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Antiviral Activity of Plants Occurring in the State of Minas Gerais (Brazil): Part III

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ABSTRACT

A total of 24 extracts from 14 plant species collected at the state of Minas Gerais, Brazil, and belonging to five botanical families (Annonaceae, Apocynaceae, Ochnaceae, Polygonaceae and Vitaceae) was screened for cytotoxicity in cultured Vero cells and for antiviral activity against human herpes virus type 1 (HSV-1), vaccinia virus (VACV) and murine encephalomyocarditis virus (EMCV). The highest cytotoxicity (CC_{50} < 10 µg/mL) was observed for the ethanol extracts from Annona coriacea fruits and seeds. Extracts from Hancornia speciosa, Ouratea castaneafolia and O. semisrrata were the only ones that have shown activity against all the three viruses assayed. Extracts from Polygonum spectabile, Hancornia speciosa, Himatanthus phagedaenica, Ouratea spectabilis and O. semiserrata were the most active against HSV-1 (EC_{50} < 50 µg/mL), with favorable SI values (8.0 to 10.0). Hancornia speciosa and Anaxagorea dolichocarpa were the most active against EMCV (EC_{50} 50 – 100 µg/mL), with reasonable SI values (5.2 to 6.1), while moderate to low activity (EC_{50} > 100 µg/mL) was observed for Ouratea spectabilis and O. semiserrata. A total of 7 plant species, Ouratea semiserrata, O. spectabilis, O. castanaeafolia, Rollinia laurifolia, Cissus erosa, Polygonum spectabile, and Hancornia speciosa, were active against VACV, disclosing EC_{50} < 50 µg/mL and SI values ranging from 6.6 to 67.3. In total, 10 out of the 14 species were selected from a literature survey on plants used to treat viral diseases in Brazil; these species were responsible for 70% of the positive results.

Keywords: Bioprospection, antiviral activity, EMCV, HSV-1, VACV.

INTRODUCTION

The ethnopharmacological knowledge is recognized as of crucial importance in the search of new drugs (new chemical entities) that is a long and high costly process. Alternatively, the traditional knowledge can lead to phytomedicines that can be developed in shorter time, at lower

costs, besides allowing the utilization of local resources for primary health. Such an approach, called "ethnopharmacy" encompasses relevant disciplines such as pharmacognosy, pharmacology, pharmaceutics (galenicals), drug delivery, toxicology, bioavailability and clinical pharmacy. Classical phytochemistry combined with bioguided isolation of bioactive natural products and advanced methods of phytochemical analysis, including metabolomics, are equally important for the development of novel extracts to be used as medicines [1, 2].

A significative number of medicinal plant extracts are reported to exhibit high levels of antiviral activity [3, 4, 5, 6, 7, 8, 9, 10] and the search for useful phytochemicals is being pursued as suitable strategy for the discovery of antiviral agents. A great variety of antiviral natural products, including flavonoids, terpenoids, lignans, coumarins, saponins, tannins, alkaloids and polysaccharides has been identified [11, 12, 13, 14]. Furthermore, several natural anti-HIV compounds, such as the calanolides, michellamines, prostatin and betulinic acid derivatives, have passed through preclinical assays [15, 16].

The present paper reports the evaluation of the susceptibility of one RNA-virus, the murine encephalomyocarditis virus (EMCV), and two DNA-viruses, the human herpes virus type 1 (HSV-1) and vaccinia virus Western Reserve (VACV-WR), to ethanol extracts of 14 plant species belonging to the families Annonaceae, Apocynaceae, Ochnaceae, Polygonaceae and Vitaceae that were collected in the state of Minas Gerais, Brazil.

Herpes viruses (HSV-1 and HSV-2) are pathogenic to humans, causing recurrent infections especially in the case of highly susceptible adults. Among HSV-related pathologies, genital herpes is an important sexually transmitted disease. In immuno-compromised patients and neonates, HSV infections can cause serious systemic illnesses. Furthermotre, HSV is involved in several ocular diseases, such as the herpetic stromal keratitis (HSK), an immunopathology which is one of the leading causes of blindness in western world [13]. Acyclovir is the most frequently used drug for treatment of HSV infections. However, resistance to acyclovir is reported [16] and the quest of new anti-herpetic drugs is necessary.

