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Comparative Study on the Antiviral Activity of Selected Monoterpenes Derived from Essential Oils

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Essential oils are complex natural mixtures, their main constituents, e.g. terpenes and phenylpropanoids, being responsible for their biological properties. Essential oils from eucalyptus, tea tree and thyme and their major monoterpene compounds α-terpinene, γ-terpinene, α-pinene, *p*-cymene, terpinen-4-ol, α-terpineol, thymol, citral and 1,8-cineole were examined for their antiviral activity against herpes simplex virus type 1 (HSV-1) *in vitro*. These essential oils were able to reduce viral infectivity by >96%, the monoterpenes inhibited HSV by about >80%. The mode of antiviral action has been determined, only moderate antiviral effects were revealed by essential oils and monoterpenes when these drugs were added to host cells prior to infection or after entry of HSV into cells. However, both essential oils and monoterpenes exhibited high anti-HSV-1 activity by direct inactivation of free virus particles. All tested drugs interacted in a dose-dependent manner with herpesvirus particles thereby inactivating viral infection. Among the analysed compounds, monoterpene hydrocarbons were slightly superior to monoterpene alcohols in their antiviral activity, α-pinene and α-terpineol revealed the highest selectivity index. However, mixtures of different monoterpenes present in natural tea tree essential oil revealed a ten-fold higher selectivity index and a lower toxicity than its isolated single monoterpenes.

Keywords: essential oils; monoterpenes; herpes simplex virus; antiviral activity; mode of action; selectivity index.

INTRODUCTION

Essential oils, odours and volatile products of plant secondary metabolism have a wide application in folk medicine as well as in fragrance industries. Essential oils are complex natural mixtures of volatile secondary metabolites, isolated from plants by distillation. The main constituents of essential oils, e.g. monoterpenes and sesquiterpenes and phenylpropanoids including carbohydrates, alcohols, ethers, aldehydes and ketones, are responsible for the fragrant and biological properties of aromatic and medicinal plants.

Herpes simplex virus (HSV) is differentiated into two antigenic types, type 1 (HSV-1) and type 2 (HSV-2), and is an important pathogen for humans, therefore the discovery of novel anti-HSV drugs deserves great effort. HSV infects and replicates in cells at the site of entry, the mucocutaneous surface. After the acute ganglionic infection subsides, HSV establishes latency and persists in the neurons for the life-time. The latent virus is reactivated spontaneously causing frequent recurrent infections in some patients, while most people experience few recurrences. HSV-1 infections are very common and reactivations mostly affect adult people (Whitley and Roizman, 2001). The primary symptoms of herpes infection include a prodromal flu-like syndrome with fever, headache, malaise, diffuse myalgias, followed by local symptoms consisting of itching and painful papules. Gingivostomatitis and pharyngitis are the most frequent clinical manifestations of the first episodes of HSV-1 infection. The clinical manifestation of the disease exhibits different severity in immunocompetent patients and in addition some patients always encounter recurrent attacks (Whitley and Roizman, 2001).

The antiviral agents acyclovir, famciclovir and valacyclovir can be used to shorten the course and decrease the severity of these clinical symptoms and may suppress the virus itself (Whitley and Roizman, 2001). Antiviral agents licensed currently for the treatment of herpesvirus infections include acyclovir and derivatives, foscarnet and cidofovir, all of which inhibit herpesvirus DNA polymerases (De Clercq, 2004). In addition, the emergence of virus strains resistant to commonly used anti-herpesvirus drugs is of importance, particularly in immunocompromised patients (Reusser, 1996; Cassady and Whitley, 1997). The development of viral resistance towards antiviral agents enhances the need for new effective compounds against viral infections. Medicinal plants produce a variety of chemical constituents with the ability to inhibit viral replication, and compounds from natural sources are of interest as possible sources to control viral infection.

Antiherpes screening experiments on medicinal plant extracts and plant derived secondary metabolites have

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been reported (Reichling *et al.*, 1999; De Logu *et al.*, 2000). The antiherpes activity of several essential oils of different plant sources as well as of some constituents of essential oils had been demonstrated previously (Farag *et al.*, 2004). 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). The antiherpes activity of eucalyptus oil, Australian tea tree oil (Schnitzler *et al.*, 2001), thyme oil (Schnitzler *et al.*, 2007) and manuka oil (Reichling *et al.* 2005) have been published previously. Some phenylpropanes (Benencia and Courrèges, 2000; Tragoolpua and Jatisatienr, 2007), triterpenes (Niedermeyer *et al.*, 2005) and sesquiterpenes (Hayashi *et al.*, 1996; Pusztai *et al.*, 2008; Rollinger *et al.*, 2008) had been tested for their antiviral activity against different herpesviruses and rhinovirus. However, monoterpenes as major constituents of essential oils, have not been analysed systematically for their antiviral potential. Only few reports describe the inhibition of viral replication by monoterpenes, e.g. isoborneol as a potent inhibitor of HSV (Armaka *et al.*, 1999), consequently there is little information about terpenes concerning the inhibition of the viral replication cycle and their mode of antiviral action.

