JPP Journal of Pharmacy and Pharmacology

JPP 2008, 60: 1545–1550
© 2008 The Authors
Received December 19, 2007
Accepted July 21, 2008
DOI 10.1211/jpp/60.11.0017
ISSN 0022-3573

Communication

Efficacy of anise oil, dwarf-pine oil and chamomile oil against thymidine-kinase-positive and thymidine-kinase-negative herpesviruses

Christine Koch, Jürgen Reichling, Roland Kehm, Mona M. Sharaf, Hanswalter Zentgraf, Jürgen Schneele and Paul Schnitzler

Department of Biology, Institute of Pharmacy and Molecular Biotechnology, University of Heidelberg, Heidelberg, Germany

Christine Koch, Jürgen Reichling, Jürgen Schneele

Department of Virology, Hygiene Institute, University of Heidelberg, Heidelberg, Germany

Christine Koch, Roland Kehm, Mona M. Sharaf, Paul Schnitzler

Applied Tumor Virology, German Cancer Research Center, University of Heidelberg, Heidelberg, Germany

Hanswalter Zentgraf

Correspondence: P. Schnitzler, Department of Virology, Hygiene Institute, University of Heidelberg, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany. E-mail: Paul_Schnitzler@med.uni-heidelberg.de

Acknowledgements: We would like to thank Prof. G. Darai for continuing support and Dr U. Bahr for sequence analysis. The authors also thank Dr A. Sauerbrei, Institute for Antiviral Chemotherapy, University of Jena, Germany, for kindly providing HSV-1 clinical isolates.

Abstract

The effect of anise oil, dwarf-pine oil and chamomile oil against different thymidine-kinase-positive (aciclovir-sensitive) and thymidine-kinase-negative (aciclovir-resistant) herpes simplex virus type 1 (HSV-1) strains was examined. Clinical HSV-1 isolates containing frameshift mutations in the thymidine kinase (TK) gene, an insertion or a deletion, yield a non-functional thymidine kinase enzyme resulting in phenotypical resistance against aciclovir. The inhibitory activity of three different essential oils against herpes simplex virus isolates was tested in-vitro using a plaque reduction assay. All essential oils exhibited high levels of antiviral activity against aciclovir-sensitive HSV strain KOS and aciclovir-resistant clinical HSV isolates as well as aciclovir-resistant strain Angelotti. At maximum noncytotoxic concentrations of the plant oils, plaque formation was significantly reduced by 96.6–99.9%, when herpesviruses were preincubated with drugs before attachment to host cells. No significant effect on viral infectivity could be achieved by adding these compounds during the replication phase. These results indicate that anise oil, dwarf-pine oil and chamomile oil affected the virus by interrupting adsorption of herpesviruses and in a different manner than aciclovir, which is effective after attachment inside the infected cells. Thus the investigated essential oils are capable of exerting a direct effect on HSV and might be useful in the treatment of drug-resistant viruses. Chamomile oil did not reveal any irritating potential on hen's egg chorioallantoic membrane, demonstrated the highest selectivity index among the oils tested and was highly active against clinically relevant aciclovir-resistant HSV-1 strains.

Introduction

Herpes simplex virus type 1 (HSV-1) is a widespread human pathogen that causes epidermal lesions in and around the oral cavity. The symptoms caused by herpes infections are usually self-limiting but can be extensive and prolonged in immunocompromised patients. All members of the family *Herpesviridae* are able to establish a latent infection (e.g. HSV in the nervous system that can be reactivated quite frequently). Several drugs are currently available for the management of HSV infections, such as aciclovir. Aciclovir, a guanine nucleoside analogue, has been widely used for the therapy of herpesvirus infections; its preferential phosphorylation by the HSV-encoded thymidine kinase (TK) makes it a selective antiviral drug (Cassady & Whitley, 1997; De Clercq 2004). The HSV-1 TK is a multisubstrate enzyme that phosphorylates a broad spectrum of purine and pyrimidine nucleoside substrate analogues. In the case of aciclovir-resistant herpetic infections, antiviral therapy with foscarnet or cidofovir is indicated. In immunocompromised patients, a prolonged antiviral therapy is required, resulting in the emergence of drug-resistant mutants in approximately 4–7% (Christophers et al 1998; Chakrabarti et al 2000; Chen et al 2000). This trend has led to the search for alternative anti-herpetic agents that have a wide range of efficacy without serious adverse effects, and which are effective against viral strains resistant to current antiviral agents.

