Evaluation of Antiviral Activity of South American Plant Extracts Against Herpes Simplex Virus Type 1 and Rabies Virus

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This paper describes the screening of different South American plant extracts and fractions. Aqueous and organic extracts were prepared and tested for antiviral (HSV-1, KOS and 29R strains) and antirabies (PV strain) activities. The evaluation of the potential antiviral activity of these extracts was performed by using an MTT assay for HSV-1, and by a viral cytopathic effect (CPE) inhibitory method for rabies virus (RV). The results were expressed as 50% cytotoxicity (CC50) for MTT assay and 50% effective (EC50) concentrations for CPE, and with them it was possible to calculate the selectivity indices (SI = CC50/EC50) of each tested material. From the 18 extracts/fractions tested, six extracts and four fractions showed antiviral action. Ilex paraguariensis, Lactoensia pacari, Passiflora edulis, Rubus imperialis showed values of SI > 7 against HSV-1 KOS and 29-R strains and Alamanda schottii showed a SI of 5.6 against RV, PV strain. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: plant extracts; antiviral activity; MTT assay; viral cytopathic effect; HSV-1; rabies virus.

INTRODUCTION

Infection by viral diseases remains as an important worldwide health problem and the control of viral diseases is the subject of constant scientific endeavor. Additionally, the appearance of viral strains resistant to antiviral agents is an emerging problem. As a consequence, there are only few antiviral drugs available for the treatment of virus diseases. Therefore, the search for more effective antiviral agents is a necessary and highly desirable task (De Clercq, 2004).

Herpes simplex viruses (HSV) are DNA viruses belonging to the family Herpesviridae and are responsible for a variety of mild to severe diseases, which are sometimes life threatening, especially in immunocompromised patients (Snoeck, 2000).

On the other hand, rabies is a neurotropic RNA virus of the Rhabdoviridae family, in an acute, progressive and, in most cases, incurable encephalitis (Rupprecht et al., 2002; Willoughby et al., 2005).

Although the incubation period varies from 1 to 3 months, it has been reported that the disease can occur days or years after exposure. Additionally, pathogenetic mechanisms remain barely understood and current care entails only palliative methods (Rupprecht et al., 2002; Hendekli, 2005).

According to the literature many traditional medicinal plants have been reported to have strong antiviral activity and some of them have already been used to treat animals and people who suffer from viral infections inhibiting the replication cycle of various types of DNA or RNA viruses. Additionally, different secondary metabolites, including lignans, tannins, saponins, flavonoids and phenolic acids exhibit promising antiviral activity (Almeida et al., 1998; Charlton, 1998; Abad et al., 2000; Chiang et al., 2003; Jassim and Naji, 2003; Palomino et al., 2005).

In the search for new antiviral agents, the antiviral activity of natural products, including Brazilian medicinal plants, was evaluated (Simões et al., 1999a, 1999b; Andrichetti-Frohner et al., 2003, 2005; Bettega et al., 2004).

This paper describes the inhibitory activity of several South American plant extracts against herpes simplex virus type 1 and rabies virus. As far as we are aware, this is the first report of the detection of antiviral activity of these plants, and the first screening of medicinal plants for antirabies activity.

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were successively partitioned (3
nol (MeOH) for 1 h. The methanol extracts of leaves
water, hydroethanol (40%), ethanol (EtOH) or metha-
of the plants were extracted with 1000 mL of distilled
extracts and fractions were filtered and concentrated
chloroform, ethyl acetate and
et al.
the tested medicinal plants were prepared according
to the procedures previously described by De Oliveira
Extract preparation.