Global erradication of smallpox was declared by WHO, more than thirty years ago. However, discontinuation of vaccination against smallpox (with vaccinia virus vaccine) has rendered most humans vulnerable to smallpox infection [16]. Re-emergence of VACV infections [17, 18] as well the threat that variola virus, the etiological agent of smallpox, might be used in warfare or terrorism, have motivated the search for measures to control or treat smallpox and poxvirus infections, in general.

EMCV (family *Picornaviridae*, genus *Cardiovirus*) is a group of closely related virus species with a wide host range. Infections with EMCV are associated with sporadic cases and outbreaks of myocarditis and encephalitis in domestic pigs, in non-humans primates and in other mammalian species. The disease is often fatal; frequently sudden death is the first indication of infection and most outbreaks have been associated with captive animals such as those in piggeries, primate research centers and zoos. Virus isolation has been reported from patients with aseptic meningitis, poliomyelitis-like paralysis, encephalomyelitis and fever of unknown origin documented by virus isolation of several specimen types. Few cases of human EMCV infection and disease have been documented; the most recent were in 2004 from two febrile patients in Peru [19]. EMCV was used as a model for RNA virus, especially for viruses from the Picornaviridae family, as it presents a safe animal model to test antiviral drugs [20].

Interferons, cell glycoproteins synthesized in response to viral infections and various nonviral inducers, have proved therapeutically effective for viral infections in experimental models and in humans. Their significance as antiviral, antiprotozoal, immunomodulatory and cell growth regulatory agents is well documented. The IFN system can impair various steps of viral replication. However EMC viruses can replicate efficiently in IFN-treated cells [21, 22] what renders these viruses useful in the investigation of compounds whose antiviral effect could be related to stimulation of IFN production. EMCV was also used by our group to study the antiviral activity of interferons [23].

In the present study, Brazilian plants belonging to 6 botanical families were selected on the basis of ethnopharmacological and taxonomic criteria which are valid and widely exploited strategies for a screening program to seek bioactive natural products [24]. This report concerns the collection of plant material, the preparation of extracts, the chromatographic characterization (TLC and HPLC) of the extracts and the *in vitro* evaluation for cytotoxic and antiviral activities. The colorimetric MTT assay was used to evaluate the cytotoxicity of the extracts in Vero cells and the susceptibility of a RNA-virus, murine encephalomyocarditis virus (EMCV), and two DNA-viruses, human herpes virus type 1 (HSV-1) and vaccinia virus (VACV) [25, 26].

EXPERIMENTAL SECTION

Plant material

Plant material was collected in the state of Minas Gerais, Brazil. Voucher species were deposited into the herbarium of UFMG (BHCB), Belo Horizonte, Brazil. The plant material was dried in an air circulating oven at 45 ^{o}C for 48 h. The different plant parts were separated, ground and exhaustively extracted by percolation with ethanol 92.8 ^{o}GL. The filtrates were combined and the solvent was completely removed under reduced pressure in a rotavapor. All the extracts were characterized by TLC, using appropriate eluents and chromogenic reagents [27] as well as by HPLC-DAD, with online registration of the UV spectra of the constituents. HPLC fingerprints were registered on a Waters 2695 apparatus with a UV-DAD detector (Waters 2996*). Conditions*: a LiChrospher 100 RP-18 column (5 µm, 250 x 4 mm i. d.) (Merck) was employed at a temperature of 40 ^{o}C, flow rate of 1.0 ml/min and detection at wavelengths of 220, 280 and 360 nm. *Sample preparation.* To an aliquot (10.0 mg) of each dried extract, HPLC grade MeOH was added, the mixture was dissolved by sonication for 15 min, followed by centrifugation at 10,000 rpm for 10 min. The supernatant was filtered through Millipore membrane (0,2 µm) and injected (10.0 µL) onto the equipment. Elution was carried out with a linear gradient of water (A) and acetonitrile (B) (from 5% to 95% of B in 60 min).

Virus and cell lines

We used Vero cells (ATCC CCl-81) from the African green monkey kidney (*Cercopthecus aethiops*) and the murine aneuploid fibrosarcoma cell line L929, which was obtained from the Roche Institute of Molecular Biology, New Jersey, USA, and was kindly provided by Dr. S. Pestka. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS), gentamicin (50 µg/mL), penicillin (100 IU/mL) and fungizone (5 µg/mL).

