Inactivation of HSV-1 and HSV-2 and prevention of cell-to-cell virus spread by Santolina insularis essential oil

Alessandro De Logu a,*, Giuseppe Loy b, Maria Luisa Pellerano a, Leonardo Bonsignore b, Maria Laura Schivo a

a Dipartimento di Scienze Chirurgiche e Trapianti d’Organo, Sezione di Microbiologia e Virologia, Università di Cagliari, Via Palabanda 14, 09123 Cagliari, Italy
b Dipartimento Farmaco Chimico Tecnologico, Università di Cagliari, Via Ospedale 72, 09124 Cagliari, Italy

Received 30 September 1999; accepted 1 September 2000

Abstract

The essential oil obtained in toto from Santolina insularis was investigated for its antiviral activity on herpes simplex type 1 (HSV-1) and type 2 (HSV-2) in vitro. The IC\textsubscript{50} values, determined by plaque reduction assays, were 0.88 and 0.7 \textmu g/ml for HSV-1 and HSV-2, respectively, while the CC\textsubscript{50} determined by the MTT test on Vero cells was 112 \textmu g/ml, indicating a CC\textsubscript{50}/IC\textsubscript{50} ratio of 127 for HSV-1 and 160 for HSV-2. Results obtained by plaque reduction assays also indicated that the antiviral activity of S. insularis was principally due to direct virucidal effects. Antiviral activity against HSV-1 and HSV-2 was not observed in a post-attachment assay, and attachment assays indicated that virus adsorption was not inhibited. Up to 80% inhibition of HSV-1 was achieved at the concentration of 40 \textmu g/ml by yield reduction assay. Furthermore, reduction of plaque formation assays also showed that S. insularis essential oil inhibits cell-to-cell transmission of both HSV-1 and HSV-2. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Santolina insularis; Essential oil; Antiherpetic activity; Virucidal agent; HSV-1; HSV-2

1. Introduction

Herpes simplex virus remains one of the most common viral infections in humans. Herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) can be distinguished by clinical manifestations, biochemical and serological criteria. Infections in immunocompromised patients are usually more severe than in the normal host. Such individuals, which include AIDS patients and immunosuppressed patients because of other iatrogenic or pathologic reasons, such as organ or bone marrow transplantation or hematological malignancies, are also prone to increased frequency of secondary herpes episodes (Greenberg et al., 1987), and the severity of herpes infection in transplant recipients correlates with the degree of immunosuppressive therapy used (Rand et al., 1997). Acyclovir, vidarabine and related drugs have proven useful for the management of specific infections such as mucocutaneous...
herpes infections, herpes simplex encephalitis, and neonatal herpes. However, recurrent episodes are less responsive and viral strains resistant to drugs commonly employed in the therapy of HSV-1 and HSV-2 infections have been increasingly isolated (Nugier et al., 1992; Safrin et al., 1994) particularly from immunocompromised patients (Erlich et al., 1989; Englund et al., 1990; Coen, 1991). Vidarabine and foscarnet are often employed to treat acyclovir-resistant strains of HSV-1 and HSV-2, since thymidine kinase activity is not required for their antiviral action. However, vidarabine and foscarnet may induce mutations in the DNA polymerase gene when used upon prolonged periods (Hwang et al., 1992), and mutants are often resistant to combination chemotherapy with existing compounds (Sacks et al., 1989; Coen, 1991; Hwang et al., 1992).

Furthermore, with increasing use of traditional antiviral agents, various cases of toxicity have been encountered. Acyclovir and ganciclovir can lead to neurotoxicity, particularly in the case of renal dysfunction (Johnson et al., 1994; Ernst and Franey, 1998), while several studies reported that therapy with foscarnet is associated with acute renal failure due to a proximal tubular toxicity extending to the collecting duct (Hoch et al., 1995).

