Inhibitory effect of essential oils against herpes simplex virus type 2

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Abstract

Essential oils from anise, hyssop, thyme, ginger, camomile and sandalwood were screened for their inhibitory effect against herpes simplex virus type 2 (HSV-2) *in vitro* on RC-37 cells using a plaque reduction assay. Genital herpes is a chronic, persistent infection spreading efficiently and silently as sexually transmitted disease through the population. Antiviral agents currently applied for the treatment of herpesvirus infections include acyclovir and its derivatives. The inhibitory concentrations (IC_{50}) were determined at 0.016%, 0.0075%, 0.007%, 0.004%, 0.003% and 0.0015% for anise oil, hyssop oil, thyme oil, ginger oil, camomile oil and sandalwood oil, respectively. A clearly dose-dependent virucidal activity against HSV-2 could be demonstrated for all essential oils tested. In order to determine the mode of the inhibitory effect, essential oils were added at different stages during the viral infection cycle. At maximum noncytotoxic concentrations of the essential oils, plaque formation was significantly reduced by more than 90% when HSV-2 was preincubated with hyssop oil, thyme oil or ginger oil. However, no inhibitory effect could be observed when the essential oils were added to the cells prior to infection with HSV-2 or after the adsorption period. These results indicate that essential oils affected HSV-2 mainly before adsorption probably by interacting with the viral envelope. Camomile oil exhibited a high selectivity index and seems to be a promising candidate for topical therapeutic application as virucidal agents for treatment of herpes genitalis.
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Introduction

Herpes simplex virus (HSV) is differentiated into two antigenic types of type 1 (HSV-1) and type 2 (HSV-2), their genome sequences are highly related for the most part and closely co-linear. HSV infects and replicates in cells at the site of entry, the mucocutaneous surface. The virus is then transported through retrograde axonal transport to cell bodies of neurons in sensory ganglion that innervates it. In the acute stage of ganglionic infection, some sensory neurons undergo lytic virus infection and are destroyed, as are cells at the site of entry. After the acute ganglionic infection subsides, the virus persists in neurons for life-time. After inoculation and limited replication at genital sites, HSV-2 ascends along neuronal axons to establish latent infection in the lumbosacral sensory ganglia. The latent virus is reactivated spontaneously or is induced to reactivate by a variety of stimuli. During the reactivation process, the virus is transported through the nerve cells axons to the original peripheral infection site, where HSV replication occurs. Infectivity is highest in pimary infections and virus excretion can persist for many weeks beyond

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clinical healing. While both types of HSV produce first episode genital infection, most cases of symptomatic primary disease are due to HSV-2 (Sucato et al., 1998). Both types of antibodies appear to be aquired earlier among women than among men and HSV-2 prevalence is usually higher in women than men in populations with higher risk sexual behavior (Howard et al., 2003; Smith and Robinson, 2002). Genital herpes is one of the most prevalent sexually transmitted disease worldwide and is the most common cause of genital ulcers. What makes HSV so difficult to control is that most sexual and perinatal transmissions occur during unrecognized or asymptomatic shedding (Koelle and Wald, 2000). The impact of genital herpes as a public health threat is augmented because epidemiological studies clearly demonstrate a strong link to the HIV epidemic. Genital herpes is a chronic, persistent infection that, on any given day, causes subclinical reactivation in about 1–2% of infected persons (Roizman and Sears, 1995; Wald et al., 1995). Since about 45 million people are infected in the USA (Prober et al., 1992), HSV-2 can spread efficiently and silently as sexually transmitted disease through the population. A dramatic increase in the prevalence of HSV-2 infection was observed in younger age cohorts (Fleming et al., 1997). In laboratory detection of genital herpes it was shown that PCR is more sensitive than virus culture, the traditional gold standard in HSV identification (Sauerbrei et al., 2000). A new real-time PCR method for the analysis of HSV-2 shedding in cervicovaginal secretions was established previously (Legoff et al., 2006). Genital herpes continues to be a public health problem in both developed and developing countries.

