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Antiviral Activity of Brazilian Plant Extracts

Jarbas A. MONTANHA [1]*, Patrícia MOELLERKE [1],
Sérgio A.L. BORDIGNON [2], Eloir P. SCHENKEL [3] & Paulo M. ROEHE [4]

[1]*Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752 (UFRGS), Porto Alegre, RS CEP 90610-000, Brasil.* [2] *Curso de Biologia da Universidade Luterana do Brasil, Gravatai, RS, Brasil; Herbário La Salle, Centro Universitário La Salle, Canoas,RS, Brasil.*
[3] *Departamento de Ciências Farmacêuticas - Universidade Federal de Santa Catarina, Trindade, Florianópolis, SC, Brasil.* [4] *Laboratório de Virologia / Instituto de Ciências Básicas da Saúde, Departamento de Microbiologia (UFRGS)*

SUMMARY. Ethanolic and aqueous extracts of fifty one plant species collected in Southern Brazil were tested for antiviral activity against herpes simplex virus type 1 (HSV-1) on Vero cell line. Nine of the species tested, namely *Aloysia gratissima* (Gill. & Hook.), *Baccharis eriоclada* DC., *Baccharis megapotamica* Hook & Arn., *Baccharis uncinella* DC., *Glechon marifolia* Benth., *Glechon spathulata* Benth., *Ilex brevicuspis* Reiss., *Ilex theezans* Mart. and *Maytenus ilicifolia* Mart. ex Reiss. exhibited antiherpetic activity.
RESUMEN. "Actividad antiviral de extractos de plantas de Brasil". Los extractos acuoso e hidroetanólico de cincuenta y una especies vegetales recolectadas en la región del Sur de Brasil fueron estudiadas en cuando a su posible actividad antiviral. De las especies ensayadas, nueve inhibhieron el efecto citopático viral (HSV-1) en células Vero: *Aloysia gratissima* (Gill. & Hook.), *Baccharis eriоclada* DC., *Baccharis megapotamica* Hook & Arn., *Baccharis uncinella* DC., *Glechon marifolia* Benth., *Glechon spathulata* Benth., *Ilex brevicuspis* Reiss., *Ilex theezans* Mart. y *Maytenus ilicifolia* Mart. ex Reissek.

INTRODUCTION

Unlike antimicrobial drugs against bacteria and fungi, only a few effective antiviral drugs are available. One of the most important reasons for the lack of success in developing antiviral drugs is due to the nature of the infectious viral agents, which totally depend upon the cell they infect for their multiplication and survival, so that many compounds that may cause the death of viruses also are very likely to injure the host cell that harbour them [1].

On of the possible method which can be used for the discovery of active substances is the screening of plant extracts for antiviral activity followed by bioassay guided fractionation of active extracts to identify the active substance.

In searching for natural products as potential antiviral agents, several medicinal plants from the South American folk medicine showed *in vitro* antiviral activity [2-4].

In an effort to discover new compounds, with relevant anti-herpes virus activity, the aim of our study was to examine the potential antiviral activity of native species from South Brazil. The species were selected mainly on the basis of their ethnopharmacological indications for treatment of viral infections such as influenza virus, bronchopulmonary infections and cold, but some others species were collected randomly.

In this study, hydroethanolic and aqueous crude extracts from fifty species were screened for in vitro antiviral activity against herpes simplex virus type I (HSV-1) in Vero cell line.

MATERIALS AND METHODS

Plant material

The plants tested were collected from the Rio Grande do Sul State area, Brazil. Herbarium specimens are deposited in the Herbarium of the Federal University of Rio Grande do Sul, Brazil (ICN). The air-dried plant materials were grounded and extracted by maceration in 50% ethanol. An aqueous extract was also prepared with dried plant material (100 g) in hot distilled water not exceeding 60 °C (200 ml). The extracts were filtered, the ethanol removed, lyophilized and stored at –20 °C until tested.

KEY WORDS: Antiviral screening, Brazilian plants, Herpes simplex virus Type 1.
PALABRAS CLAVE: Tamizaje antiviral, Plantas de Brasil, Virus del Herpes Simplex Tipo 1.