Essential oils of many plants have been widely used in traditional medicine (Reichling 1999). Recently, the anti-herpes activity of several essential oils from different plant sources, as well as of various constituents of essential oils, was demonstrated (Sivropoulou et al 1997; Benencia & Courrèges 1999; Bourne et al 1999; De Logu et al 2000). The anti-herpes activity of Australian tea tree oil and peppermint oil has been published (Carson et al 2001;

Schuhmacher et al 2003). Here we report studies on the antiviral activity of three essential oils against TK-positive and TK-negative clinical HSV-1 isolates where therapy with aciclovir failed.

Materials and Methods

Essential oils and aciclovir

Different essential oils were investigated – anise oil (*Illicium verum*), dwarf-pine oil (*Pinus mugo*) and chamomile oil (*Matricaria recutita*) (purchased from Caelo, Hilden, Germany). To confirm the pharmaceutical quality and identity of the essential oils tested their chemical composition was analysed quantitatively and qualitatively by GC and GC/MS methods as described previously (Schnitzler et al 2007). All essential oils tested met the standard demands of either current pharmacopoeias or literature data (Blaschek et al 2006); they were dissolved in ethanol and added to the cell culture medium. For each essential oil one defined batch was ordered for all antiviral assays, thus always the same batch of oil was used. To guarantee the reproducibility of the essential oil composition, each sample of each batch was analysed by GC and GC/MS. All analysed essential oil samples contained the same spectrum and same amount of individual components with negligible differences. Thus a high quality and reproducibility of the essential oils used in antiviral assays was assured. Aciclovir was purchased from Glaxo Smith Kline (Bad Oldesloe, Germany) and dissolved in sterile water.

Cell culture and viruses

RC-37 cells (African green monkey kidney cells), Vero cells and MCDK cells were grown with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 100 U mL^{-1} penicillin and 100 $\mu g\ mL^{-1}$ streptomycin. Aciclovir-sensitive herpes simplex virus type 1 (HSV-1) strain KOS and aciclovir-resistant strain Angelotti, as well as clinical isolates 1246/99 and 496/02, were used (Schnitzler et al 2007). Strain Angelotti exhibits a single point mutation in the pyrophosphate binding site of the DNA polymerase gene, where alanine is replaced by valine in the 14-amino acid conserved binding site (Knopf 1987).

Polymerase chain reaction and DNA sequencing

Amplification of HSV DNA was performed using 5 μL of the eluted DNA in a total reaction volume of 50 μL as described previously (Chibo et al 2004). PCR products were purified and sequenced in the forward and reverse direction.

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 oils was added onto subconfluent cells (Söderberg et al 1996). The cytotoxic concentration of the drug, which reduced viable cell number by 50% (TC50), was determined from dose–response curves. Each experiment was performed with ten replicates and repeated three times.

Mode of antiviral activity

Inhibition of virus replication was measured using a plaque reduction assay with RC-37 cells (African green monkey kidney cells), Vero cells and MCDK cells. By reference to the number of plaques observed in virus-infected controls, treated with 1% ethanol but no addition of essential oil, the concentration of test compound that inhibited the plaque number by 50% (IC50) was determined from dose–response curves. Each experiment was performed with six replicates and repeated three times. To determine the mode of antiviral action, cells were pretreated with essential oils before viral infection, viruses were incubated with essential oils before infection and cells and viruses were incubated together during adsorption or after penetration of the virus into the host cells as previously described (Reichling et al 2005). Essential oils were always used at the maximum noncytotoxic concentration. Each test was run with six replicates and repeated three times.

