Antiviral Effect of *Guazuma ulmifolia* and *Stryphnodendron adstringens* on Poliovirus and Bovine Herpesvirus

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Crude extract (CE) and aqueous (AqF) and ethyl acetate (EtOAcF) fractions of *Guazuma ulmifolia* Lam., *Sterculiaceae* and the corresponding AqF, EtOAcF of *Stryphnodendron adstringens* (Mart.) Coville, *Leguminosae* were tested for their antiviral activity against poliovirus 1 (P-1) and bovine herpesvirus 1 (BHV-1) in HEp-2 cultured cells. The antiviral activity was monitored by plaque assay and immunofluorescence assay (IFA) under virucidal and therapeutic protocols. The therapeutic protocol demonstrated statistically significant positive results with both plants and for both virus strains. The highest percentages of viral inhibition were found for *G. ulmifolia* EtOAcF which inhibited BHV-1 and P-1 replication by 100% and 99%, respectively ($p<0.05$, Student's *t*-test). For *S. adstringens*, AqF was the most efficient, inhibiting BHV-1 and P-1 by 97% and 93%, respectively ($p<0.05$). In the virucidal protocol, *G. ulmifolia* CE inhibited the replication of BHV-1 and P-1 by 60% and 26%, respectively ($p<0.05$), while, for *S. adstringens*, inhibition of 62% ($p<0.05$) was demonstrated only with EtOAcF for P-1. IFA demonstrated that the greatest reduction in fluorescent cell number occurred with *G. ulmifolia*, under the therapeutic protocol for both virus strains. However, AqF and EtOAcF of *S. adstringens* were most efficient with the virucidal protocol for P-1. In conclusion, we demonstrated that *G. ulmifolia* and *S. adstringens* inhibited BHV-1 and P-1 replication, as well as, blocked the synthesis of viral antigens in infected cell cultures.

Key words antiviral activity; plant extracts; poliovirus; bovine herpesvirus; cell culture

Works on antiviral compounds date back to the 1950s, but for several reasons, only a couple of drugs were approved for clinical use about a decade later. To date, many antiviral drugs have been developed after extensive research and demanding trials, some of them with selective mechanisms against viral replication.[1)] Although the primary focus has been on synthetic products, the number of natural compounds with antiviral action for different DNA and RNA viruses that are being studied is increasing. Many phytochemical compounds are being investigated based primarily on ethnopharmacologic knowledge and have been considered of great importance in new drug development.[2)]

Extracts of many plant species, such as *Tridax procumbens*, *Carissa carandas*, *Mallotus philippensis*, *Streblus aspere*, *Terminatta alata*, *Macaranga pustulata*, *Sibbaldia micropetala*, *Hypericum cordifolium*, *H. uralum* and *Maesa macrophylla* were found to be active against the replication of poliovirus and herpes simplex-1 (HSV-1), with some of them also active against Sindbis virus.[3,4)] A methanol extract of *Annona muricata* and aqueous extract of *Petunia myctaginiflora* were shown to inhibit HSV-1 cytopathic effect in Vero cells, at a concentration of 1 mg/ml.[5)] Methanol extracts of Indonesian plants were tested for cytotoxicity and antiviral activity (HSV-1 and poliovirus) in murine and human cell lines. *Piper aduncum* was found to be active on poliovirus, while *Elytranthe tubaeflora* and *Melastoma malabathricum* inhibited HSV-1.[6)] Dengue virus was inhibited by an aqueous extract of *Azadirachta indica* when assayed in mice.[7)]

Stem bark of *Stryphnodendron adstringens* (Mart.) Coville, *Leguminosae*, popularly known in our country as *barbatimão*, has been empirically used as wound healing, astringent, antimicrobial, antifungal, antidiarrheal and hypoglycemic agents.[8)] Similarly, *Guazuma ulmifolia* Lam., *Sterculiaceae*, popularly known as *mutamba*, is known to have medicinal properties, such as wound healing, antiulcerogenic, hypoglycemic and antimicrobial.[9)] *G. ulmifolia* aqueous and methanol fractions were found to inhibit HSV-1 replication, respectively, by 16.8% and 4.5%, at a concentration of 100 μg/ml, in a study of various plants. Those showing antiviral activity *in vitro* were also tested in HSV-1 infected mice. *Melaleuca leucadendron* and *Nephelium lappaceum* reduced mortality and delayed the appearance of lesions.[10)]