Encouraging results have been obtained with new approaches for the prevention and therapy of experimental infection by HSV (Sanna et al., 1996; De Logu et al., 1998), but new molecules and alternative tools are needed for the clinical treatment of HSV-1 and HSV-2 infections.

Many plant extracts have been described as potential antiviral agents (Vanden Berghe et al., 1986). In particular, several flavones (Hayashi et al., 1992, 1997), anthraquinones and anthraquinone derivatives have been studied (Anderssen et al., 1991; Sydiskis et al., 1991). Some naphthodianthrones, such as hypericin and pseudohypericin, obtained from Hypericum species, show an interesting activity against retroviruses (Meruelo et al., 1988) and polyphenolic- and poly-sulfonate-substituted anthraquinones inhibit HIV-1 reverse transcriptase (Schinazi et al., 1990). Yet, other plant extracts or molecules isolated from plant extracts show antiviral activity (Ferrea et al., 1993), such as the glycoside acemannan that is active against the replication of HSV, HIV-1 and Newcastle disease virus (Kemp et al., 1990), and oryzacystatin (Aoki et al., 1995).

Several products obtained from Santolina species have been investigated for their biological activities. Apolar extracts of Santolina chamaccyparissus antagonize the effects induced by spasmoden agonists (Giner et al., 1989) and coumarine obtained from Santolina oblongifolia show interesting anti-inflammatory properties (Silvan et al., 1996), but until now the antiviral properties of Santolina insularis essential oil against HSV-1 and HSV-2 have not been described.

2. Materials and methods

2.1. Essential oil

The aerial parts of S. insularis were collected during full blossom (May) at Marganai massif (Sardinia, Italy). Plants were identified and voucher specimens deposited in the herbarium of the Institute of Botany and Botanical Garden, University of Cagliari, Italy. Up to 1500 g of fresh aerial parts were distilled in a Clevenger-type apparatus for 5 h (Poli et al., 1997). The essential oil was dried over anhydrous sodium sulfate and stored at 4°C.

2.2. Virus and cells

African green monkey kidney cells (Vero) were obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia (Brescia, Italy). Cells were grown in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and penicillin, streptomycin and fungizone (100 U/ml, 100 and 2.5 μg/ml, respectively). Overlay medium for the plaque assays of HSV consisted of modified Eagle’s medium (MEM) without phenol red (Gibco) plus 2% FBS containing antibiotics as described above and 0.5% agarose.

The strains of HSV type 1 (HSV-1 strain F) and HSV type 2 (HSV-2 strain G) used in this study were obtained from the American Type Culture
Collection (ATCC), Rockville, MD. HSV-1 and HSV-2 were propagated in Vero cells. Virus titers were determined by plaque assay in Vero cells and are expressed as plaque forming units (PFU) per ml. The viruses were stored at −70°C until used.

2.3. Cellular toxicity

Cellular toxicity of S. insularis essential oil was tested in vitro by two methods. For cell growth inhibition studies, \(2 \times 10^5\) Vero cells were seeded in 6-well tissue culture plates and incubated for 72 h at 37°C in the presence of S. insularis essential oil in RPMI 1640 with 10% FBS. The medium was then removed, the cells trypsinized and viable cells counted by the trypan blue dye exclusion test (Hayashi et al., 1997). The 50% cytotoxic concentration (CC\(_{50}\)) was calculated by regression analysis of the dose–response curves generated from the data.

In the second method, a cell viability assay previously reported (Mosmann, 1983; Denizot and Lang, 1986) was used. Monolayers of Vero cells in 96-multiwell plates were incubated with the essential oil in RPMI 1640 for 48 h and the medium replaced with 50 \(\mu\)l of a 1 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, (MTT, Sigma) in RPMI without phenol red (Sigma). Cells were incubated at 37°C for 3 h, the untransformed MTT removed and 50 \(\mu\)l of acid-isopropanol (0.04 N HCl in isopropanol) was added to each well. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read using an automatic plate reader with a 570 nm test wavelength and a 690 nm reference wavelength.