The goal of the present study is the evaluation of the antiviral activity of monoterpenes, major constituents of essential oils, against HSV-1 and the mode of antiviral action of these monoterpenes during the viral multiplication cycle.

MATERIALS AND METHODS

Essential oils and monoterpenes. All essential oils tested met the standard demands of either current pharmacopoeias or literature data (Blaschek *et al.*, 2005). Eucalyptus oil and tea tree oil were provided by Primavera Life (Sulzberg, Germany) and thyme oil was obtained from Caelo (Hilden, Germany). Monoterpenes α-terpinene, γ-terpinene, terpinen-4-ol, α-pinene, *p*-cymene, α-terpineol, citral (*cis*-citral and *trans*-citral), 1,8-cineole and thymol were purchased from Roth (Karlsruhe, Germany). All monoterpenes met high purity standards. High contents of about 88% 1,8-cineole in eucalyptus oil, 10% α-terpinene, 20% γ-terpinene and 40% terpinen-4-ol in tea tree oil, 35% *p*-cymene and 38% thymol in thyme oil and 64% citral in lemon oil have been reported previously (Reichling *et al.*, 2005). Structural formulas of these selected monoterpene constituents are presented in Fig. 1. Essential oils and monoterpenes were dissolved in ethanol and further diluted in medium for cell culture experiments, always resulting in an ethanol concentration below 1% which has no effect on cells and viruses (Schnitzler *et al.*, 2007).

Acyclovir. Acyclovir was purchased from GlaxoSmith-Kline (Bad Oldesloe, Germany) and dissolved in sterile water.

Cell culture and herpes simplex virus type 1 (HSV-1). 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 U/mL penicillin and 100 µg/mL streptomycin. The monolayers were removed from their plastic surfaces and serially passaged whenever they became confluent. The cells were plated out onto 96-well and 6-well culture plates for cytotoxicity and antiviral assays, respectively, and propagated at 37 ºC in an atmosphere of 5% CO_2. Herpes simplex virus type 1 (HSV-1) strain KOS was used for all experiments. Viruses were routinely grown on RC-37 cells and virus stock cultures were prepared from supernatants of infected cells and stored at −80 ºC. Infectivity titers were determined by a standard plaque assay on confluent RC-37 cells (Schnitzler *et al.*, 2008).

Cytotoxicity assay. For cytotoxicity assays, the cells were seeded into 96-well plates and incubated for 24 h at 37 ºC. The medium was removed and fresh DMEM containing the appropriate dilution of the essential oils or monoterpenes was added onto subconfluent cells in eight replicates for each concentration of the drugs. Wells containing medium with 1% ethanol but no drug were also included on each plate as controls. After 3 days of incubation, the growth medium was removed and the viability of the drug treated cells was determined in a standard neutral red assay (Söderberg *et al.*, 1996). 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-control wells was assigned a value of 100%. The cytotoxic concentration of the drug which reduced the viable cell number by 50% (TC_{50}) was determined from dose-response curves. Additionally the maximum noncytotoxic concentration of each drug was determined.

Dose-response assays. Inhibition of HSV replication was measured by a plaque reduction assay. Usually 2×10^3 plaque forming units (pfu) were incubated with different concentrations of essential oils or monoterpenes for 1 h at room temperature, then the virus was allowed to adsorb to the cells for 1 h at 37 ºC. The residual inoculum was discarded and the infected cells were overlaid with medium containing 0.5% methylcellulose. Each concentration was performed in three replicates, virus-infected cells in wells containing medium with 1% ethanol but no drug were also included on each plate as controls. After incubation for 3 days at 37 ºC, the 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% (IC_{50}) was determined from dose-response curves.

