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Comparative study on the cytotoxicity of different *Myrtaceae* essential oils on cultured Vero and RC-37 cells

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Medicinally and commercially important essential oils from the family Myrtaceae, i.e. cajuput, clove, kanuka and manuka were phytochemically analysed by GC-MS. Cytotoxicity of these essential oils was evaluated in a standard neutral red assay. Maximum noncytotoxic concentrations for cajuput oil and clove oil were determined at 0.006%, kanuka oil and manuka oil were more cytotoxic with a maximum noncytotoxic concentration of 0.001%. The compounds α-pinene, eugenol and leptospermone demonstrated maximum noncytotoxic concentrations at dilutions of 0.001%, 0.003% and 0.001%, respectively. However, the terpene 1,8-cineole was about 100 times less toxic to cultured cells with a maximum noncytotoxic concentration of 0.1% and a TC_{50} value of 0.44%. Manuka essential oil exhibited high levels of virucidal activity against HSV-1 as well against drug-resistant HSV-1 isolates in viral suspension tests. Determination of cytotoxicity of natural products is an important prerequisite for application in cosmetic and health care products and in antiviral tests.

1. Introduction

Medicinal plant extracts have been widely used in traditional medicine and plant-derived secondary metabolites are increasingly of interest as antimicrobial and antiviral agents. Extracts from plants as well as essential oils of different plant families have been implicated with immune modulatory (Fang et al. 2005), antioxidant (Aruoma et al. 1996; Bozin et al. 2007), antibacterial (Harkenthal et al. 1999; Fu et al. 2007) and antiviral effects (Kucera and Herrmann 1967; Schnitzler et al. 2001, 2008). Recently, Jassim and Naji (2003) published a review on novel viral agents derived from medicinal plants. The pharmaceutical market offers a wide range of drug products for topical application that contain essential oils or terpenes as their isolated compounds. The terpene camphor has been eliminated from essential oil products as it manifests toxicity on the central nervous system, kidney and liver and is considered as one of the most dangerous terpenes (Poppenga 2002). The present widespread use of essential oils conifer oleoresins in pharmacy and industry necessitates research on their cytotoxicity. Essential oils are nowadays found in many products like antiseptics, soaps, deodorants, flavors, and dentistry products. Australian tea tree oil is considered non-poisonous and is included in a large range of products for skin and wound care (Carson and Riley 1993) and is becoming increasingly popular as a naturally occuring antimicrobial agent. It has become popular with the general public and paramedical practitioners as a non-prescriptive medicament. However, only a few reports have been published concerning cytotoxic effects on cultured cell lines (Södergerg et al. 1996; Hayes et al. 1997; Minami et al. 2003). Essential oils are used in traditional as well as in complementary medicine, especially in phytotherapy (Wölbling and Leonhardt 1994; Saller et al. 2001).

The common name tea tree is not only typical for *Melaleuca alternifolia*, but is also attributed to various other members of the family Myrtaceae. The aromatic foliage of several myrtaceous species were used by early settlers in Australia as a tea substitute. Leaves and terminal branchlets of different tea trees are steam distilled for production of essential oils, e.g. cajuput oil (plant species: *Melaleuca cajuputi*), clove oil (plant species: *Syzygium aromaticum*), kanuka oil (plant species: *Kunzea ericoides*) and manuka oil (plant species: *Leptospermum scoparium*). These essential oils are widely used in various health care products, cosmetics and household disinfectants (Harnischfeger and Reichling 1998; Saller and Reichling 1996). Manuka oil, a β-triketone-rich essential oil, is commercially applied as anti-infective agent in New Zealand and has been shown to possess antibacterial activities (Harkenthal et al. 1999; Porter and Wilkins 1998; Christoph et al. 2000; Douglas et al. 2004). Reports about the toxicity of tea tree oil encompass contact dermatitis from the application of the oil and oral toxicity resulting in dizziness, disorientation, swelling, dermatitis and/or exacerbation of existing dermatitis.