Among plant antiviral compounds, anthraquinones found in *Aloe barbadensis* inhibited HSV-1, HSV-2, varicela-zoster, pseudorabies and influenza.[11)] *Stevia rebaudiana* polysaccharides inhibited adsorption of human rotavirus in MA-104 cells.[12)] Flavonoids present in *Troillius chinensis* flowers also inhibited parainfluenza in HEp-2 cells.[13)] *Quassia africana* extracts were tested for HSV-1, coxsackie B2, polio-1, measles, Semliki Forest virus and vesicular stomatitis virus, where simalikalactone D was found to be responsible for the antiviral effect.[14)] Anagyrine, oxymatrine, sophoranol, wogonin and oroxylin found in *Sophora flavescens* and *Scutellaria baicalensis* were found to be responsible for inhibiting respiratory syncytial virus.[15)]

Herpesviruses are widespread, enveloped and double-stranded DNA agents which cause various infections in human and animals, especially in immunocompromised individuals. Bovine herpesvirus type 1 (BHV-1), a member of the family *Herpesviridae*, and sub-family *Alphaherpesviri-*

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nae, is an important pathogen in cattle. It is responsible for a variety of clinical signs and especially for a respiratory disease called infectious bovine rhinotracheitis (IBR). Conventional attenuated vaccines have contributed to disease regression. However, disease control is still difficult due to the establishment of life-long latency after primary infection or after vaccination with attenuated viruses.[16)]

Poliovirus is a single-stranded RNA, non-enveloped virus and a member of the *Picornaviridae* family. When the virus spreads to the central nervous system, it may develop paralytic poliomyelitis. The incidence of paralytic poliomyelitis has been reduced over the last decades, especially by the systematic use of a vaccine; however, the disease is still endemic in Asia and Africa.[17)]

The current work investigated the *in vitro* antiviral activity of extracts of *Guazuma ulmifolia* LAM. and *Stryphnodendron adstringens* (MART.) COVILLE against bovine herpesvirus-1 and polio-1.

MATERIALS AND METHODS

Plant Materials Stem bark of *Guazuma ulmifolia* LAM. and *Stryphnodendron adstringens* (MART.) COVILLE was collected in the rural area of Ibiporã and São Jerônimo da Serra in Paraná State (Brazil), during the period of 2001—2002. The specimens were authenticated by Prof. Cássia Mônica Sakuragui (Dept. of Biology, UEM) and vouchers were deposited at UEM herbarium, identified as HUM 1182 and HUM 3800, respectively, for *G. ulmifolia* and *S. adstringens.*

Extracts Bark samples from both species were submitted to extraction with acetone/water (7 : 3, v/v) for 15 min in an Ultra-Turrax (UTC5KT). Briefly, the solvent was removed under reduced pressure, and the residue was dried and identified as crude extract (CE). CE was dissolved in water (50 g/500 ml) and liquid–liquid partition carried out with ethyl acetate (EtOAc) (10×500 ml).[18)] EtOAc and aqueous phases were separated and concentrated, and the solvent removed; these two fractions were designated EtOAcF and AqF, respectively. *S. adstringens* CE was not available for testing.

EtOAc and Aq fractions of both plants, and CE of *G. ulmifolia* were dissolved in sterile water at a concentration of 100 μg/ml, and dimethyl sulfoxide was added to EtOAcF at a final concentration of 0.25%. Fractions and extract were stored at −8 °C.

HPLC Characterization of EtOAc Fraction HPLC was carried out with a Gilson apparatus (France) consisting of a 321 pump, Gilson UV/VIS detector 156 and Rheodyne manual injector (20.0 μl). A LiChrospher® 100 RP-18 column (250×4 mm I.D., 5 μm) was employed (Merck, Germany) at 30 °C with a flow rate of 0.5 ml/min. Analyses were performed at 210 and 280 nm. A gradient mobile phase system consisting of MeOH (A) and acetic acid (pH 2.1) (B) was employed: 0—20 min: 0.05→1% A; 20—27 min: 1.0→6.0% A; 27—35 min: 6.0→15.0% A; 35—40 min: 15→80% A; 40—45 min: isocratic 80% A; 45—50 min: 80→0.05% A. HPLC-grade solvents (Mallickrodt, U.S.A.) were degassed with a Gilson degasser 184. The EtOAcF sample was dissolved in MeOH at a concentration of 1 mg/ml. Epigallocatechin (UEM, Brazil), gallocatechin (provided by the Institut für Pharmazeutische Biologie und Phytochemie der Universität Münster, Germany), catechin, and epicatechin (Sigma Chem. Co., U.S.A.) were used as reference standards at 1 mg/ml.

