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Screening of Brazilian medicinal plants for antiviral activity against rotavirus

Alzira Batista Cecílio[a,*], Déborah Behr de Faria[a], Pollyana de Carvalho Oliveira[a], Sérgio Caldas[a], Dario Alves de Oliveira[b], Marcos Eduardo Guerra Sobral[c], Maria Gorette Resende Duarte[a], Carolina Paula de Souza Moreira[a], Cláudia Gontijo Silva[a], Vera Lúcia de Almeida[a]

[a] Diretoria de Pesquisa e Desenvolvimento, Fundação Ezequiel Dias, Rua Conde Pereira Carneiro, 80, Gameleira, CEP 30510-010, Belo Horizonte, Minas Gerais, Brazil
[b] Centro de Ciências Biológicas e da Saúde, Universidade Estadual de Montes Claros, Rua Dr. Rui Braga s/n, Vila Mauricéia, CEP 39401-089, Montes Claros, Minas Gerais, Brazil
[c] Departamento de Ciências Naturais, Universidade Federal de São João Del-Rei, Praça Dom Helvecio, 74, CEP 36301-160, São João Del-Rei, Minas Gerais, Brazil

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ABSTRACT

Ethnopharmacological relevance: Brazilian medicinal plants traditionally used for the treatment of diarrhoea were investigated for their *in vitro* antiviral activity against the simian rotavirus SA11.
Materials and methods: The ethanolic crude extracts of plants collected in the *cerrado* of Minas Gerais, Brazil were submitted to phytochemical screening. The cytotoxicity of the extracts was inferred by cellular morphologic alterations. Antiviral activity was assessed by the ability of the extracts to inhibit the cytopathic effect (CPE) of rotavirus on the treated cells. RT-PCR was performed to confirm and/or confront antiviral assay data.
Results: The maximum non-toxic concentration ranged from 50 to 500 μg/mL. All extracts were toxic at a concentration of 5000 μg/mL but no extract showed cytotoxicity at 50 μg/mL. The species *Byrsonima verbascifolia*, *Myracrodruon urundeuva*, *Eugenia dysenterica* and *Hymenaea courbaril* exhibited the strongest *in vitro* activity against rotavirus. Their extracts prevented the formation of CPE, and RT-PCR analysis detected no amplification of genetic material from rotavirus. Tannins, flavonoids, saponins, coumarins and terpenes were the major classes of natural products found in the leaf extracts that showed antiviral activity.
Conclusion: Among the species studied, *Byrsonima verbascifolia*, *Eugenia dysenterica*, *Hymenaea courbaril* and *Myracrodruon urundeuva* showed potential activity against rotavirus and are worthy of further study. The present study corroborates ethnopharmacological data as a valuable source in the selection of plants with antiviral activity and to some extent validates their traditional uses.

1. Introduction

Rotavirus is the leading cause of severe diarrhoea in newborn and young children with a worldwide distribution and a significant public health impact (Black et al., 2010; Walker et al., 2011). Overall, two million children are hospitalized, and more than 500,000 deaths occur annually due to rotavirus disease in the poorest countries (Atherly et al., 2009). The virus is transmitted by the faecal-oral route and its replication causes damage to cells in the small intestine resulting in diarrhoea with vomiting, and an increased loss of electrolytes in the liquid stool and dehydration (Tallett et al., 1977; Malek et al., 2006). The only known treatment for rotavirus gastroenteritis is the replacement of fluids and electrolytes, since there is no specific drug against the virus (Farthing, 2001; D'Agostino, 2006; Walker et al., 2011). In 1999, a highly efficacious rotavirus vaccine licensed in the United States, RotaShield, was withdrawn from the market after 14 months because of its association with intussusceptions. Two new live, oral, attenuated rotavirus vaccines were licensed in 2006: RotaRix (GlaxoSmithKline) and RotaTeq (Merck/CSL) but vaccine effectiveness in preventing rotavirus infections in populations at highest risk in developing countries remains to be assessed (Dennehy, 2008). In this scenario, there is growing interest in the search for novel compounds for the treatment of diarrhoeal diseases as well as alternative methods to control rotavirus infection. Despite the advances made with synthetic products, natural medicine is a valuable field of research, since the biological diversity of nature is the source of a wide range of bioactive molecules (De Clercq, 2005). It is known that secondary metabolites have a wide spectrum of biological activities, including potential antiviral effect (Vlietinck and Vanden Berghe,

* Corresponding author. Tel.: +55 31 33144778; fax: +55 31 33144768.
E-mail address: alzira@funed.mg.gov.br (A.B. Cecílio).