The battery of viruses was composed of two DNA viruses: herpes simplex virus type 1 (HSV-1), a clinical isolate obtained at the *Laboratório de Virus*, UFMG, Belo Horizonte, Brazil. *Vaccinia virus* Western Reserve (VACV-WR) and one RNA-virus, murine *encephalomyocarditis virus* (EMCV); were kindly supplied by Dr. C. Jungwirth, Würzburg University, Germany, and Dr. I. Kerr, Cancer Research, UK, London Research Institute, London, United Kingdom, respectively.

Multiplication of the viruses was performed in Vero cells (ATCC CCL-81) (HSV-1 and VACV-WR) and murine aneuploid fibrosarcoma cells (L929 cell) in 150 cm^3 (EMCV) bottles; cells were infected with 0.01 UFP per bottle and examined daily by light microscopy for the cythopathic effect (CPE). Cultures showing 90-100% CPE were centrifuged at 3,000 rpm for 5 min at 4 ^oC. Aliquots of the supernatants were kept at -70 ^oC until further use.

Cytotoxicity assay

Vero cell monolayers were trypsinized, washed with culture medium, plated in 96-well flat-bottomed plates (6 x 10^4 cells per well) and incubated in a humidified atmosphere with 5% CO_2 at 37 ^oC. After a 24-h incubation, serial two-fold dilutions of the plant extracts (500 – 0.125 µg/mL) made in DMEM were added to appropriate wells, and the plates were incubated for an additional 48 and 72 h. The supernatants were removed from the wells, and MTT (28 µl of a 2 mg/mL solution in PBS) was added to each well; the plates were incubated for 90 min at 37 ^oC; then, DMSO (130 µL) was added to each well to dissolve the formazan crystals. After shaking the plates to ensure complete dissolution of formazan, the optical density was determined at 492 nm in a multiwell spectrophotometer (Start Fax, mod. 2100). The percentage of cytotoxicity was calculated as (A – B)/A x 100, where A and B are the OD_{492} of untreated and treated cells, respectively. The 50% cytotoxic concentration (CC_{50}) of the test extracts is defined as the concentration that reduces the OD_{492} of treated cells to 50% of that of untreated cells and was determined by a dose-response curve (not shown) [28].

Antiviral assay

Titration of the virus stocks. The titers of the virus stocks were determined by the 50% tissue culture infectious dose ($TCID_{50}$) obtained by the endpoint dilution of a virus in cultured Vero cells. Cells were seeded into 96-well microtiter plates with 6x10^4 cells per well and incubated at 37 ^oC in growth medium (DMEM containing 5% fetal bovine serum plus antibiotics). Monolayers were infected with 11-fold dilutions of cell-free virus in 0.2 ml of DMEM containing 1% fetal bovine serum plus antibiotics and incubated in a humidified atmosphere with 5% CO_2 at 37 ^oC; the development of the CPE was monitored every 24-hours for a week and compared to the control cells [29]. The titers determined were 1.0 x $10^{8.2}$, 1.0 x $10^{10.2}$ and 1.0 x 10^9 $TCID_{50/mL}$ for HHV-1, EMCV and VACV-WR, respectively.

MTT assay. Viral samples were titrated using the method of the microculture assay (TCID) for the virus dilutions that caused a 100% cytopathic effect in the cell monolayer after 48 h for HSV-1/EMCV and 72 h for VACV-WR [27]. The titers of the viral samples were 2.5 x 10^6 $TCID_{100}/_{mL}$ for HSV-1, and 1.0 x 10^6 $TCID_{100}/_{mL}$ for both VACV-WR and EMCV. The antiviral activity of the extracts was evaluated in Vero cells by the MTT colorimetric assay as described [25, 26]. Controls for cytotoxicity (uninfected treated cells), cells (uninfected untreated cells), virus (infected untreated cells) and positive controls (acyclovir/Calbiochem and α-2a interferon/Bergamo) were run in parallel during each experiment. The 50% antiviral concentration (EC_{50}) is expressed as the concentration that achieves 50% protection of treated infected cells from virus induced destruction.