Time of addition assay. In order to determine the mode of antiviral action for oils and monoterpenes, the cells were pretreated with essential oils or components before viral infection, viruses were incubated with drugs before infection or infected cells were incubated immediately after penetration of the virus into cells. Essential oils and components were always used at the maximum noncytotoxic concentration. Cell monolayers were pretreated with drugs prior to inoculation with virus by adding the essential oils or components to the culture medium followed by incubation for 1 h at 37 ºC. The drugs were

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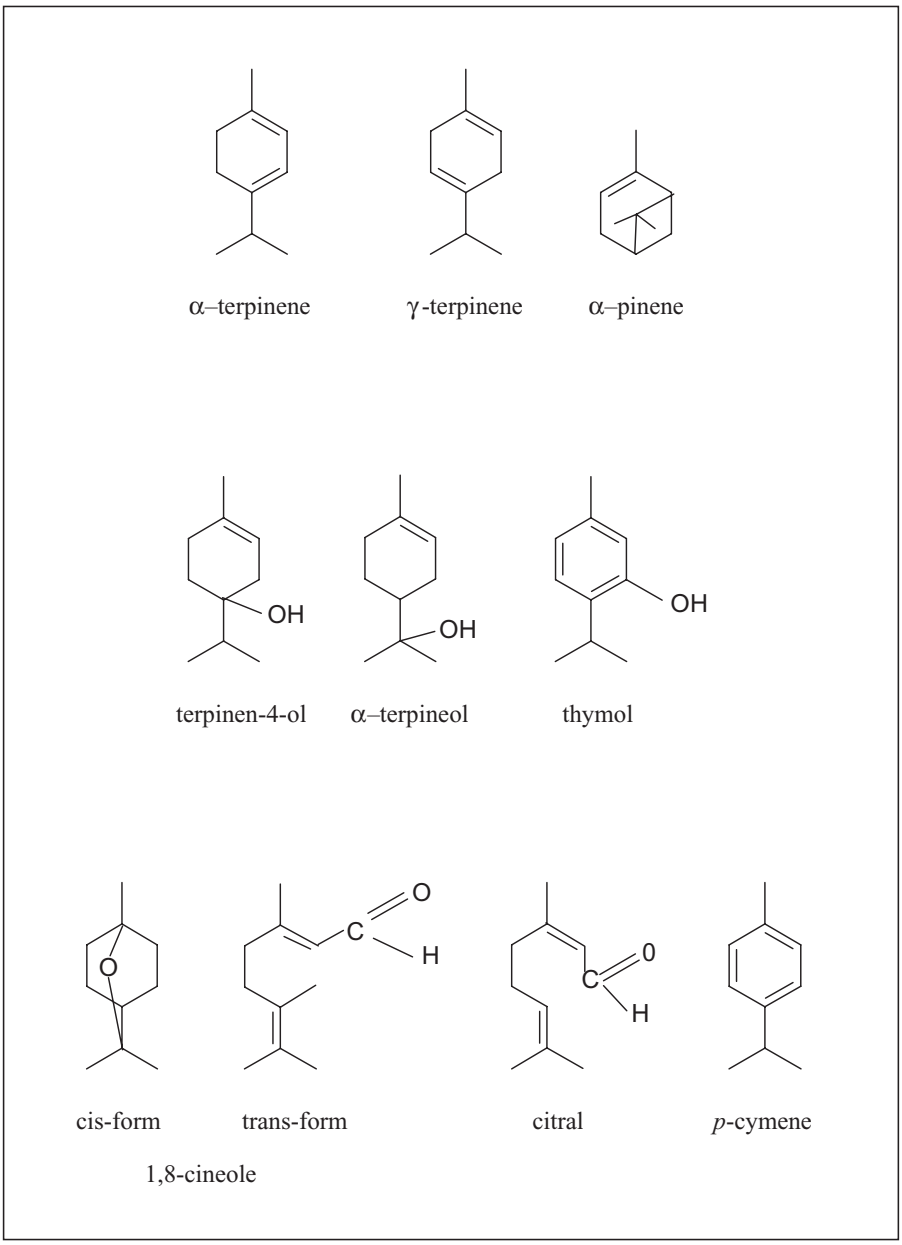

Figure 1. Structural formulas of monoterpenes.

aspirated and the cells were washed before the HSV inoculum was added. For pretreatment of herpes simplex virus with drugs, about 2×10^3 pfu of HSV was incubated in medium containing the maximum noncytotoxic concentration of the drugs for 1 h at room temperature prior to infection of RC-37 cells. The effect of essential oils and components against HSV was also tested during the replication period by addition of drugs after cell infection to the overlay medium, as typically performed in antiviral susceptibility studies. Each assay was run in three replicates. Plaque reduction assays were carried out as described above and the number of plaques of drug-treated cells and viruses were compared with untreated controls. Wells containing medium with 1% ethanol but no drug were also included on each plate as controls.

