

**EVALUATION OF THE ANTIVIRAL ACTIVITY OF BRAZILIAN CERRADO
PLANTS AGAINST ANIMAL VIRUSES**

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ABSTRACT

Medicinal plants are abundant in the Brazilian flora including the Cerrado region. Extracts of sixteen plant species of the Brazilian Cerrado were investigated *in vitro* for antiviral activity against bovine herpesviruses type 1 (BoHV-1), infectious bursal disease virus (IBDV) and avian reovirus. Eight plants presented potent antiviral activity. *Erythroxylum deciduum*, *Lacistema hasslerianum* and *Xylopia aromatica* against BoHV-1; *Gochnatia polymorpha* and *Lithraea molleoides* against avian reovirus; and *Banisteriopsis variabilis*, *Byrsonima intermedia* and *Campomanesia xanthocarpa* against both viruses. No extract was active against IBDV.

INTRODUCTION

Viral infections still remain a serious worldwide problem. There is no known specific treatment for viral diseases and limited therapeutic efficacy of most drugs has led to a dependence on preventive measures using vaccines. Nucleoside analogues and other synthetic compounds have traditionally been the primary sources for antiviral agents. The use of antiviral synthetic drugs is often unsatisfactory and limited. Mutant viruses resistant to the existing antiviral agents arise upon treatment or these agents may cause side or toxic effects besides their high costs (Kott et al. 1999, Rates 2001, Cos et al. 2006). Higher plants may serve as promising sources of novel viral prototypes (Cordell 1995, Cowan 1999, Rates 2001, Jassim & Naji 2003).

Although natural products have been used by civilization since ancient times, only in recent decades has there been growing research into alternative therapies and the therapeutic use of natural products, especially those derived from plants (Cordell 1995, Calixto 2000, Rates 2001). The Brazilian flora is particularly rich in medicinal plants including the Cerrado

region where only recently has there been an interest in scientific researches or sustainable development and conservation (Rossi-Bergmann et al. 1997, Alves et al. 2000).

Herbal preparations are frequently used not only in rural areas in developing countries but also in developed countries in human and veterinary medical practices (Calixto 2000, Waihenya et al. 2002, Veiga Jr. et al. 2005, Cos et al, 2006). As result, a number of studies have been carried out on antiviral activity against several animal and human viruses in all continents. Several screenings using local medicinal plant extracts have been done (Semple et al., 1998, Kott, et al., 1999, Mtambo et al., 1999, Simões, et al., 1999, Lopez et al. 2001, Abad et al. 1999, Schmitt et al. 2001).

Bovine herpesvirus 1 (BoHV-1) is a major pathogen of cattle. Primary infection is accompanied by various clinical manifestations such as infectious bovine rhinotracheitis, abortion, infectious pustular vulvovaginitis, and systemic infection in neonates (Muylkens et al. 2007). Infectious bursal disease virus (IBDV) is an acute, highly contagious viral infection of young chickens of economic importance in poultry farms (Lukert & Saif 2003). Avian reoviruses are recognized as a cause of viral arthritis in chickens although also related to other disease conditions (Jones 2003, Rosenberger 2003).

The aim of the present work was assess the *in vitro* antiviral activity of sixteen plants from the Brazilian Cerrado against these viruses that cause important diseases in veterinary medicine.

MATERIAL AND METHODS

Plant material.

Leaves from Cerrado plant species were collected in Mogi-Guaçu, São Paulo State, Brazil. The specimens were identified by comparison with exsiccates deposited in the

Herbarium of the Instituto de Botânica, São Paulo, by botanist, Eduardo Luis Martins Catharino. The scientific and popular names of 16 plant species are listed in Table 1.

Table 1. Species of Brazilian Cerrado plants collected in Mogi-Guaçu, São Paulo State, Brazil, family and popular names.

