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The *in vitro* antiviral activity of an aliphatic nitro compound from *Heteropteris aphrodisiaca*

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Summary

We investigated the antiviral activity of an aliphatic nitro compound (NC) isolated from *Heteropteris aphrodisiaca* O. Mach. (Malpighiaceae), a Brazilian medicinal plant. The NC was tested for its antiviral activity against poliovirus type 1 (PV-1) and bovine herpes virus type 1 (BHV-1) by plaque reduction assay in cell culture. The NC showed a moderate antiviral activity against PV-1 and BHV-1 in HEp-2 cells, and the 50% inhibitory concentration (IC50) were 22.01 μg/ml (selectivity index (SI) = 2.83) and 21.10 μg/ml (SI = 2.95), respectively. At the highest concentration of the drug (40 μg/ml) a reduction of approximately 80% in plaque assay was observed for both viruses. The treatment of cells or virus prior to infection did not inhibit the replication of virus strains.

Introduction

Viral diseases remain as an important worldwide problem, 50 yr after the beginning of the works with antiviral agents, due in part to the toxicity of many drugs developed (Ernst and Franey, 1998; De Clercq, 2001) and rapid emergence of drug resistant strains (Gilbert et al., 2002; Field, 2001; Zoulim, 2001). In order to find new alternatives and efficient antiviral compounds, many traditional medicinal plants have been screened (Vlietinck and Vanden Berghe, 1991; Jassim and Naji, 2003), including Brazilian medicinal plants (Simões et al., 1999; Schmitt et al., 2001; Esquenazi et al., 2002).

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A great diversity of compounds isolated from plants show antiviral activity including flavonoids, tannins, proteins, polysaccharides, alkaloids and others (Jassim and Naji, 2003). Bovine herpesvirus type 1 (BHV-1), a DNA virus member of the family Herpesviridae, is responsible for infections in bovines, such as those involved with the upper respiratory (rhinotracheitis) and genital tracts (vulvovaginitis and balanopostitis) and is resistant to acyclovir (Hinkley et al., 1998; Babiuk et al., 1983). Poliovirus is an RNA virus, member of the family Picornaviridae and is an appropriate model for study of viral replication. The virus is the etiological agent of poliomyelitis, disease under control in most of the countries; however, it has been faced as a serious threat worldwide. Besides, the virus belongs to the genus *Enterovirus*, in which members are found causing a wide array of illnesses, such as, meningitis, myocarditis, encephalitis and respiratory diseases (Mueller et al., 2005).

Heteropteris aphrodisiaca O. Mach. (Malpighiaceae) is a plant, endemic to the Brazilian scrubland regions, traditionally used in folk medicine as an aphrodisiac, stimulant and in the treatment of nervous weaknesses (Pott and Pott, 1994). Mattei et al. (2001) demonstrated that its extract, BST0298, reduced the oxidative stress in young and old rat brains. Galvão et al. (2002) found out that the treatment with the same extract improved learning and memory in aged rats. In this work we reported the antiviral activity of a nitro compound (NC) from *H. aphrodisiaca* against poliovirus and bovine herpesvirus in cell culture. To our knowledge this biological activity was never studied for this NC.

Materials and methods

Plant material

Roots of *H. aphrodisiaca* O. Mach. (Malpighiaceae) were collected in October 2000, in Santo Antônio do Leverger, Mato Grosso State, Brazil, and identified by Prof. Miramy Macedo (Universidade Federal de Mato Grosso, UFMT). A voucher specimen (UFMT-22181) was deposited at the UFMT Central Herbarium in Cuiabá, Mato Grosso, Brazil.