The effect of essential oils and defined components on cell growth and viability was evaluated by the neutral red

uptake assay. The neutral red bioassay quantifies the number of viable uninjured cells after their exposure to toxicants and is based on the cellular uptake of neutral red, a water-soluble supravital dye, which passes through intact membranes of viable cells and is concentrated in the lysosomes. The uptake is measured spectrophotometrically after extraction from the lysosomes and compared with neutral red recovered from untreated control cultures and the results are therefore quantifiable. The assay can replace cell replication and DNA analog incorporation studies with a direct measure of cell viability and is advantageous for the evaluation of the sensitivity of different cell types to chemical agents (Söderberg et al. 1996).

Herpes simplex virus type 1 (HSV-1) causes very common infections producing recurrent orofacial lesions. 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 (Cassady and Whitley 1997), especially in the immunocompromised host where viral resistance to acyclovir represents a particular problem. This trend has led to search for completely novel-anti-herpes virus compounds, e.g. plant derived secondary metabolites and essential oils (Reichling 1999; Koch et al. 2008).

In the present study, we examined the cytotoxic potential of several myrtaceous essential oils on different cell lines, which are commonly used for propagation of herpesviruses. These cell lines are also commonly applied for antiviral tests of plant extracts and essential oils against herpesviruses. The determination of cytotoxicity of plant-derived products is an important prerequisite prior to the analysis of their antiviral activity.

2. Investigations and results

The chemical composition of myrtaceous essential oils was characterized phytochemically. The major components of all essential oils were identified by comparing their 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. About a dozen different major compounds were identified in cajuput essential oil, the most prominent compound comprising 61.16% of the total oil volume was 1,8-cineole (Table 1). In clove essential oil, only a few components were identified, with 74.98% eugenol, that is not present in any other tested myrtaceous essential oils. In kanuka essential oil, similar compounds were found as in cajuput oil, and represents an α-pinene rich (70.63%) essential oil. Manuka oil consists of a few different compounds, and is characterized by a high triketone chemotype (14.36% leptospermone), that is only present in manuka oil and was not identified in other tested myrtaceous essential oils.

Essential oils represent complex mixtures of different chemical substances and are not water soluble. When the lipophilic essential oil is mixed directly to the aqueous cell culture medium, it floats on top and is not in solution. The insolubility of essential oils in water rendered these agents unsuitable for cytotoxicity tests with standard cultured cell lines. Lipophilic solvents such as ethanol are able to dissolve essential oils, consequently all essential oils and components of essential oils had been dissolved in ethanol. However, ethanol itself demonstrates a cytotoxic ability and plain ethanol without any drug was tested on Vero cells and RC-37 cells in serial dilutions. 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. 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. 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%. The concentration of the drug which reduced viable cell number by 50% (TC_{50}) was determined from dose-response curves (Fig. 1). The maximum noncytotoxic concentration of ethanol was determined at 1.5% for both cell lines and neutral red uptake was half-maximally inhibited at concentrations (TC_{50}) of 3.2% and 2.6% for Vero cells and RC-37 cells, respectively. In all following experiments, essential oils as well as single lipophilic components were dissolved in ethanol followed by further dilution in aqueous cell culture medium. The concentration of the cell culture medium was at least 99%, and the highest final concentration of ethanol in diluted test compounds never exceeded 1%, which is still nontoxic by itself.

Lipophilic myrtaceous essential oils from cajuput, clove, kanuka and manuka were first dissolved in ethanol and

Table 1: Major compounds of Myrtaceae essential oils in % (v/v)