HET-CAM assay

The HET-CAM assay (hen's egg test – chorioallantoic membrane) was used to determine the irritation potential of chamomile oil (Möller et al 2006). Fresh, fertile eggs were incubated at 37.8°C and 70% humidity for 72 h. Five millilitres of albumin were removed from the eggs with a syringe followed by another 96-h period of incubation. For the HET-CAM assay the eggs were used on day 9 of incubation. A 50% mixture of cream and water was injected under the CAM to increase visibility of the CAM's vessel system. Essential oils were applied to the CAM at different concentrations until the irritation threshold of the individual oils were detected. For documentation, the eggs were placed under a stereo microscope, equiped with a digital camera. Olive oil, the non-irritating solvent for essential oils, served as negative control. HET-CAM assays were performed with six replicates and repeated three times.

Statistical analysis

Cytotoxicity assays with different essential oils, mode of antiviral action at different steps of viral replication and HET-CAM assays with different essential oils were compared statistically. Results were expressed as means ± standard deviation (s.d.) and differences between treatments were examined using a nonparametric test (Mann–Whitney method).

Results

Characterization of thymidine-kinase-negative HSV-1 clinical isolates

RC-37 cells were infected with thymidine-kinase-negative (aciclovir-resistant) HSV-1 clinical isolates 1246/99 and 496/02, strain Angelotti and the thymdine-kinase-positive

(aciclovir-sensitive) strain KOS, which served as a control. Infected cells were incubated with medium containing 25 μg mL^{-1} aciclovir for 4 days. Viral replication of the aciclovir-resistant HSV-1 isolates 1246/99, 496/02 and strain Angelotti was not significantly inhibited by the drug aciclovir whereas the plaque formation of strain KOS was reduced by about 90% (data not shown). Foscarnet was effective against all HSV-1 strains and no mutations in the polymerase gene were detected. Both clinical isolates revealed a single point mutation in the coding sequence of the TK gene. An insertion was found in strain 1246/99 at position 436, a deletion was detected in the middle of the TK gene at position 551 in strain 496/02. Both frameshifts probably result in deficiency of TK activity, in strain 1246/99 by changing more than half of the amino acid sequence and in strain 496/02 by a premature stop codon in the new reading frame at position 916.

Cytotoxicity

All essential oils were dissolved in ethanol and added to the medium at a final concentration of 1% ethanol. The effect of the oils on the growth of eukaryotic cells was examined at dilutions from 1 mg mL^{-1} to 0.1 μg mL^{-1}. The toxic concentration (TC50) of the tested essential oils was determined as 160 μg mL^{-1} for anise oil, 40 μg mL^{-1} for dwarf-pine oil and 30 μg mL^{-1} for chamomile oil (Table 1). The MTT formazan assay for cytotoxicity of essential oils was conducted in parallel but revealed very similar results for toxic and maximum nontoxic concentrations. Maximum noncytotoxic concentrations for the essential oils are shown in Table 1; cells incubated for four days with maximum noncytotoxic oil concentrations did not reveal any influence on morphology and viability.

Efficacy of essential oils against HSV-1 strains

Pretreatment of cells with the essential oils did not reduce virus production; however, pretreatment of HSV with each essential oil before infection caused a significant reduction in infectivity ranging from 96.6% (anise oil) to 99.9% (chamomile oil) for strain KOS and from 98.5% (anise oil) to 99.9% (chamomile oil) for the aciclovir-resistant isolate 1246/99. HSV strains incubated in 1% ethanol without essential oil served as untreated control (Table 2). When these essential oils were added only during the adsorption period, virus titres were reduced by about 70–80%. Interestingly, no significant effect on viral growth could be observed when the essential oils were added exclusively to the overlay medium after viral adsorption during the replication period immediately following the removal of the unadsorbed virus inoculum. A clearly concentration-dependent activity of chamomile oil was demonstrated in the dose–response curves (Figure 1). Similar concentration-dependent results were obtained with anise oil and dwarf-pine oil; IC50 values for all oils tested are shown in Table 1. The effect of essential oils was also investigated in Vero cells and MDCK cells, two other permissive cell lines for HSV. Similar results to RC-37 cells were obtained; a reduction of virus titres between 84% and 92% was detected when HSV strains were incubated with essential oils during the adsorption phase and even a more than 98% reduction