Plants selected for antiviral screening

<table>
<thead>
<tr>
<th>Family</th>
<th>Botanical name</th>
<th>Local name</th>
<th>Plant part used</th>
<th>Traditional use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apocynaceae</td>
<td>Allamanda blanchetti A. DC.</td>
<td>Almamanda</td>
<td>Leaves and flowers</td>
<td>Ornamental (Pio Correa, 1978)</td>
</tr>
<tr>
<td></td>
<td>Allamanda schottii Pohl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aquifoliaceae</td>
<td>Ilex paraguariensis A. St.Hil.</td>
<td>Erva-mate</td>
<td>Leaves</td>
<td>Stimulant (Gosmann et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>L. and S. guianensis Aubl.</td>
<td>Mangava-brava</td>
<td>Leaves</td>
<td>Tonic and febrifuge; for gastric</td>
</tr>
<tr>
<td></td>
<td>Benth</td>
<td></td>
<td></td>
<td>ulcers and inflammations</td>
</tr>
<tr>
<td>Lythraceae</td>
<td>Lactoemia pacari St.Hil.</td>
<td></td>
<td></td>
<td>(Solon et al., 2000)</td>
</tr>
<tr>
<td>Passifloraceae</td>
<td>Passiflora edulis Deg.</td>
<td>Maracujá-zedo</td>
<td>Leaves</td>
<td>Sedative (Pio Correa, 1978)</td>
</tr>
<tr>
<td>Rosaceae</td>
<td>Rubus imparatus Deg. Sch.</td>
<td>Amora-branca,</td>
<td>Aerial parts</td>
<td>Diabetes (Niero et al., 1999)</td>
</tr>
<tr>
<td>Elaeocarpaceae</td>
<td>Sloanea guianensis Abl.</td>
<td>Amora-do-mato</td>
<td>Leaves and stems</td>
<td>For wood extraction</td>
</tr>
<tr>
<td></td>
<td>Benth</td>
<td>Sapopema</td>
<td></td>
<td>(Pio Correa, 1978)</td>
</tr>
<tr>
<td>Leguminosae</td>
<td>Glycine max L.</td>
<td>Soja</td>
<td>Seeds, soybeans</td>
<td>Food; to treat menopause</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>symptoms (Murphy et al., 1999)</td>
</tr>
</tbody>
</table>

MATERIAL AND METHODS

Plant material. Allamanda blanchetti A. DC., Allamanda schottii Pohl, Ilex paraguariensis St.Hil., Lactoemia pacari St.Hil., Passiflora edulis Deg., Rubus imparatus Sch. Schl., Sloanea guianensis L. and Glycine max L. were collected in Brazil. The plant materials were identified and voucher specimens have been deposited at the Herbariums of the Universidade de Passo Fundo (RSPF/UPF/RS), Universidade Federal de Santa Catarina (FLOR/UFS/SC) and Herbario Barbosa Rodrigues (HBR/ITA/AS/SC). General information about these plants is listed in Table 1.

Extract preparation. Aqueous and organic extracts of the tested medicinal plants were prepared according to the procedures previously described by De Oliveira et al. (2005) with modifications. Briefly, different parts of the plants were extracted with 1000 mL of distilled water, hydroethanol (40%), ethanol (EtOH) or methanol (MeOH) for 1 h. The methanol extracts of leaves were successively partitioned (3 × 50 mL) with hexane, chloroform, ethyl acetate and n-BuOH yielding HX, CH, EA and BuOH fractions, respectively. These extracts and fractions were filtered and concentrated under reduced pressure (Büchi®-R200) – organic extracts – or lyophilized (Edwards®) – aqueous extracts. The extracts were suspended in DMSO 1%, dissolved in culture medium, filtered (Millipore® 0.22 µm) and stored (4 °C) until used.

Cell culture and viruses. The used cell lines were VERO (ATCC: CCL81) and McCoy (ATCC: CRL1696) grown, respectively, in MEM Medium (Sigma®) and DMEM (Gibco® BRL) both supplemented with 10% fetal bovine serum (FBS – Gibco® BRL), penicillin G (100 U/ mL), streptomycin (100 µg/mL) and amphotericin B (0.002 µg/mL) (Gibco® BRL). Cell cultures were maintained at 37 °C under a humidified 5% CO2 atmosphere. The following viruses were used: herpes simplex virus type 1 (HSV-1), strains KOS and 29-R/acyclovir resistant (Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Rennes, France), and rabies virus, PV strain (Pasteur Institute, Sao Paulo, Brazil). HSV-1 and rabies virus were propagated in VERO and McCoy cells, respectively. Stock viruses were prepared as previously described (Simões et al., 1999a) and the supernatant fluids were collected, titrated and stored at −80 °C until used. Virus titers were obtained by the limit-dilution method and expressed as 50% tissue culture infections dose per mL (TCID50/mL) (Reed and Muench, 1938).