Extraction and isolation

The roots (500 g) were macerated with acetone for 1 week at room temperature of approximately 25 °C. The extract was filtered and the solvent evaporated under vacuum resulting in the crude extract (13 g). One portion of the extract (10 g) was chromatographed on silica gel by vacuum liquid chromatography using toluene and CHCl3/MeOH (97:3; 19:1; 9:1; 17:3; 1:1) to yield six fractions. Fractions (200 ml) were collected and checked by thin-layer chromatography [silica gel 60 F254 plates, using solvent systems: *n*-BuOH:CHCl3:MeOH (55:40:5)]. Fraction 4 (1.0 g) was crystallized with methanol:water to yield pure compound 2,3,4,6-*tetra-O*-(3-nitropropanoyl)-*O*-$\widehat{a}$-D-glucopyranoside (200 mg) (Roman Jùnior et al., 2005) (Fig. 1).

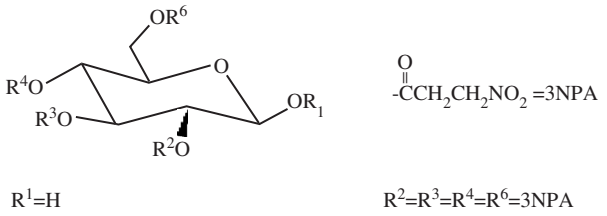

Figure 1. Chemical structure of 2,3,4,6-tetra-*O*-(3-nitropropanoyl)-*O*-$\widehat{a}$-D-glucopyranoside.

Cells and virus

HEp-2 cells (human larynx carcinoma, ATCC, CCL-23) were cultured at 37 °C with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, Penicillin (100 IU/ml), Streptomycin (100 μg/ml) and Amphotericin B (0.25 μg/ml).

The clinical isolate strain of bovine herpes virus type 1 (BHV-1) was supplied by Prof. A. Alfieri, DMVP-UEL. The poliovirus type 1 (PV-1) (ATCC, VR-58) was provided by Departamento de Virologia, IMPPG-UFRJ. Both virus strains were propagated in HEp-2 cells and stored at −80 °C. The virus titers were determined by plaque assay.

Cytotoxicity test

The NC cytotoxicity activity was evaluated by the alteration cell morphology and by trypan blue exclusion staining. Briefly, HEp-2 cells grown in 96-well culture plates were submitted to treatment with the compound at different concentrations and observed daily for cytotoxic alterations. Alternatively, cell cultures submitted to 48 h treatment with NC were dispersed with 0.2% trypsin and after staining with trypan blue the percentage of viable cells was calculated as follows: cell viability (%) = (viable cell number/total cell number) × 100.

Plaque reduction assay

Confluent cell monolayers grown in 24-well plates were inoculated with 50–100 pfu/ml of virus strains and after 1 h adsorption at 37 °C cells were washed with phosphate-buffered saline, pH 7.3, and overlaid with DMEM with 0.75% agarose containing two-fold dilutions of the NC, and for poliovirus 25 mM $MgCl_2$ were added (Wallis et al., 1966). After 48 h, the monolayers were fixed with 10% formaldehyde and nutrient agarose layer removed. Cell monolayers were stained with 0.5% crystal violet in 20% ethanol, and plaques counted. The antiviral activity was determined as the percent of plaque inhibition in comparison to controls.

Assay of NC either on virus strains or on the cells before infection

The stock of virus strains (10^6 pfu/ml) was diluted with equal volumes of DMEM containing 10, 20, 30 or 40 μg/ml of the NC, and incubated for 1 h at 37 °C. Virus strains without treatment were used for control. Virus titers were determined, as before. Confluent cell monolayers were incubated during 1 h at 37 °C with DMEM containing the same concentrations of the NC. Cells were washed with PBS and followed by infection as described before for plaque reduction assay.

Table 1. The 50% cytotoxic concentration (CC_{50}) and the 50% inhibitory concentration (IC_{50}) of a nitro compound from *Heteropteris aphrodisiaca* against bovine herpesvirus type 1 (BHV-1) and poliovirus type 1 (PV-1) in HEp-2 cells by plaque reduction assay

Virus	CC_{50} (μg/ml)[a]	IC_{50} (μg/ml)[b]	SI (CC_{50}/IC_{50})[c]
PV-1	62.29	22.01	2.83
BHV-1	62.29	21.10	2.95

[a]50% Cytotoxic concentration.
[b]50% Inhibitory concentration.
[c]Selectivity index.