Table 1. Plant species assayed for antiviral activity: voucher numbers, extractives and results of the TLC and HPLC-DAD phytochemical screening

Extract Number	Family/Species (Selection method)	Voucher	Plant part used	Extractives (%)	NPC/TLC	NPC/HPLC-DAD
	Annonaceae					
1	*Anaxagorea dolichocarpa* Sprague & Sandwith[a]	BHCB 34332	Stems	7.8	Ta, Alk, Sa, Co, TS, Fla, Ant	Proac
2	*Annona coriacea* Mart.[a]	BHCB 25383	Leaves	11.7	Ta, TS, Fla, Ant	Fla
3			Fruits	10.7	Ta, TS, Fla, Alk	Proac
4			Seeds	15.3	Ta, Alk, Co, TS, Fla	Cin
5	*Rollinia laurifolia* Schltdl.[b]	BHCB 23289	Leaves	11.9	Ta, Alk, Sa, Cu, TS, Fla	Fla
6			Stems	15.0	Alk, Co, TS, Fla	Fla
	Apocynaceae					
7	*Aspidosperma cylindrocarpon* Müll.Arg.[b]	BHCB 25332	Stems and leaves	12.0	Ta, Alk, TS, Fla	Fla
8	*Aspidosperma parvifolium* A.DC.[b]	BHCB 106776	Stems	5.3	Ta, Alk, Co, TS, Fla	Alk, Fla
9	*Aspidosperma tomentosum* Mart.[a]	BHCB 25383	Fruits	23.0	Alk, Sa, TS, Fla	Fla
10			Seeds	12.0	Alk, Sa, TS, Fla	Ant
11	*Hancornia speciosa* Müll.Arg.[a]	BHCB 49319	Leaves	16.0	Ts , Fla	Fla, Xant
12			Stems	22.0	TS, Fla	Proac
13	*Himatanthus phagedaenicus* (Mart.) Woods[a]	BHCB 112116	Leaves	20.0	Ta, TS	Fla
14			Stems	6.2	Alk, Ta, TS	Fla
15	*Tabernaemontana laeta* Mart.[a]	BHCB 94263	Leaves	8.2	Alk, Sa, TS, Fla	Fla
16			Stems	15.0	Alk, Sa, TS, Fla	Fla
17			Latex	-	Ta, Alk , TS	
	Ochnaceae					
18	*Ouratea castaneifolia* (DC.) Engl.	BHCB 25577	Leaves		Ta, Sa, TS, Fla	Fla, Proac
19	*Ouratea semiserrata* (Mart. & Nees) Engl.[b]	BHCB 42166	Leaves		Ta, Sa, TS, Fla	Fla, Proac
20			Stems		Ta, Sa, Co, TS, Fla	Fla, Proac
21	*Ouratea spectabili*s (Mart.) Engl.[a]	BHCB 48940	Leaves		Ta, TS, Fla	Fla, Proac
	Polygonaceae					
22	*Polygonum spectabile* Mart.[a]	PAMG 55256	Aerial parts	15.7	Ant, Ta, TS, Fla	Fla, Proac
	Vitaceae					
23	*Cissus erosa Rich.*[a]	BHCB 48733	Leaves	13.4	Ta, TS, Fla	Fla, Xant
24			Stems	8.2	TS	Fla

[a]Ethnopharmacological selection; [b]Taxonomic selection; Fla – flavonoids, TS – triterpenes and steroids, Alk – alkaloids, Ta – tannins, Sa – saponins, Ant – anthraquinones, Proac – proanthocyanidins, Cin – cinnamic acid derivative

Table 2. Cytotoxicity and antiviral activity of ethanol extracts from plants occurring in the state of Minas Gerais, Brazil