Cytotoxicity evaluation – Cell viability test. The cytotoxicity evaluation was performed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method, according to Takeuchi et al. (1991) and Sieuwers et al. (1995) with minor modifications. Briefly, VERO and McCoy cell cultures (2 × 105 cells/mL) were prepared in 96-well tissue culture plates (Corning®). After a 24 h period of incubation at 37 °C under a humidified 5% CO2 atmosphere, the cell monolayers were confluent, the medium was removed from the wells, and 200 µL of each extract/fraction dilutions (1:2 – ranging from 2000 to 15.6 µg/mL prepared in cell culture medium) was added to each well. As a cell control only 200 µL of medium was added to the cells. The plates were incubated under the same conditions cited above. After 4 days, the medium was removed by suction from all wells and 50 µL of MTT solution (Sigma®, 1 mg/ mL) solution prepared in cell culture medium were added to each well and the plates were incubated once more for 4 h. After the MTT solution was removed without disturbing the cells and 100 µL of DMSO was added to each well to dissolve the formazan crystals. After gently shaking the plates, the crystals were completely dissolved, and the absorbances were read on a multiwell spectrophotometer (Bio-Tek®, Elx 800) at 540 nm. The CC50 was defined as the cytotoxic concentration of each extract/fraction that reduced the absorbance of treated cells to 50% when compared with that of the cell control.

Antiherpes assay. VERO cell cultures (2 × 105 cells/ mL) were prepared in the same way as described above and, when the cell monolayers were confluent, the medium was removed from the wells and 100 µL of non-cytotoxic concentrations (≤CC50) values of the extracts/fractions and 100 µL/well of HSV-1 (KOS and 29-R strains) at a MOI of 0.5 were added simultaneously to the cells. Cell and viral controls were performed by adding only 200 µL of MEM medium or 200 µL of viral suspension, respectively. The plates were incubated for 96 h. The same MTT method used to evaluate cell viability was followed. The percentages of protection

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were calculated as \([A - B] \times 100/(C - B)\), where \(A\), \(B\) and \(C\) indicate the absorbances of the extracts/fractions, virus and cell controls, respectively. Each obtained EC\(_{50}\) value was defined as the effective concentration that reduced the absorbance of infected cells to 50% when compared with cell and virus controls. Acyclovir [9-(2-hydroxyethoxymethyl) guanosine, Sigma\(^a\), 10 \(\mu\)g/mL] was used as a positive control for HSV-1 (KOS strain) inhibition.

**Antirabies virus assay.** The viral cytopathic effect (CPE) inhibitory assay as previously described by Simões et al. (1999b) was used with minor modifications. McCoy cell culture was prepared similarly to VERO cell culture. 100 \(\mu\)L/well of non-cytotoxic concentrations (\(<EC\(_{50}\)) values) of the extracts/fractions and 100 \(\mu\)L/well of rabies virus (PV strain) at a MOI of 1.0 were added simultaneously to the cells. Cell and viral controls were performed by adding, respectively, 200 \(\mu\)L of DMEM medium or 200 \(\mu\)L of viral suspension. Isopropinoine (UNIBIOS\(^b\), 1800 \(\mu\)m) and ketamine (DOPALEN\(^c\), 3000 \(\mu\)m) were used as positive controls for rabies virus inhibition, according to Hernandez-Jaurégui et al. (1980) and Lockhart et al. (1992). After 96 h, the cells were visually scored for the inhibition of CPE and EC\(_{50}\) values were estimated in relation to the controls. The results were expressed by using the selectivity index (SI = CC\(_{50}/EC\(_{50}\)) of each tested extract.

**Data analysis.** The 50% cytotoxic (CC\(_{50}\)) and 50% effective (EC\(_{50}\)) concentrations were calculated from concentration-effect curves after linear regression analysis. The results represent the mean ± standard error of the mean values of three different experiments.

### RESULTS AND DISCUSSION

Eighteen plant extracts and fractions were investigated for their antiviral activity against herpes simplex virus type 1 (HSV-1, KOS and 29-R/acyclovir resistant strains). Additionally, four of these extracts were tested against rabies virus (PV strain). Although there is no evidence that these plants have been used as antiviral agents, several medicinal plants belonging to this genus have long been used in folk medicine (Pio Correa, 1978; DerMarderosian and Beutler, 2002).

Before the evaluation of the antiviral activity, the cytotoxic effects of the selected extracts/fractions on VERO and McCoy cells were investigated. For this purpose, the MTT colorimetric assay was used, and for each tested material a CC\(_{50}\) value after 96 h of incubation was calculated. This assay has several advantages: it is easy to perform, the evaluations are objective, it can be automated using a personal computer and the cytotoxicity evaluation can be made in parallel with antiviral activity evaluation (Takeuchi et al., 1991; Andrighetti-Frohner et al., 2005; Palomino et al., 2005). The results of the cytotoxicity evaluation of the tested extracts and fractions are shown in Tables 2 and 3, respectively.

