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Antiviral activity of Australian tea tree oil and eucalyptus oil against herpes simplex virus in cell culture

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The antiviral effect of Australian tea tree oil (TTO) and eucalyptus oil (EUO) against herpes simplex virus was examined. Cytotoxicity of TTO and EUO was evaluated in a standard neutral red dye uptake assay. Toxicity of TTO and EUO was moderate for RC-37 cells and approached 50% (TC_{50}) at concentrations of 0.006% and 0.03%, respectively. Antiviral activity of TTO and EUO 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 (IC_{50}) of TTO for herpes simplex virus plaque formation was 0.0009% and 0.0008% and the IC_{50} of EUO was determined at 0.009% and 0.008% for HSV-1 and HSV-2, respectively. Australian tea tree oil exhibited high levels of virucidal activity against HSV-1 and HSV-2 in viral suspension tests. At noncytotoxic concentrations of TTO plaque formation was reduced by 98.2% and 93.0% for HSV-1 and HSV-2, respectively. Noncytotoxic concentrations of EUO reduced virus titers by 57.9% for HSV-1 and 75.4% for HSV-2. Virus titers were reduced significantly with TTO, whereas EUO exhibited distinct but less antiviral activity. In order to determine the mode of antiviral action of both essential oils, either cells were pretreated before viral infection or viruses were incubated with TTO or EUO before infection, during adsorption or after penetration into the host cells. Plaque formation was clearly reduced, when herpes simplex virus was pretreated with the essential oils prior to adsorption. These results indicate that TTO and EUO affect the virus before or during adsorption, but not after penetration into the host cell. Thus TTO and EUO are capable to exert a direct antiviral effect on HSV. Although the active anti-herpes components of Australian tea tree and eucalyptus oil are not yet known, their possible application as antiviral agents in recurrent herpes infection is promising.

1. Introduction

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) cause very common infections in man producing recurrent orofacial and genital lesions, respectively. HSV-1 causes epidermal lesions in and around the oral cavity. The hallmark of an HSV infection is the ability of the virus to establish a latent infection in the nervous system, to reactivate and to cause recrudescent lesions. Several drugs are currently available for the treatment of HSV infections. Acyclovir is a compound that interferes with viral DNA replication through its activation by viral thymidine kinase. Although extremely effective when given orally or intravenously for the treatment of primary or encephalitic HSV infections, acyclovir can be less effective topically. The incidence and severity of disease produced by herpes simplex virus have been increasing in recent years [1], especially in the immunocompromised host where viral resistance to acyclovir (ACV) represents a particular problem. The prevalence of resistance to acyclovir in treated immunocompromised individuals is approximately 6% [2]. This trend has led to search for completely novel anti-herpes virus compounds, e.g. plant derived secondary metabolites [3].
While many essential oils possess high levels of antibacterial [4, 5] and antifungal [6] activity, there is only little information on the effects of essential oils on viruses or viral infections. Recently, the virucidal activity of oregano oil and clove oil as well as the essential oil of *Salvia fructicosa* and other plant derived products against herpes simplex virus type 1 was demonstrated [7, 8, 9]. Australian tea tree oil (TTO), a yellowish essential oil derived from leaves of *Melaleuca alternifolia* L. (Myrtaceae), has also been reported to exhibit anti-HSV-1 activity [4]. Since TTO is becoming increasingly popular in a wide range of health care products, the efficacy of tea tree oil as an antimicrobial agent was investigated frequently [4, 5]. However all reports on the antiviral activity of TTO are anecdotal descriptions. Only its antiviral activity against tobacco mosaic virus was previously reported [10]. Nevertheless, TTO is propagated worldwide in various lipsticks as a potent antiviral agent against herpes labialis. Neither experimental data nor clinical studies about the anti-herpes activity are available. Eucalyptus oil, which possesses antimicrobial activity, is mainly derived from leaves of *Eucalyptus globulus* LABILL. (Myrtaceae) and also used worldwide in various health care products, cosmetics and household disinfectants. Hitherto, it is unknown whether eucalyptus oil is even active against viruses such as herpes simplex virus.
In the present study, we examined the antiviral activity of both essential myrtaceous oils, Australian tea tree oil and eucalyptus oil, against infection with HSV-1 and HSV-2 *in vitro*.