Extract Number	Family Species	Plant part used	Vero Cells CC_{50} (μg/mL)	HSV-1 EC_{50} (μg/mL)	SI	VACV-WR EC_{50} (μg/mL)	SI	EMCV EC_{50} (μg/mL)	SI
	Annonaceae								
1	*Anaxagorea dolichocarpa*	Stems	> 500	NA		NA		90.5 ± 5.7	> 5.5
2	*Annona coriacea*	Leaves	> 500	NA		NA		NA	
3		Fruits	6.8 ± 1.9	NA		NA		NA	
4		Seeds	4.5 ± 0.0	NA		NA		NA	
5	*Rollinia laurifolia*	Leaves	111.9 ± 5.3	NA		13.3 ± 0.4	8.4	NA	
6		Stems	169.7 ± 1.8	NA		NA		NA	
	Apocynaceae								
7	*Aspidosperma cylindrocarpon*	Stems and Leaves	152.4 + 5.0	NA		NA		NA	
8	*A. parvifolium*	Stems	> 500	NA		NA		NA	
9	*A. tomentosum*	Fruits	> 500	304.1 ± 11.5	> 1.6	NA		NA	
10		Seeds	80.4 + 1.1	NA		NA		NA	
11	*Hancornia speciosa*	Leaves	323.4 ± 33.7	56.5 ± 1.6	5.7	NA		NA	
12		Stems	> 500	38.2 ± 0.5	> 10	37.6 ± 1.1	> 13.3	81.9 ± 4.4	> 6.1
13	*Himatanthus phagedaenica*	Leaves	197.2 + 11.0	NA		NA		NA	
14		Stems	> 500	48.2 ± 5.5	> 10.4	NA		NA	
15	*Tabernaemontana laeta*	Leaves	416.5 ± 8.3	NA		NA		NA	
16		Stems	336.9 ± 29.4	NA		NA		NA	
17		Latex	> 500	NA		NA		NA	
	Ochnaceae								
18	*Ouratea castaneifolia*	Leaves	> 500	56.5 ± 3.2	> 8.8	39.6 ± 1.3	> 12.6	465.7 ± 32.5	> 1.0
19	*O. semiserrata*	Leaves	> 500	8.4 ± 0.7	> 59.5	9.2 ± 0.8	> 54.3	254.4 ±10.7	> 2.0
20		Stems	> 500	57.8 ± 2.4	> 8.7	7.4 ± 0.1	> 67.3	185.9 ± 9.8	> 2.7
21	*O. spectabilis*	Leaves	247.0 ± 14.7	8.9 ± 1.1	27.7	11.4 ± 0.8	21.7	NA	
	Polygonaceae								
22	*Polygonum spectabile*	Aerial parts	153.4 ± 13.6	24.2 ± 2.6	6.3	30.5 ± 1.9	5.0	NA	
	Vitaceae								
23	*Cissus erosa*	Leaves	305.3 ± 31.1	97.2 ± 6.6	3.1	NA		NA	
24		Stems	384.4 ± 11.0	121.7 ±17.0	3.2	27.9 ± 0.3	13.8	NA	
	Aciclovir			[a]40					
	Interferon α					[ab]2.5 x 10^2		[ab]1.5 x 10^2	

CC_{50} – 50 % cytotoxic concentration (μg/ml) for vero cells; EC_{50} – effective concentration (μg/ml) required to inhibit by 50 % the cytopathic effect (CPE) at a viral title of 2.5 x 10^6, 1.0 x 10^6 and 1.0 x 10^6 $TCID_{100}$/ml for HSV-1, VACV-WR and EMCV respectively; SI – selectivity index = CC_{50}/EC_{50}; HSV - 1 – human herpes virus type 1, EMCV – encephalomyocarditis virus, VACV- WR – vaccinia virus Western Reserve; NA – not active

The percent protection is calculated as [(A – B)/(C – B)] x 100, where A, B and C are the OD_{492} of treated infected, untreated infected and untreated uninfected cells, respectively. The 50% antiviral concentration (EC_{50}) was determined by a dose-response curve (not shown).

RESULTS AND DISCUSSION

A total of 24 extracts from 14 botanical species belonging to six families (Anacardiaceae, Annonaceae, Apocynaceae, Ochnaceae, Polygonaceae and Vitaceae) was assayed for their antiviral activity. To increase the likelihood of success, we followed the recommendations that help to develop a stronger *in vitro* "proof-of-concept" in the evaluation of the antiviral potential of natural products [30], including: 1) selection of plant species with documented information on their traditional use to treat symptoms related to viral infections or taxonomic related species; 2) phytochemical characterization of the extracts by examining their TLC and HPLC-DAD profiles and on line registration of the UV-VIS spectra of the constituents; and 3) using cell-based *in vitro* assays with reference virus strains as well appropriate controls. Data on the collected plant species, the extracts prepared and classes of natural products detected by TLC and HPLC-DAD are shown on Table 1.

The results of the screening for antiviral activity of the plant extracts at non-cytotoxic concentrations are expressed as EC_{50} (µg/mL). Cytotoxicity in Vero cell cultures is expressed as CC_{50} (µg/mL), which is the 50% cytotoxic concentration of the test extracts on cultured Vero cells. Selectivity Index (SI), represented by $CC_{50}/EC_{50,}$ is a useful parameter for the preliminary evaluation of the selectivity of compounds or extracts and it is very useful for guiding bioactivity-directed fractionation and defining prioritizations for *in vivo* studies. The data are shown on Table 2.