Cell Cultures HEp-2 (human larynx carcinoma) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (*), supplemented with 8% fetal bovine serum (Gibco BRL, U.S.A.), 100 μg/ml streptomycin (*), 100 IU/ml penicillin (*Sigma Chem. Co., U.S.A.) and 2.5 μg/ml amphotericin B (Bristol-Myers Squibb, Brazil). Cultured cells were incubated at 37 °C in a 5% CO_2 atmosphere.

Virus The strain of poliovirus type 1 (P-1), Sabin (ATCC VR-192), was provided by the Department of Virology, IMPPG-UFRJ. The strain of bovine herpesvirus type 1 (BHV-1) was provided by Laboratory of Virology, DMVP, UEL. Both virus strains were propagated in HEp-2 cell cultures to yield titers of 0.8×10^7 pfu/ml and 0.6×10^7 pfu/ml for BHV-1 and P-1, respectively,

Cytotoxicity Assay Cytotoxicity of crude extracts and fractions at concentrations of 5, 25 and 50 μg/ml was determined as described by Huheihel *et al.*[19)] The cultures were observed daily for seven days for evidence of any morphological changes.

Virus Strains Treatment with Interferon and Cytosine Arabinoside (Ara-C) Virus titers of 10^8 pfu/ml (P-1) and 10^7 pfu/ml (BHV-1) were submitted to treatment with human alfa-2 B interferon (Meizler Com. Intern. SA, Brazil) at concentrations of 1000 U/ml, 10000 U/ml and 100000 U/ml. Additionally, BHV-1 was also assayed with Ara-C (Pharmacia NV/SA, Belgium) at the concentration of 50 mg/ml. These compounds were used as positive control.

Antiviral Activity Sub-confluent cell cultures grown in 24-well microplates (Corning, U.S.A.) were infected with viruses at ten-fold serial dilutions (six wells for each dilution) and tested for antiviral activity in triplicate using the virucidal and therapeutic protocols. For virucidal activity, virus strains at a titer of 10^6 pfu/ml were incubated at 37 °C for 1 h with DMEM containing extracts or fractions at a concentration of 5 μg/ml. Thereafter, treated virus strains were diluted tenfold in DMEM and added to sub-confluent cell cultures grown in 24-well microplates for 1 h adsorption by cells, followed by the removal of the excess inoculum and cell washing. Cells were overlaid with 0.75% nutrient agarose, and for P-1, 25 mM $MgCl_2$ was added. After 2 days incubation at 37 °C, cells were fixed with 10% formaldehyde in phosphate-buffered saline (PBS), pH 7.3, nutrient agarose layer removed, and cells stained with 0.5% crystal violet in 20% ethanol. Plaques were counted and antiviral activity defined as percent of viral inhibition, as follows: % VI=[1−(number of plaques test/number of plaques control)]×100. For therapeutic activity, virus strains were diluted tenfold in DMEM and added to cell cultures for 1 h adsorption. Cultures were overlaid with nutrient agarose containing 5 μg/ml of the test substance and maintained throughout. Antiviral activity was calculated as previously described.