Table 1 Toxic concentrations, selectivity indices and irritation threshold of different essential oils for HSV-1

Essential oil	Maximum noncytotoxic concn (μg mL^{-1})	TC50 (μg mL^{-1})	IC50 (μg mL^{-1})	Selectivity index (SI)	Irritation threshold
Anise oil	100 ± 8	160 ± 19.2	40 ± 4.0	4	90%
Dwarf-pine oil	30 ± 3.6	40 ± 4.4	7 ± 0.84	6	100%
Chamomile oil	10 ± 1.2	30 ± 4.2	0.3 ± 0.045	100	Not detectable

Table 2 Effect of anise oil, dwarf-pine oil and chamomile oil on infection of HSV-1 strains

	Anise oil				Dwarf-pine oil				Chamomile oil			
	KOS	Ang.	1246/99	496/02	KOS	Ang.	1246/99	496/02	KOS	Ang.	1246/99	496/02
Pretreatment cells	101.4 ± 7.4	102.4 ± 5.9	108.7 ± 3.6	93.9 ± 3.2	102.2 ± 9.0	95.9 ± 18.3	112.2 ± 12.6	91.3 ± 9.0	96.6 ± 9.7	89.6 ± 8.4	105.2 ± 14.1	94.4 ± 12.3
Pretreatment virus	3.4 ± 2.4	0.5 ± 0.3	1.5 ± 0.5	0.4 ± 0.3	1.3 ± 0.8	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.2
Adsorption	28.5 ± 4.0	23.2 ± 3.2	19.8 ± 4.4	22.7 ± 2.9	22.2 ± 3.2	28.1 ± 4.1	19.9 ± 3.3	24.5 ± 3.9	18.7 ± 1.9	29.7 ± 3.0	21.0 ± 2.8	22.3 ± 4.1
Replication	97.7 ± 19.1	114.2 ± 18.4	114.6 ± 16.0	111.7 ± 13.2	96.3 ± 15.2	105,7 ± 17.5	105,7 ± 11.5	108.2 ± 16.8	92.0 ± 11.2	89.7 ± 9.0	92.9 ± 10.5	96.4 ± 14.0

Remaining infectivity is indicated in % compared with untreated controls. HSV-1 strain KOS (aciclovir-sensitive), Angelotti (Ang.; aciclovir-resistant), and aciclovir-resistant HSV-1 strains 1246/99 and 496/02 derived from patients who failed therapy with aciclovir were used.

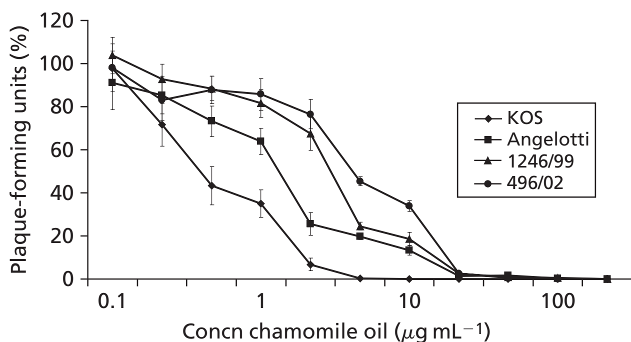

Figure 1 Dose–response curves for chamomile oil against indicated HSV-1 strains. Virus strains were preincubated for 1 h at room temperature with increasing concentrations of chamomile oil and immediately tested in a plaque reduction assay. Reduction of viral titres are shown as percentage of plaque-forming units (pfu) compared with untreated controls.

could be achieved by preincubating the virus before infection. These results indicate an antiviral effect of essential oils on HSV independent of the cell line used. When 1000-fold higher herpesvirus titres than mentioned above were preincubated for 1 h with different essential oils and subsequently highly diluted viral suspensions were tested in plaque assays, the viral infectivity was reversed. This indicates adsorption inhibition rather than viral inactivation by a virucidal mechanism.

