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Antiviral activity of monoterpenes beta-pinene and limonene against herpes simplex virus in vitro

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ABSTRACT

Background and Objectives: Essential oils are complex mixtures containing compounds of several different functional-group classes. Depending on the structure, we can distinguish monoterpenes, phenylpropanes, and other components. Here in this study two monoterpene compounds of essential oils, i.e. β-pinene and limonene were examined for their antiviral activity against herpes simplex virus type 1 (HSV-1) in vitro.

Material and Methods: All antiviral assays were performed using RC-37 cells. Cytotoxicity was determined in a neutral red assay, antiviral assays were performed with HSV-1 strain KOS. The mode of antiviral action was evaluated at different periods during the viral replication cycle. Acyclovir was used as positive antiviral control.

Results: Beta-pinenene and limonenen reduced viral infectivity by 100%. The mode of antiviral action has been determined, only moderate antiviral effects were revealed by monoterpenes when these drugs were added to host cells prior infection or after entry of HSV into cells. However, both monoterpenes exhibited high anti-HSV-1 activity by direct interaction with free virus particles. Both tested drugs interacted with HSV-1 in a dose-dependent manner thereby inactivating viral infection.

Conclusions: These results suggest that monoterpenes in essential oils exhibit antiherpetic activity in the early phase of viral multiplication and might be used as potential antiviral agents.

Keywords: monoterpenes, herpes simplex virus, antiviral activity, mode of action

INTRODUCTION

Medicinal plants have been widely used to treat a variety of infectious and non-infectious ailments and represent an abundant source of new bioactive secondary metabolites. Besides small molecules from medicinal chemistry, natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases. Essential oils containing monoterpenes have a wide application in folk medicine as well as in fragrance industries. The main constituents of essential oils, e.g. monoterpenes and sesquiterpenes and phenylpropanoids are responsible for the fragrant and biological properties of aromatic and medicinal plants.

Herpes simplex virus (HSV) is differentiated into two antigenic types of 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 efforts. HSV infects and replicates in cells at the site of entry, the mucocutaneous surface. 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 mostly affect adult people (1). 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 (2). Some of these antiviral

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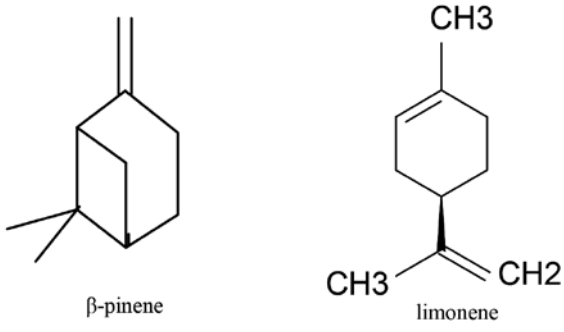

Fig. 1. Structural formulas of monoterpenic compounds.

agents might produce toxic side-effects. In addition, the emergence of virus strains resistant to commonly used anti-herpesvirus drugs is of importance, particularly in immunocompromised patients (3-5). The development of viral resistance towards antiviral agents enhances the need for new effective compounds against viral infections. Antiherpes screening experiments on medicinal plant extracts and plant derived secondary metabolites have been reported (6, 7). Antibacterial, antifungal, immunomodulatory, antiinflammatory, and antirheumatic activities have been described for essential oils (8-14). The antiherpes activity of several essential oils of different plant sources as well as of some constituents of essential oils had been demonstrated previously (15-20). Some phenylpropanes (17), triterpenes (21), and sesquiterpenes (22-25) had been tested for their antiviral activity against different herpesviruses and rhinoviruses. Monoterpenes as major constituents of essential oils, e.g. alpha-terpinene, gamma-terpinene, alpha-pinene, p-cymene, terpinen-4-ol, alpha-terpineol, thymol, citral and 1, 8-cineole were examined for their antiviral activity against herpes simplex virus type 1 (HSV-1) in vitro (26). Isoborneol, a monoterpene and a component of several plant essential oils was evaluated against HSV-1 (27). Antiviral activity of beta-pinene a monoterpene compound of many essential oils was analysed against infectious bronchitis virus (28), and antiviral activity of limonene, also a monoterpenic compound, was tested against yellow fever virus and tobacco mosaic virus (29, 30).