The antiviral activity was also evaluated by MTT assay in VERO cells inoculated with both virus strains at a MOI of 0.5. Nevertheless, considering that for antirabies activity the MTT assay did not show significant differences between the absorbances of cell control and viral infected cells (data not shown), for PV rabies strain, the studies were based on the viral cytopathic effect inhibitory method by using McCoy cells and the PV strain at a MOI of 1.0 (Consales et al., 1990; Nogueira, 1992).

From the crude tested extracts, *Ilex paraguariensis* (aqueous extract – leaves), *Lafoensia pacari* (MeOH extract – leaves), *Passiflora edulis* (aqueous extract – leaves), *Ilex paraguariensis* (aqueous extract – leaves), *Lafoensia pacari* (MeOH extract–leaves) and *Sloanea guianensis* (MeOH extract – leaves) were the most active against both strains of HSV-1. Their EC\(_{50}\) values ranged from 60 to 170 \(\mu\)g/mL, and their SI were higher than 7. Nevertheless, the antirabies virus activity was detected only for *Allamanda schottii* (MeOH extract – leaves) with a SI = 5.6. The results of the antiviral evaluation of the tested extracts are shown in Table 2.

For aqueous extract from the leaves of *Ilex paraguariensis*, it was possible to verify strong antiviral activity against HSV-1 KOS (IS = 15.8) and 29-R (IS = 12.6) strains. It has been reported that caffeoyl acids and triterpenoid saponins possess strong antiviral

<table>
<thead>
<tr>
<th>Plant Used</th>
<th>Extract</th>
<th>CC(_{50}) ((\mu)g/mL)</th>
<th>EC(_{50}) ((\mu)g/mL)</th>
<th>SI</th>
<th>CC(_{50}) ((\mu)g/mL)</th>
<th>EC(_{50}) ((\mu)g/mL)</th>
<th>SI</th>
<th>Rabies virus (PV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alomanda blanchetti</td>
<td>Roots</td>
<td>EtOH</td>
<td>1900 ± 0.2</td>
<td>719.7 ± 0.6</td>
<td>2.6</td>
<td>1290 ± 0.3</td>
<td>1542 ± 0.5</td>
<td>500 ± 0.11</td>
</tr>
<tr>
<td>Alomanda schottii</td>
<td>Leaves</td>
<td>MeOH</td>
<td>1190 ± 0.2</td>
<td>1220 ± 0.4</td>
<td>2.4</td>
<td>1542 ± 0.5</td>
<td>500 ± 0.11</td>
<td>3.1</td>
</tr>
<tr>
<td>Ilex paraguariensis</td>
<td>Leaves</td>
<td>Aqueous</td>
<td>1260 ± 0.6</td>
<td>80.0 ± 0.2</td>
<td>15.8</td>
<td>100.0 ± 0.4</td>
<td>12.6</td>
<td>NT</td>
</tr>
<tr>
<td>Glycine max</td>
<td>Seeds</td>
<td>EtOH 40%</td>
<td>3000 ± 0.8</td>
<td>4877.7 ± 0.4</td>
<td>2.4</td>
<td>1542 ± 0.5</td>
<td>500 ± 0.11</td>
<td>3.1</td>
</tr>
<tr>
<td>Laofoensia pacari</td>
<td>Leaves</td>
<td>MeOH</td>
<td>1140 ± 0.6</td>
<td>60.0 ± 0.5</td>
<td>19.0</td>
<td>170.1 ± 0.7</td>
<td>6.7</td>
<td>NT</td>
</tr>
<tr>
<td>Passiflora edulis</td>
<td>Roots</td>
<td>EtOH 40%</td>
<td>1220 ± 0.6</td>
<td>1600 ± 0.3</td>
<td>5.5</td>
<td>89.9 ± 0.5</td>
<td>17.8</td>
<td>1713 ± 0.7</td>
</tr>
<tr>
<td>Rubus imperialis</td>
<td>Leaves</td>
<td>MeOH</td>
<td>1390 ± 0.8</td>
<td>70.0 ± 0.2</td>
<td>19.8</td>
<td>90.0 ± 0.8</td>
<td>15.4</td>
<td>NT</td>
</tr>
<tr>
<td>Sloanea guianensis</td>
<td>Leaves</td>
<td>MeOH</td>
<td>1400 ± 0.5</td>
<td>318.2 ± 0.6</td>
<td>4.4</td>
<td>140.0 ± 0.6</td>
<td>10.0</td>
<td>NT</td>
</tr>
<tr>
<td>Stems</td>
<td>MeOH</td>
<td>610.0 ± 0.5</td>
<td>381.2 ± 0.8</td>
<td>1.6</td>
<td>160.5 ± 0.7</td>
<td>3.8</td>
<td>1543 ± 0.5</td>
<td>3.8</td>
</tr>
</tbody>
</table>