Immunofluorescence Assay (IFA) Sub-confluent HEp-2 cell cultures grown in 24-well microplates provided with slides were infected with virus strains at a multiplicity of infection of 1. At time intervals of 7 h and 8 h post-infection for P-1 and BHV-1, respectively, cultures treated with extract and fractions according to the therapeutic protocol, as described above, were submitted to IFA as detailed else-

Table 1. Antiviral Activity of *Guazuma ulmifolia* and *Stryphnodendron adstringens* Extracts for Bovine Herpesvirus-1 (BHV-1) and Poliovirus-1 (P-1)

Extracts	Therapeutic activity							
	BHV-1				P-1			
	Viral titer[a)]	Viral titer[b)]	% VI[c)]	*p*	Viral titer[a)]	Viral titer[b)]	% VI[c)]	*p*
CE	4.50	0.13	97	<0.05	8.50	3.21	62	<0.05
AqF[a]	7.06	0.02	99	<0.05	9.54	0.36	96	<0.05
EtOAcF[a]	7.20	Zero	100	<0.05	7.20	0.03	99	<0.05
AqF[b]	10.97	0.26	97	<0.05	4.90	0.30	93	<0.05
EtOAcF[b]	7.69	3.48	54	<0.05	3.56	0.33	90	<0.05

Extracts	Virucidal activity							
	BHV-1				P-1			
	Viral titer[a)]	Viral titer[b)]	% VI[c)]	*p*	Viral titer[a)]	Viral titer[b)]	% VI[c)]	*p*
CE	8.80	3.60	60	<0.05	5.01	3.70	26	<0.05
AqF[a]	4.41	1.98	55	<0.05	0.58	0.44	23	<0.05
EtOAcF[a]	5.49	3.95	28	<0.05	3.64	2.71	25	<0.05
AqF[b]	6.01	7.06	0	<0.05	5.10	5.45	0	<0.05
EtOAcF[b]	6.87	6.12	10	<0.05	4.86	1.82	62	<0.05

a) Control viral titer ($\times 10^6$ pfu/ml); *b*) viral titer after the treatment with extracts ($\times 10^6$ pfu/ml); *c*) percent of viral inhibition; *p* by Student's *t*-test. CE, *G. ulmifolia* crude extract; AqF[a], *G. ulmifolia* aqueous fraction; EtOAcF[a], *G. ulmifolia* ethyl acetate fraction; AqF[b], *S. adstringens* aqueous fraction; EtOAcF[b], *S. adstringens* ethyl acetate fraction.

where.[20)] Inhibition of virus replication was scored and represented as percent reduction in the number of cells with specific fluorescence.

RESULTS AND DISCUSSION

The HPLC profile was obtained at two different wavelengths, before and after adding the reference compounds (epigallocatechin, gallocatechin, catechin and epicatechin). EtOAcF of *S. adstringens* was much more complex than that of *G. ulmifolia*. EtOAcF demonstrated a wide variety of condensed tannins, which have been previously isolated and identified.[18,21)] The same was true for *G. ulmifolia*.[9,22)] The retention times recorded for epigallocatechin, gallocatechin, catechin and epicatechin were 8.0, 5.1, 5.1 and 7.4 min, respectively, at the respective concentrations of 2.0, 4.0, 6.9 and 11.1% for the EtOAcF.

P-1 and BHV-1 strains were inhibited in 100% when treated with interferon at the concentrations used. Additional treatment of BHV-1 with Ara-C resulted in an inhibition of 60%. Both antivirals were assayed by plaque assay.

CE, AqF and EtOAcF, when assayed for cytotoxicity at concentrations of 5, 25 and 50 μg/ml, did not cause any morphological changes in HEp-2 cells with both plants (*S. adstringens* CE was not available for assay). Therefore, extract and fractions were tested for antiviral activity at a concentration of 5 μg/ml throughout.

Following the virucidal protocol, we demonstrated that CE of *G. ulmifolia* inhibited BHV-1 and P-1 by 60% and 26%, respectively. AqF also inhibited BHV-1 by 55%, but EtOAcF inhibited BHV-1 and P-1 by 28% and 25%, respectively. Percent of virus inhibition varied from 25 to 60% and was statistically significant ($p<0.05$) (Table 1). With regard to *S. adstringens* using the same protocol, EtOAcF inhibited P-1 by 62% and BHV-1 by 10%, while AqF showed no effect.