Antiviral agents licensed currently for the treatment of herpesvirus infections include acyclovir and derivatives, ganciclovir, foscarnet and cidofovir, all of which inhibit herpesvirus DNA polymerases. Acyclovir, ganciclovir and cidofovir are nucleoside analogues which function as DNA chain terminators, ultimately preventing elongation of viral DNA. Foscarnet inhibits the viral DNA polymerase by binding to the pyrophosphate binding site. Acyclovir has been widely used for the management of herpes virus infections, its preferential phosphorylation by the HSV-encoded thymidine kinase (TK) makes it a selective antiviral drug (De Clercq, 2004). Some of these antiviral agents, e.g, ganciclovir and foscarnet might produce toxic side-effects. In addition, the emergence of virus strains resistant to commonly used anti-herpesvirus drugs is a growing problem, particularly in immunocompromised patients (Reusser, 1996; Cassady and Whitley, 1997; Whitley, 2001; Whitley et al., 1984).

A large number of antiherpes screening experiments on medicinal plant extracts and plant derived secondary metabolites (e.g. flavonoids, anthraquinones, naphthodianthrones, polyphenolics) have been reported (Reichling, 1999; De Logu et al., 2000). Essential oils obtained from fruits, leaves, seeds, stem bark, and roots of many plants have been widely used in traditional medicine. Among others, antibacterial, antifungal, immunomodulatory, antiinflammatory, and antirheumatic activities have been described (Messager et al., 2005; Carson et al., 2002, 2006; Harkenthal et al., 1999; Hammer et al., 2002; Koch et al., personal communication). Recently, the antiherpes activity of several essential oils of different plant sources as well as of various constituents of essential oils was demonstrated (Bourne et al., 1999; Sivropoulou et al., 1997; Benencia and Courrèges, 1999). The application of tea tree oil, the essential oil of *Melaleuca alternifolia*, for the treatment of recurrent herpes labialis has been reported recently (Carson et al., 2001). For the treatment of patients colonized with methicillin-resistant *Staphylococcus aureus*, nasal ointment and body wash containing 4–5% tea tree oil were applied (Caelli et al., 2000). The antiherpes activity of Australian tee tree oil, peppermint oil and manuka oil have previously been published (Schnitzler et al., 2001; Davies et al., 2002; Schuhmacher et al., 2003; Reichling et al., 2005). The application of topical microbicides for the prevention of genital herpes infection was described recently (Keller et al., 2005). Multicentre trials demonstrated a higher risk of acquisition of HIV following frequent use of the microbicide N-9 relative to placebo (Van Damme et al., 2002). While the preclinical data indicate that SDS has less cytotoxicity than N-9 (Krebs et al., 2000), its clinical safety and efficacy have yet to be established, and there are concerns that frequent use of any surfactant or detergent will have a deleterious effect on the vaginal environment. Thus, there is an urgent need for novel prophylactic methods, such as topical virucidal constituents designed for genital application. These include compounds that inactivate virus directly and drugs that block viral binding and entry.

In the present study the *in vitro* virucidal activity of essential oils of anise, hyssop, thyme, ginger, camomile and sandalwood against HSV-2 and the mode of action of these essential oils at different steps in the viral infection cycle are described.

Materials and methods

Essential oils

Essential oils from anise (*Illicium verum*), hyssop (*Hyssopus officinalis*), thyme (*Thymus vulgaris*), ginger (*Zingiber officinale*), camomile (*Matricaria recutita*) and sandalwood (*Santalum album*) were investigated. Anise oil, hyssop oil, thyme oil, ginger oil, camomile oil and sandalwood oil were 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 (Harkenthal et al., 1999). All essential oils tested met the standard demands of either current pharmacopoeias or literature data (Blaschek et al., 2006). All essential oils were dissolved in ethanol and added to the cell culture medium.

Acyclovir

Acyclovir (ACV) was purchased from Glaxo Smith Kline (Bad Oldesloe, Germany) and dissolved in sterile water.

Cell cultures

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), $100\,\mu g/ml$ penicillin and $100\,\mu g/ml$ 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 $37\,^{\circ}C$ in an atmosphere of 5% CO_2.

Herpes simplex virus type 2 (HSV-2)

HSV-2 strain HG52 was used for all experiments (Dolan et al., 1998). Viruses were routinely grown on RC-37 cells as described previously (Rösen-Wolff et al., 1988). Herpes simplex virus stock cultures were prepared from supernatants of infected cells and stored at $-80\,^{\circ}C$. Infectivity titers were determined by a standard plaque assay on confluent RC-37 cells.