RESULTS

Eucalyptus oil, tea tree oil, thyme oil and some selected monoterpenic compounds (Fig. 1) were serially diluted in ethanol and added to the cell culture medium to examine the effect on the growth and viability of tissue culture cells, always resulting in an ethanol concentration below 1% which had no effect on cells and viruses. Cell monolayers were grown in medium containing different concentrations of these drugs. After 3 days of incubation, the cell viability of RC-37 cells was determined with the neutral red assay (Table 1). The maximum noncytotoxic concentrations of these drugs were determined between 20 μg/mL for citral and 1250 μg/mL for 1,8-cineole, the least toxic compound tested. Essential oils and monoterpenes revealed low cytotoxicity.

The potential antiviral effect of different essential oils and some selected components was determined against herpes simplex virus type 1 (HSV-1) *in vitro*. HSV-1 was incubated for 1 h at room temperature with various concentrations of eucalyptus oil, tea tree oil, thyme oil, α-terpinene, γ-terpinene, α-pinene, *p*-cymene, terpinen-4-ol, α-terpineol, thymol, citral and 1.8-cineole. In all assays untreated virus-infected cells were used as a

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control. Subsequently, aliquots of each dilution were added on the cells for 1 h, afterwards the cells were washed and overlaid with drug-free medium and incubated for 3 days at 37 ºC. The 50% inhibitory concentrations (IC_{50}) for HSV-1 were determined in a wide range between 2.0 µg/mL for tea tree oil and 1200 µg/mL for 1,8-cineole (Table 2). The results are presented as virus reduction and represent the average of three independent experiments. In plaque reduction assays, all essential oils and monoterpenes exhibited a concentration-dependent antiviral effect, tea tree oil was the most effective drug. All essential oils were able to suppress viral multiplication by >96% (Table 2), all monoterpenes inhibited HSV by >80%, with the exception of the less active 1,8-cineole. In Table 2, the results for up to 100 µg/mL are shown, for some monoterpenes with maximum noncytotoxic concentrations beyond 100 µg/mL the data are not shown. Selectivity indices for oils and different monoterpenes were calculated as the TC_{50}/IC_{50} ratio (Table 3), the highest selectivity index of 60 was determined for tea tree oil. Low selectivity indices were found for eucalyptus oil, thyme oil and most monoterpenes.

Herpesvirus replication is characterized by a complex sequence of different steps at which antiviral agents might interfere. In order to investigate the inhibitory effects on herpes simplex virus in detail, all drugs were added at different stages during viral infection. For comparison, all untreated controls contained the same concentration of ethanol as the drug-treated viruses, in order to exclude any influence of ethanol. When the host cells were pretreated with drugs prior to infection, some of the tested drugs showed minor effects on viral infection, e.g. eucalyptus oil, tea tree oil and α-pinene (Fig. 2). On the other hand, pretreatment of HSV-1 with the oils or monoterpenic compounds for 1 h prior to infection caused a significant reduction in plaque formation. At maximum non-cytotoxic concentrations of the tested drugs, infectivity was reduced by >96% for all essential oils as well as for three of the monoterpenes, α-terpinene, γ-terpinene and α-pinene (Fig. 3). With the exception of 1,8-cineole, all other constituents revealed a plaque reduction of herpes simplex virus between about 80% and 90%. Acyclovir showed the highest antiviral activity when added during the replication period with inhibition of the viral replication of 98.6%. This drug inhibits specifically the viral DNA polymerase during the replication cycle when new viral DNA is synthesized. However, only minor effects on viral infection were detected when cells or viruses were pretreated with acyclovir (data not shown). In contrast, when the oils or compounds were added to the overlay medium

Table 1. Cytotoxicity of essential oils and selected compounds on RC-37 cells. Viability of the drug-treated cells was determined in a standard neutral red assay. Results are given in % compared with untreated controls and represent the mean of three independent experiments