2. Investigations and results

2.1. Chemical characterisation of Australian tea tree oil and eucalyptus oil

Since the chemical composition of TTO as well as EUO is important for their antiviral activity, each essential myrtaceous oil was chemically characterized before using it in the bioassay. The major components of both essential oils 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. TTO consisted mainly of terpinen-4-ol (39.3%), γ-terpinene (20.7%), and α-terpinene (10.5%) whereas eucalyptus oil exhibited a very high 1,8-cineol content (80,12%). For more details see Harkenthal *et al.* [5].

2.2. Cytotoxicity

Tea tree oil and eucalyptus oil were dissolved in ethanol and added to the medium at a final concentration of 1%

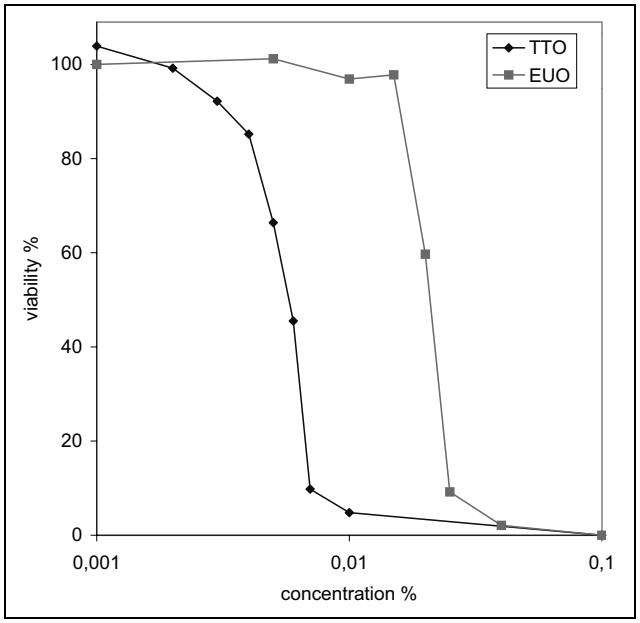

Fig. 1: Cytotoxicity of tea tree oil (TTO) and eucalyptus oil (EUO) on RC-37 cells as determined with the neutral red assay. Cells were seeded in 24-well microtiter plates and equal numbers of cells were incubated with 1% ethanol or increasing concentrations of tea tree oil and eucalyptus oil for 4 days. Medium was removed and the cells were incubated with neutral red for 3 h. Cell viability was determined at 540 nm and optical density was compared with the uptake of the dye from the ethanol controls. The optical densities of drug-treated cells are expressed as a percentage of control cells. The values are the mean average of three independent experiments