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 $37\,^{\circ}C$. The medium was removed and fresh DMEM containing the appropriate dilution of the essential oils was added onto subconfluent RC-37 cells in ten replicates for each concentration of the drug. All essential oils were dissolved in ethanol and added to the medium at a final concentration of 1% ethanol, wells containing 1% ethanol but without 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 dye assay (Söderberg et al., 1996). The neutral red dye uptake was determined by measur-ing the optical density (OD) of the eluted neutral red at 540 nm in a spectrophotometer. The cytotoxic concentration of the drug which reduced viable cell number by 50% (TC_{50}) was determined from dose–response curves.

Direct plaque assay

Inhibition of virus replication was measured by plaque reduction assay. All essential oils were dissolved in ethanol and added to the medium at a final concentration of 1% ethanol, wells containing 1% ethanol but without drug were also included on each plate as controls. Serial dilutions of the oil-treated virus were adsorbed to RC-37 cells for 1 h at $37\,^{\circ}C$. The residual inoculum was discarded and infected cells were overlaid with medium containing 0.5% methylcellulose. After incubation for 4 days at $37\,^{\circ}C$, monolayers were fixed with 10% formalin. The cultures were stained with 1% crystal violet and subsequently plaques were counted. The concentration of test compound which inhibited plaque numbers by 50% (IC_{50}) was determined from dose–response curves.

Mode of antiviral activity

Cells and viruses were incubated with acyclovir or essential oils at different stages during the viral infection cycle in order to determine the mode of antiviral action. Cells were pretreated with acyclovir or essential oils before viral infection, viruses were incubated with acyclovir or essential oils before infection and cells and viruses were incubated together with acyclovir or essential oils during adsorption or after penetration of the virus into the host cells. All essential oils were dissolved in ethanol and added to the medium at a final concentration of 1% ethanol, wells containing 1% ethanol but without drug were also included on each plate as controls. Essential oils or acyclovir were always used at the maximum noncytotoxic concentration. Cell monolayers were pretreated with the oils prior to inoculation with virus by adding the essential oils or acyclovir to the culture medium and by incubation for 1 h at $37\,^{\circ}C$. The compound was aspirated and cells were washed immediately before the HSV-2 inoculum was added. For pretreatment of herpes simplex virus about 2×10^3 pfu of HSV-2 were incubated in medium containing the maximum noncytotoxic concentration of the oils for 1 h at room temperature prior to infection of RC-37 cells. For analysing the antiviral inhibition during the adsorption period, HSV-2 was mixed with the drug and added to the cells immediately. After 1 h of adsorption at $37\,^{\circ}C$, the inoculum was removed and cells were overlaid with medium containing 0.5% methylcel-lulose. The effect of essential oils against HSV was also tested during the replication period by adding the

compounds after adsorption to the overlay medium, as typically performed in antiviral susceptibility studies.

Results

Chemical characterization of essential oils

Essential oils are lipophilic multi-component systems with a characteristic pattern of mainly monoterpenes, sesquiterpines and phenylpropanoids. The specific combination of these compounds determines their different biological activities. To confirm the identity and pharmaceutical quality the chemical composition of each essential oil was quantitatively and qualitatively analyzed by GC and GC–MS methods. Anise oil was characterized mainly by *trans*-anethol (89.1%), estragol (3.6%), linalool (1.1%), α-terpineol (0.2%) and *cis*-anethol (0.2%), camomile oil by bisabolol (27.4%), β-farnesene (23%), bisabololoxide A (14.9%) and bisabololoxide B (5.3%) and ginger oil contained mainly zingiberene (18.9%), limonene/cineol (15.5%), β-sesquiphellandrene (6.8%), camphene (6.2%) and pinocamphene (6.8%)%). Hyssop oil yielded mainly isopinocamphone (42.8%), pinocamphone (38.4%), spatulenol (7.9%) and linalool (2.7%), sandalwood oil α-santalol (52.5%), *trans*-α-santalol (33.1%), *cis*-lanceol (2.6%) and α-santalene (0.8%) and thyme oil was characterized by thymol (40.5%), *p*-cymene (23.6%), carvacrol (3.2%), linalool (5.4%), β-caryphyllene (2.6%) and terpinen-4-ol (0.7%).

Cytotoxicity

All essential oils were dissolved in ethanol and added to the medium at a final concentration of 1% ethanol. Ethanol by itself did not exhibit any toxic effect on RC 37 cells when applied up to 5% (data not shown). The effect of the oils on the growth of eucaryotic cells was examined. Monolayer cultures of RC-37 cells were grown in 0.00001–0.1% drug-containing medium and after 4 days of incubation, cell viability was determined in the neutral red assay. The toxic concentration (TC_{50}) of the essential oils for RC-37 cells was 0.016% for anise oil, 0.0075% for hyssop oil, 0.007% for thyme oil, 0.004% for ginger oil, 0.003% for camomile oil and 0.0015% for sandalwood oil (Fig. 1).