To gain more insight into the mode of action against HSV, the virus was incubated with chamomile oil concentrations ranging from 1 μg mL^{-1} to 10 mg mL^{-1} for 1 h at room temperature and several thousand viral particles were subsequently analysed by electron microscopy. No obvious effect on the viral envelope or structure could be observed compared with untreated virus. However, this method was not sufficiently precise enough to affirm that the envelope is not affected by a lipidic compound.

Selectivity index and irritation potential

Selectivity indices for different essential oils were calculated as the ratio TC50/IC50 and are given in Table 1. Anise oil and dwarf-pine oil exhibited low selectivity indices; chamomile oil revealed the highest selectivity index of 100 among the essential oils tested. In view of their topical application and to assess the possible irritation potential of these essential oils to skin and mucous membranes, their irritation threshold concentration was determined using the HET-CAM (hen's egg test – chorioallantoic membrane) assay. Plant oils were applied to the chorioallantoic membrane of embryonated eggs at concentrations ranging from 10% to 100%. Anise oil displayed a threshold of 90%, dwarf-pine oil a threshold of 100% and chamomile oil did not reveal any irritating behaviour, either diluted or undiluted (Table 1, Figure 2).

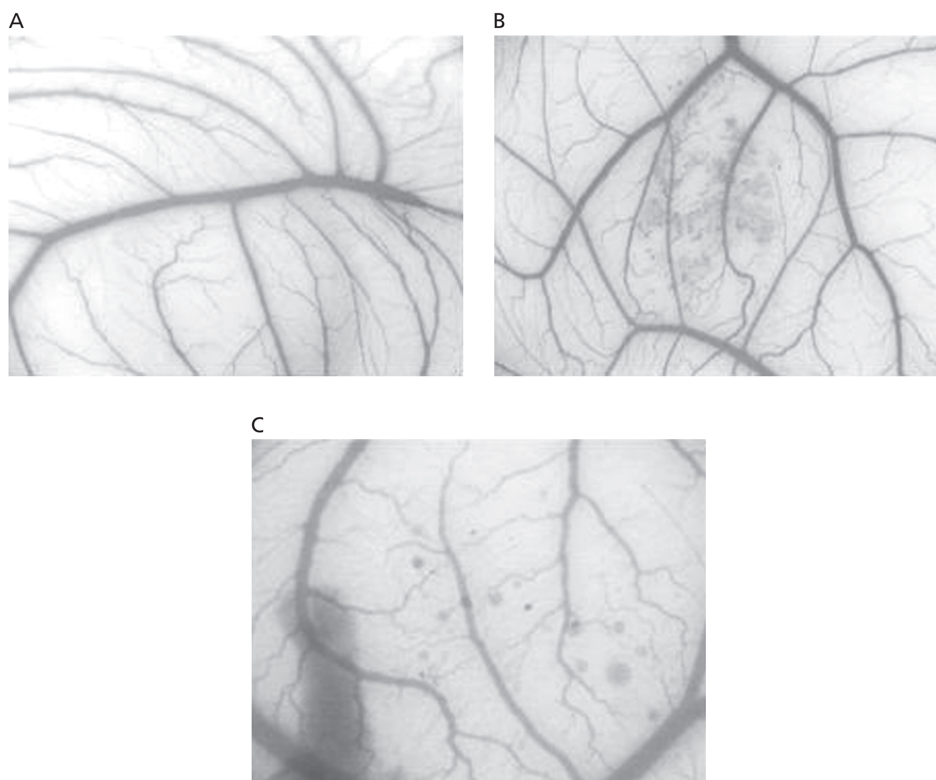

Figure 2 HET-CAM assay (hen's egg test – chorioallantoic membrane) was used to determine the irritation potential of chamomile oil; drugs were applied to the chorioallantoic membrane. A. Olive oil, the solvent for essential oils, served as negative control and caused no irritation of the chorioallantoic membrane. B. 0.5% SDS-solution represents a positive control and induces visible haemorrhage. C. 100% Chamomile oil showed no irritation potential on the chorioallantoic membrane.