2.4. Plaque reduction assay

S. insularis essential oil was first tested for antiviral activity against HSV-1 and HSV-2 by a plaque reduction assay with monolayer cultures of Vero cells grown in RPMI. Cells were infected with 200–250 PFU of HSV-1 or HSV-2. After 1-h adsorption at 37°C the plates were washed and the medium replaced with MEM containing agarose 0.5%, FBS 2% and different concentrations of S. insularis. After 72-h incubation, the monolayers were fixed with 10% formaldehyde in PBS, nutrient agar removed, and cells stained with a 1% solution of crystal violet in 70% methanol.

Some experiments were also performed by incubating about 200–250 PFU of HSV-1 and HSV-2 with S. insularis essential oil at concentrations of 30–0.03 \(\mu\)g/ml at 37 or 4°C for varying time periods up to 2 h. Viruses were then adsorbed at 37°C on Vero cells for 1 h, the cells washed and the medium replaced with MEM containing agarose 0.5% and FBS 2% in the presence of the same concentrations or in absence of S. insularis. After 72-h incubation at 37°C the monolayers were fixed and processed as described above. Experiments in which Vero cells were pre-incubated with S. insularis essential oil for 1 h at 37°C before HSV-1 or HSV-2 adsorption were also performed. The IC\(_{50}\) values were calculated by regression analysis of the dose–response curves generated from the data.

2.5. Yield reduction assay

Monolayers of Vero cells grown in 6-well plates were infected by adsorption of HSV-1 at a multiplicity of infection (MOI) of 1 PFU per cell for 1 h at 37°C. Cells were washed with warm medium and S. insularis essential oil at concentrations ranging between 40 and 2.5 \(\mu\)g/ml in minimum essential medium with 2% FBS added immediately after adsorption. At 16 h after virus inoculation, cells were thoroughly washed with warm medium to remove the essential oil. Cells in the culture medium were lysed by freezing and thawing (three times), and supernatant consisting of culture medium and cell lysate was obtained by centrifugation at 400 \(\times\) g for 10 min at 4°C. Virus titer was determined by plaque forming assay in Vero cells as described above.

Experiments in which Vero cells were pre-incubated with 40 \(\mu\)g/ml S. insularis essential oil for 1 h at 37°C before HSV-1 adsorption were also performed.
2.6. Inhibition of plaque development assay

Reduction of plaque development assays were performed as previously described (Navarro et al., 1992) with modifications. Monolayers of Vero cells were infected with about 100 PFU of HSV-1 or HSV-2 for 3 h at 37°C. Cells were then washed and the medium was replaced with nutrient agar containing 40, 20, 10 or 2.5 μg/ml of essential oil and 10 μg/ml of HSV-1 and HSV-2 neutralizing antibody (Chemicon International Inc., Temecula, CA) to ensure that plaque development was actually due to cell-to-cell virus spread. After 24, 48, 72 and 90 h, the plates were fixed with 10% formaldehyde in phosphate-buffered saline (PBS) for 30 min, the nutrient agar overlay was removed, and the cells were stained with 1% solution of crystal violet in 70% methanol for 30 min. The stained monolayers were then washed and the plaque diameter was measured with a digital caliper (Mitutoyo, Japan). Reduction of plaque size by 50% was considered as significant inhibition. At least 30 plaques were measured per well. Plaques < 0.2 mm in diameter were considered abortive and therefore were not counted.

2.7. Attachment assay

Vero monolayers grown in 6-well plates were pre-chilled at 4°C for 15 min and infected with HSV-1 and HSV-2 diluted in serum-free MEM to 200 PFU/ml for varying time periods up to 3.0 h at 4°C in the presence or absence of serial dilutions of S. insularis essential oil. Unadsorbed virus was then removed and cells overlaid with nutrient agar. After 72 h the cells were fixed and stained as described above.