Compound	Cajuput oil	Clove oil	Kanuka oil	Manuka oil
α-Thujene	0.80		0.88	
α-Pinene	9.05		70.63	1.14
β-Pinene	3.82		0.59	0.11
Myrcene	0.88		0.17	0.42
α-Phellandrene	0.18			
α-Terpinene	0.63			
p-Cymene	2.00		5.09	0.15
Limonene	5.99		1.28	
1,8 Cineole	61.16		3.48	0.29
γ-Terpinene	3.10		3.94	0.73
Terpinolene	1.25		0.76	
Linalool			1.15	
Terpinene-4-ol	1.25		1.09	
α-Terpineol	3.50		0.29	
Eugenol		74.98		
β-Cubebene			0.36	
Longifolene	0.39			
β-Caryophyllene	1.46	14.95	0.17	2.79
Aromadendrene			0.34	2.11
Viridoflorene	0.34		1.05	4.40
δ-Cadinene			0.30	6.02
α-Cubene			0.17	4.38
α-Copaene			0.23	6.58
α-Gurjunene			0.20	1.05
α-Humulene	0.56	1.79		0.30
Alloaromadendrene			0.43	0.79
Selinene				6.10
Calarene			1.10	
Calamene			1.04	12.93
Caryophyllenoxide		0.34		
Leptospermone				14.36
Isoleptospermone				3.94

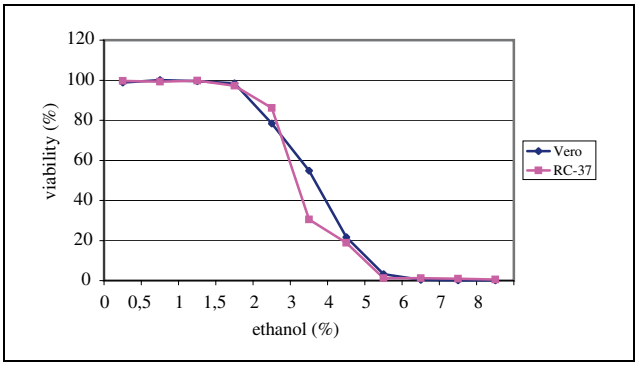

Fig. 1: Cytotoxicity of ethanol on Vero cells and RC-37 cells as determined with the neutral red assay. Cells were seeded in 96-well microtiter plates and equal numbers of cells were incubated with increasing concentrations of ethanol for 4 days. Medium was removed and the cells were incubated with neutral red for 3 hours. Cell viability was determined at 540 nm and optical density was compared with the uptake of the dye of untreated cell controls. The optical densities of drug-treated cells are expressed as a percentage of control cells. The values are the mean of three independent experiments

subsequently serially diluted in cell culture medium with final concentrations of the essential oils from 1% to 0.0001%. The highest concentration of ethanol never exceeded 1%, at this ethanol concentration no toxic effect on Vero cells and RC 37 cells was observed. 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. The cytotoxic concentration of the essential oil which reduced viable cell number by 50% (TC_{50}) was determined from dose-response curves for Vero cells (Fig. 2) and RC-37 cells (Fig. 3). Cytotoxicity is expressed as the toxic concentration, which is required to reduce cell growth by 50% of the control. The toxic concentration (TC_{50}) for essential oils from cajuput, clove, kanuka, and manuka on RC-37 cells were determined at 0.015%, 0.02%, 0.0043%, and 0.0042%, respectively (Table 2). Similar results were obtained for Vero cells (Fig. 2). Cajuput oil and clove oil were about half as toxic compared to kanuka oil and manuka oil, this finding was observed for both cell lines tested.

Essential oils are complex mixtures of many different organic components, the most prominent single substances are 1,8-cineole (61.16%) in cajuput oil, eugenol (74.98%)

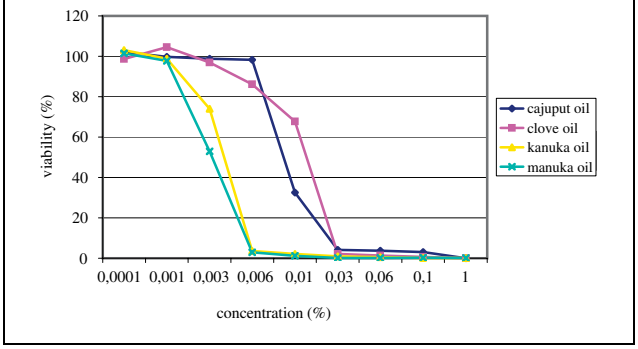

Fig. 2: Cytotoxicity of increasing concentrations of myrtaceous essential oils from cajuput, clove, kanuka and manuka on Vero cells. Essential oils were diluted in ethanol and viability of the cells was determined with the neutral red assay. The values are the mean of three independent experiments