I, inactive; NT, not tested; SI = CC\(_{50}/EC\(_{50}\)). MeOH, methanol; EtOH, ethanol. DOI: 10.1002/ptr

activity, respectively, against HSV-1 and adenovirus (Chiang et al., 2002) and HSV-1 (Hostettmann and Marston, 1995; Simões et al., 1999b). In view of that caffeoylquinic acids and derivatives (Filip et al., 2001) and triterpenoid saponins (Gosmann et al., 1995) are considered the major compounds present in this plant, the antiviral effect observed for *Illex paraguariensis* could be associated with the presence of these compounds.

Otherwise, the detected antitherpes activity of MeOH extract obtained from the leaves of *Lafoensia pacari* (SI / KOS = 19 and SI / 29R = 6.7) could be related to the presence of tannins, as other authors have also reported the antiviral activity of tannins (Fukuchi et al., 1989; Solon et al., 2000; Fortin et al., 2002; Cheng et al., 2004).

For *Passiflora edulis*, the presence of saponins was described (Zucolotto et al., 2006; Yoshikawa et al., 2000) and flavonoids (Petry et al., 2001; De Paris et al., 2002). So, the observed antiviral effect (SI / KOS = 5 and SI / 29R = 17.8) could be associated with these compounds, since for these groups of metabolites similar antitherpes activity has been already reported (Almeida et al., 1998; Simões et al., 1999b; Gonçalves et al., 2001; Goss et al., 2002). *Rubus imperialis* is the crude extract that showed higher activity against HSV-1 (SI / KOS = 19.8 and SI / 29-R = 15.4). For this genus, the occurrence of tannins and flavonoids was reported (Gudej and Tomczyk, 2000) leading us to propose that these compounds could be related to the detected antitherpes activity for this extract (Hudson, 1990).

The methanol extracts from leaves that showed highest activity against HSV-1 were partitioned with solvents of increasing polarity: hexane (HX), chloroform (CH), ethyl acetate (EA) and n-butanol (BuOH). From the seven tested fractions, EA fractions of *Lafoensia pacari* (anti-HSV-1/29R strain, SI = 10.3) and *Sloanea guianensis* (anti-HSV-1/KOS strain, SI = 7.8) showed promising antitherpes activity, and the n-BuOH fraction of *Rubus imperialis*, with polar substances as the main components (Niero et al., 1999), was the most active against HSV-1 (anti-HSV-1/29R, SI = 26.2). The results of the antiviral evaluation of the tested fractions are shown in Table 3.

Considering the results obtained, it can be stated that these extracts protect against viral infection, but the mechanism of their antiviral action and the active substances are not yet identified. Further studies are needed in order to verify which compounds could be responsible for this activity and how they exert their antitherpes effects. Studies with these goals are under development in our laboratory.

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**REFERENCES**


**Table 3. Antiviral activity of fractions of selected South American plants against herpes virus (HSV-1)**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Fraction</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (µg/mL) VERO cells</th>
<th>HSV-1 (KOS) Acyclovir-sensitive</th>
<th>HSV-1 (29R) Acyclovir-resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lafoensia pacari</em></td>
<td>HX</td>
<td>1050 ± 0.2</td>
<td>I –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CH</td>
<td>600.0 ± 0.2</td>
<td>500.0 ± 0.8</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>EA</td>
<td>1030 ± 0.4</td>
<td>490.5 ± 0.5</td>
<td>2.1</td>
</tr>
<tr>
<td><em>Sloanea guianensis</em></td>
<td>HX</td>
<td>380.0 ± 0.2</td>
<td>290.1 ± 0.7</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>CH</td>
<td>640.0 ± 0.2</td>
<td>5 ± I</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>EA</td>
<td>500.0 ± 0.2</td>
<td>63.9 ± 0.6</td>
<td>7.8</td>
</tr>
<tr>
<td><em>Rubus imperialis</em></td>
<td>BuOH</td>
<td>1390 ± 0.6</td>
<td>300 ± 0.2</td>
<td>4.8</td>
</tr>
</tbody>
</table>

I, inactive; SI = CC<sub>50</sub>/EC<sub>50</sub>; HX; CH; EA; BuOH (see experimental section).
ANTIVIRAL ACTIVITY OF SOUTH AMERICAN PLANTS