Concentration (µg/mL)	10	25	50	75	100	250	500	750
	Cell viability (%) ± SD							
Eucalyptus oil	102.5 ± 8.3	104.1 ± 6.5	104.8 ± 5.4	102.8 ± 3.4	94.6 ± 7.4	83.2 ± 7.4	4.9 ± 2.0	4.4 ± 2.0
Tea tree oil	98.1 ± 5.4	103.3 ± 3.5	103.3 ± 5.6	101.2 ± 8.3	72.2 ± 18.8	5.2 ± 0.9	5.0 ± 1.0	6.0 ± 1.0
Thyme oil	104.4 ± 10.0	102.0 ± 9.1	102.2 ± 16.2	18.5 ± 4.1	4.1 ± 2.5	n.d.	n.d.	n.d.
α-Terpinene	106.3 ± 10.2	101.0 ± 11.0	96.8 ± 11.2	75.7 ± 6.0	6.0 ± 0.8	5.8 ± 2.1	5.2 ± 0.9	5.5 ± 0.2
γ-Terpinene	116.4 ± 19.8	110.2 ± 16.9	9.6 ± 2.4	11.6 ± 4.0	7.9 ± 2.1	n.d.	n.d.	n.d.
α-Pinene	116.5 ± 4.4	112.8 ± 5.5	99.9 ± 3.2	65.4 ± 4.5	5.3 ± 0.4	n.d.	n.d.	n.d.
p-Cymene	95.5 ± 9.3	96.8 ± 10.5	79.0 ± 14.8	35.1 ± 11.2	20.1 ± 6.6	19.8 ± 4.5	15.9 ± 4.6	14.2 ± 3.3
Terpinen-4-ol	101.5 ± 6.9	93.0 ± 1.4	98.5 ± 0.7	91.3 ± 0.7	98.9 ± 17.1	95.3 ± 11.7	78.0 ± 14.0	31.1 ± 2.5
α-Terpineol	115.5 ± 8.7	103.8 ± 11.6	111.8 ± 5.9	109.3 ± 12.9	104.3 ± 15.3	73.5 ± 17.7	41.6 ± 12.4	5.7 ± 0.4
Thymol	98.7 ± 5.8	96.6 ± 12.1	85.3 ± 8.3	66.1 ± 13.5	30.9 ± 12.5	n.d.	n.d.	n.d.
Citral	93.2 ± 4.2	89.7 ± 2.6	36.3 ± 10.6	5.3 ± 0.8	6.2 ± 1.1	n.d.	n.d.	n.d.
1,8-Cineole	104.9 ± 4.2	99.6 ± 12.3	95.5 ± 4.9	96.1 ± 5.2	97.7 ± 2.8	101.4 ± 6.2	95.5 ± 8.4	98.5 ± 2.1

n.d., not determined.

Table 2. Antiviral effect of serial dilutions of essential oils and selected compounds against herpes simplex virus type 1. Results are given in % compared with untreated virus controls and represent the mean of three independent experiments

Concentration (µg/mL)	1	2.5	5	7.5	10	25	50	75	100
	Remaining infectivity (% of control) ± SD								
Eucalyptus oil	115.5 ± 0.7	120.0 ± 14.1	106.5 ± 2.1	109.0 ± 16.1	96.5 ± 2.1	86.0 ± 5.7	63.5 ± 16.3	0.0 ± 0.0	0.3 ± 0.4
Tea tree oil	103.0 ± 14.1	43.1 ± 4.2	15.0 ± 5.7	2.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	n.d.
Thyme oil	81.6 ± 12.9	72.0 ± 20.4	60.6 ± 12.9	53.0 ± 15.6	51.6 ± 13.3	7.0 ± 2.7	1.0 ± 1.6	n.d.	n.d.
α-Terpinene	109.0 ± 4.2	91.5 ± 3.5	102.0 ± 11.3	58.5 ± 9.2	33.5 ± 3.3	0.0 ± 0.0	0.0 ± 0.0	n.d.	n.d.
γ-Terpinene	96.5 ± 7.8	100.2 ± 9.9	91.0 ± 8.5	42.0 ± 1.4	1.5 ± 2.1	0.0 ± 0.0	n.d.	n.d.	n.d.
α-Pinene	90.4 ± 10.9	73.2 ± 17.3	46.6 ± 3.5	4.7 ± 0.6	2.6 ± 2.8	0.0 ± 0.0	0.0 ± 0.0	n.d.	n.d.
p-Cymene	99.3 ± 0.6	97.0 ± 1.0	100.4 ± 9.0	93.7 ± 17.5	78.0 ± 11.8	31.0 ± 12.7	n.d.	n.d.	n.d.
Terpinene-4-ol	101.5 ± 6.9	93.0 ± 1.4	98.5 ± 0.7	91.3 ± 0.7	98.9 ± 17.1	95.3 ± 11.7	78.0 ± 14.0	31.1 ± 2.5	0.4 ± 0.1
α-Terpineol	97.5 ± 12.0	106.0 ± 7.8	95.0 ± 7.1	96.0 ± 8.5	92.5 ± 16.3	66.0 ± 16.2	52.5 ± 13.4	38.6 ± 7.1	33.4 ± 6.0
Thymol	99.2 ± 8.8	89.5 ± 12.1	83.3 ± 14.8	74.0 ± 5.7	72.7 ± 16.3	54.6 ± 6.5	n.d.	n.d.	n.d.
Citral	70.9 ± 20.3	55.9 ± 10.1	39.7 ± 9.3	25.2 ± 3.2	12.1 ± 1.2	14.2 ± 2.3	n.d.	n.d.	n.d.
1,8-Cineole	114.9 ± 4.2	11.6 ± 15.6	110.5 ± 10.6	103.0 ± 7.1	103.4 ± 6.4	105.2 ± 9.9	94.0 ± 5.7	89.1 ± 6.0	85.2 ± 7.1