Table 3. Traditional uses of some of the plant species assayed for antiviral activity

Plants	Local name	Traditional uses	Ref.
Annonaceae			
Anaxagorea dolichocarpa	Bananinha	Leaves, stem-bark and fruits: against grippes and cold	[31]
Annona coriacea	Cabeça-de-negro, Araticum-do-campo	Leaves and seeds: chronic diarrhea and dysentery	[32]
Apocynaceae			
Aspidosperma tomentosum	Pereira-do-campo	Stem-bark: for fevers, stimulants and antiseptic	[33, 34]
Hancornia speciosa	Mangaba, mangabeira	Latex and stem-bark: against tuberculosis, cramps and respiratory diseases	[32, 33, 34, 35]
Himatanthus phagedaenicus	Angélica-da-mata, leiteiro	Latex and green fruit: against external ulcers, diabetes, inflammations, liver disorders and warts	[35]
Tabernaemontana laeta	Esperta, leiteira	Stem-bark and latex: tonic, against external ulcers and warts	[36]
Ochnaceae			
Ouratea spectabilis	Folha-de-serra	Oil epicarp fruit: Diseases of the liver and skin	[31]
O. castaneifolia	Farinha-seca, mangue-do-mato	Bark: tonic and adstringent	[37]
Polygonaceae			
Polygonum spectabile	Erva-de-bicho	Aerial parts: stimulant, against helminthes, hemorrhoids, diarrhoea, ulcers, gingivitis	[33, 38]
Vitaceae			
Cissus erosa	Cipó-fogo	Entire plant: for warts and external ulcers	[35, 39]

Ethnopharmacological selection of plant species for this antiviral screening was based on literature data documenting the medicinal use mainly for treatment of sympthoms that might be related to virus infections. The traditional use and local names are described on Table 3.

All the extracts were firstly evaluated for cytoxicity in Vero cells. Only two extracts, those from *Annona coriacea* fruits and seeds, were highly cytotoxic to Vero cells, with CC_{50} values lower than 10 µg/mL. *A. coriacea* contains cytotoxic tetrahydrofuran acetogenins [40, 41], but extracts from its leaves have shown low cytoxicity on Vero cells (CC_{50} > 500 µg/mL). The extract of *Aspidosperma tomentosum* seeds has demonstrated moderate cytotoxicity (CC_{50} 80.36 $\pm$ 1.1 µg/mL). For 10 extracts, the CC_{50} values ranged from 152.4 $\pm$ 5.0 to 416.5 ± 8.3 µg/mL; for the 10 remaining extracts no cytotoxicity was observed up 500 µg/mL.

It is well known that the virus titer can influence the EC_{50} in the in vitro bioassays. Plant extracts that are able to protect cells from the CPE of a virus with a $TCID_{50}$/ml > 103 are considered relevant and deserve further investigation for the isolation of their active compounds [42]. It should be emphasized that we have used viruses at TCID $\geq$ 106 in the present screening what provides us with confidence in our positive results. It would be expected to observe lower EC_{50} values in experiments with viruses having TICD < 106. Extracts showing EC_{50} < 100 µg/mL might be considered as promising sources of antiviral drugs.

Five plant extracts have show pronounced activity against the HSV-1, with EC_{50} values lower than 50 µg/mL: *Ouratea spectabilis* (leaves), *O. semiserrata* (leaves), *Polygonum spectabile* (aerial parts), *Hancornia speciosa* (stems) and *Himatanthus phagedaenica* (stems). Favorable SI coefficients (8.0 to 10.0) were observed for this plant group. Leaves extracts of *Hancornia speciosa, Ouratea castaneifolia, O. semiserrata* and *Cissus erosa* were moderately active, with EC_{50} values ranging from 56.5 ± 1.6 to 97.24 ± 6.6 µg/mL and SI coefficients ranging from 2.0 to 8.8. Extracts of *Aspidosperma tomentosum* (fruits) and *Cissus erosa* (stems) have shown a low antiviral effect, with EC_{50} values of 121.7 ± 17.0 and 304.1 $\pm$ 11.5 µg/mL. Thirteen extracts were completely inactive against HSV-1.

At lower concentrations (EC_{50} < 50 µg/mL), no plant extract was active against EMCV, an RNA-virus. Stem extracts from *Hancornia speciosa* and *Anaxagorea dolichocarpa* have shown activity within the range of 50 – 100 µg/mL with reasonable SI coefficients (5.5 to 6.1). Moderate to low antiviral activity (EC_{50} 185.9 ± 9.8 µg/mL to 465.7 $\pm$ 32.5 µg/mL) was observed for *Ouratea castaneaefolia* and *O. semiserrata.* All the others were inactive.