The highest levels of virus inhibition were obtained with the therapeutic protocol (Table 1). CE of *G. ulmifolia* inhibited BHV-1 and P-1 by 97% and 62%, respectively, while the extent of inhibition varied from 96 to 100% with AqF and EtOAcF for both viruses. AqF and EtOAcF of *S. adstringens* also inhibited both virus strains and the percent inhibition varied from 54% to 97% ($p<0.05$). As the best inhibitions were obtained with *G. ulmifolia* EtOAcF and *S. adstringens* AqF, at the concentration of 5.0 μg/ml, under therapeutic protocol, for both virus strains, dose–response experiment was carried out. These fractions were used at the concentrations varying from 2.5 μg/ml to 40.0 μg/ml and dose-dependent results are shown in Table 2. Table 3 shows the effect of *G. ulmifolia* and *S. adstringens* on the replication of the two viruses by IFA. The least effect on the number of fluorescent cell was demonstrated for BHV-1 under the virucidal protocol for both plants. However, in the therapeutic approach, the test substances demonstrated reductions of 14 to 65% for

Table 2. Dose-Dependent Response of the *Guazuma ulmifolia* Ethyl Acetate Fraction (EtOAcF) and *Stryphnodendron adstringens* Aqueous Fraction (AqF) in the Replication of Bovine Herpesvirus-1 (BHV-1) and Poliovirus-1 (P-1)

Fractions	(μg/ml)	Viral inhibition (%)	
		BHV-1	P-1
EtOAcF	2.5	97.5±1.0[a)]	85.7±5.6
	5.0	98.3±0.5	95.7±2.1
	10.0	100.0	100.0
	20.0	99.2±0.5	100.0
	40.0	100.0	100.0
AqF	2.5	12.6±4.2	44.2±0.7
	5.0	24.9±11.3	84.2±0.7
	10.0	27.7±16.9	98.5±0.7
	20.0	60.2±1.4	100.0
	40.0	91.8±4.9	100.0

Percent of virus inhibition under therapeutic protocol monitored by plaque assay. *a*) Standard deviation.

Table 3. The Effect of *Guazuma ulmifolia* and *Stryphnodendron adstringens* Extracts on the Number of Fluorescent Cells Infected with Bovine Herpesvirus-1 (BHV-1) and Poliovirus-1 (P-1) after 8 h and 7 h p.i., Respectively, Monitored by Immunofluorescence Assay

Extracts	BHV-1		P-1	
	Virucide	Therapeutic	Virucide	Therapeutic
CE	—	4+	2+	2+
AqF[a]	—	4+	—	4+
EtOAcF[a]	1+	4+	1+	5+
AqF[b]	2+	3+	5+	—
EtOAcF[b]	—	1+	5+	2+

Reduction in number of fluorescent cells (score, % range): —, none; 1+, 1—20%; 2+, 21—40%; 3+, 41—60%; 4+, 61—80%; 5+, 81—100%. CE, *G. ulmifolia* crude extract; AqF[a], *G. ulmifolia* aqueous fraction; EtOAcF[a], *G. ulmifolia* ethyl acetate fraction; AqF[b], *S. adstringens* aqueous fraction; EtOAcF[b], *S. adstringens* ethyl acetate fraction.

both plants. The decrease in fluorescent cells with P-1 varied from 6 to 96% under virucidal protocol and 27 to 82% in the therapeutic procedure, for both plants. We also demonstrated that the inhibition of both viruses was more pronounced under the therapeutic protocol. This finding was supported by IFA; however, conflicting results were found for Aq and EtOAc fractions of *S. adstringens* against P-1 with the virucidal method.

Tannins, flavonoids, anthraquinones and polysaccharides, among others, are compounds found in plant extracts and have been implicated in the inhibition of virus replication *in vitro*.[6,10,12,13,15] These compounds have been shown to act either in the early stage of adsorption or on virus surface structures thereby inhibiting penetration into susceptible cells. These modes of action favor the basis of the virucidal protocol as the more relevant approach for determining antiviral effect (eventually, prophylactic, where cells are treated before the infection). However, our results demonstrated that overall the therapeutic protocol was more consistent, although we still do not know the nature of the antiviral compounds. The identification of these compounds is crucial for better understanding of their antiviral mechanism. In conclusion, crude extract and fractions of *G. ulmifolia* and *S. adstringens*, were shown to inhibit BHV-1 and P-1 replication when assayed for effect on viral infectivity and presence of viral antigens in infected cells. We suggest that the antiviral effect is more likely to occur after the entry of the virus strains in the subsequent stages of their replication in cell culture.

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