n.d., not determined.

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Table 3. Selectivity indices (SI) of essential oils and selected monoterpenes for HSV-1. Experiments were repeated independently two times and data presented are the mean of three experiments ± SD (%)

Essential oil/ monoterpene	Max. noncytotoxic concentration (μg/mL) ± SD (%)	TC_{50} (μg/mL) ± SD (%)	IC_{50} (μg/mL) ± SD (%)	Selectivity index
Eucalyptus oil	200 ± 3.5%	290 ± 5.8%	55 ± 8.2%	5.3
Tea tree oil	75 ± 8.3%	120 ± 9.6%	2 ± 4.2%	60.0
Thyme oil	50 ± 16.2%	70 ± 2.1%	11 ± 13.3%	6.4
α-Terpinene	50 ± 11.2%	55 ± 8.1%	8.5 ± 16.3%	6.5
γ-Terpinene	35 ± 8.1%	38 ± 12.7%	7 ± 2.8%	5.4
α-Pinene	50 ± 3.2%	80 ± 5.1%	4.5 ± 10.4%	17.8
p-Cymene	30 ± 12.7%	65 ± 13.0%	16 ± 16.2%	4.1
Terpinen-4-ol	250 ± 11.7%	650 ± 13.3%	60 ± 17.7%	10.8
α-Terpineol	150 ± 16.5%	400 ± 19.9%	22 ± 14.9%	18.2
Thymol	35 ± 8.1%	85 ± 12.8%	30 ± 6.5%	2.8
Citral	20 ± 1.7%	45 ± 8.9%	3.5 ± 10.1%	12.9
1,8-Cineole	1250 ± 9.6%	2000 ± 8.4%	1200 ± 8.9%	1.7

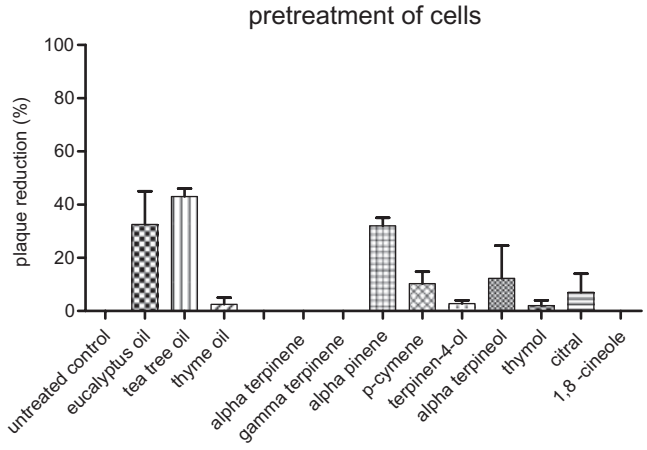

Figure 2. Antiviral activity of essential oils and selected monoterpenes against herpes simplex virus type 1 after pretreatment of cells with drugs. Prior to viral infection, the cells were incubated with maximum noncytotoxic concentrations of drugs for 1 h at 37 °C. The number of virus plaques was determined 3 days after infection and compared with the untreated control. The results are expressed as percentage of plaque reduction. These experiments were repeated independently and data presented are the mean of three experiments.