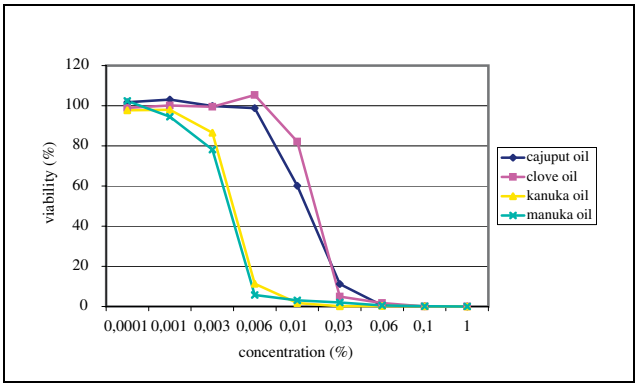

Fig. 3: Cytotoxicity of increasing concentrations of myrtaceous essential oils from cajuput, clove, kanuka and manuka on RC-37 cells. Essential oils were diluted in ethanol and viability of the cells was determined with the neutral red assay. The values are the mean of three independent experiments

Table 2: Maximum noncytotoxic concentrations and TC_{50} values of ethanol, myrtaceous essential oils from cajuput, clove, kanuka and manuka and their main compounds for RC-37 cells

	Maximum noncytotoxic concentration (%)	TC_{50} (%)
Ethanol	1.5	2.6
Cajuput essential oil	0.006	0.015
Clove essential oil	0.006	0.02
Kanuka essential oil	0.001	0.0043
Manuka essential oil	0.001	0.0042
1,8-Cineole	0.1	0.44
α-Pinene	0.001	0.002
Eugenol	0.003	0.01
Leptospermone	0.001	0.04

in clove oil, α-pinene (70.63%) in kanuka oil and the triketone leptospermone (14.36%) in manuka oil (Table 1, Fig. 4). In order to find out if the moderate toxic nature of the tested myrtaceous essential oils corresponds to the most prominent single component in the essential oil,

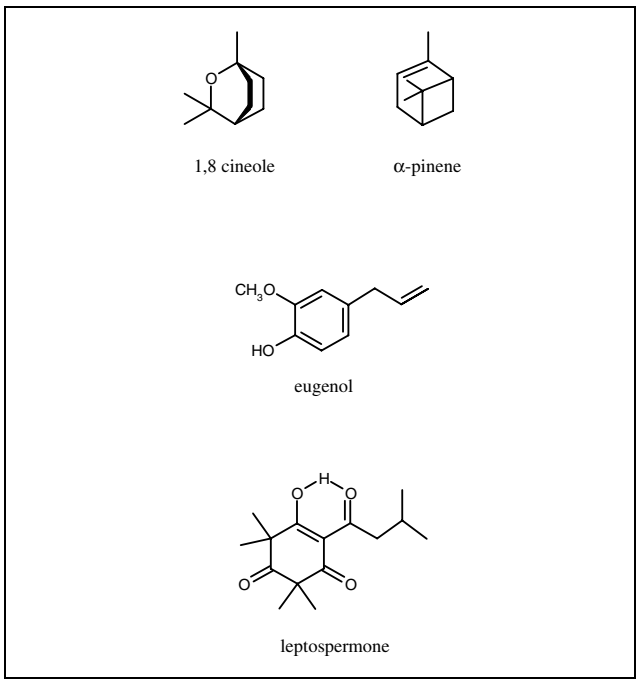

Fig. 4: Structural formulas of essential oils major components, 1,8-cineole, α-pinene, eugenol, and leptospermone

