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Antiherpetic activity of the traditionally used complex essential oil Olbas®

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Essential oils of medicinal plants are increasingly of interest as novel drugs for antiherpetic agents, since herpes simplex virus (HSV) might develop resistance to commonly used antiviral drugs. The antiviral effect of Olbas®, a traditionally used complex essential oil, and of cajuput oil, a major constitutent of Olbas®, against HSV type 1 was examined. The antiviral activity of these essential oils was tested *in vitro* on monkey kidney cells using a plaque reduction assay. The 50% inhibitory concentration (IC_{50}) of Olbas® and cajuput oil for herpes simplex virus plaque formation was determined at 1.8 μg/ml and 7.5 μg/ml, respectively. At noncytotoxic concentrations of these oils, plaque formation was significantly reduced by 99% for Olbas® and 66% for cajuput oil. The selectivity index of 150 for Olbas® against herpes simplex virus was superior to a rather low selectivity index for cajuput oil. The mode of antiviral action of these essential oils was assessed by time-on-addition assays. Herpesvirus was significantly inhibited by pretreatment with Olbas® essential oil prior to infection of cells. These results indicate that Olbas® affected the virus before adsorption, but not after penetration into the host cell, thus Olbas® exerted a direct antiviral effect on HSV. A clearly time-dependent antiviral activity for Olbas® and cajuput oil could be demonstrated. Considering the lipophilic nature of the Olbas® complex essential oil mixture, which enables it to penetrate the skin, and a high selectivity index, Olbas® might be suitable for topical treatment of herpetic infections.

1. Introduction

Medicinal plants produce a variety of chemical constituents with the potential to inhibit viral replication and compounds from natural sources are of interest as possible sources to control viral infection. These plants have been widely used in traditional medicine to treat a variety of infectious diseases and cancer (Efferth 2009) and represent an abundant source of new bioactive secondary metabolites. Olbas is a complex essential oil distillate traditionally used for the treatment of headaches, colds, cough, gastrointestinal complaints, and of localised muscle pain. Olbas is composed of three major components, i.e. peppermint oil (53%), eucalyptus oil (21%) and cajuput oil (21%) and two minor constituents, juniper berry oil and wintergreen oil (Hamoud et al. 2012). According to anecdotal reports, the origin of Olbas can be traced back to **Ol**eum **bas**ileum, a complex essential oil distillate well known in Central Europe in the 16th century. Besides other biological effects, the major individual essential oil ingredients of Olbas are also known for their antimicrobial activity (Mulyaningsih et al. 2010; Hamoud et al. 2012) and antiviral activity (Schuhmacher et al. 2003; Reichling et al. 2009; Astani et al. 2011). Thus Olbas, as a complex mixture of different essential oils, seemed to be a potential candidate to assess antiherpetic activity.

Herpes simplex virus type 1 (HSV-1) is a wide spread human pathogen, which causes epidermal lesions in and around the mouth. The symptoms caused by herpes infections are usually self-limiting in immunocompetent individuals, but can be extensive and prolonged in immunocompromised patients. 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, the virus persists in neurons for life-time. 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 (Whitley and Roizman 2001). Antiviral agents licensed currently for the treatment of herpes virus infections include acyclovir and derivatives, nucleoside analogues which function as DNA chain terminators, ultimately preventing elongation of viral DNA. Some of these antiviral agents 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 (Chakrabarti et al. 2000; Chen et al. 2000). A number of essential oils and plant extracts have been shown to induce an antiherpetic effect, acting before virus penetration and revealed a different mode of action than the commonly used drugs. They all represent promising antiviral agents for topical therapeutic application (Koch et al. 2008; Schnitzler et al. 2008a, b; Reichling et al. 2008, 2009). Still there is a need for antiviral agents with different or combined modes of action from natural sources.

In the present study we have analysed the antiviral property of Olbas and cajuput essential oil against herpes simplex virus type

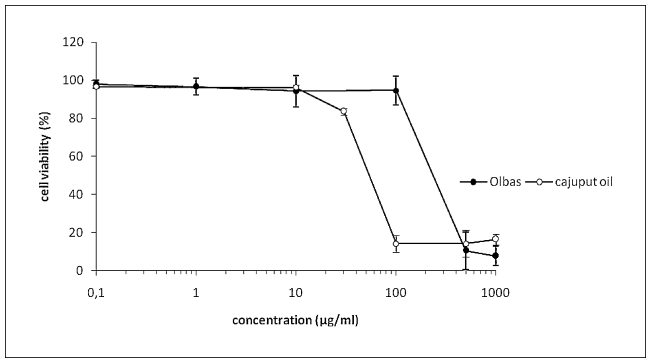

Fig. 1: Cytotoxicity of increasing concentrations of Olbas essential oil and cajuput essential oil on RC-37 cells. Essential oils were diluted in ethanol and viability of the cells was determined with the neutral red assay. The values are the mean of three independent experiments.