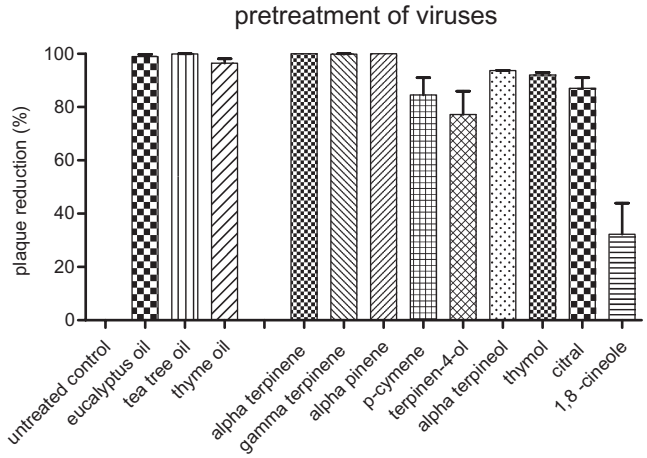

Figure 3. Antiviral activity of essential oils and selected monoterpenes against herpes simplex virus type 1 after incubation of HSV with drugs. HSV was incubated for 1 h at room temperature with maximum noncytotoxic concentrations of drugs. The number of virus plaques was determined 3 days after infection and compared with untreated control. Results are expressed as percentage of plaque reduction. These experiments were repeated independently and data presented are the mean of three experiments.

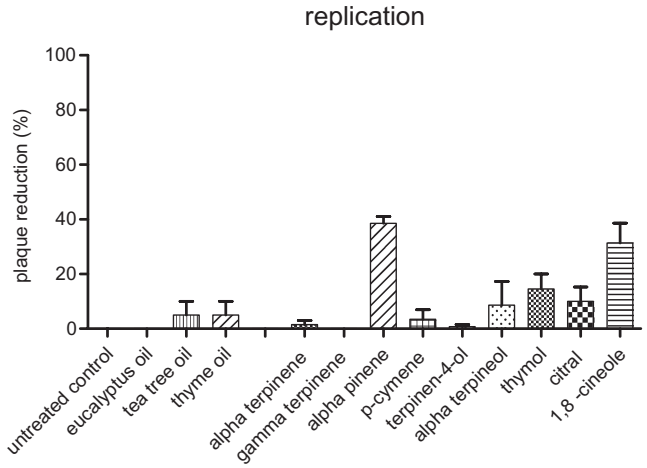

Figure 4. Antiviral activity of essential oils and selected monoterpenes against herpes simplex virus type 1 during intracellular virus replication. Drugs were applied to HSV-1 infected cells after penetration of the viruses into cells for 3 days. Number of virus plaques was determined 3 days after infection and compared with untreated control. Results are expressed as percentage of plaque reduction. These experiments were repeated independently and data presented are the mean of three experiments.

after penetration of the viruses into the host cells, plaque formation was not significantly reduced, only α-pinene and 1,8-cineole being moderately effective (Fig. 4).

DISCUSSION

The pharmaceutical industry is increasingly targeting medicinal plants with the aim of identifying lead compounds, focusing particularly on suitable alternative antiviral agents. Several drugs are currently available for the management of HSV infections such as acyclovir. Topical treatment of herpes labialis infection is standard, for the most part carried out with acyclovir creams, but also with phytotherapeutic preparations containing sage oil and lemon balm oil (Saller *et al.*, 2001; Wölbling and Leonhardt, 1994; Koytchev *et al.*, 1999). Both plant essential oils were shown to be significantly superior to placebo and equivalent to acyclovir (Wölbling and Leonhardt, 1994). Our previous *in vitro*

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experiments revealed similar results for essential oils from eucalyptus, tea tree and thyme (Schnitzler *et al.*, 2001; Schuhmacher *et al.*, 2003; Koch *et al.*, 2008).