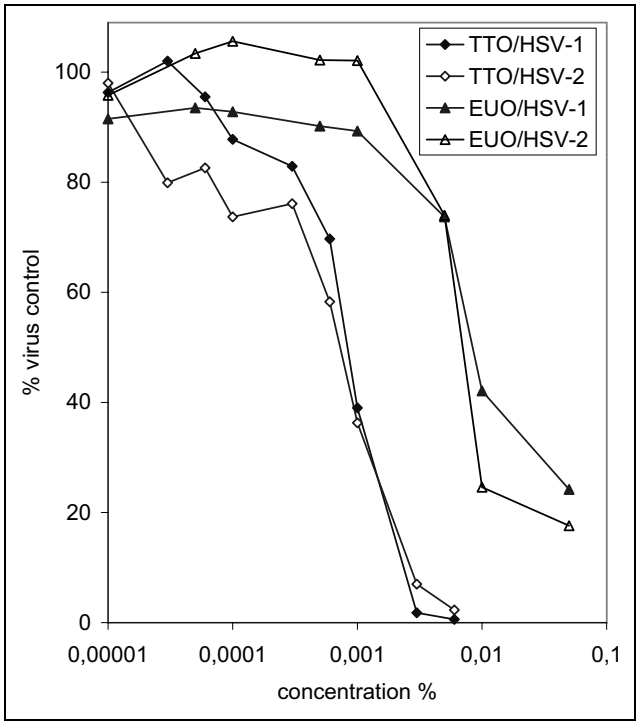

Fig. 2: Determination of the 50% inhibitory concentration (IC_{50}) of tea tree oil (TTO) and eucalyptus oil (EUO) against HSV-1 and HSV-2. Viruses were incubated for 1 h at room temperature with increasing concentrations of tea tree oil or eucalyptus oil and immediately tested in a plaque reduction assay. The percent reduction was calculated relative to the amount of virus in the ethanol treated virus control. These experiments were repeated independently two times and data presented are the mean of three experiments

ethanol. Ethanol by itself did not exhibit any toxic effect on RC 37 cells. The effect of tea tree oil and eucalyptus 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. Tea tree oil concentrations up to 0.003% did not show any visible changes in cell morphology and cell density, whereas complete cell death was observed at a concentration of 0.01% tea tree oil (Fig. 1). Cytotoxicity is expressed as the toxic concentration, which is required to reduce cell growth by 50% of the control. The toxic concentration (TC_{50}) of TTO for RC-37 cells is 0.006%. Eucalyptus oil was less cytotoxic than TTO, concentrations of 0.01% EUO or less did not reduce the viability for drug treated cells (Fig. 1). The TC_{50} value of eucalyptus oil was determined at 0.03%.

2.3. Virucidal activity

The virucidal action of tea tree oil and eucalyptus oil against HSV-1 and HSV-2 was evaluated. The viruses were exposed for 1 h to various concentrations ranging from 0.00001–0.1% of these compounds in suspension assays. Tubes containing 2×10^3 plaque forming units (pfu) of HSV-1 strain KOS and HSV-2 strain HG52 were incubated for 1 h at room temperature in Dulbecco's modified Eagle's medium (DMEM), supplemented with various concentrations of the essential oils. 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 control. After 1 h, an aliquot was removed from each tube, serially diluted and assayed for remaining infectivity on confluent monolayers of RC-37 cells in 6 well plates by plaque assay. Briefly, the samples were diluted in medium and aliquots of each dilution were adsorbed on cells for 1 h at 37 °C. At the end of the adsorption period the virus inoculum was removed and the cells were replenished with fresh medium containing 0.5% methylcellulose. The infected cells were incubated at 37 °C in a 5% CO_2 atmosphere, and viral plaques were stained and counted after 4 days. Ethanol at a final concentration of 1% had no effect on virus titers. The 50% inhibitory concentration (IC_{50}) of TTO was determined at 0.0009% and 0.0008% and of EUO at 0.009% and 0.008% for HSV-1 and HSV-2, respectively (Fig. 2). The results are presented as a percentage of virus control and are the mean values from three independent experiments. Eucalyptus oil showed less virucidal effect in this assay (Fig. 2). However tea tree oil and eucalyptus oil inhibited plaque formation of HSV-1 and HSV-2 in a dose-dependent manner. At a concentration of 0.003% tea tree oil, which is still not cytotoxic, the titres of HSV-1 and HSV-2 are reduced by 98.2% and 93.0%, respectively. Noncytotoxic concentration of eucalyptus oil reduced the titers of HSV-1 by 57.9% and of HSV-2 by 75.4%.