Virucidal activity of essential oils against HSV-2

The potential inhibitory effect against HSV-2 of different essential oils was determined by pretreatment of the virus with the corresponding oils for 1 h at room temperature and subsequent infection of RC-37 cells. The dose–response curves are shown in Fig. 2 demonstrating a dose-dependent activity of the tested essential oils. Selectivity indices for different essential oils were calculated as the ratio TC_{50}/IC_{50} and are given in Table 1. The lowest selectivity index was found for the relative cytotoxic sandalwood oil, high selectivity indices could be demonstrated for hyssop oil, thyme oil and ginger oil with an SI of 13, 10 and 40, respectively.
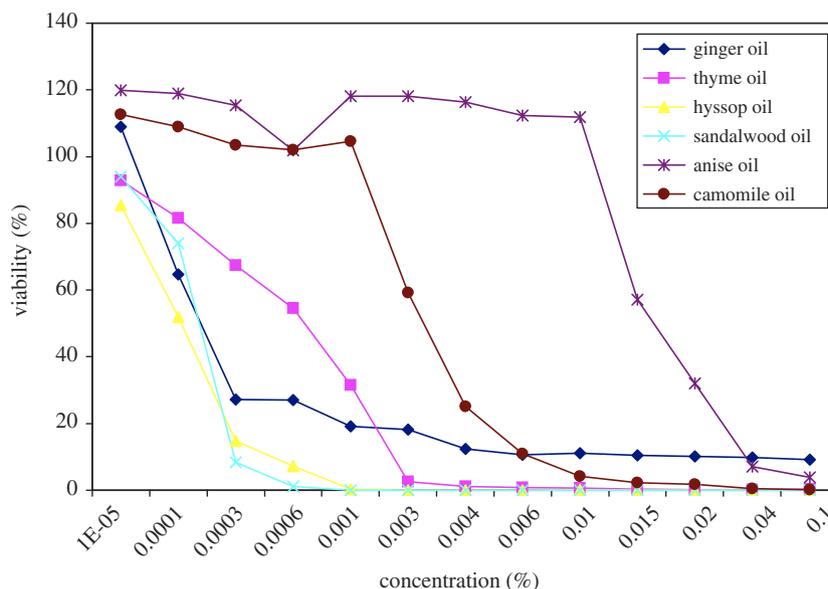

Fig. 1. Cytotoxicity of essential oils from anise, hyssop, thyme, ginger, camomile and sandalwood on RC-37 cells. For cytotoxicity assays, cells were incubated with different concentrations of the essential oils for 4 days. Viability of the drug-treated cells was determined in a standard neutral red assay. Experiments were repeated independently two times and data presented are the mean of three experiments.
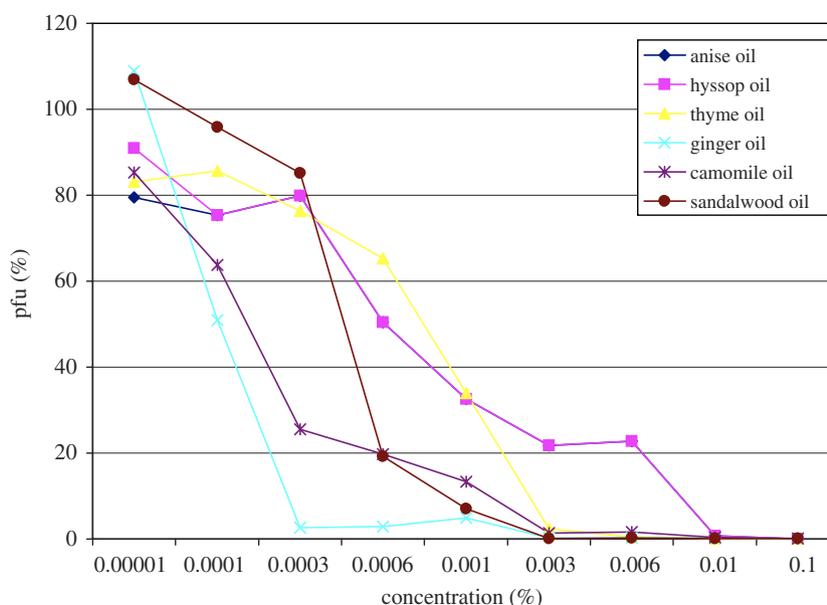
Fig. 2. Determination of the 50% inhibitory concentration (IC_{50}) of essential oils from anise, hyssop, thyme, ginger, camomile and sandalwood against HSV-2. Viruses were incubated for 1 h at room temperature with increasing concentrations of the essential oils and immediately tested in a plaque reduction assay. Experiments were repeated independently two times and data presented are the mean of three experiments.

