Herpes virus inhibitory substances from *Hypericum connatum* Lam., a plant used in southern Brazil to treat oral lesions

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Abstract

*Hypericum connatum* (Guttiferae) is used in southern Brazil in the treatment of lesions in the mouth, often related to acute herpetic gingivostomatitis. The chemical investigation of the plant revealed the presence of phloroglucinol derivatives and flavonoids. From the n-hexane extract of the aerial parts a phloroglucinol derivative, hyperbrasilol B, was isolated, while the methanolic extract afforded four flavonoids: amentoflavone, hyperoside, guaijaverine and luteoforol. The crude methanolic extract and fractions (n-hexane, dichloromethane and methanol) as well as the isolated compounds were tested for antiviral activity against herpes simplex viruses (HSV). Among the tested samples, luteoforol was the most active inhibiting the cytopathic effect (CPE) and reducing the viral titer of HSV-1 DNA viral strains KOS and VR733 (ATCC).

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1. Introduction

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are responsible for a broad range of human infectious diseases. Herpes simplex virus type 1 infection can cause several clinical conditions besides gingivo-stomatitis, such as keratitis, cutaneous herpes, genital herpes and encephalitis. These pathologies may result from a primary infection or, alternatively, from a reactivation of a latent infection. The illness is more serious in patients with deteriorated cellular immunity, e.g. human immunodeficiency virus (HIV) infected patients (Hook et al., 1992), who must receive chronic therapy with antiviral agents, favoring the selection of resistant variants (Timbury, 1997). Drugs with clinically relevant activity against HSV infections include interferons (IFNs), acyclovir (ACV), vidarabine (ara-A), gancyclovir (DHPG) and phosphonoformic acid (foscarnet, PFA). However, undesirable complications and the emergence of drug-resistant viruses urge the development of new antitherpetic agents.

Efforts have been made to evaluate the antiviral activity of a wide array of natural products, including those from plants, in order to isolate and characterize new compounds which could inhibit virus replication and/or treat viral infection, or even serve as models for new molecules (Serkedjieva and Ivancheva, 1999). Members of the Guttiferae family, for example, have been used in traditional medicine to treat wounds, lymphatitis, parotitis, hepatitis, gastrointestinal disorders and tumors, which could be related to viral agents. Many compounds have been isolated from plants of this family and have had their antiviral activity studied. The USA National Cancer Institute has done extensive research in this family and some substances as benzophenones and pyranocoumarins have demonstrated an excellent anti-retroviral activity. Calanolide-A, a coumarin isolated from tropical plants of the genus *Calophyllum*, has exhibited both in vitro and in vivo activity against HIV-1 and is under clinical investigation (Xu et al., 1999). Hypericin and pseudohypericin, isolated from plants of the genus *Hypericum* also received attention due to...
the antiviral action on lipid enveloped and non-enveloped DNA and RNA viruses. These substances are highly effective in preventing viral-induced manifestations that follow infection with a variety of viruses in vitro and in vivo, and reduce the spread of HIV-1 in vitro (Vlietinck et al., 1998).

Considering the presence of antiviral compounds in the Gut-tiferae family and the long traditional reputation of many species of the Hypericum genus as medicinal plants for the treatment of a variety of conditions, commonly related to viral infections, the extracts and isolated compounds from one of these species, Hypericum connatum, were tested for antitherapeutic activity. In Brazil, this plant is popularly known as ‘orelha-de-gato’ (cat’s ear) and is used in traditional medicine as tonic and adstringent (Correa, 1984), while in southern Brazil the main use is in the treatment of oral lesions, frequently caused by herpes viruses (Mentz et al., 1997). The indigenous people know the plant as Ka’avotory and to them, the flower is abortive. In Argentina this plant is widely used as a cardiac tonic and is commercialized under the name cabotoril. Previous investigations of the extracts of Hypericum connatum showed them to be active against the feline immunodeficiency virus (FIV), a widespread lentivirus of domestic cats sharing numerous biological and pathogenic features with the human immunodeficiency virus (HIV) (Schmitt et al., 2001).

Hypericum species often present phenolic compounds with the phloroglucinol substitution pattern. The most common compounds isolated from plants of this genus are polycyclic quinones, xanthones, flavonoids, phloroglucinol derivatives and, less frequently, benzopyrans and benzophenones (Fritz, 2006).