2.4. Mode of antiviral action

Herpes virus replication is characterized by a cascade of coordinately regulated events. The inhibitory effect of tea tree oil was determined following addition at different times relative to virus infection. To identify the step at which replication might be inhibited, cells were infected with HSV after preincubation of the cells with TTO and EUO, pretreatment of the virus with essential oils prior to infection, addition of the essential oils during adsorption

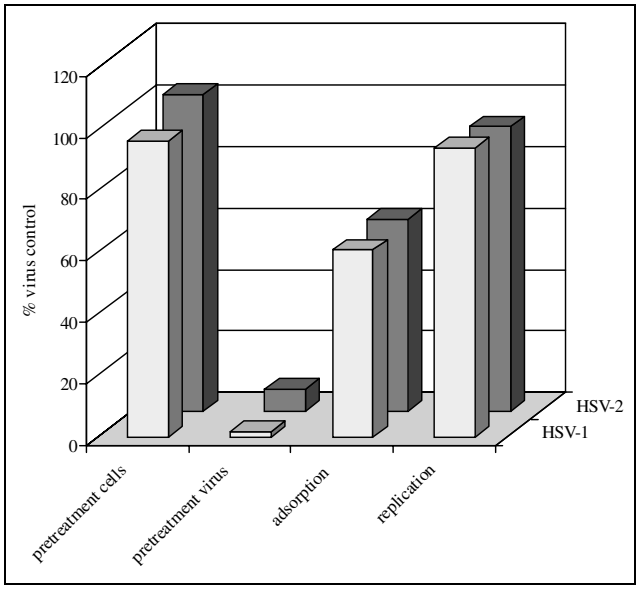

Fig. 3: Antiviral effect of tea tree oil against HSV-1 and HSV-2 by incubation of cells or viruses at different periods of time. The amount of the remaining plaque forming units was determined in a plaque reduction assay. Tea tree oil was added at the noncytotoxic concentration of 0.003%. Cells were pretreated with TTO prior to virus infection (pretreatment cells), viruses were pretreated prior to infection (pretreatment virus), TTO was added during the adsorption period (adsorption) or after penetration of the viruses into cells (replication). The percent reduction was calculated relative to the amount of virus of the control

or after the adsorption period. In all experiments virus infected cells were used as control. The percent reduction was calculated relative to the amount of virus produced in the absence of the compounds. In all assays the noncytotoxic concentrations of the essential oils were used.

For the pretreatment of cells, RC-37 monolayers were incubated for 1 h at 37 °C with DMEM medium containing 0.003% TTO or 0.01% EUO. Medium was removed, cells were washed with phosphate buffered saline (PBS) and incubated with HSV-1 or HSV-2 for 1 hour at 37 °C. Unadsorbed virus was removed, followed by the addition of fresh medium containing methylcellulose and incubation

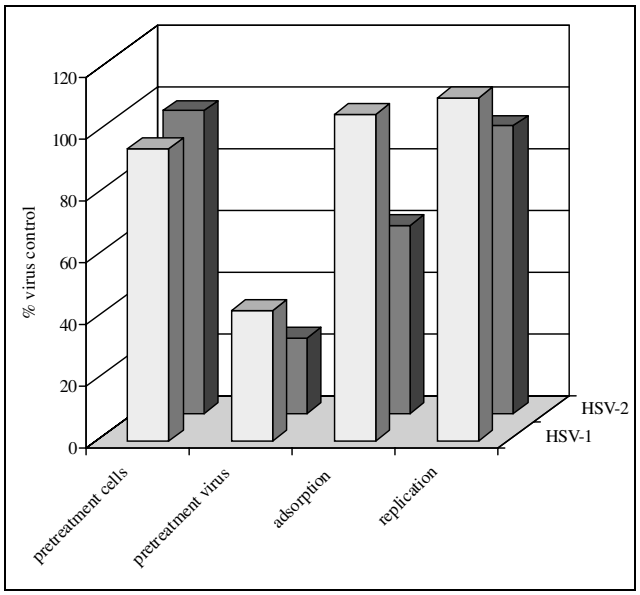

Fig. 4: Antiviral effect of eucalyptus oil against HSV-1 and HSV-2 by incubation of cells or viruses at different periods of time. For details see Fig. 3