* Author to whom correspondence should adressed. *E-mail:* Montanha@farmacia.ufrgs.br

Cells and viruses

African green monkey kidney cells (Vero cell line ATCC CCL-81) were grown in Eagle's minimum essential medium (MEM) supplemented with 10 % newborn calf serum, 2 µg. ml^{-1} of amphotericin B and 10 $mg.ml^{-1}$ of enrofloxacin. A virus stock of herpes simplex virus type I, strain KOS (University of Rennes/France), ATCC-VR733 and 29R-acyclovir resistant were prepared on Vero cells infected at a low multiplicity of infection, incubated for 1 to 2 days, frozen/thawed three times, before clearing the preparation by centrifugation at low speed to remove the cell debris. Virus stocks were maintained in liquid nitrogen until use. Virus titration was performed by Kärber method [5] using a 96-well microtitre plates. The virus titre was estimated from cytopathogenicity and expressed as 50% tissue culture infectious doses ($TCID_{50}.ml^{-1}$). It was 10^{-6} TCID50.ml^{-1} for strain KOS; 10^{-4} $TCID_{50}.ml^{-1}$ for strain ATCC and 10^{-4} $TCID_{50}.ml^{-1}$ for strain 29R-acyclovir resistant.

Evaluation of cytotoxicity

To assess the effect of extracts on uninfected Vero cells, dilutions ranging from 40 $mg.ml^{-1}$ to 0.15 µg.ml^{-1} in the MEM medium, were added to Vero monolayers (using a 96-well microplate with 4.0 x 10^4 cells per well). After 72 h of incubation at 37 °C, cytotoxicity was determined by microscopic examination of cell morphology in treated and untreated cultures. The concentra-

Species	Type of Extract	CC_{50} mg/ml	MTC mg/ml	Active Concentration mg/ml (CPE)		
				KOS	ATCC	AC-R
Aquifoliaceae						
Ilex brevicuspis Reiss.	aqueous	10	5	5	5	NA
Ilex theezans Mart.	aqueous	2	0.25	0.25	0.25	NA
Asteraceae						
Baccharis erioclada DC.	aqueous	2.5	1.25	1.25 0.625 0.312	1.25 0.625 NA	1.25 NA NA
	hydroethanolic	1	0.5	0.5 0.25	0.5 NA	0.5 NA
Baccharis megapotamica Hook. & Arn.	hydroethanolic	0.0097	0.0048	0.0048	NA	NA
Baccharis uncinella DC.	hydroethanolic	2.5	1.25	1.25 0.625 0.312	NA NA NA	1.25 NA NA
Celastraceae						
Maytenus ilicifolia Mart. ex Reiss.	aqueous	2.5	1.25	1.25	1.25	NA
	hydroethanolic	2	0,5	0.5	0.5	NA
Lamiaceae						
Glechon marifolia Benth.	hydroethanolic	5	1.25	1.25 0.625	1.25 0.625	NA NA
Glechon spathulata Benth.	hydroethanolic	2	0,5	0.5 0.25	0.5 0.25	0.5 0.25
Verbenaceae						
Aloysia gratissima (Gill. & Hook.) Troncoso	hydroethanolic	2.5	1.25	1.25	1.25	NA

Table 2. Plant extracts which showed antiherpetic activity (HSV–1) strains KOS, ATCC-VR733 and 29 R-acyclovir resitant. CC_{50}: 50% cytotoxic concentration (mg/ml). MTC: maximum tolerated concentration (mg/ml). NA: not active.