The goal of the present study is the evaluation of the antiviral activity of two monoterpenes, β-pinene and limonene, against HSV-1. The mode of antiviral action of these monoterpenes has been determined during the viral multiplication cycle.

MATERIALS AND METHODS

Compounds. Monoterpenes β–pinene and limonene were purchased from Roth (Karlsruhe, Germany). These monoterpenes met high purity standards. Structural formulas of these selected monoterpene constituents are presented in Fig. 1. Compounds 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 (18).

Acyclovir. Acyclovir was purchased from GlaxoSmithKline (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. Cells were plated out onto 96-well and 6-well culture plates for cytotoxicity and antiviral assays, respectively, and propagated at 37°C 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°C. Infectivity titers were determined by a standard plaque assay on confluent RC-37 cells (31).

Cytotoxicity assay. For cytotoxicity assays, cells were seeded into 96-well plates and incubated for 24 h at 37°C. The medium was removed and fresh DMEM containing the appropriate dilution of the 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 viability of the drug treated cells was determined in a standard neutral red assay (32). 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 viable cell number by 50% (TC_{50}) was determined from dose-response curves. Additionally the

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

concentration (µg/ml)	10	25	50	75	100
	cell viability (%) ± SD				
β-pinene	100.0 ± 2.7	95.5 ± 8.4	99.8 ± 1.0	61.0 ± 9.9	5.4 ± 0.7
limonene	100.0 ± 3.5	98.4 ± 6.2	81.8 ± 6.6	18.1 ± 15.3	15.1 ± 6.5

maximum noncytotoxic concentration of each drug was determined.

Dose-response assays. Inhibition of HSV replication was measured by plaque reduction assay. Usually 2×10^3 plaque forming units (pfu) were incubated with different concentrations of monoterpene compounds for 1 h at room temperature, then virus was allowed to adsorb to the cells for 1 h at 37°C. The residual inoculum was discarded and 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°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% (IC_{50}) was determined from dose-response curves.

Time of addition assay. In order to determine the mode of antiviral action for compounds, cells were pretreated with monoterpenes before viral infection, viruses were incubated with drugs before infection or infected cells were incubated with monoterpenes immediately after penetration of the virus into cells. Components were always used at the maximum noncytotoxic concentration.

Pretreatment of cells with drugs. Cell monolayers were pretreated with drugs prior to inoculation with virus by adding the components to the culture medium followed by incubation for 1 h at 37°C. The drugs were aspirated and cells were washed before the HSV inoculum was added.

Pretreatment of viruses with drugs. For pretreatment of herpes simplex virus with drugs, about 2×10^3 pfu of HSV were incubated in medium containing the maximum noncytotoxic concentration of the drugs for 1 h at room temperature prior to infection of RC-37 cells.

Addition of drugs during viral intracellular replication. The effect of components against HSV was also tested during the replication period by addition of drugs after cell infection to the overlay medium, as typical performed in antiviral susceptibility studies. Each assay was run in three replicates. Plaque reduction assays were carried out as described above and number of plaques of drug-treated cells and viruses were compared to untreated controls. Wells containing medium with 1% ethanol but no drug were also included on each plate as controls.

Viruses are synchronized at each stage of infection. The drug-pretreated cell free viruses are added at the same time to host cells, thus infection starts at the same time. When intracellular viruses are drug-treated, the infection had started simultaneously, all non-infecting virus particles had been washed off and all intracellular viruses are in the same phase of replication.