2. Materials and methods

2.1. Plant material

The aerial parts of Hypericum connatum Lam. were collected in São Francisco de Paula in the state of Rio Grande do Sul, Brazil, in December 2004. The voucher specimen was deposited in the Herbarium of the Federal University of Rio Grande do Sul (ICN/UFRGS) (Hypericum connatum, Bordignon and Salazar, 1527). The plant material was carefully dried and powdered.

2.2. Extraction and isolation of compounds

Air dried and powdered plant material was extracted with methanol (ratio = 1:10, w/v) by maceration (3 × 24 h) yielding 15% of crude extract. Another portion of the plant material (85 g) was extracted in a Soxhlet apparatus during 12 h each, with n-hexane (2.6%), dichloromethane (3.0%) and methanol (6.5%). The extracts were evaporated to dryness under reduced pressure. Aqueous extract was obtained by maceration with water at room temperature and subsequent lyophilization.

The n-hexane extract (2.2 g) was analyzed by TLC on silica gel and the main product was isolated by column chromatography on silica gel using an ethyl acetate/methanol gradient system affording four compounds. The compounds were identified by direct comparison with authentic samples, by ultraviolet and 1H NMR and 13C NMR spectroscopy.

2.3. Cells and viruses

African green monkey kidney cells (Vero cell line CCL-81-ATCC) were grown in Eagle’s minimum essential medium (MEM) supplemented with 10% newborn calf serum, 2 µg/ml of amphotericin B and 10 mg/ml of enrofloxacin. A virus stock of herpes simplex virus type I, strain KOS (University of Rennes/France) and VR733 (ATCC) were prepared on Vero cells infected at a low multiplicity of infection, incubated for 1–2 days, frozen/thawed, before clearing the preparation by centrifugation at low speed to remove the cell debris. Virus stocks were maintained in liquid nitrogen until use. Virus titration was performed by the Kärber method using 96-well microtitre plates (Payment and Trudel, 1989). The virus titer was estimated from cytopathogenicity and expressed as 50% tissue culture infectious doses (TCID50/50 µl). It was 103.5 TCID50/50 µl for strain KOS and 105.25 TCID50/50 µl for strain VR733 (ATCC).

2.4. Evaluation of cytotoxicity

The solutions to be tested in the antiviral experiments were prepared by dissolving the extracts in sterilized bidestilled water and when necessary, DMSO at sub toxic concentration (maximum of 0.019% was added).

To assess the effect of extracts and compounds on uninfected Vero cells, dilutions ranging from 20 mg/ml to 0.019 mg/ml in the maintenance medium, were added to Vero monolayers (using a 96-well microplate with 4.0 × 104 cells per well). After 72 h of incubation at 37 °C, cytoxicity was determined by microscopic examination of the cell morphology in treated and untreated cultures. The maximum concentration at which no reduction of cell numbers was observed (compared to controls) was considered as the maximum tolerated concentration (MTC) (Montanha et al., 2004). The MTC was determined for all extracts and compounds before proceeding to the antiviral activity assays. All assays were carried out in triplicate.

2.5. Antiviral activity

Dilutions of the extracts and compounds were prepared starting from the previously determined MTC. The samples from MTC, MTC/2, MTC/4, MTC/8 and MTC/16 were added on confluent 24 h old monolayer of Vero cells grown in microtitre tissue culture plates just before virus inoculation. One hundred tissue culture infection doses per 50 µl (TCID50) of the HSV-1 KOS and ATCC-VR733 strains were added to each of the wells. Toxicity controls, cell and virus controls titration were run simultaneously. Plates were incubated for 72 h at 37 °C, and then examined for the presence of cytopathic effects (CPE). Acyclovir, Sigma, at 0.01 mg/ml was used as positive control for HSV-1 inhibition.
In order to quantify the antiviral activity, the contents of the four identical wells were harvested, mixed, and clarified by low speed centrifugation, and virus titration was performed on the supernatant fluid by the Kärber method (Payment and Trudel, 1989), using a 96-well microtitre plate. The antiviral activity of each extract was determined as the viral titer reduction factor ($\log_{10}$) by comparison with untreated controls.