Species	Type of Extract	CC_{50} (mg/ml)	MTC (mg/ml)
Anacardiaceae			
Schinus lentiscifolius Marchand	aqueous	5	2.5
	hydroethanolic	0.12	0.03
Aquifoliaceae			
Ilex brevicuspis Reiss.	hydroethanolic	0.25	0.125
Ilex dumosa Reiss.	aqueous	0.125	0.0312
	hydroethanolic	0.25	0.0625
Ilex microdonta Reiss.	aqueous	7.5	2.5
	hydroethanolic	0.5	0.25
Ilex paraguariensis A.St.-Hil.	aqueous	10	5
	hydroethanolic	2	0.5
Ilex theezans Mart.	hydroethanolic	0.5	0.25
Asteraceae			
Baccharis articulata Pers.	aqueous	7.5	2.5
	hydroethanolic	0.5	0.25
Baccharis cylindrica DC.	aqueous	1	0.5
	hydroethanolic	0.625	0.3125
Baccharis conyzoides DC.	aqueous	0.465	0.15
	hydroethanolic	0.31	0.15
Baccharis erigeroides DC.	aqueous	0.0037	0.00092
	hydroethanolic	0.00048	0.00024
Baccharis megapotamica Hook & Arn.	aqueous	0.0039	0.0019
Baccharis mesoneura DC.	aqueous	2.5	1
	hydroethanolic	2	0.5
Baccharis patens Baker	aqueous	62.5	0.03125
	hydroethanolic	62.5	0.0156
Baccharis pseudotenuifolia L. Teodoro	aqueous	2.5	1.25
	hydroethanolic	1	0.25
Baccharis punctulata DC.	aqueous	2	0.5
	hydroethanolic	1	0.5
Baccharis radicans DC.	aqueous	0.125	0.031
	hydroethanolic	0.062	0.031
Baccharis semiserrata Steud. ex Baker	aqueous	0.125	0.0625
	hydroethanolic	0.125	0.0625
Baccharis spicata Hieron.	aqueous	2.5	1.25
	hydroethanolic	2	0.5
Baccharis trimera Less.	aqueous	2.5	1
	hydroethanolic	1	0,25
Baccharis trinervis Pers.	aqueous	0.062	0.031
	hydroethanolic	0.031	0.015
Baccharis uncinella DC.	aqueous	5	2.5
Baccharis usterii Heering	aqueous	0.5	0.12
	hydroethanolic	2	1
Mikania involucrata Hook & Arn.	aqueous	1	0.25
	hydroethanolic	0.625	0.15
Solidago chilensis Meyen	aqueous	2	1
	hydroethanolic	0.5	0.125
Vernonia tweedieana Baker	aqueous	1	0.25
	hydroethanolic	0.5	0.125
Bromeliaceae			
Tillandsia aeranthos Loisel	hydroethanolic	0.25	0.125
	aqueous	2.5	1.25
Tillandsia usneoides L.	aqueous	2.5	1.25
	hydroethanolic	0.156	0.078
Flacourtiaceae			
Casearia sylvestris Sw.	aqueous	5	2.5
	hydroethanolic	1.25	0.625
Lamiaceae			
Cunila menthiformis Epling	aqueous	1	0.25
	hydroethanolic	0.25	0.06
Glechon ciliata Benth.	aqueous	0.25	0.12
	hydroethanolic	1	0.25
Glechon marifolia Benth.	aqueous	0.5	0.12
Glechon spathulata Benth.	aqueous	0.25	0.12
Glechon thymoides Spreng	aqueous	0.5	0.12
	hydroethanolic	1	0.25
Passifloraceae			
Passiflora actinia Hook.	aqueous	2.5	0.06
	hydroethanolic	0.06	0.03
Passiflora alata Curtis	aqueous	0.3	0.15
	hydroethanolic	0.15	0.08
Passiflora capsularis L.	aqueous	10	2.5
	hydroethanolic	5	1.25
Passiflora edulis Sims	aqueous	16	1
	hydroethanolic	5	1.25
Passiflora misera Kunth	aqueous	5	2.5
	hydroethanolic	5	1.25
Passiflora suberosa L.	aqueous	10	5
	hydroethanolic	5	2.5
Passiflora warmingii Mast.	aqueous	10	5
	hydroethanolic	5	1.25
Poaceae			
Brachiaria decumbens Stapf	aqueous	1	0.5
	hydroethanolic	0.125	0.0625
Sapotaceae			
*Chrysophyllum marginatum (*Hook. & Arn.) Radlk.	aqueous	2.5	1.25
	hydroethanolic	0.031	0.015
Solanaceae			
Solanum cernuum Vell.	aqueous	20	5
	hydroethanolic	10	2.5
Solanum nigrescens M. Martens & Galeotti	aqueous	10	2.5
	hydroethanolic	0.12	0.03
Styracaceae			
Styrax leprosum Hook. & Arn.	aqueous	5	2.5
	hydroethanolic	0.12	0.06
Symplocaceae			
Symplocos uniflora Benth.	aqueous	5	2.5
	hydroethanolic	0.25	0.06
Tiliaceae			
Luehea divaricata Mart.	aqueous	2.5	0.625
	hydroethanolic	1.25	0.625
Verbenaceae			
Aloysia gratissima (Gill. & Hook.) Troncoso	aqueous	5	2.5
Lantana camara L.	aqueous	0.04	0.02
	hydroethanolic	0.01	0.005

Table 1. Plant extracts which proved inactive against HSV-1 strain KOS. CC_{50}: 50% cytotoxic concentration (mg/ml). MTC: maximum tolerated concentration (mg/ml).

tion of the extract at which the cell number was reduced to 50% of the controls, was taken as the 50% cytotoxic concentration (CC_{50}). The maximum concentration at which no reduction on cell numbers was observed (compared to controls) was considered as the maximum tolerated concentration (MTC) [6-9]. The MTC was determined for each extract before proceeding to make antiviral activity assays. All assays were carried out in quadruplicate.