Statistical analysis

All experiments were performed in quadruplicate, and data were analyzed by ANOVA, followed by Dunnett's test. $P = 0.05$ values were considered significant. The 50% cytotoxic concentration (CC_{50}) and the 50% inhibitory concentration (IC_{50}) were calculated by regression analysis of the dose–response curves.

Results and discussion

The antiviral activity of plant extracts against poliovirus and members of the Herpesvirida family has been demonstrated by several authors. Medicinal plants from Brazilian Atlantic tropical forest showed antiviral activity against herpes simplex type 1 (HSV-1) and poliovirus type 2 (PV-2) (Andrighetti-Fröhner et al., 2005). The antiviral activity of an aqueous extract of *Phyllanthus orbicularis* against BHV-1 and HSV-2 was found to be dependent on the extract concentration in tissue culture (Barrio and Parra, 2000).

NCs, such as hiptagin, were found in several plants of the genus *Heteropteris*, but no biological activity had been attributed to them (Stermitz et al., 1975). However, a synthetic NC, 5-nitropyrimidine derivative, was demonstrated to possess activity against HSV-1 suggesting its effect in the stage of virus attachment or fusion (Schmidtke et al., 2002).

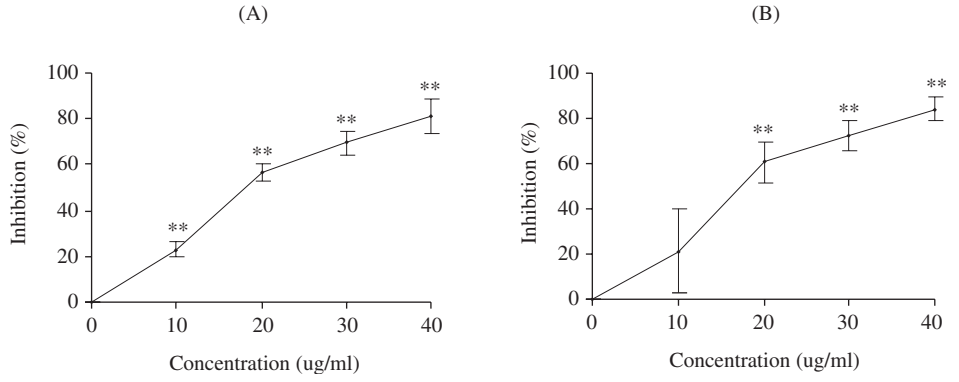

Figure 2. Dose–response curve of the inhibition of poliovirus type 1 (A) and bovine herpesvirus type 1 (B) by the aliphatic nitro compound from *Heteropteris aphrodisiaca*, in HEp-2 cell cultures. Data are expressed as mean ± S.D. ($n = 4$). **$P = 0.01$, no treatment vs. different concentrations of the nitro compound.

We found no cytotoxicity of the NC at concentrations lower than 50 μg/ml. However, at 50 μg/ml cells demonstrated discrete cytopathic changes revealed by abnormal granules and vacuoles. Nevertheless, trypan blue staining resulted in CC_{50} of 62.29 μg/ml (Table 1).

The effect for both virus strains was evaluated at the concentrations of the NC below 40 μg/ml. Table 1 shows the IC_{50} and selectivity index (SI) defined by the ratio CC_{50}/IC_{50}. The NC showed a moderate antiviral activity with selectivity indexes of 2.83 and 2.95 for PV-1 and BHV-1, respectively. The dose–response curves are shown in Fig. 2. We demonstrated a direct correlation of compound concentrations and viral inhibition, and at the highest drug concentration (40 μg/ml) a reduction of approximately 80% in the number of plaques was observed for both viruses. No effect was observed when both viruses and cells were pre-treated (data not shown), suggesting that the NC neither inactivated virus particles directly (both strains) nor protected the cell from infection, but interfered with the virus replication after the penetration step. Nevertheless, further experiments are required to elucidate the mechanism of action for this compound.

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