3. Results and discussion

From the \textit{n}-hexane extract of the aerial parts of \textit{Hypericum connatum} hyperbrasilol B (1) was obtained, a phloroglucinol derivative previously isolated from this species and from \textit{Hypericum caprifoliatum} (Nör et al., 2004) and from \textit{Hypericum brasiliense} (Rocha et al., 1996). The methanolic extract yielded the flavan-4-ol luteoforol (2), the glycosides hyperoside (3) and guaijaverin (4) and the biflavonoid amentoflavone (5).

The extracts and fractions of \textit{Hypericum connatum}, as well as the isolated compounds were evaluated for the in vitro antiviral activity in order to establish a correlation with the traditional use in the treatment of oral lesions (Mentz et al., 1997) that could be caused by herpes simplex virus.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (mg/ml)</th>
<th>Yield reduction ($\log_{10}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane fraction root</td>
<td>0.078</td>
<td>1.33 ± 0.38</td>
</tr>
<tr>
<td>Crude root extract</td>
<td>0.078</td>
<td>1.33 ± 0.28</td>
</tr>
<tr>
<td>Hyperbrasiliol B</td>
<td>0.039</td>
<td>1.08 ± 0.14</td>
</tr>
<tr>
<td>Amentoflavone</td>
<td>0.078</td>
<td>1.58 ± 0.14</td>
</tr>
<tr>
<td>Luteoforol</td>
<td>0.625</td>
<td>2.94 ± 0.17</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>0.010</td>
<td>3.16 ± 0.28</td>
</tr>
<tr>
<td>Hyperoside</td>
<td></td>
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<tr>
<td>Guaijaverin</td>
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<tr>
<td>Amentoflavone</td>
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<td>Luteoforol</td>
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<td>Acyclovir</td>
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Experiments were performed with herpes simplex virus type 1 growing on Vero cells. The data represent the mean ± standard deviation for four replicate samples of three separated experiments.

The anti-HSV-1 effect of the extracts and compounds isolated from \textit{Hypericum connatum} was determined as described in the experimental section. The concentrations of samples that did not inhibit cell growth nor induced granularity, retraction or lyses of Vero cells (MTC) are indicated in Table 1.

The isolated compounds hyperbrasiliol B, amentoflavone and luteoforol were found to inhibit the cytopathic effect (CPE) and to reduce the viral titer of the HSV-1 DNA viral strains KOS and VR733 (ATCC) (Table 1). This effect on HSV-1 replication was quantified through the reduction of the infectious titer after several rounds of multiplication, the culture being inoculated at 100 infectious doses. To be considered active, a
sample should induce at least a $2\log_{10}$ decrease in virus titer in comparison with untreated virus control (Vanden Berghe et al., 1986). According to these criteria only luteoforol was active since this compound reduced the virus titer by $2.9\log_{10}$ against Strain KOS and by $3\log_{10}$ against strain VR733 (ATCC). The reduction of the titer by $0.5$–$0.9\log_{10}$, as shown by hyperbrasilol B and amentoflavone, can be considered a moderate activity using the criteria of Sidwell and Huffman (1971). Hyperbrasilol B was not investigated before but amentoflavone, in agreement with our result, exhibited moderate anti-HSV-1 anti-HSV-2 activities (Lin et al., 1999). In this work hyperoside did not show antiviral activity against HSV-1. Nevertheless this flavonoid when tested in a mixture with quercetin-3-O-β-glucoside (isoquercitrin) exhibited antiviral activity against HSV-1 acyclovir resistant strains at the concentration of 17 µg/ml reducing by 58.3% the effect of the virus (Amaral et al., 1999).

The antiviral activity detected in this study could have taken place at many different steps of the viral replication cycle. The tested compounds could have inhibited viral infection by affecting the virus particle itself (virucidal effect), by interfering with the virus entry across the cell membrane and subsequent uncoating or with the integrity and transport of released viral genome, or even by interfering with the replication of viral genome, or other early steps of intracellular viral replication. Our findings support the assertion that traditional medicine remains a valuable source for the discovery of natural pharmaceutical compounds that ethnopharmacological data can lead to the discovery of potential new drug candidates.

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References