Antiviral activity

Dilutions of the extracts were prepared starting from the previously determined MTC. Extracts from MTC, MTC/2, MTC/4, MTC/8 and MTC/16 were added on confluent 24 h old monolayers of Vero cells grown in microtitre tissue culture plates just before virus inoculation. One hundred tissue culture infection doses per 50 ml ($TCID_{50}$) of the HSV-1 KOS strain were added to each of the wells. Toxicity controls, cell and virus controls titration were rum simultaneously. All assays were carried out in quadruplicate. When some antiviral activity was detected on HSV-1 strain KOS, the extracts were also tested against strain ATCC-VR733 and 29 R-acyclovir resistant. Plates were incubated for 72 h at 37 °C, and then examined for the presence of cytopathic effects (CPE). The concentration of extract which inhibited 100% of the viral CPE (compared to the controls) was considered as the active concentration.

RESULTS AND DISCUSSION

In the course of studies for the evaluation of natural products with antiviral properties we have investigated anti-HSV-1 activity *(in vitro)* of the water and hydroethanolic extracts, prepared from fifty one plant species of fifteen different botanical families collected in Southern Brazil. The antiviral activity was evaluated by inhibition of CPE in cultures inoculated with 100 tissue culture infecting doses ($TCID_{50}$). The concentrations ($mg.ml^{-1}$) causing 50% cytotoxicity in uninfected Vero cells (CC_{50}) and the maximal tolerated concentration (MTC) for inactive plant extracts are indicated in Table 1, and the active extracts concentration required for a 100% CPE inhibition are indicated in Table 2. We found that the aqueous extracts of *Maytenus ilicifolia, Ilex theezans, Ilex brevicuspis, Baccharis eriocla-da* and the hydroethanolic extracts of *Aloysia gratissima, Baccharis eriocla*d*a, Baccharis megapotamica, Baccharis uncinella, Glechon marifolia, Glechon spathulata,* and *Maytenus ilicifolia* showed inhibition of herpesvirus CPE at a MTC and in some cases MTC/2 (Table 2). Aqueous extract of *Baccharis eriocla*d*a* and hydroethanolic extracts of *Baccharis eriocla*d*a* and *Glechon spathulata* also inhibited the CPE of HSV-1 acyclovir-resistant and ATCC strain, thus we concluded that these extracts have an important antiviral activity and are potential candidates for further studies of activity-monitored fractionation to identify the active principles.

Our results gives conclusive evidence that nine species tested have antiviral activity. Present research in our laboratory is focused on the fractionation of the active extracts and isolation of the active principles (s) from these extracts.

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REFERENCES

1. Vlietinck, A.J, T. De Bruyne & D.A. Vanden Berghe (1997) *Curr. Org. Chem*. **1**: 307-44.
2. Abad, M.J., P. Bermejo, S. Palomino, X. Chiriboga& L. Carrasco (1999) *Phytother. Res.* **13**: 142-6.
3. Betancur-Galvis, La., J. Saez, H. Granados, A. Salazar & Je. Ossa (1999) *Mem. Inst. Oswando Cruz*. **94**: 531-5.
4. Simões, C.M.O., M. Falkenberg, L.A. Mentz, E.P. Schenkel, M. Amoros & L. Girre (1999) *Phytomedicine* **6**: 205-14.
5. Payment, P. & M. Trudel (Ed.) *"Manuel De Techniques Virologiques"*. Québec: Presses De L'université Du Québec, 1989.
6. Wilson, A.P. (1986) *"Cytotoxicity And Viability Assays"*. In: "Animal Cell Culture: A Practical Approach" (R.I. Freshney, Ed.). Oxford: IRL Press.
7. Walum, E., K. Stenberg & D. Jenssen (1990) *"Understanding Cell Toxicology. Principles And Practice"*. Ellis Horwood, New York.
8. Montanha, J.A. (1995) *"Recherche De Molecules Naturelles Antivirales: Étude Particuliere De Carraghenanes Et D'alcaloides"* Rennes, France: Université De Rennes I,. Thése De Doctorat Mencion Sciences Biologiques Et Santé.
9. Montanha, J.A., M. Amoros, J. Boustie & L. Girre (1995) *Planta Med*. **61**: 419-24.