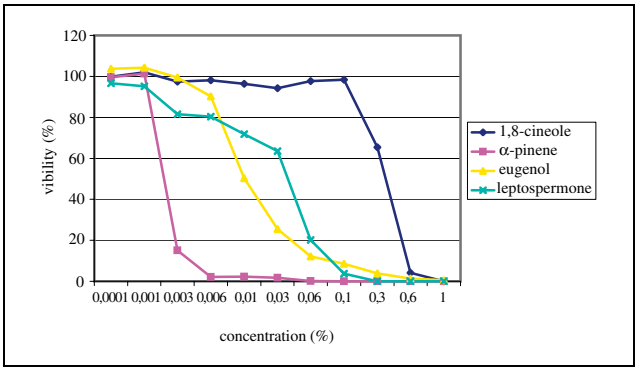

Fig. 5: Cytotoxicity of increasing concentrations of 1,8-cineole, α-pinene, eugenol, and leptospermone. Viability of the cells was determined with the neutral red assay, the values are the mean of three independent experiments

these constituents were tested for cytotoxicity in cultured cells. The toxic concentrations (TC_{50}) for 1,8-cineole, α-pinene, eugenol and leptospermone on RC-37 cells were determined at 0.44%, 0.002%, 0.01%, and 0.04%, respectively (Fig. 5). A striking high cytotoxic ability was determined for α-pinene, whereas eugenol and leptospermone exhibited an intermediate cytotoxicity. However, 1,8-cineole was about 100-fold less cytotoxic than α-pinene and all myrtaceous essential oils tested.

The antiviral potential of manuka essential oil against herpes simplex virus type 1 (HSV-1) and three HSV-1 acyclovir-resistant isolates (HSV-1 isolates A, B, and C) was evaluated. The viruses were exposed for 1 h to the maximum noncytotoxic concentration of manuka essential oil (0.001%) in suspension assays. Since the initial dilution of the essential oil was always performed in ethanol and all assays contained up to 1% ethanol final concentration, 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 a plaque reduction assay. The infected cells were incubated at 37 °C, and viral plaques were stained and counted after 4 days. Ethanol at a final concentration of 1% had no effect on virus titers. The results are presented as a percentage of virus control and are the mean values from three independent experiments. Manuka essential oil reduced the infectivity of acyclovir-sensitive as well as acyclovir-resistant virus isolates by $> 99\%$ (Fig. 6).

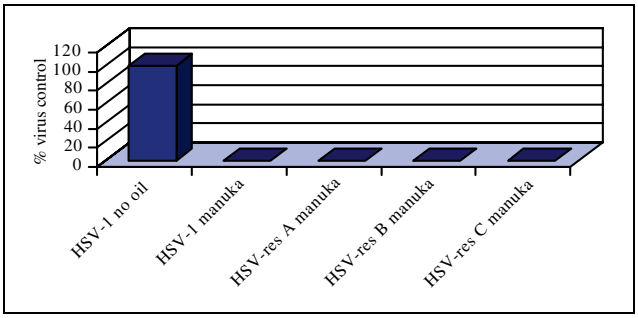

Fig. 6: Antiviral effect of manuka oil against herpes simplex virus type 1 (HSV-1) and different acyclovir-resistant HSV-1 strains abbreviated A, B and C. Essential oil was diluted to the maximum noncytotoxic concentration and incubated with herpesvirus strains for 1 h at room temperature prior to infection of RC-37 cells. Infected cell cultures were incubated for 4 days, then fixed and stained with crystal violet to visualize and count plaques, results are the mean of three independent experiments

3. Discussion

The pharmaceutical market offers a wide range of drug products that contain essential oils or terpenes as their isolated compounds in antiseptics, soaps, deodorants and flavors. A widespread use of essential oils in pharmacy and industry necessitates research on their cytotoxicity. Essential oils consist of many different compounds, e.g. monoterpenes, sesquiterpenes, monoketones and triketones. Myrtaceous essential oils from cajuput, clove, kanuka and manuka were examined phytochemically and 1,8-cineole, α-pinene, eugenol and leptospermone characterized as their main constituents, respectively. Two different cell lines were used in cytotoxicity assays, these cell lines are often applied for propagation of herpes viruses and antiviral tests against herpesviruses *in vitro*.