2.8. Penetration assay

Penetration assays were performed using published procedures with modifications (Rosenthal et al., 1985). Briefly, about 200 PFU of HSV-1 and HSV-2 were adsorbed for 3 h at 4°C on Vero cells grown on 6-well plates. The temperature was then abruptly increased to 37°C to maximize penetration of virus. Penetration proceeded for various time periods (30 min, 1, 1.5 and 2 h) in the absence or presence of S. insularis essential oil (final concentrations 40, 20, 10, 5 and 2.5 μg/ml). Monolayers were then treated with PBS, pH 3 for 1 min to neutralize any remaining attached virus and after several washes with serum-free medium cells were overlaid with MEM-0.5% agarose to quantitate surviving virus versus time of essential oil exposure.

2.9. Postattachment virus neutralization assay

Postattachment virus neutralization assays were carried out using published procedures with modifications (Highlander et al., 1987; Burioni et al., 1994). About 250 PFU of HSV-1 and HSV-2 in 0.5 ml of MEM were adsorbed to Vero cells for 2 h at 4°C. Cells were then washed, medium replaced with DMEM containing the essential oil of S. insularis (40–2.5 μg/ml) and incubated for 2 h at 4°C. Cell monolayers were again washed and overlaid with DMEM containing 0.5% agarose and incubated at 37°C until plaques were fully developed. As a control, HSV-1 and HSV-2 were incubated with the essential oil for 2 h at 4°C prior to adsorption to cells. Cells were fixed and stained as described above, and the number of plaques obtained with control HSV-1 and HSV-2 pretreated with S. insularis essential oil was compared with the number of plaques obtained when the essential oil was added after virus adsorption.

3. Results

3.1. Cellular toxicity

The CC₅₀ of S. insularis essential oil against Vero cells, determined by the trypan blue exclusion test, was 105 μg/ml in the cell growth assay. Similar CC₅₀ values (112 μg/ml) were obtained by the MTT test carried out on confluent monolayers.

3.2. Antiviral activity

The activity of S. insularis essential oil against HSV-1 and HSV-2 was first evaluated by a plaque reduction assay. As reported in Fig. 1, when
HSV-1 and HSV-2 were exposed for 1 h at 37°C to the essential oil before and during adsorption. *S. insularis* exhibited a concentration-dependent inhibition of plaque formation compared with the controls. A 50% inhibition was observed at 0.88 µg/ml and 80% inhibition at 1.87 µg/ml for HSV-1, while for HSV-2 the 50 and 80% inhibition values were determined at 0.7 and 1.25 µg/ml, respectively. HSV-1 and HSV-2 inactivation were clearly dependent on the length of exposure to the essential oil and an higher inhibition was observed when HSV-1 and HSV-2 were pre-incubated for 2 h at 37°C (50% inhibition at 0.31 and 0.26 µg/ml; 80% inhibition at 0.78 µg/ml and 0.89 µg/ml for HSV-1 and HSV-2, respectively). Inactivation of HSV-1 occurred more efficiently than HSV-2 when viruses were pre-incubated for 15 min before adsorption (50% inhibition at 6.39 and 7.66 µg/ml; 80% inhibition at 12.1 and 21.29 µg/ml for HSV-1 and HSV-2, respectively). No differences were observed when HSV-1 and HSV-2 were pre-incubated with *S. insularis* essential oil, adsorbed on Vero cells in the presence of the same concentrations of essential oil, and cells were overlaid with nutrient agar still containing *S. insularis* essential oil at the same concentrations used during the virus pre-treatment and adsorption. No inhibition was observed by plaque reduction assay when cells were pre-incubated with the essential oil and then infected with untreated HSV-1 or HSV-2. Furthermore, no differences of antiviral activity of *S. insularis* essential oil were detected when HSV-1 and HSV-2 were incubated at 4 or 37°C before virus adsorption (data not shown).