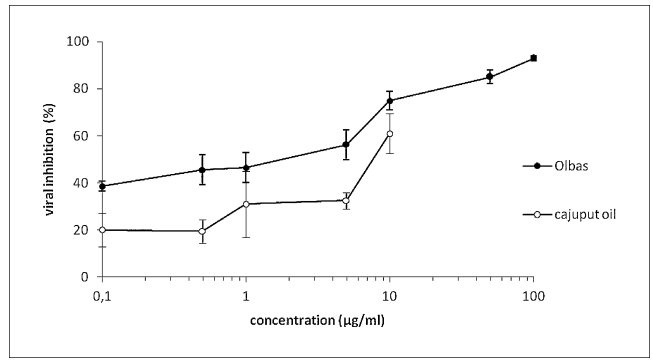

Fig. 2: Determination of the 50% inhibitory concentration (IC_{50}) of Olbas and cajuput essential oil for herpes simplex virus. Viruses were incubated with increasing concentrations of the extracts for 1 h and inhibition of viral infectivity was tested in a plaque reduction assay. Data represented are the mean of three independent experiments.

1. The infectivity of HSV was significantly reduced *in vitro*, and the mode of antiviral action was analysed at different steps in the viral infection cycle.

2. Investigations and results

Olbas essential oil and cajuput essential oil were serially diluted and added to cell culture medium to examine the effect on the growth and viability of tissue culture cells, always resulting in an ethanol concentration below 1%, which had no effect on cells and viruses. The concentration range tested for Olbas and cajuput essential oil was 0.1 – 1 000 µg/ml. The maximum non-cytotoxic concentrations of these drugs were 100 µg/ml and 10 µg/ml, respectively (Fig. 1). CC_{50} values were 270 µg/ml for Olbas and 54 µg/ml for cajuput oil. For antiviral assays, cell monolayers were infected with 100 pfu/well of drug-pretreated HSV-1. The highest concentration of these drugs was always the maximum noncytotoxic concentration. After incubation for 3 days at 37 °C, monolayers were fixed and plaques were counted. The minimal concentration of drugs required to reduce plaques by 50% (IC_{50}) was calculated by regression analysis of the dose-response curves. IC_{50} for Olbas oil and cajuput oil were determined at 1.8 µg/ml and 7.5 µg/ml, respectively (Fig. 2). The selectivity index (SI) was determined as ratio of CC_{50}/IC_{50}. Olbas essential oil revealed a higher selectivity index of 150 compared to a low SI of 7.2 for cajuput oil.

To identify the step at which viral replication might be inhibited, cells were infected with HSV and treated with essential oils at different steps during the viral replication cycle. In all experiments untreated virus incubated with 1% ethanol were used as control, to exclude any influence of ethanol on viral replication. The percent reduction was calculated relative to the amount of virus produced in the absence of the drug. Acyclovir showed the highest antiviral activity when added during the intracellular replication period, thus inhibiting DNA synthesis of virus progeny (data not shown). When the tested essential oils were added prior infection to host cells or during intracellular replication, plaque formation was not significantly inhibited (Fig. 3). However, pretreatment of HSV-1 with maximum noncytotoxic concentrations of Olbas and cajuput oil caused a significant decline in viral infectivity by 99% and 66%, respectively (Fig. 3). Thus Olbas essential oil and cajuput oil are effective against free herpes simplex virus particles. We next evaluated the ability of Olbas essential oil and cajuput oil to affect viral attachment and penetration. The attachment assay was carried out at 4 °C, which allows viral binding but not viral entry. Our results suggest that Olbas oil and cajuput oil did not interfere with HSV-1 attachment phase (data not shown). To further assess the effect of these essential oils on the viral penetration step, HSV-1 particles were allowed to first attach to RC-37 cells at 4 °C and later allowed to penetrate into the cells in the presence or absence of oils, followed by a temperature shift to 37 °C. Viral penetration was not significantly affected during the viral infection (data not shown). However, a clearly time-dependent antiviral activity for both oils could be demonstrated, when the essential oils were incubated with HSV for 1, 5, 10, 15, 20, 30 or 60 min (Fig. 4).