Seven plant species have inhibited the replication of VACV-WR, a DNA-virus, with EC_{50} values below 50 µg/mL; most of them had favorable SI coefficients (6.6 – 67.3): *Ouratea spectabilis, O. castaneaefolia, O. semiserrata, Rollinia laurifolia, Cissus erosa, Polygonum spectabile, and Hancornia speciosa.* Among the active extracts that one from *O. semiserrata* should be highlight with EC_{50} of 7.4 ± 0.1 and SI coefficients greater than 67.3, the highest determined SI coefficients obtained in this study. All the other extracts have not inhibited the cytopathic effect of VACV-WR.

In general, plant extracts were found to be highly selective towards the viruses assayed, as only three species were active against all the three viruses: *Hancornia speciosa* (stems), with an EC_{50} < 100 µg/mL for all the three viruses; *Ouratea semiserrata* (leaves), with an EC_{50} < 50 µg/mL for HSV-1 and VACV-WR and EC_{50} > 100 µg/mL for EMCV; *O. castaneafolia* (leaves), with EC_{50} values of 56.5 ± 3.2 µg/mL (HSV-1), 39.6 ± 1.3 µg/mL (VACV-WR) and 465.7 $\pm$ 32.5 µg/mL (EMCV).

The lowest EC_{50} values were observed against VACV-WR for *Ouratea semiserrata* stems and leaves (EC_{50}=7.4±0.1µg/mL and EC_{50}=9.2±0.8µg/mL, respectively) and *O. spectabilis* (leaves) (EC_{50} = 11.4 ± 0.8 µg/mL); *Polygonum spectabile* (EC_{50} = 24.2 ± 2.6 µg/mL), *O. spectabilis* (leaves) (EC_{50} = 8.9 ± 1.1 µg/mL) and *O. semiserrata* (leaves) (EC_{50} = 8.4 ± 0.7 µg/mL) against HSV-1, and *Hancornia speciosa* (stems) against EMCV (EC_{50} = 81.9 ± 4.4 µg/mL). These species are being further investigated by bioactivity-directed fractionation aiming to isolating antiviral compounds.

More than 50% of the 24 extracts tested showed some inhibitor effect on the CPE of the three viruses. Thirteen extracts (54.2 %) were active, with EC_{50} values in the ranging of 7.4 ± 0.1 to 465.7 ± 32.5 µg/mL. Nine of the active extracts (64.3 %) can be considered promising source of antiviral compounds as they showed $EC_{50} \leq 50$ µg/mL in high viral titer cultures (TCID 10^6). Virus inhibitions were observed for 8 out of the 10 ethnopharmacologically selected plant species (80.0 %) while for those that were taxonomically selected, this percentage was 50.0 % (2 active species out of 4 species tested).

In general, *in vitro* antiviral assays are based on the cytopathic effect (CPE) in a cell culture. In these assays, the activity is expressed by the 50% endpoint titration technique (EPTT) [30]. However, over the last two decades, the colorimetric MTT assay, in which the MTT dye is reduced by viable cells, has been frequently used. This assay is semi-automated, rapid, requires only a small amount of test sample and directly assesses cell viability [24, 43].

This paper is the first to report on the antiviral activity of the selected plant species. Informations on the phytochemistry and other biological effects is available for 13 out of the 14 species evaluated (Table 4). Annonaceae are considered as sources of alkaloids [44] and acetogenins [45, 46]. Until now, alkaloids have not been obtained from the three annonaceous plant species that were evaluated in the present work, although characteristic alkaloid spots, as well as saponins, triterpenes/steroids, coumarins, anthraquinones and flavonoids, have been observed on TLC. The two otherannonaceous species presently assayed, *Annona coriacea* and *Rollinia laurifolia*, afforded highly cytotoxic acetogenins [40, 41, 47, 48, 49], and anti-HSV-1 activity has been reported for this class of natural products [46]. A phytochemical investigation of several Brazilian *Aspidosperma* species (Apocynaceae), including *A. cylindrocarpon, A. parvifolium* and *A. tomentosum*, was intensively conducted in the 1960s, when many indolomonoterpenoid alkaloids were isolated [50]. Information on the biological activities of these alkaloids is very scarce, however. Only one, *A. tomentosum*, of the three *Aspidosperma* species evaluated, has shown antitumoral [51] and antimycobacterial activities [52]. Alkaloids were also isolated from *Tabernaemontana laeta* [53, 54], but no registration was found on their occurrence in *Himatanthus phagedaenicu*s and *Hancornia speciosa*. Iridoids were reported to occur in *H. phagedaenicu*s; flavonoids and myoinositol derivatives were isolated from *H. speciosa* [55, 56, 57]. The ethanol extract from this last species has gastroprotective and antimicrobial activity [58, 59].