RESULTS

Cytotoxicity. Selected monoterpenic compounds beta-pinene and limonene were serially diluted in ethanol and added to 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, cell viability of RC-37 cells was determined with the neutral red assay (Table 1). The maximum noncytotoxic concentrations of these drugs were determined at 60 µg/ml and 40 µg/ml for β-pinene and limonene, respectively (Table 2). These monoterpenes revealed low cytotoxicity**.**

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

concentration (µg/ml)	1	2.5	5	7.5	10	25	50
	remaining infectivity (% of control) ± SD						
β-pinene	82.3 ± 18.0	67.5 ± 26.9	23.1 ± 6.2	6.1 ± 12.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Limonene	94.7 ± 8.5	99.0 ± 9.6	64.3 ± 16.1	27.8 ± 19.8	8.2 ± 13.8	0.0 ± 0.0	0.0 ± 0.0

Antiherpetic activity. The potential antiviral effect of selected components was determined against herpes simplex virus type 1 (HSV-1) in vitro. HSV-1 was incubated for 1 hour at room temperature with various concentrations of β-pinene and limonene. In all assays untreated virus-infected cells were used as a control. Subsequently, aliquots of each dilution were added on the cells for 1h, afterwards cells were washed and overlaid with drug-free medium and incubated for 3d at 37°C. The results are presented as virus reduction and represent the average of three independent experiments. In plaque reduction assays, these compounds exhibited a concentration-dependent antiviral effect. Both compounds were able to suppress viral multiplication completely (Table 2). The 50% inhibitory concentrations (IC_{50}) for HSV-1 were determined 3.5 µg/ml for β-pinene and 5.9 µg/ml for limonene. Selectivity indices for compounds were calculated as the TC_{50}/IC_{50} ratio (Table 3).

Mode of antiviral action. 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, compounds 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 host cells were pretreated with drugs prior to infection, both tested drugs showed minor effects on viral infection (Fig. 2). On the other hand, pretreatment of HSV-1 with the monoterpenic compounds for 1h prior to infection caused a significant reduction in plaque formation. At maximum non-cytotoxic concentrations of the tested compounds, infectivity was reduced by 100% (Fig. 3). Acyclovir showed the highest antiviral activity when added during the replication period with inhibition of the viral replication of 98.6% (Fig. 4). 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. In contrast, when the compounds were added to the overlay medium after penetration of the viruses into the host cells, plaque formation was not significantly reduced (Fig. 4).

DISCUSSION

In the present study, the inhibitory effect of two compounds against herpes simplex virus infection was analysed. Experiments to assess the cytotoxicity of monoterpenes for cultured eucaryotic cells indicate a moderate toxic behaviour in cell cultures according to Halle and Göres (33). Both tested monoterpenes exhibited high levels of antiviral activity against HSV-1 in viral suspension tests. At maximum noncytotoxic concentrations plaque formation was reduced by 100% for β-pinene and limonene.

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. Pretreatment of the cells

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

monoterpene	max. noncytotoxic concentration (µg/ml) ± SD	TC_{50} (µg/ml) ± SD	IC_{50} (µg/ml) ± SD	selectivity index (SI)
β-pinene	60 ± 6.9	85 ± 5.3	3.5 ± 4.0	24.3
limonene	40 ± 6.4	60 ± 11.0	5.9 ± 5.3	10.2

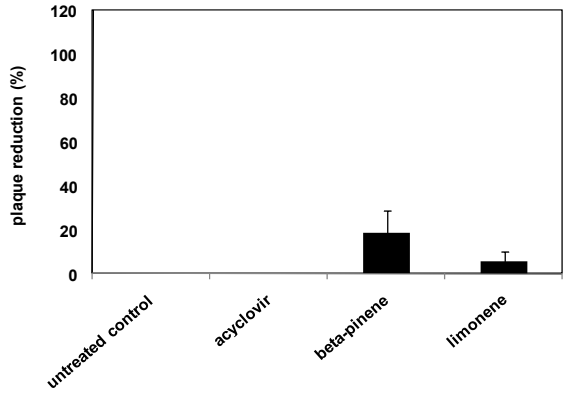

Fig 2. Pretreatment of cells. Antiviral activity of selected monoterpenes against herpes simplex virus type 1 after pretreatment of cells with drugs. Prior to viral infection, cells were incubated with maximum noncytotoxic concentrations of drugs for 1h at 37°C. Number of virus plaques was determined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