3. Discussion

The pharmaceutical industry is increasingly targeting natural products focusing particularly on suitable alternative antiviral agents (Khan et al. 2005; Cos et al. 2006). 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 (Brady and Bernstein 2004; De Clercq 2004). The incidence and severity of disease produced by herpes simplex virus have been increasing in recent years, especially in the immunocompromised host where viral resistance to acyclovir represents a particular problem (Christophers et al. 1998; Stranska et al. 2005). Topical treatment of herpes labialis infection is standard, for the most part carried out with acyclovir creams, but also with phytotherapeutics preparations containing lemon balm aqueous extract (Wölbling and Leonhardt 1994). HSV-1 is transmitted through contact with saliva and causes recurrent herpes labialis (Whitley and Roizman 2001). Consequently, plant-derived antivirals are of increasingly interest for the development of new, more effective and specific anti-herpesvirus agents. Antiviral activity of some essential oils against HSV, e.g. peppermint oil (Schuhmacher et al., 2003) and eucalyptus oil (Astani et al. 2010) have been reported previously. Besides cajuput oil, these essential oils are major compounds of Olbas.

Experiments to assess the cytotoxicity of Olbas indicate a quite low toxic behaviour in cell cultures according to Halle and Göres (1987). A low cytotoxic effect and a high inhibitory effect result in high selectivity index for Olbas essential oil. We have analysed the inhibitory effect of Olbas and cajuput essential oil on HSV-1 *in vitro* using plaque reduction assays. At maximum noncytotoxic concentration of these oils, plaque formation of herpesvirus was significantly reduced. In order to determine the mode of antiviral action, time-on addition experiments have been performed at different steps in the herpesvirus replication cycle. Pretreatment of host cells with both essential oils and addition of these drugs after the penetration of the viruses into host cells revealed no or minor effects on viral replication. No antiviral effect was observed during the entra and attachment phase of HSV. However a drastic decrease in viral infectivity

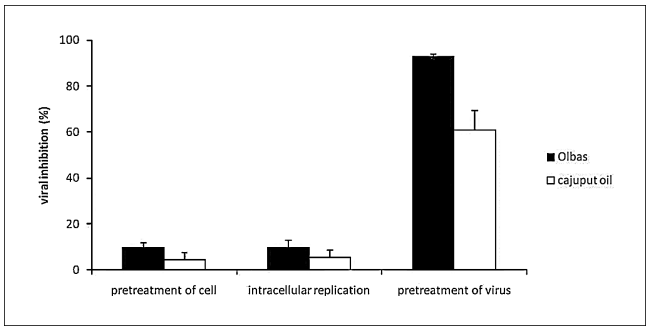

Fig. 3: Antiviral activity of Olbas and cajuput oil against herpes simplex virus in time-on-addition assays. Number of virus plaques was determined 3 d after infection and compared to untreated control. Results are presented as percentage of plaque reduction and are the mean of three independent experiments.

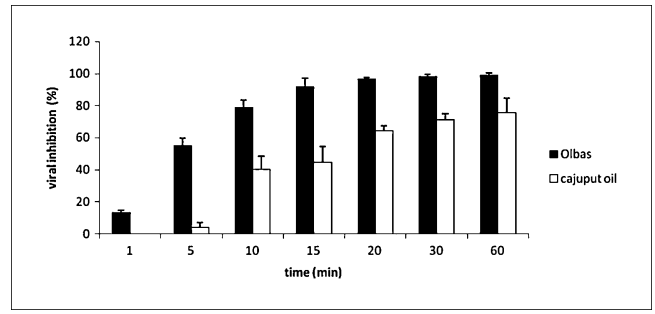

Fig. 4: Time-dependent activity of Olbas and cajuput oil against herpes simplex virus. Virus was incubated with maximum noncytotoxic concentrations of these oils for indicated time periods. Experiments were repeated independently and performed in triplicate assays, data are presented as mean of three independent experiments.

was detected for HSV-1, when viruses were treated with Olbas or cajuput oil prior to infection, thus a high antiviral activity probably due to direct drug-virus interaction was detected. Similar results have been reported for other constituents of Olbas, e.g. peppermint oil (Schuhmacher et al. 2003) and eucalyptus oil (Astani et al., 2010). A similar report showed a dissolution of the HSV envelope by treatment with oregano essential oil (Siddiqui et al. 1996). Our results indicate that Olbas oil affected viruses before adsorption and in a different manner than acyclovir. It remains to be determined whether the inhibitory effect of Olbas and cajuput 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. The application of tea tree oil, the essential oil of *Melaleuca alternifolia*, for the treatment of recurrent herpes labialis has been reported recently as well as preparations containing lemon balm aqueous extracts (Carson et al. 2001; Wölbling and Leonhardt 1994). Some essential oils, e.g. from thyme, were shown to be effective even against acyclovir-resistant HSV (Schnitzler et al. 2007).
Since all compounds of Olbas contribute to the antiviral activity against herpes simplex virus, these compounds might act synergistically in the complex essential oil. Interactions between phytochemicals and a multi-target therapeutic concept of phytotherapy have been demonstrated recently (Astani et al. 2010; Efferth 2011). Similar results have been shown for the antiherpetic effect of monoterpenes and sesquiterpenes (Astani et al. 2011). The complex mixture of Olbas revealed a high selectivity index of 150, whereas single essential oil constituents demonstrated lower selectivity indices. The topical use of Olbas essential oil for the treatment of HSV infections seems promising, especially for those patients who experience frequent recurrences.