The Ochnaceae family is chemically little known, the presence of catechins and biflavonoids, triterpenes, steroids and lignans in the genus *Ouratea* is reported [81]. Some biflavonoids, as well as extracts from *Ouratea hexasperma and O. semiserrata* showed anticancer activity [77]. From *Lophira alata*, an African species, tetrameric chalcones were isolated [82]. The present screening has detected tannins, saponins, flavonoids and terpenoids in the extracts of the Ochnaceae species evaluated. Several compounds belonging to these classes of natural products exhibit antiviral activity *in vitro* and *in vivo* [14, 83].

Table 4. Chemistry and biological activities reported for some of the plant species assayed for antiviral activity

Family Species	Compounds/extracts	Biological activity	Ref.
Annonaceae			
Anaxagorea	Monoterpenes		[60]
dolichocarpa	Sesquiterpenes		[61]
Annona coriacea	Ethanol extract	Genotoxic effect	[62]
	Acetogenins	Cytotoxic activity	[40, 63]
	Acetogenins	Cytotoxicity (*Artemia salina*)	[41]
	Diterpenes		[64]
	Lectin	Neutrophil induced migration in mice	[65]
	Lectin	Insecticidal activity	[66]
Rollinia laurifolia	Acetogenins	*In vitro* anticancer activity	[47, 48, 49]
Apocynaceae			
Aspidosperma	Alkaloids		[50, 67,
cylindrocarpon	Volatile oil		68]
			[69]
Aspidosperma	Alkaloids		[50, 70]
parvifolium	Triterpenos, steroids		[70]
Aspidosperma	Dichloromethane extract	Antimycobacterial	[52]
tomentosum	Dichloromethane and ethanol extracts		[51]
	Alkaloids		[50]
Hancornia speciosa	Ethanol extract	In vitro inhibition of angiotensin converting enzyme (ACE), vasodilation	[71]
	Ethanol extract	Gastroprotective	[72, 73]
	Ethanol extract		[58]
	Ethanol extract	Antimicrobial	[59]
	Flavonoids, *myo*-inositol		[56, 57]
	derivative	NF-κB inhibitory activity	[74]
	Proanthocyanidins		
Himatanthus	Iridoids		[55]
phagedaenicus			
Tabernaemontana laeta	Alkaloids		[53, 54]
Ochnaceae			
Ouratea castanaefolia	Ethanol extract	Vasodilatation	[75]
	Flavonoids, triterpenes, steroids		[76]
O. semiserrata	Ethanol extract	Vasodilatation	[75]
	Biflavonoids, diterpenes, isoflavones	Antitumor activity	[77]
*O. spectabili*s	Biflavonoids	Inhibition of lens aldose reductase	[78]
Polygonaceae			
Polygonum spectabile	Extracts, flavones, chalcones, steroids	Antimicrobial and antiviral activities	[79, 80]

Polygonum spectabile and *Cissus erosa*, the only evaluated representatives of the families Polygonaceae and Vitaceae, respectively, are both active against HHV-1 and VACV-WR. Flavonoids, anthraquinones, sesquiterpenes, tannins, and stilbene C-glucosides are some of the classes of secondary metabolites isolated from species of these genera [84, 85, 86, 87, 88]. There are several reports describing the antiviral activity of *Polygonun* spp. extracts; recently, the anti-HIV activity of a quercetin glycoside and a sesquiterpene, viscoazulone, isolated from *Polygonum viscosum*, was reported [89]. A methanol extract from *C. subaphylla*, in the genus *Cissus*, has exhibited anti-HHV-1 activity [90].

CONCLUSION

This screening discloses the high potential of ethnopharmacogically selected plant species as sources of antiviral agents. Further studies are on progress aiming at the identification of antiviral constituents from the most promising plant species, particularly those which have disclosed high activity against vaccinia and herpes viruses.

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