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Table 1
Brazilian medicinal plant species studied for their antiviral activity against rotavirus.

Species	Voucher	Collection site	Family	Vernacular name	Traditional uses	Reference
Anacardium humile A. St. -Hil.	Duarte 1	Santana do Pirapama	Anacardiaceae	Cajuzinho, cajueiro-do-campo	Diarrhoea, external ulcers	Agra et al. (2007)
Annona crassiflora Mart.	Duarte 3	Santana do Pirapama	Annonaceae	Araticum, marolo	Cronic diarrhoea	Brandão et al. (1992) and Rodrigues and Carvalho (2001)
Astronium fraxinifolium Schott	Sobral et al. 12207	Lagoa Santa	Anacardiaceae	Gonçalo-alves, aroeira-vermelha	Diarrhoea, skin ulcers, toothaches	Almeida et al. (1998) and Agra et al. (2007)
Byrsonima coccolobifolia Kunth	Sobral et al. 12206	Lagoa Santa	Malpighiaceae	Murici de flor rósea, murici-do-cerrado	Diarrhoea	Lorenzi (2002) and Brandão et al. (1992)
Byrsonima verbascifolia (L.) DC.	Sobral et al. 12205	Lagoa Santa	Malpighiaceae	Murici de flor amarela, murici-cascudo	Fever, diarrhoea, astringent, mild laxative	Brandão et al. (1992) and Rodrigues and Carvalho (2001)
Campomanesia pubescens (DC.) O. Berg.	Gontijo 3	Mateus Leme	Myrtaceae	Gabiroba, guabiroba	Diarrhoea, urinary tract infections	Rodrigues and Carvalho (2001)
Curatella americana L.	Sobral et al. 12209	Lagoa Santa	Dilleniaceae	Lixeira, cajueiro-bravo	To wash wounds	Brandão et al. (1992)
Eugenia dysenterica DC.	Sobral et al. 12208	Lagoa Santa	Myrtaceae	Cagaiteira, cagaita	Diarrhoea, purgative	Almeida et al. (1998)
Eugenia uniflora L.	Gontijo 1	Juatuba	Myrtaceae	Pitangueira, pitanga	Diarrhoea, bronchitis, fever, verminosis	Panizza (1997) and Lorenzi and Matos (2002)
Hymenaea courbaril L.	Sobral et al. 12204	Lagoa Santa	Fabaceae	Jatobá, farinheira	Diarrhoea, dysentery, intestinal colic, pulmonary weakness, chronic cystitis	Panizza (1997) and Lorenzi and Matos (2002)
Luehea paniculata Mart.	Sobral et al. 12210	Lagoa Santa	Tiliaceae	Açoita-cavalo, ivitinga	Dysentery, rheumatism	Almeida et al. (1998)
Myracrodruon urundeuva (Allemão) Engl.	Duarte 2	Santana do Pirapama	Anacardiaceae	Aroeira, aroeira-do-sertão	Female genital tract antiinflammatory, urinary and respiratory diseases, diarrhoea	Lorenzi and Matos (2002) and Viana et al. (2003)
Psidium guajava L.	Gontijo 2	Juatuba	Myrtaceae	Goiabeira, araçá-das-almas	Acute diarrhoea, intestinal colic, gastrointestinal disorders	Matos (2000), Lorenzi and Matos (2002) and Gilbert et al. (2005)
Stryphnodendron adstringens (Mart.) Coville	Duarte 4	Santana do Pirapama	Leguminosae	Barbatimão, casca-da-virgindade	Gynaecological problems, diarrhoea, decubitus ulcers healing	Lorenzi and Matos (2002) and Gilbert et al. (2005)

1991; Longanga et al., 2000; Goncalves et al., 2005; Palombo, 2006).