Discussion

Resistance to aciclovir in HSV infection is often caused by mutations in the viral thymidine kinase genes (Chibo et al 2004; Andrei et al 2005). An insertion for isolate 1246/99 located between the ATP-binding site and the nucleotide-binding site and a deletion in the open reading frame of the thymidine kinase gene of isolate 496/02 located about 30 nucleotides downstream of the nucleotide-binding site have been detected. Both frameshifts result in expression of a non-functional enzyme, which leads to phenotypical aciclovir resistance.

We have analysed the inhibitory effect of several essential oils against herpes simplex virus infection in-vitro. Pretreatment of the cells with the essential oils had no effect on the production of infectious virus and plaque formation was not affected. The same results were found when the essential oils were added during the replication period of the infection cycle. However, all tested essential oils exhibited high levels of antiviral activity against aciclovir-sensitive and aciclovir-resistant HSV-1 strains in viral suspension tests. At maximum noncytotoxic concentrations of the compounds, plaque formation was significantly reduced by 96.6–99.9%, when herpesviruses were preincubated with essential oils. Addition of the essential oils during the 1-h adsorption period also resulted in virus inhibition up to 90%. These results indicate that anise oil, dwarf-pine oil and chamomile oil affected the virus before attachment and in a different manner to aciclovir since plaque formation of aciclovir-resistant isolates HSV-1 1246/99 and 496/02 was significantly reduced. Preincubated viruses were exposed for 2 h to the drugs and were more inhibited than viruses treated with essential oils for only 1 h during the adsorption period. During adsorption, viral and cellular receptors interact and during viral replication, herpesviruses spread from cell to cell and form plaques. An inhibition of cell-to-cell infection via lateral diffusion by the essential oils of *Artemisia arborescens* has been reported recently (Saddi et al 2007). However, inhibition of plaque development or reduction of plaque size was not observed for the essential oils of anise oil, dwarf-pine oil and chamomile oil. These essential oils probably interacted directly with viral particles and inhibited attachment to host cells. When 1000-fold higher herpesvirus titres than mentioned above were preincubated for 1 h with different essential oils and subsequently highly diluted viral suspensions were tested in plaque assays, the viral infectivity was reversed. This indicates adsorption inhibition rather than viral inactivation by a virucidal mechanism. Electron microscopy studies did not reveal any obvious change in viral structure after incubation with the plant-derived oils. This is in accordance with the hypothesis of an interfering effect of essentials oils with the virus that prevents binding or uptake of the virus into the host cell. These results suggest that the investigated essential oils interfere with virion envelope structures or might mask viral compounds that are necessary for adsorption or entry into host cells. An interaction of tea tree oil, which has similar properties against HSV in-vitro, with the cell membrane of *Escherichia coli* was reported recently (Cox et al 2000). A dissolution of the HSV envelope by treatment with oregano essential oil has been described (Siddiqui et al 1996). Sandalwood oil was reported to show no virucidal effect when incubated with herpesvirus before infection but inhibited the replication significantly when it was added after the adsorption period. Thus, different mechanisms of antiviral activity of different essential oils seem to be present. The inhibition of HSV by anise oil, dwarf-pine oil and chamomile oil appears to occur before, or during, 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.

Interestingly, TK-negative strains were significantly inhibited by the essential oils: the titre of HSV was reduced by about 99%. Since essential oils are able to inhibit aciclovir-resistant HSV-1 isolates, the mechanism of interaction between these compounds and aciclovir with HSV must be different. Aciclovir inhibits virus replication by interference with the DNA polymerase inside the cell, whereas essential oils probably interact with HSV before it enters the cell. Viral resistance to aciclovir represents a particular problem – the prevalence of resistance in aciclovir-treated immunocompromised individuals is approximately 4–7% (Christophers et al 1998; Stranska et al 2005). Therefore, other anti-herpetic agents that are effective against viral mutants resistant to current antiviral agents are of great interest as topical additional treatment. The successful application of tea tree essential oil against herpetic infections in patients for the treatment of recurrent herpes labialis has been reported recently (Carson et al 2001, 2006). Essential oils could be used as topical treatment for herpetic infections and might even be effective against aciclovir-resistant strains that had been described previously for labial and genital herpes (Gaudreau et al 1998; Chibo et al 2004; Stranska et al 2005). The results of our study support the concept that the antiviral effect of essential oils is due to direct interaction with viruses before attachment. This interaction might also affect extracellular herpesviruses released from host cells and lead to effective antiviral treatment for herpes labialis, as demonstrated previously (Carson et al 2001).