3.3. Yield reduction assay

Yield reduction assays showed a dose-dependent antiviral activity of *S. insularis* essential oil against HSV-1. An 80% inhibition was observed at a concentration of 40 µg/ml, and 42.7% inhibition was still observed at a concentration of 10 µg/ml. When Vero cells were pre-incubated for 1 h at 37°C in the presence of *S. insularis* essential oil at the concentration of 40 µg/ml the percent inhibition was slightly higher, and a 84.2 and 55.7% inhibition was observed at 40 and 10 µg/ml, respectively (Fig. 2).

3.4. Inhibition of plaque development assay

As described above, preliminary experiments on the antiviral activity of *S. insularis* were carried out allowing the essential oil to act during the virus adsorption and incubating the infected cells with nutrient agar containing the essential oil. In such experiments, a sharp difference in the plaque

---

**Fig. 1.** Effects of *Santolina insularis* essential oil on plaque formation by HSV-1 (A) and HSV-2 (B). About 200–250 PFU of HSV-1 or HSV-2 were pre-incubated for 15 min (■), 1 h (●) or 2 h (○) at 37°C in the presence of serial dilutions of essential oil and then adsorbed on Vero cells. *S. insularis* showed a 50% inhibition of plaque formation with respect to the control at 0.88 µg/ml and 80% inhibition at 1.87 µg/ml against HSV-1, while against HSV-2 50 and 80% inhibition were observed at 0.7 and 1.25 µg/ml, respectively, when viruses were pre-incubated at 37°C for 1 h. The data represent the means for four replicate samples of three separate experiments.
Fig. 2. Antiviral activity of *S. insularis* essential oil against HSV-1 as determined by yield reduction assay. Vero cells were infected by adsorption of HSV-1 (MOI 1) for 1 h, and then incubated with serial dilutions of essential oil in RPMI 1640 for 24 h (●). Alternatively, Vero cells were treated for 1 h at 37°C with *S. insularis* 40 μg/ml before virus adsorption (■), and then infected with HSV-1. After 16 h the essential oil was removed, cells were lysed by three cycles of freezing and thawing, and HSV-1 titer was determined by plaque forming assay. The data represent the means for four replicate samples of two separate experiments.

3.5. Effects on virus adsorption and penetration

Attachment assays indicated a 50% inhibition with respect to the untreated controls at concentrations higher than 30 μg/ml for both HSV-1 and HSV-2. However, these values were significantly higher than the values obtained with the controls of HSV-1 and HSV-2 pre-incubated for 2 h at 4°C in the presence of the same concentrations of *S. insularis* essential oil, indicating that attachment was not affected and effects were mainly due to a direct effect on the virion. Furthermore, no inhibition was observed when Vero cells were infected with HSV-1 and HSV-2 at 4°C to prevent penetration of viruses and the temperature was then increased to 37°C in the presence of *S. insularis* essential oil. In addition, no inhibition with respect to the controls was detected by post-attachment virus neutralization assays. The number of plaques obtained with HSV-1 and HSV-2 adsorbed on Vero cells at 4°C and then incubated at 4°C in the presence of *S. insularis* was similar to the number of plaques obtained if HSV-1 and HSV-2 were incubated at 4°C in the presence of essential oil before virus adsorption (data not shown).

4. Discussion

This report clearly demonstrates the antiviral activity of *S. insularis* essential oil in toto against HSV-1 and HSV-2 in vitro. CC₅₀ as determined by the MTT test on Vero cells was 112 μg/ml,
indicating a CC_{50}/IC_{50} ratio of 127 and 160 for HSV-1 and HSV-2, respectively.