Family	Species	Popular name
Malpighiaceae	<i>Banisteriopsis variabilis</i>	Cipó – prata
Malpighiaceae	<i>Byrsonima intermedia</i>	Murici - mirim
Myrtaceae	<i>Campomanesia xanthocarpa</i>	Guabiroba
Flacourtiaceae	<i>Casearia sylvestris</i>	Erva de teiú, Guaçatonga
Vitaceae	<i>Cissus erosa</i>	Uva do diabo
Caesalpiniaceae	<i>Copaifera langsdorffii</i>	Copaíba
Erythroxylaceae	<i>Erythroxylum deciduum</i>	Fruta de- pomba
Asteraceae	<i>Gochnatia polymorpha</i>	Cambará
Lacistemataceae	<i>Lacistema hasslerianum</i>	Pau de largarto
Anacardiaceae	<i>Lithraea molleoides</i>	Aroeira
Lauraceae	<i>Ocotea pulchella</i>	Canela
Mimosaceae	<i>Stryphnodendron adstringens</i>	Barbatimão
Styracaceae	<i>Styrax ferrugineus</i>	Laranjinha do cerrado
Trigoniaceae	<i>Trigonia eriosperma</i>	NK
Trigoniaceae	<i>Trigonia nivea</i>	NK
Annonaceae	<i>Xylopiia aromatica</i>	Pimenta de macaco

NK: not known

Extract preparation

The crude aqueous extracts were prepared by grinding dried leaves with de-ionized distilled water (10%, w/v) in a mixer and maintenance at 4°C overnight. The aqueous extracts were filtered on filter paper and freeze-dried. Lyophilized extracts were dissolved in equal parts of sterile de-ionized distilled water and Eagle minimum essential medium (MEM) at a concentration of 10,000, 4,000 or 2,000µg/ml. The extracts were centrifuged at 2,500g/10 min and sterilized by filtration (0.22µm filter).

Virus and cells.

The Los Angeles strain of BHV-1 and S1133 strain of avian reovirus were propagated in MDBK and Vero cells respectively. An isolate of IBDV (BA) of our laboratory (Simoni et al. 2002) was replicated in RK-13 cells. All cells were grown in MEM with 10% fetal bovine serum (FBS). RK-13 cells were supplemented with 2.95% of tryptose phosphate broth (TPB).

Cytotoxicity assays.

The assays were performed using 96-well microtiter plates with 30,000 cells/well. After 24 h of incubation at 37°C in a humidified 5% CO₂ atmosphere, each cell type was exposed to decreasing concentrations of plant extract in triplicate. Any cell morphology alteration was observed during the next 3 days to determine the maximum non-toxic concentration (MNTC). Monolayers of cells incubated only with MEM were used as a control.

Antiviral assays.

The antiviral activity of plant extracts was also assayed in 96-well microtiter plates. After 24 h of incubation, the medium was poured off and 100 µl of extracts at dilutions corresponding at MNTC maximum non-toxic concentration were added. The cells were incubated for 1h. After this period, 50µl of logarithmic dilutions of viruses were added in triplicate or quadruplicate. The plates were incubated again for 96 h. Controls consisted of untreated infected (virus titer), treated non-infected (extract control) and untreated non-infected (cell control) cells. Viral titers were determined by 50% infective doses in tissue culture-TCID₅₀ (Reed & Muench 1938). Antiviral activities were calculated as the difference of virus titer between treated and untreated infected control cultures. Values were expressed

as viral inhibition index (VII) and inhibition percentage (IP) as described in Koseki et al. (1990).

RESULTS




The cytotoxicity of plant extracts in three cell lines is showed in Table 2. The plants were grouped by maximum non-toxic concentration (MNTC) in three groups: G1 with MNTC above 625µg/mL, G2 between 125–625µg/mL and G3 below 125µg/mL. Eight plants presented equal MNTC group for three cell lines; six remained in the same group for at least two cell lines and only two showed different MNTC group for each cell line. *Cissus erosa* was the least cytotoxic whereas *Byrsonima intermedia*, *Ocotea pulchella*, *Styrax ferrugineus* and *Trigonia eriosperma* were the most cytotoxic for three cell lines. *Campomanesia xanthocarpa*, *Trigonia nivea* and *Xylopiya aromatica* presented intermediate cytotoxicity (G2) for three cells.