Table 1. Selectivity indices (SI) of essential oils for HSV-2

Essential oil	TC_{50} (%)	IC_{50} (%)	Selectivity index (SI)
Anise oil	0.016	0.003	5
Hyssop oil	0.0075	0.0006	13
Thyme oil	0.007	0.0007	10
Ginger oil	0.004	0.0001	40
Camomile oil	0.003	0.00015	20
Sandalwood oil	0.0015	0.0005	3

Mode of antiviral activity

To identify the step at which replication might be inhibited, cells were infected with HSV-2 after preincubation of the cells with essential oils, pretreatment of the virus with the essential oils prior to infection, addition of the essential oils during adsorption or after adsorption during the intracellular replication period. In all experiments cells infected with untreated virus as well as acyclovir 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 maximum noncytotoxic concentrations of the essential oils were used and the results are shown in Fig. 3. Pretreatment of cells with the essential oils and addition of all four essential oils during the replication phase did not reduce virus production. However pretreatment of HSV with the analysed essential oils prior to infection caused a significant reduction of infectivity ranging from about 65% for camomile oil to >90% for hyssop oil, thyme
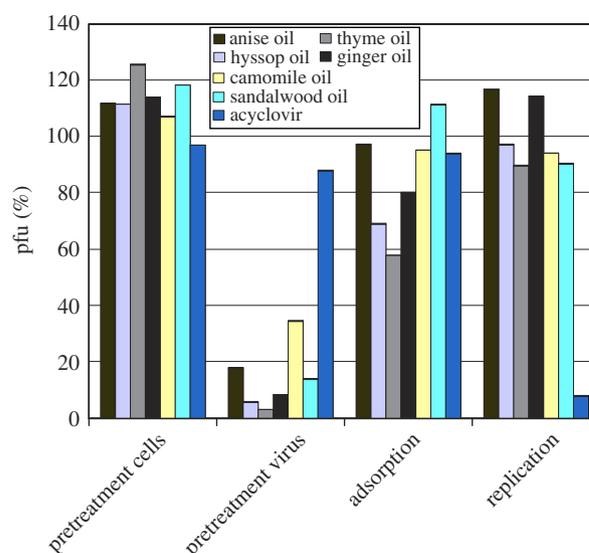

Fig. 3. Mode of inhibitory effect of anise oil, hyssop oil, thyme oil, ginger oil, camomile oil and sandalwood oil against HSV-2 during different periods of the viral replication cycle. Virus or cells were treated with the maximum noncytotoxic concentration of the essential oils, acyclovir was used as a control. Experiments were repeated independently two times and data presented are the mean of three experiments.

oil and ginger oil. When these essential oils were added only during the adsorption period, virus titres were either not reduced or only moderately reduced up to about 40% for thyme oil. These results indicate that the virucidal effect of the investigated essential oils is mainly exerted prior to adsorption of HSV to the host cells.

Acyclovir was effective against HSV-2 and showed the highest antiviral activity when added during the replication period (Fig. 3).

Discussion

Genital herpes is a critical global health problem because of its devastating impact on young adults and infants and its association with the HIV epidemic. Only little information on the effects of essential oils against viral infections is available. Antiviral activity of tea tree oil against tobacco mosaic virus was reported previously (Bishop, 1995). Most reports on the inhibitory activity of essential oils against HSV are anecdotal reports. Several drugs are currently available for the management of HSV infections such as acyclovir. Acyclovir and related synthetic nucleosides interfere with viral DNA replication through activation by viral thymidine kinase. Genital herpes is a chronic, persistent infection that might reactivate quite frequently. A dramatic increase in the prevalence of HSV-2 infection was observed in younger age cohorts (Fleming et al., 1997). Genital herpes continues to be a public health problem in both developed and developing countries.