4. Experimental

4.1. Olbas essential oil and cajuput essential oil

Olbas is a complex oil mixture, containing peppermint oil, eucalyptus oil, and cajuput oil as major constituents. Olbas essential oil mixture and cajuput essential oil were provided by Schoenenberger company, Magstadt, Germany.

4.2. Cell culture and herpes simplex virus

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, 100 u/ml penicillin and 100 µg/ml streptomycin. The monolayers were removed from their plastic surfaces and serially passaged. Cells were plated out onto 96-well and 6-well culture plates for cytotoxicity and antiviral assays, respectively, and propagated at 37 °C in an atmosphere of 5% CO_2. Herpes simplex virus type 1 strain KOS was used for antiviral assays (Schnitzler et al. 2008a).

4.3. Cytotoxicity test and plaque inhibition assay

RC-37 cells were grown in monolayer cultures, and then plated onto 96-well plates. After 24 h cells were treated with a serial dilution of the essential oils for 72 h. Cells were fixed with formalin then stained with neutral red. Cell viability was measured photometrically at 540 nm wavelength. For plaque inhibition assay, cells were plated onto 6-well plates, infected with herpes simplex virus type-1 (HSV-1) for 1 h and then incubated at 37 °C. Medium was removed after 72 h, cells were fixed with 10% formalin, stained with crystal-violet and plaques were counted. Extracts were added at different time periods to the viral infection (Schnitzler et al. 2008b).

4.4. Mode of antiviral activity

Cells and viruses were incubated with the essential oils at different stages during viral infection cycle in order to trace the mode of antiviral action. Cells were pretreated with diluted oils prior to infection with HSV, or viruses were incubated with the extract for 1 h at room temperature, or the infected cells were incubated for 1 h after penetration of HSV into host cells with the essential oils for 72 h (Koch et al. 2008). Both essential oils were always used at the maximum non-cytotoxic concentration.
For analysis of the mode of antiviral action, cells were pretreated with essential oils before viral infection, or viruses were incubated with extracts before cell infection or after penetration of the virus into the host cells. After 3 days of incubation the monolayers were fixed with 10% formalin, stained with 1% crystal violet and plaques were counted. The essential oils were used at their maximum non-cytotoxic concentrations. In all experiments untreated virus infected cells were used as control. The number of plaques of treated cells and viruses were compared to untreated controls to calculate the extent of plaque reduction and acyclovir was used as positive control in all assays. Untreated controls always contained 1% ethanol in order to exclude any effect of ethanol on cells or viruses.

4.5. Attachment and penetration assay

The attachment assay according to Cheng et al. (2004) and de Sousa Cardozo (2011) was used in this study with a minor modification. Briefly, RC-37 cell monolayers were grown in 6-well culture plates and then prechilled at 4 °C for 1 h. The medium was aspirated and the cell monolayer was infected with 100 pfu/well of in the absence or presence of diluted drugs at the maximum noncytotoxic drug concentrations. After further incubating the infected cell monolayer at 4 °C for another 2 h to allow attachment, the medium was aspirated to remove unadsorbed virus. Cell monolayer was then washed with PBS three times and overlaid with medium containing 0.5% methylcellulose and plaque assayed.
To determine the effect of the essential oils on entry of virus, a penetration assay was conducted according to Gescher et al. (2011). Cells were prechilled at 4 °C for 1 h followed by infecting with 100 pfu of HSV per well for 2 h at 4 °C. After washing off the unbound virus, the essential oils were added for another 30 min at 4 °C. Then, temperature was shifted to 37 °C to allow penetration. After 30 min at 37 °C, cells were treated with citrate buffer (pH = 3) to stop penetration and to inactivate attached, unpenetrated virions.

4.6. Statistical analysis

All experiments were performed in triplicate and statistical analysis was performed by SPSS software (SPSS for Windows, 11.0, 2001, SPSS Chicago, Illinois). The means and standard errors were recorded.

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