Eight of sixteen plants presented potent antiviral activity (IP≥97%). *Erythroxylum deciduum*, *Lacistema hasslerianum* and *Xylopiya aromatica* against BoHV-1 only; *Gochnatia polymorpha* and *Lithraea molleoides* against avian reovirus only; *Banisteriopsis variabilis*, *Byrsonima intermedia* and *Campomanesia xanthocarpa* against both viruses. *Styrax ferrugineus*, *Cissus erosa*, *Ocotea pulchella* and *Stryphnodendron adstringens* plant extracts presented antiviral activity (IP between 96-90%) against avian reovirus and/or BoHV-1. The other four plants showed IP<90% for both viruses.

Table 2: Cytotoxicity of Brazilian Cerrado plant extracts in MDBK, RK-13 and Vero cell lines.

Plant species	Cell line		
	MDBK	RK-13	Vero
<i>Banisteriopsis variabilis</i>	Group 2	Group 2	Group 1
<i>Byrsonima intermedia</i>	Group 3	Group 3	Group 3
<i>Campomanesia xanthocarpa</i>	Group 2	Group 2	Group 2
<i>Casearia sylvestris</i>	Group 3	Group 3	Group 2
<i>Cissus erosa</i>	Group 1	Group 1	Group 1
<i>Copaifera langsdorffii</i>	Group 3	Group 1	Group 3
<i>Erythroxylum deciduum</i>	Group 3	Group 2	Group 2
<i>Gochnatia polymorpha</i>	Group 2	Group 1	Group 2
<i>Lacistema hasslerianum</i>	Group 2	Group 2	Group 3
<i>Lithraea molleoides</i>	Group 1	Group 2	Group 3
<i>Ocotea pulchella</i>	Group 3	Group 3	Group 3
<i>Stryphnodendron adstringens</i>	Group 3	Group 2	Group 1
<i>Styrax ferrugineus</i>	Group 3	Group 3	Group 3
<i>Trigonía eriosperma</i>	Group 3	Group 3	Group 3
<i>Trigonía nivea</i>	Group 2	Group 2	Group 2
<i>Xylopiá aromática</i>	Group 2	Group 2	Group 2

MNTC: maximum non-toxic concentration

-  = Group 1: MNTC above 625 µg/mL
-  = Group 2: MNTC between 624-125µg/mL
-  = Group 3: MNTC bellow 125 µg/mL

The Table 3 shows the results of antiviral assays of plant extracts against BoHV-1 and avian reovirus. No extract was active against IBDV.

Table 3: Antiviral activity of Brazilian Cerrado plant extracts against BoHV-1 and reovirus.

Plant species	BoHV-1		Avian reovirus	
	VII	IP(%)	VII	IP(%)
<i>Banisteriopsis variabilis</i>	2.79	99	2.69	99
<i>Byrsonima intermedia</i>	2.67	99	1.50	97
<i>Campomanesia xanthocarpa</i>	2.08	99	1.50	97
<i>Casearia sylvestris</i>	0	0	0.50	68
<i>Cissus erosa</i>	1.41	96	0	0
<i>Copaifera langsdorffii</i>	0.59	74	0.76	83
<i>Erythroxylum deciduum</i>	1.75	98	0.83	85
<i>Gochnatia polymorpha</i>	0.83	85	1.62	98
<i>Lacistema hasslerianum</i>	3.10	99	0.62	76
<i>Lithraea molleoides</i>	0.59	74	2.96	99
<i>Ocotea pulchella</i>	1.20	93	0.26	45
<i>Stryphnodendron adstringens</i>	1.27	93	0.50	68
<i>Styrax ferrugineus</i>	0	0	1.00	90
<i>Trigonia eriosperma</i>	0.08	15	0.50	68
<i>Trigonia nivea</i>	0.58	73	0.50	68
<i>Xylopia aromatica</i>	2.58	99	0.26	45