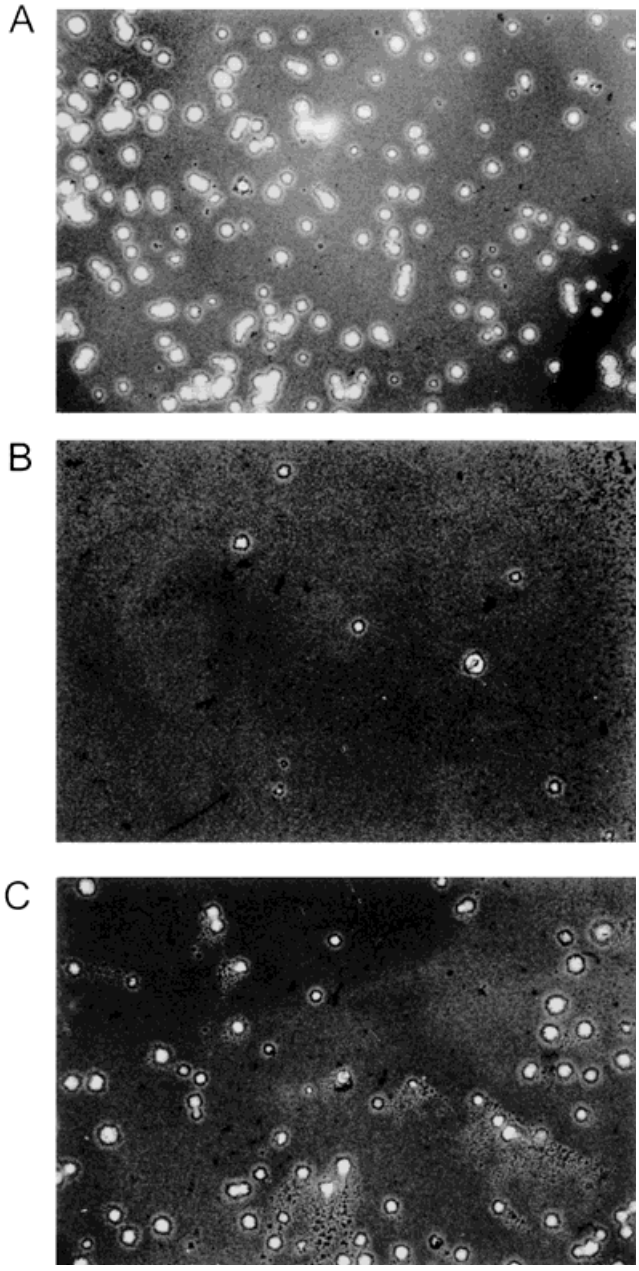

Fig. 5: Virucidal activity of tea tree oil against HSV-1. Tea tree oil was added at the nontoxic concentration for 1 h to cells prior to infection (A), to virus prior to infection (B) or during the adsorption period (C). Infected cell cultures were incubated for 4 days, then fixed and stained with crystal violet to visualize plaques

for 4 days. Pretreatment of cells with the essential oils did not reduce virus production (Fig. 3, Fig. 4). However pretreatment of HSV-1 and HSV-2 prior to infection with TTO caused a drastic reduction in the amount of plaques (Fig. 3) and a significant reduction with EUO (Fig. 4). Tea tree oil caused a reduction of infectivity of 98.2% and 93.0% for HSV-1 and HSV-2, respectively. Noncytotoxic concentrations of EUO reduced virus titers by 57.9% for HSV-1 and 75.4% for HSV-2. Both essential oils were also added for 1 h only during the adsorption period of HSV-1 and HSV-2. Afterwards cells were washed with PBS, fresh medium was added and infected cells were incubated for 4 days. When TTO was added only during the adsorption period, virus titres were reduced by 39.0% for HSV-1 and by 37.7% for HSV-2 (Fig. 3) and by about 40% reduced with EUO for HSV-2 (Fig. 4). In contrast,

no significant effect on viral growth could be observed when the essential oils were added only to the overlay medium after the adsorption period immediately following the removal of the unadsorbed virus inoculum. These results suggest that the antiviral effect of both essential oils is exerted prior to viral infection of host cells.