Attempts were made to overcome methodological problems encountered with lipophilic essential oils and compounds which have limited solubility in aqueous media and adequate solubilizing agents were examined. Solubility and turbidity problems associated with the essential oils were solved by using ethanol as solvent and allowed to quantify cytotoxicity reproducibly. Ethanol itself displayed cytotoxic properties when applied in higher concentrations with a TC_{50} of about 3%. However, a final concentration of 1% ethanol did not reveal any influence on cell viability of Vero cells and RC-37 cells as determined with a neutral red uptake assay. Therefore lipophilic agents like essential oils can be dissolved in ethanol, diluted with aqueous cell culture medium and added to cell lines at a final concentration of 1% ethanol without affecting cell viability or cytotoxicity assays for essential oils.

Experiments to assess the toxicity of myrtaceous essential oils revealed a moderate toxic behaviour in cell cultures. Kanuka oil and manuka oil are about fivefold more toxic for both cell lines than cajuput oil and clove oil. Maximum noncytotoxic concentrations for these myrtaceous oils were similar for both cell lines. Suschke et al. (2007) examined the cytotoxicity of essential oils from catnip, lemon catnip and lemon balm to keratinocytes and bronchial epithelial cells. All tested oils revealed similar cytotoxic properties for both cell lines. The essential oil of *Origanum vulgare* against Vero cells revealed a TC_{50} value of 0.0027% (Sivropoulou et al. 1996), which is about twofold up to tenfold more toxic than myrtaceous oils. When *Melaleuca alternifolia*, another member of the family Myrtaceae, essential oil was tested against epithelial cells, a TC_{50} of 0.032% was determined (Hayes and Markovic 2002), which is about in the same range of toxicity as clove essential oil. Prashar et al. (2006) determined the toxicity of clove oil for three different cell lines and came up with the same TC_{50} value that was examined with Vero and RC-37 cells.

Essential oils are complex mixtures of many different organic components, the most prominent single substances are the cyclic monoterpeneether 1,8-cineole in cajuput oil, eugenol in clove oil, α-pinene in kanuka oil and the triketone leptospermone in manuka oil. The toxic concentration (TC_{50}) for 1,8-cineole, α-pinene, eugenol and leptospermone on RC-37 cells were determined at 0.44%, 0.002%, 0.01%, and 0.04%, respectively. A strikingly high cytotoxic ability was determined for α-pinene, whereas eugenol and leptospermone exhibited intermediate cytotoxicity. However, 1,8-cineole was about 100-fold less cytotoxic than α-pinene and all myrtaceous essential oils tested. A similar moderate toxicity was described for 1,8-cineole when human keratinocytes and a bronchial cell

line were used (Suschke et al. 2007; Reichling et al. 2006). Porter and Wilkins (1998) described the β-triketone fraction of manuka oil as the main active one. The generally accepted view is that 1,8-cineole is thought to be responsible for skin irritation. Eucalyptol (1,8-cineole) is one of the most present terpenes in drug products. There are very few reports on side effects relevant to use of eucalyptol or eucalyptus essential oil. It is perceived to be non-allergizing, non-irritant, and of low toxicity when administered orally. It was noted that eucalyptol induces hepatic microsomal enzymes belonging to the P-450 cytochrome group (Balacs 1997). This activity can make drugs, which are taken simultaneously with eucalyptol, act less effectively and for shorter time. The ability of eucalyptol to increase percutaneous absorption of drugs and other compounds present in vehicle is well known (Jain et al. 2002; Narisetty and Panchagnula 2005). The triketone chemotype of manuka essential oil is commercially important because of its antimocrobial activity (Douglas et al. 2004; van Klink et al. 1999).

According to the correlation of toxicity *in vitro* and *in vivo* LD_{50} values for animals might be predicted (Halle and Göres 1987). According to these criteria, the tested myrtaceous oils exhibit moderate to low cytotoxicity, and 1,8-cineole is very little toxic. However, cytotoxicity might be underestimated and *in vitro* data have to be verified in animals. Limitations of cell cultures are the lack of distribution, biotransformation, metabolism and excretion of the applied drugs and lack of concentration of the drugs in certain tissues or organs. However, the cytotoxicity for topical application of essential oils on the skin or mucous membrane might be much lower, since the stratum corneum, the outmost layer of the epidermis, consists of dead cells.