Chamomile oil did not cause any irritation even when applied undiluted to the highly sensitive chorioallantoic membrane. In comparison with other tested essential oils chamomile oil revealed the highest selectivity index of 100. Considering the lipophilic nature of chamomile oil, which enables it to penetrate the skin, antiviral activity against both aciclovir-sensitive and aciclovir-resistant HSV-1, lack of irritating potential and high selectivity index, chamomile oil might be a promising topical therapeutic agent in the treatment of recurrent herpes infection.

References

Andrei, G., Balzarini, J., Fiten, P., De Clercq, E., Opdenakker, G., Snoeck, R. (2005) Characterization of herpes simplex virus type 1 thymidine kinase mutants selected under a single round of high-dose brivudin. *J. Virol.* **79**: 5863–5869

Benencia, F., Courrèges, M. C. (1999) Antiviral activity of sandalwood oil against herpes simplex viruses -1 and -2. *Phytomedicine* **6**: 119-123

Blaschek, W., Ebel, S., Hackenthal, E., Holzgrabe, U., Keller, K., Reichling, J., Schulz, V. (2006) *Hagers Handbuch der Drogen und Arzneistoffe, Hager ROM*. Springer Verlag, Berlin, Heidelberg, New York

Bourne, K. Z., Bourne, N., Reising, S. F. Stanberry, L. R. (1999) Plant products as topical microbicide candidates: assessment of *in vitro* activity against herpes simplex virus type 2. *Antivir. Re.s* **42**: 219–226

Carson, C. F., Ashton, L., Dry, L., Smith, D. W., Riley, T. V. (2001) *Melaleuca alternifolia* (tea tree) oil gel (6%) for the treatment of recurrent herpes labialis. *J. Antimicrob. Chemother.* **48**: 450–451

Carson, C. F., Hammer, K. A., Riley, T. V. (2006) *Melaleuca alternifolia* (Tea Tree) oil: a review of antimicrobial and other medicinal properties. *Clin. Microbiol. Rev.* **19**: 50–62

Cassady, K. A., Whitley, R. J. (1997) New therapeutic approaches to the alphaherpesvirus infections. *J. Antimicrob. Chemother.* **3**: 119–128

Chakrabarti, S., Pillay, D., Ratcliffe, D., Cane, P. A., Collinghan, K. E., Milligan, D. W. (2000) Resistance to antiviral drugs in herpes simplex virus infections among allogeneic stem cell transplant recipients: risk factors and prognostic significance. *J. Infect. Dis.* **181**: 2055–2058

Chen, Y., Scieux, V., Garrait, V., Socié, G., Rocha, V., Molina, J. M., Thouvenot, D., Morfin, F., Hocqueloux, I., Garderei, L., Esperou, H., Selimi, F., Dervergie, A., Lelen, G., Aymard, M., Morinet, F., Gluckman, E., Ribaud, P. (2000) Resistant herpes simplex virus type 1 infection: an emerging concern after allogeneic stem cell transplantation. *Clin. Infect. Dis.* **31**: 927–935

Chibo, D., Druce, J., Sasadeusz, J., Birch, C. (2004) Molecular analysis of clinical isolates of acyclovir-resistant herpes simplex virus. *Antiviral Res.* **61**: 83–91

Christophers, J., Clayton, J., Craske, J., Ward, R., Collins, P., Trowbridge, M., Darby, G. (1998) Survey of resistance of herpes simplex virus to acyclovir in north-west England. *Antimicrob. Agents Chemother.* **42**: 868–872

Cox, S. D., Mann, C. M., Markham, J. L., Bell, H. C., Gustafson, J. E., Warmington, J. R., Wyllie, S. G. (2000) The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *J. Appl. Microbiol.* **88**: 170–175