VII: inhibition viral index; IP: inhibition percentage

DISCUSSION

Research into biological activities of plant extracts must investigate their toxicity for safety of their future use as a therapeutic agent (Harbell et al. 1997, Rates 2001). Therefore, plant extracts were first assayed for cell toxicity in three susceptible cell lines to three viruses. The maximum non-toxic concentration (MNTC) was used for an *in vitro* antiviral screening program that evaluated a large number of plants. Variations in a MNTC group of plant species did not interfere with antiviral tests, once these were done using the extracts in their respective MNTCs in each cell line used.

Of 16 extracts tested, seven are used in Brazilian popular medicine and four of them were positive for at least one of the viruses used (Sciesserre 2003, Manha 2004). Ethnobotany or ethnopharmacology have been the most common strategies for selecting a plant for antiviral study (Vlietinck & Vanden Berghe 1991, Kott et al. 1999, Rates 2001, Cos et al. 2006). Thus, our data confirmed the importance of the choice of plant resources based on traditional use.

The plant species presenting strong antiviral activity for one or more viruses are promising as an antiviral component source. No information including antiviral activity of *Banisteriopsis variabilis*, *Erythroxylum deciduum* and *Lacistema hasslerianum* has formerly been described. Leaf methanolic extracts from *Byrsonima intermedia* and aqueous extracts from *Campomanesia xanthocarpa* were studied for the presence of mutagenic activity (Fernandes & Vargas 2003, Sannomiya et al. 2007). *Xylopia aromatica* is rich in components like diterpenes and acetogenins that showed in vitro activity against parasites and bacteria (de Mesquita et al. 2007, Takahashi et al. 2006). Searching for these components in leaves could elucidate the antiviral activity observed against BoHV-1.

Stem bark from *Stryphnodendron adstringens* is an important source of tannins used in traditional medicine for the treatment of various diseases. Felipe et al. (2006) demonstrated a viral inhibition of 97% using an aqueous fraction from *S. adstringens* barks against BoHV-1 identifying catechins and tannins. In this work the leaves presented moderate (93%) activity against this virus. *Casearia sylvestris* did not exhibit antiviral activity in the present experiment against BoHV-1, as was formerly reported in Simões et al. (1999) against *Herpes simplex* virus type 1 and 2 (HVS-1 and 2).

Several reports showed antiviral activity against BoHV-1 of several plant species (Simoni et al. 1996, Summerfield et al. 1997, Barrio & Parra, 2000, Biltoveni et al. 2005, Felipe et al. 2006, Melo et al. 2006) but few against the two avian viruses (IBDV and

reovirus). Meyer et al. (1997) verified an inhibition of reovirus with galangin isolated from *Helichrysum aureonitens* and Hady et al. (2002) observed antiviral activity for both viruses investigating different propolis samples. *Lithraea molleoides* exhibited potent antiviral activity against avian reovirus. Resorcinols from *L. molleoides* showed *in vitro* strong paralyzing effects on nematodes (Valcic et al. 2002). Aqueous extracts from *Gochnatia polymorpha* leaves have demonstrated significant antiinflammatory activity (Moreira et al. 2000). In screening studies of Simões et al. (1999), the hydromethanolic extracts of *G. polymorpha* were not active against five different viruses including HVS-1 and HVS-2. Kott et al. (1999) showed antiviral activity against HVS-1 and respiratory syncytial virus (RSV) using hot leaf extracts. Under our experimental conditions (cold aqueous extracts), *G. polymorpha* was active against avian reovirus but not against BoHV-1.

Studies of suid herpesvirus type 1 (SuHV-1) are also in process. Those plants that present activity against the most viruses will have priority for further studies.

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