of this essential oil approached 50% (TC50) at concentrations of 0.014%. In plaque reduction assays peppermint oil exhibited a concentration-dependent virucidal effect, when HSV was mixed with the essential oil prior to inoculation. Using noncytotoxic concentrations of peppermint oil in viral suspension assays, plaque formation was reduced by 82% and 92% for HSV-1 and HSV-2, respectively. Higher concentrations of peppermint oil reduced viral titers of both herpesviruses by more than 90%, which is as effective as tea tree oil (Schnitzler et al. 2001).

A clearly time-dependent activity could be demonstrated, after 2 h of incubation the peppermint oil exhibited an antiviral activity of about 98% and more than 99% after an incubation period of 3 h. Time- and dose-dependent reduction of infectious HSV-1 titers by the essential oil of Houttuynia cordata could be demonstrated recently by interfering with the function of virus envelope (Hayashi et al. 1995).

In order to determine the mode of antiviral action, either cells were pretreated before viral infection or viruses were incubated with noncytotoxic concentration of ACV or peppermint oil before infection, during adsorption or after penetration into the host cells. ACV reduced plaque formation most when added during the replication period when this drug is incorporated into viral DNA. Pretreatment of the cells with the essential oil had no effect on the production of infectious virus and plaque formation was not affected. However, pretreatment of HSV-1 and HSV-2 with peppermint oil prior to infection resulted in a concentration-dependent reduction of plaques, suggesting that peppermint oil interferes with virion envelope structures or is masking viral compounds which are necessary for adsorption or entry into host cells. When peppermint oil was added during the adsorption period, the amount of plaques for HSV-1 and HSV-2 was reduced by 18% and 25%, respectively. A similar antiadsorption effect was demonstrated for the milkprotein lactoferrin inhibiting the attachment of HSV-1 to Vero cells (Marchetti et al. 1996). After the adsorption period, peppermint oil exhibited only a minor antiviral effect. These results suggest that free virus is very sensitive to the virucidal effect of peppermint oil. The inhibition of HSV appears to occur before adsorption or during adsorption but not after penetration of the virus into the cell. Peppermint oil reduced the infectivity of the virus possibly due to direct interaction with the viral envelope and glycoproteins. An interaction of tea tree oil, which has similar properties against HSV in vitro, with the cell membrane of Escherichia coli was reported recently (Gustafson et al. 2001). The ability of tea tree oil to disrupt the permeability barrier of cell membrane structures in E. coli could be demonstrated (Cox et al. 2000). The antiviral effect of tea tree oil against tobacco mosaic virus, a nonenveloped plant virus, has been reported previously (Bishop, 1995). However, the mechanism of this antiviral action was not analysed. A dissolution of the HSV envelope by treatment with oregano essential oil has been described (Siddiqui et al. 1996). Sandalwood oil was reported to show no virucidal effect when incubated with herpesvirus prior to infection but inhibited the replication significantly when it was added after the adsorption period. Thus different mechanisms of antiviral activity of different essential oils seem to be present. It remains to be determined whether the inhibitory effect of peppermint oil is due to binding of the essential oil to viral proteins involved in host cell adsorption and penetration or is due to damage to the virions, possibly their envelopes, thereby impairing their ability to infect host cells.

HSV-1-ACV was significantly inhibited by the essential oil, the titre of HSV-1-ACV was reduced by 99%. Since peppermint oil is able to inhibit an acyclovir-resistant HSV-1 strain, the mechanism of interaction between peppermint oil and acyclovir with HSV must be different. Acyclovir inhibits virus replication by interference with the DNA polymerase inside the cell, whereas peppermint oil probably inactivates HSV virus, a nonenveloped plant virus, has been reported previously (Bishop, 1995). However, the mechanism of this antiviral action was not analysed. A dissolution of the HSV envelope by treatment with oregano essential oil has been described (Siddiqui et al. 1996). Therefore alternative antiviral agents which are effective for viral strains resistant to current antiviral agents are of great interest.

The application of tea tree oil, the essential oil of Melaleuca alternifolia, for the treatment of recurrent herpes labialis has been reported recently (Carson et al. 2001). Both peppermint oil and tea tree oil possess virucidal activity against herpes simplex virus in vitro. Time-of-addition experiments suggested that the inhibitory action of peppermint oil was through blocking of virus adsorption. The topical use of this essential oil for the treatment of HSV infections would be ideal, especially for those patients who experience frequent recurrences.

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References


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