Major components of tea tree oil, i.e. terpinen-4-ol, α-terpineol and α-pinene were found to be active against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Candida albicans* and *Propionibacterium acnes* whereas 1,8-cineole was inactive against these bacteria, α-pinene had the highest MIC (Raman et al. 1995; Carson et al. 1995). The primary target of triketones against antibiotic-resistant bacteria is the cytoplasmic membrane of these bacteria (van Klink et al. 2005). The results from Söderberg et al. (1996) on the cytotoxicity of tea tree oil support the view that antibacterial activity parallels cytotoxic activity which suggests a similar mode of action, most probably exerted by membrane-associated reactions. Therefore, manuka oil, the most cytotoxic essential oil tested, was assayed in antiviral tests against herpes simplex virus. In plaque reduction assays manuka oil exhibited a significant antiviral effect, when HSV was mixed with this essential oil at maximum noncytotoxic concentrations. Infectivity of HSV-1 as well as of acyclovir-resistant HSV-1 isolates was reduced by more than 99%. The prevalence of resistance to acyclovir in treated immunocompromised individuals is approximately 6% (Christophers et al. 1998) and is of increasing importance. 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, a yellowish essential oil derived from leaves of *Melaleuca alternifolia* L. (*Myrtaceae*) and manuka oil have also been reported to exhibit anti-HSV-1 activity against wild-type herpes virus (Schnitzler et al. 2001; Reichling et al. 2005). An virucidal effect against drug-resistant herpes virus has been reported recently (Schnitzler et al. 2007). Since tea tree oil is becoming increasingly popular as a naturally occuring agent for a wide range of health care products, the efficacy of tea tree oil as an antimicrobial agent was investigated frequently. Antibacterial activity of cajuput oil, kanuka oil, manuka oil and clove oil against several grampositive and gramnegative bacteria had been demonstrated previously (Harkenthal et al. 1999; Fu et al. 2007). Methicillin-resistant *Staphylococcus aureus* (MRSA) is susceptible to tea tree oil (Carson et al. 1995).

In summary, determination of cytotoxicity of essential oils and their main constituents is a prerequisite for comparison of results from different groups and only noncytotoxic concentrations of solvents, essential oils and single compounds should be used in order to guarantee optimal cell viability. These results may have significant implications for the future development of myrtaceous essential oils as antimicrobial and antiviral agents.

4. Experimental

4.1. Essential oils

The essential oils tested were commercial products: cajuput essential oil (Spinnrad, Gelsenkirchen, Germany), clove essential oil, kanuka essential oil (Colimex, Köln, Germany) and manuka essential oil (ALVA, Wallenhorst, Germany). Essential oils were dissolved in ethanol and added to cell culture medium.

4.2. Chemical compounds

Four major compounds of the essential oils were further analysed, e.g. 1,8-cineole, α-pinene, eugenol and leptospermone and have been supplied by Roth (Karlsruhe, Germany).

4.3. Phytochemical analysis of essential oils

Essential oils were analysed by gas chromatography 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 as described previously (Harkenthal et al. 1999). The GC column was a 15 m × 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 40 °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.

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 programm: 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.

4.4. Cell culture

Vero cells and RC-37 cells are both epithelial cells derived from African green monkey and 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 as described previously (Schnitzler et al. 2001). The monolayers were 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.

4.5. 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 Vero cells or RC-37 cells in ten replicates for each concentration of the drug as described previously (Koch et al. 2008). 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 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.

4.6. Virus strains

Herpes simplex virus type 1 (HSV-1) strain KOS and acyclovir-resistant HSV-1 isolates A, B, and C were used for the experiments. Virus was routinely grown as described previously (Rösen-Wolff et al. 1988). 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 cells as described previously (Nolkemper et al. 2006).

4.7. Antiviral assay

Inhibition of virus replication was measured by plaque reduction assays. Usually 2×10^3 plaque forming units (pfu) were incubated with different concentrations of manuka essential 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 as described previously (Schnitzler et al. 2008).

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