Cytotoxicity of the essential oils was tested on African green monkey kidney cells, revealing anise oil as low cytotoxic and sandalwood as the most cytotoxic among the tested essential oil. However the cytotoxic range of all essential oils is still in the same order of magnitude. Sandalwood oil is only twice as cytotoxic in vitro as the well tolerated camomile oil and based on the CC_{50} classification system of Halle and Göres (1987) the cytotoxicity of all essential oils tested can be rated as low to moderate. In order to determine the mode of antiviral action, either cells were pretreated before viral infection or viruses were incubated with noncytotoxic concentration of essential oils or before infection, during adsorption or after penetration into the host cells. 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. A moderate reduction in infectivity could be demonstrated for thyme oil by treating the virus during adsorption to the cells. An antiviral activity of Acanthospermum hispidum extracts could be demonstrated during the attachment phase of the alphaherpesvirus pseudorabies virus, but not during the replication period (Summerfield et al., 1997). The assay of virus-pretreatment did not exclude the inhibitory effect on virus adsorption, since after incubation of the virus-oil mixtures there is no dilution of the sample, then the compounds remain present during virus infection of the cells at a concentration able to interfere with adsorption. Essential oils seem to be mostly efficient on cell-free virus but with limited effect on virus replication in cells and on cell to cell spread of the virus. Antiviral activity of a polysaccharide derived from hyssop against HIV was described previously (Gollapudi et al., 1995). However, all tested essential oils exhibited significant levels of virucidal activity against HSV-2 in viral suspension tests. At maximum noncytotoxic concentrations of the essential oils, plaque formation was significantly reduced by more than 90% when HSV-2 was preincubated with hyssop oil, thyme oil or ginger oil. These results indicate that essential oils derived from anise, hyssop, thyme, ginger, camomile and sandalwood affected the virus before the adsorption period. A similar effect of peppermint oil and manuka oil against HSV-1 has been published recently (Schuhmacher et al., 2003; Reichling et al., 2005). A high level of virucidal activity could be detected during the pretreatment of HSV-1 by using the essential oil of Salvia fructicosa (Sivropoulou et al., 1997). Putranjivain A from Euphorbia jolkini inhibits both virus entry and late stage replication of herpes simplex virus type 2 in vitro (Cheng et al., 2004).

These results suggest that the investigated essential oils interfere with virion envelope structures or might mask viral compounds which are necessary for adsorption or entry into host cells. A dissolution of the HSV-1 envelope by treatment with oregano essential oil has been described (Siddiqui et al., 1996). Sandalwood oil was reported previously to exhibit antiviral effect against HSV-1 and HSV-2 (Benencia and Courrèges, 1999). The inhibition of HSV appears to occur before adsorption or during adsorption but not after penetration of the virus into the cell. All essential oils tested reduced the infectivity of the virus possibly due to direct interaction with the viral envelope and glycoproteins. 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 or is due to damage to the virions, possibly their envelopes, thereby impairing their ability to infect host cells. Nonoxynol-9, a surfactant microbicide, has been demonstrated to disrupt the envelope of HSV-2 (Rapp and Wrzos, 1985) and an extract from Cassia javanica disturbed the attachment of HSV-2 to host cells but the inhibitory effect was minor (Cheng et al., 2006).

Recently, a new therapeutic approach against genital herpes has been described in an animal model. Local vaginal delivery of immunostimulatory CpG containing oligodeoxynucleotides, a synthetic mimic of bacterial DNA, induces innate immunity in genital herpes infection (Harandi, 2004). Since application of DNA vaccination is still not approved in humans, alternative products for the treatment of genital herpes are of great interest. Plant products as topical microbicide candidates against HSV-2 in an animal model have been reported recently (Bourne et al., 1999). Topical

application of essential oils that block transmission of HSV-2 at the mucosal surface seems to be a therapeutic treatment option since the lipophilic nature of the essential oils enables them to penetrate the skin. Carson et al. (2001) applied a 6% tea tree oil gel for the treatment of recurrent herpes labialis. At this concentration, the essential oil seems to penetrate the skin and might also act inside the cell to block viral replication. The reduction in time to re-epitheliazation seen in the tea tree oil patient group was similar to reductions reported for other topical therapies (Carson et al., 2001). Essential oils may be a potentially useful cheaper alternative and do pose only little threat to induce resistance. In our study camomile oil exhibited a high selectivity index, therefore it seems to be a promising candidate for additional topical treatment of herpes genitalis.

Acknowledgements

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