The data presented in this report also indicate that S. insularis is effective in inactivating HSV-1 and HSV-2. When the viruses were exposed to the essential oil before adsorption, a concentration-dependent inhibition of plaque formation was observed. The virus inactivation is time-dependent and temperature-independent, since no differences were observed when HSV-1 and HSV-2 were incubated in the presence of the essential oil at 37 or 4°C. Inactivation by S. insularis was also dependent on the amount of virus input (data not shown). No differences were detected in the plaque reduction assays when cells were treated with the essential oil before HSV-1 and HSV-2 adsorption. These data support the conclusion that S. insularis directly inactivates the virus particles, thus preventing adsorption of virion to host cells. This conclusion is also supported by other experimental data. We found that attachment of HSV-1 and HSV-2 to cells is not inhibited and S. insularis is unable to prevent virus penetration when added after adsorption. Furthermore, post-attachment assays indicated that the essential oil is ineffective in reducing infectivity after HSV-1 and HSV-2 attachment. Reduction of plaque development assays in the presence of neutralizing antibodies shows that S. insularis essential oil is capable of preventing cell-to-cell virus spread in HSV-1 and HSV-2 infected cells. Furthermore, treatment of cells with S. insularis before virus adsorption led to a slight enhancement of inhibition as determined by a yield reduction assay, indicating that an intracellular effect might also be involved.

Further studies are in progress to isolate the compound or compounds involved in the antiviral activity of S. insularis essential oil. Preliminary experiments indicate the presence of flavones, which have been previously isolated from several plant extracts and described for their antiviral activity (Li et al., 1993; Critchfield et al., 1996). The ability of flavones to increase TNF-induced antiviral activity against encephalomyocarditis virus (ECMV) and vesicular stomatitis virus (VSV) and synergistic inhibition of HSV-1 in combination with acyclovir have also been investigated (Ohnishi and Bannai, 1993; Hayashi et al., 1997). However, flavones exhibit mechanisms of action different from that delineated for S. insularis. In fact, some flavonoid compounds, such as quercetin and quercitrin, reduced the yield of HSV-1 and elevated the intracellular level of cyclic AMP, indicating a relation between antiviral effect and the cAMP-enhancing activity (Mucsi and Pragai, 1985). Furthermore, it has been demonstrated that 5,6,7-trimethoxyflavone is unable to prevent HSV-1 adsorption, penetration and viral protein synthesis, and that its inhibitory
effects involve a virucidal activity (Hayashi et al., 1997), while the mode of antiviral activity of SP-303, a natural plant flavonoid polymer, against HSV-1 is through inhibition of virus penetration into cells (Barnard et al., 1993a,b).

It is interesting to compare the mechanism of inhibition by S. insularis with that of hypericin, a polycyclic anthrone. In fact, hypericin inactivates enveloped viruses, and in particular HSV-1 is inactivated at a concentration (1 µg/ml) comparable with that of S. insularis. However, the neutralizing activity of hypericin is observed when HSV-1 is pre-incubated at 37°C, while it is lost when the pre-incubation is carried at 4°C (Tang et al., 1990). On the contrary, the neutralizing activity by S. insularis against HSV-1 and HSV-2 was comparable when the pre-incubation was carried out at 37 or 4°C. Furthermore, it must be pointed out that S. insularis is an essential oil in toto and thus the antiviral activity in terms of concentration is difficult to compare with that of hypericin.

The mode of antiviral activity of S. insularis essential oil may be rather unique in that no compounds obtained from plant extracts concomitantly inactivate the virus and inhibit cell-to-cell virus spread. Furthermore, the IC₅₀ values of S. insularis essential oil in toto for HSV-1 and HSV-2 compare favorably with those of flavonoids (usually between 3 and 10 µg/ml) and approach those of approved drugs for the treatment of herpes simplex infections, such as acyclovir and ganciclovir.

The results obtained encourage further exploration of the antiviral properties of S. insularis such as the exploration of the in vivo activity in animal models infected with HSV, and the isolation of the ingredient(s) responsible for the antiviral activity.

Acknowledgements

The authors thank Luisa Cappai for technical assistance. This work was partially supported by Ministero dell’Università e della Ricerca Scientifica e Tecnologica (MURST) funds.

References