The virucidal activity of tea tree oil against HSV-1 is also shown in Fig. 5. Tea tree oil was added at the nontoxic concentration for 1 h to cells prior to infection (Fig. 5A), to virus prior to infection (Fig. 5B) or during the adsorption period (Fig. 5C). Infected cell cultures were incubated in 6 well plates for 4 days, then fixed and stained with crystal violet to visualize plaques.

3. 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 (TTO) against tobacco mosaic virus was reported previously [10]. All reports on the antiviral activity of TTO against HSV are anecdotal descriptions. Therefore we analysed the possible antiviral effect of TTO and EUO against herpes simplex virus infection *in vitro*.

Experiments to assess the toxicity of TTO and EUO indicate a moderate toxic behaviour in cell cultures. The toxicity of TTO and EUO approached 50% (TC_{50}) at concentrations of 0.006% and 0.03%, respectively. In plaque reduction assays TTO and EUO exhibited a concentration-dependent antiviral effect, when HSV was mixed with essential oils prior to inoculation. In contrast to the significant reduction of infectivity caused by tea tree oil, eucalyptus oil exhibited a moderate virucidal effect. At noncytotoxic concentrations of TTO plaque formation was reduced by 98.2% and 93.0% for HSV-1 and HSV-2, respectively. Noncytotoxic concentrations of EUO reduced virus titers by 57.9% for HSV-1 and 75.4% for HSV-2.

In order to determine the mode of antiviral action, either cells were pretreated before viral infection or viruses were incubated with noncytotoxic concentrations of TTO or EUO before infection, during adsorption or after penetration into the host cells. Pretreatment of the cells with both essential oils had no effect on the production of infectious virus and plaque formation was not affected. However pretreatment of HSV-1 and HSV-2 with both essential oils prior to infection resulted in a concentration-dependent reduction of plaques, suggesting that TTO and EUO bind to virion envelope structures or are masking viral compounds which are necessary for adsorption or entry into host cells. When TTO was added during the adsorption period, the amount of plaques for HSV-1 and HSV-2 was reduced by 39.0% and 37.7%, respectively. A similar anti-adsorption effect was demonstrated for the milkprotein lactoferrin inhibiting the attachment of HSV-1 to Vero cells [11]. After the adsorption period, TTO exhibited only a minor antiviral effect. These results suggest that free virus is very sensitive to the antiviral effect of TTO. The inhibition of HSV appears to occur before adsorption or during adsorption but not after penetration of the virus into the cell. Tea tree oil and eucalyptus oil reduced the infectivity of the virus possibly due to direct interaction with the viral envelope and glycoproteins. The active component of both essential oils is not known at present. The antiviral effect of TTO against tobacco mosaic virus, a nonenveloped plant virus, has been reported previously [10]. However, the mechanism of this antiviral action was not analysed. Siddiqui et al. [7] described a dissolution of the HSV envelope by treatment with oregano essential oil. It remains to be determined whether the inhibitory effect is due to binding of the essential oils 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.

Australian tea tree oil and eucalyptus oil possess antiviral activity against herpes simplex virus *in vitro*. The topical use of essential oils for the treatment of HSV infections would be ideal, especially for those patients who experience frequent recurrences. Studies on the further evaluation of the active constituents of tea tree oil and eucalyptus oil and clinical trials are in progress.

4. Experimental

4.1. Apparatus and chemicals

4.1.1. Essential oils

The essential oils tested were commercial products: Australian tea tree oil (ALVA, Wallenhorst, Germany) and Oleum Eucalypti Ph. Eur. (eucalyptus oil) (Caesar Loretz, Hilden, Germany). Essential oils were dissolved in ethanol and added to cell culture medium.