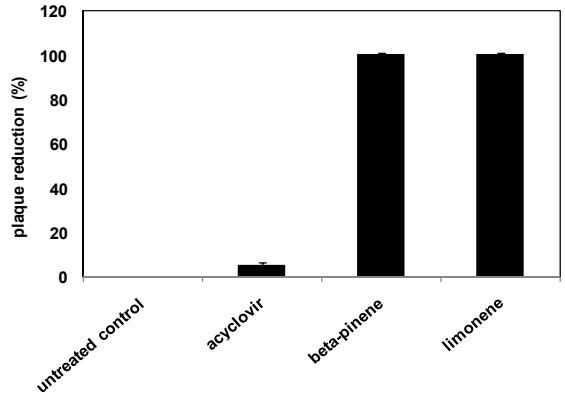

Fig 3. Pretreatment of virus. Antiviral activity of selected monoterpenes against herpes simplex virus type 1 after incubation of HSV with drugs. HSV was incubated for 1 h at roomtemperature with maximum noncytotoxic concentrations of drugs. Number of virus plaques was determined 3 d after infection and compared to untreated control. Results are expressed as percentage of plaque reduction. These experiments were repeated independently and data presented are the mean of three experiments.

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 were added during the replication period of the infection cycle. However, high antiviral activity was observed for monoterpenes 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 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 compounds is due to binding of the compounds to viral proteins involved in host cell adsorption and penetration. De Logu et al (7) 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 for monoterpenes. Isoborneol, a monoterpene and a component of several plant essential oils, showed virucidal activity against HSV-1 and specifically inhibited glycosylation of viral proteins (27). The application of the monoterpene cineole protected mice against infection with HSV-2 (15). Since essential oils are able to inhibit acyclovir-resistant HSV-1 isolates (18), the mechanism of interaction between essential oils and these compounds and acyclovir with HSV must be different. Acyclovir inhibits virus replication by interference with the DNA polymerase inside the cell, whereas monoterpenes probably inactivate HSV before it enters the cell. Astani *et al.* (26) 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% (34). Therefore

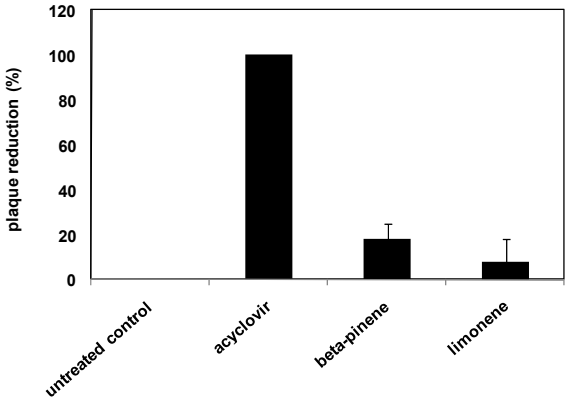

Fig 4. Replication. Antiviral activity of selected monoterpenes against herpes simplex virus type 1 during intracellular virus replication. Drugs were applied to HSV-1 infected cells after penetration of the viruses into cells for 3 d. Number of virus plaques was determined 3 d after infection and compared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

other antiherpetic agents which are effective for viral mutants resistant to current antiviral agents are of great interest for topical treatment.

In a recent study, the most active antibacterial constituents of essential oils were cinnamaldehyde, phenols, monoterpene alcohols and aldehydes. Mono- and sesquiterpene hydrocarbons as well as sesquiterpene alcohols, were inactive against the tested bacteria. In previous study by Astani *et al.* (26), monoterpenic hydrocarbons were more effective than other monoterpenes. Here in our study, both monoterpenes hydrocarbons showed a good antiviral activity as well. These findings are in contrast to results in antiviral activity, where the complex mixture of the essential oil revealed a higher antiviral activity (26) than single monoterpenes. However, the antiviral activity of single monoterpenes does not contribute equally to the antiviral activity of the essential oil. Cos *et al.* (35) 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 compounds in our study revealed IC_{50} values of 3.5 μg/ml and 5.9 μg/ml for β-pinene and limonene, respectively, and are far below the recommended cut off and present a 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.* (36) recommended a selectivity index of at least 4 as appropriate. According to this suggestion, both monoterpenes might be suitable agents. These results suggest that the monoterpenes beta-pinene and limonene exhibit antiherpetic activity and might be used as a potential antiviral agents in recurrent herpes labialis.

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