De Clercq, E. (2004) Antiviral drugs in current clinical use. *J. Clin. Virol.* **30**: 115–133

De Logu, A., Loy, G., Pellerano, M. L., Bonsignore. L., Schivo, M. L. (2000) Inactivation of HSV-1 and HSV-2 and prevention of cell-to-cell virus spread by *Santolina insularis* essential oil. *Antivir. Res.* **48**: 177–185

Gaudreau, A., Hill, E. L., Balfour, H. H. J., Erice, A., Boivin, G. (1998) Phenotypic and genotypic characterization of acyclovir-resistant herpes simplex viruses from immunocompromised patients. *J. Infect. Dis.* **178**: 297–303

Knopf, C. W. (1987) The herpes simplex virus type 1 DNA polymerase gene: site of phosphonoacetic acid resistance mutation in strain Angelotti is highly conserved. *J. Gen. Virol.* **68**: 1429–1433

Möller, M., Suschke, U., Nolkemper, S., Schneele, J., Distl, M., Sporer, F., Reichling, J., Wink, M. (2006) Antibacterial, antiviral, antiproliferative and apoptosis-inducing properties of *Brackenridgea zanguebarica* (Ochnaceae). *J. Pharm. Pharmacol.* **58**: 1131–1138

Reichling, J. (1999) Plant–microbe interactions and secondary metabolites with antiviral, antibacterial and antifungal properties. In: Wink M. (ed.) *Functions of plant secondary metabolites and their exploitation in biotechnology. Annual Plant Review, Vol. 3,* pp. 187–273, Sheffield Acad. Press, Sheffield

Reichling, J., Koch, C., Stahl-Biskup, E., Sojka, C., Schnitzler, P. (2005) Virucidal activity of a beta-triketone-rich essential oil of *Leptospermum scoparium* (manuka oil) against HSV-1 and HSV-2 in cell culture. *Planta Med.* **71**: 1123–1127

Saddi, M., Sanna, A., Cottiglia, F., Chisu, L., Casu, L., Bonsignore, L., De Logu, A. (2007) Antiherpesvirus activity of *Artemisia arborescens* essential oil and inhibition of lateral diffusion in Vero cells. *Ann. Clin. Microbiol. Antimicrob.* **6**: 6–10

Schnitzler, P., Koch, C., Reichling, J. (2007) Susceptibility of drug-resistant clinical herpes simplex virus type 1 strains to essential oils of ginger, thyme, hyssop, and sandalwood. *Antimicrob. Agents Chemother.* **51**: 1859–1862

Schuhmacher, A., Reichling, J., Schnitzler, P. (2003) Virucidal effect of peppermint oil on the enveloped viruses herpes simplex virus type 1 and type 2 *in vitro*. *Phytomedicine* **10**: 504–510

Siddiqui, Y. M., Ettayebi, M., Haddad, A. M., Al-Ahdal, M. N. (1996) Effect of essential oils on the enveloped viruses: antiviral activity of oregano and clove oils on herpes simplex virus type 1 and Newcastle disease virus. *Med. Sci. Res.* **24**: 185–186

Sivropoulou, A., Nikolaou, C., Papanikolaou, E., Kokkini, S., Lanaras, T., Arsenakis, M. (1997) Antimicrobial, cytotoxic, and antiviral activities of *Salvia fructicosa* essential oil. *J. Agric. Food Chem.* **45**: 3197–3201

Söderberg, T., Johannson, A., Gref, R. (1996) Toxic effects of some conifer resin acids and tea tree oil on human epithelial and fibroblast cells. *Toxicology* **107**: 99–109

Stranska, R., Schuurman, R., Nienhuis, E., Goedegebuure, I. W., Polman, M., Weel, J. F., Wertheim-Van Dillen, P. M., Berkhout, R. J. M., van Loon, A. M. (2005) Survey of acyclovir-resistant herpes simplex virus in the Netherlands: prevalence and characterization. *J. Clin. Virol.* **32**: 7–18