In the present study, the inhibitory effects of several essential oils against herpes simplex virus infection were compared with the antiviral potential of their major monoterpenic compounds. All tested essential oils and most monoterpenes exhibited high levels of antiviral activity against HSV-1 in viral suspension tests. At maximum noncytotoxic concentrations plaque formation was significantly reduced by >96% by eucalyptus oil, tea tree oil and thyme oil, most monoterpenic constituents were able to suppress viral infection by >80%. High antiviral activity was observed for essential oils and isolated monoterpenes when herpesvirus was incubated with these drugs prior to host cell infection. These results suggest that the investigated drugs directly inactivate herpes virus and might interfere with virion envelope structures or mask viral structures which are necessary for adsorption or entry into host cells. A virucidal activity of *Melaleuca armillaris* essential oil has been reported recently (Farag *et al.*, 2004) and dissolution of the HSV envelope by treatment with oregano essential oil has been described (Siddiqui *et al*., 1996). Thus different mechanisms of antiviral activity of different essential oils seem to be present. The inhibition of HSV by the tested essential oils and monoterpenes 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 essential oils is due to binding of the essential oil to viral proteins involved in host cell adsorption and penetration. De Logu *et al.,* (2000) 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 with our results for essential oils and monoterpenes. Adenovirus, a virus without envelope, was not affected by eucalyptus essential oil due to the lack of a viral envelope (Cermelli *et al.*, 2008). Sesquiterpenes, e.g. triptofordin C-2 and sesquiterpene coumarins inhibit cytomegalovirus, (Hayashi *et al.*, 1996), severe acute respiratory syndrome coronavirus (Wen *et al.*, 2007) and rhinovirus (Rollinger *et al.*, 2008). Pusztai *et al.* (2008) reported a specific inhibition of the CMV immediate early gene expression, whereas other sesquiterpenes are moderately virucidal against different enveloped viruses, e.g. herpes simplex virus, cytomegalovirus, measles virus and influenza virus (Hayashi *et al.*, 1996). Isoborneol, a monoterpene and a component of several plant essential oils, showed virucidal activity against HSV-1 and specifically inhibited glycosylation of viral proteins (Armaka *et al.*, 1999). The application of cineole protects mice against infection with HSV-2 (Bourne *et al.*, 1999). Since essential oils are able to inhibit acyclovir-resistant HSV-1 isolates (Schnitzler *et al.*, 2007), the mechanism of interaction between these compounds and acyclovir with HSV must be different. Acyclovir inhibits virus replication by interference with the DNA polymerase inside the cell, whereas essential oils and monoterpenes probably inactivate HSV before it enters the cell. Viral resistance to acyclovir represents a particular problem, the prevalence of resistance in acyclovir-treated immunocompromised individuals is approximately 4% to 7% (Christoph *et al.*, 1998; Stranska *et al.*, 2005). Therefore other antiherpetic agents which are effective for viral mutants resistant to current antiviral agents are of great interest for topical treatment. 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, 2006).

Terpinen-4-ol, the principal active component of tea tree oil, was more active in antibacterial time-kill assays as an isolated compound than when present in tea tree oil (Cox *et al.*, 2001). These findings are in contrast to results of antiviral activity, where the complex mixture of the essential oil revealed a higher antiviral activity and selectivity index of 60, whereas single major monoterpenes α-terpinene, γ-terpinene and terpinen-4-ol revealed low selectivity indices of 6.5, 5.4 and 10.8, respectively. These rather low selectivity indices of monoterpenes will not permit topical application on mucous membranes. The tested monoterpenes dominate the toxicity of essential oils, e.g. eucalyptus oil consists of about 80% 1,8-cineole, both revealed very low toxicity but also low selectivity indices. For thyme oil, the TC_{50} of 70 μg/mL corresponds well to its major compounds *p*-cymene and thymol, with TC_{50} values in the same range of 65 μg/mL and 85 μg/mL. The same is true for tea tree oil, the main constituents α-terpinene, γ-terpinene and terpinen-4-ol contribute equally to a final TC_{50} of 120 μg/mL for the mixture in this essential oil. Taken together, the composition of monoterpenes in these essential oils dominates their toxic activity. However, the antiviral activity of single monoterpenes does not contribute equally to the antiviral activity of the essential oil. Tea tree oil is composed of about 10% α-terpinene, 20% γ-terpinene and 40% terpinen-4-ol. Although terpinen-4-ol is the major compound in tea tree oil and revealed a much lower antiviral activity than α-terpinene and γ-terpinene, the complex oil demonstrated a high antiviral activity. Cos *et al.* (2006, 2008) recommended IC_{50} values for promising natural products against infectious diseases, e.g. for extracts below 100 μg/mL and below 25 μM for pure compounds. The essential oils in our study revealed IC_{50} values of 55 μg/mL, 2 μg/mL and 11 μg/mL for eucalyptus, tea tree and thyme, respectively, and are far below the recommended cut off. However, only the monoterpene citral revealed an IC_{50} value below the suggested cut off with 23 μM, all other monoterpenes displayed higher IC_{50} values and do not present promising antiinfective agents according to this suggestion. However the most important predictive value for future application of these drugs is their selectivity index, Amoros *et al.* (1992) recommended a selectivity index of at least 4 as appropriate. According to this suggestion, all essential oils and monoterpenes, with the exception of thymol and 1,8-cineole might be suitable agents. However, drugs with a higher selectivity are preferable (Vanden Berghe *et al.*, 1986), e.g. tea tree oil which had already been successfully applied in labial herpes infections. Other compounds of essential oils need to be screened but a mixture of different compounds in essential oils seems to be superior and preferable to single compounds. Considering the lipophilic nature of tea tree oil which enables penetration of the skin, it is a promising topical therapeutic agent in the treatment of recurrent herpes infection.

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