4.1.2. GC method

The essential oil was analysed as 1% solution in n-hexane containing tridecane as the internal standard. Gas chromatography was performed using a Carlo Erba GC 6000 chromatograph equipped with a Spectra Physics Integrator SP 4290. The GC column was a 15 cm × 0.25 mm fused silica capillary column coated with OV 1 (phase thickness 0.25 μm) and with helium as the carrier gas (flow rate: 2 ml/min). The initial column temperature was 42 °C for 4 min. Subsequently, the temperature rate was programmed from 40 °C to 300 °C in two steps, first 4 °C/min up to 120 °C followed by 10 °C/min up to 300 °C. Injector temperature was 250 °C and the detector temperature 300 °C.

4.1.3 GC-MS method

A gas chromatograph Carlo Erba HRGC 4160 was coupled via an open, split interface to a Finnigan MAT 4500 mass spectrometer. GC column: 30 m × 0.25 mm (i.d.) fused silica capillary column coated with OV 1 (phase thickness: 0.25 μm). Split: 1 : 20. Temperature programe: 46 °C for 4 min; 3 °C/min up to 76 °C, then 4 °C/min up to 136 °C and 6 °C/min up to 300 °C. EI ionizing voltage 70 eV.

4.2. Virology

4.2.1. Cells and viruses

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 μg/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 24-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 [12] and HSV-2 strain HG52 [13] were used for the experiments. HSV-2 was kindly provided by Dr. G. Darai, University of Heidelberg. Virus was routinely grown on RC-37 cells as described previously [14]. Herpes simplex virus stock cultures were prepared from supernatant fluids of infected cells and stored at −80 °C. Infectivity titers were determined by a standard plaque assay on confluent RC-37 cells.

4.2.2. In vitro cytotoxicity assay

For cytotoxicity assays, cells were seeded into 24-well plates at a density of 5×10^4 cells per well and incubated for 24 h at 37 °C. The medium was removed and fresh DMEM containing the appropriate dilution of the essential oil or isolated compound 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 [15]. This assay quantifies the number of viable cells after their ex-

posure 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 μg/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% $CaCl_2$ for 1 min. Finally the cells were dissolved in 50% ethanol with 1% acetic acid, incubated for 5 min on a shaker and the neutral red dye incorporated by the viable cells eluted. The neutral red uptake was determined by measuring the optical density (OD) of the solutions at 540 nm in a spectrophotometer. The mean OD of the cell-control wells was assigned a value of 100%. Uptake and accumulation of the supravital dye neutral red has been shown to be linear with cell numbers. The cytotoxic concentration of the drug which reduced viable cell number by 50% (TC_{50}) was determined from dose-response curves.

4.2.3. Antiviral activity

Inhibition of virus replication was measured by plaque reduction assay. Usually 2×10^3 plaque forming units (pfu) were incubated with different concentrations of tea tree oil and eucalyptus 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 overlaid 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% (IC_{50}) was determined from dose-response curves.

4.2.4. Mode of antiviral action

In order to determine the mode of antiviral action, cells were pretreated with TTO or EUO before viral infection, viruses were incubated with TTO or EUO before infection and cells and viruses were incubated together during adsorption or after penetration of the virus into the host cells. The essential oils were always used at the noncytotoxic concentrations of 0.003% and 0.01%, respectively. Cell monolayers were pretreated with drugs prior to inoculation with virus by adding the essential oils to the culture medium and by incubation for 1 h at 37 °C. The compounds were aspirated and cells were washed immediately before the HSV inoculum was added. For pretreatment of herpes simplex virus about 100 pfu of HSV-1 and HSV-2 were incubated in medium containing 0.003% TTO or 0.01% EUO for 1 h at room temperature prior to infection of RC-37 cells. For analysing the antiviral activity during the adsorption period, the same amount of HSV-1 or HSV-2 was mixed with the drugs 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 TTO and EUO against HSV was also tested during the replication period by adding TTO or EUO after adsorption to the overlay medium, as typically performed in antiviral susceptibility studies. 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.

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