Estimates suggest that 70% of drugs for infectious diseases and 67% of anticancer are either natural products or derived from them (Cragg and Newman, 2005). Natural products, either pure compounds or as standardized plant extracts, provide unlimited opportunities for the development of new leads (Cos et al., 2006). The chemical composition of most Brazilian plants is still unknown, representing a potential to be exploited. Phytochemical studies of species within the Fabaceae, Myrtaceae, and others families have been carry out in order to obtain compounds with antidiarrhoeal activity (Lima et al., 2011).

Furthermore, the study of medicinal plants based on popular use, can provide useful information for the discovery of new drugs with less financial burden. An ethnopharmacological approach was chosen for screening extracts derived from plants used in traditional medicine to treat infectious diseases such as gastrointestinal disorders (Simões et al., 1999; Rajbhandari et al., 2001; Cos et al., 2002; Alanís et al., 2005).

The aim of this study is to investigate whether leaf extracts of 14 medicinal plants traditionally used for the treatment of diarrhoea show *in vitro* antiviral activity against the simian rotavirus SA-11. Their aerial parts (leaves, fruits, stem barks and seeds) are used in Brazilian traditional medicine to treat diarrhoea even though other uses have also been reported (Table 1).

2. Methodology

2.1. Plant material

The species were selected based on their ethnopharmacological use for treating diarrhoea. The 14 Brazilian medicinal plants studied are presented in Table 1. They were identified by Dr. Marcos Eduardo Guerra Sobral from the Departamento de Ciências Naturais, Universidade Federal de São João Del-Rei. Voucher specimens were deposited at the Herbarium BHCB from the Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais. The leaves

were collected from adult plants in the *cerrado* area of Minas Gerais State, Brazil, between September 2006 and February 2007.

2.2. Preparation of extracts and stock solution

The leaves were dried at 40 °C and subsequently powdered. The extracts were prepared by percolation of material with ethanol 95° GL (Vetec Química Fina), until exhaustion at room temperature. The ethanolic extracts were evaporated under reduced pressure at 40 °C. For *in vitro* experiments, each 50 mg of extract was solubilized in 1 mL dimethylsulfoxide (DMSO), centrifuged at 9000 rpm to given a concentration of 50 mg/mL.

2.3. Cells and viruses

The rhesus monkey kidney cell line MA-104 was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL (Gibco) and 100 U/mL penicillin G (Invitrogen). The cell cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Simian rotavirus SA11 were activated with 10 μg/mL trypsin for 60 min at 37 °C and propagated in MA-104 cells monolayers in presence of 10 μg/mL trypsin. The virus titers were estimated from cytopathogenicity by the limit-dilution method and expressed as 50% tissue culture infectious dose per mL ($TCID_{50}$/mL) by Reed and Muench (1938).

2.4. Determination of the maximum non-toxic concentration

The cytotoxicity of each extract was determined using the method described by Miranda et al. (1997). This method is based on cellular morphologic alterations. Concentrations 5000, 500 and 50 μg/mL of each extract were placed in contact with confluent MA-104 cells monolayers prepared in 12-well microplates and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 48 h. After the incubation period, the cells were examined using an inverted optical microscope (Nikon) and treated and untreated cultures (control) were compared. The higher concentration of each extract showing no cellular morphologic changes was considered as the maximum non-toxic concentration (MNTC).

2.5. Antiviral assay

Antiviral activity was assessed by the ability of the extracts to inhibit the cytopathic effect (CPE) of rotavirus on the treated MA-104 cells monolayers cultivated in 96-well microplates. The MNTC of crude extracts were tested in an antiviral assay. The extracts non-toxic at 500 μg/mL were also tested at 50 μg/mL. Afterwards, 10 $TCID_{50}$ and 20 $TCID_{50}$ of activated rotaviruses were added to treated and untreated cells. The plates were incubated for 48 h in a humidified 5% CO_2 atmosphere at 37 °C. Experiments were carried out in duplicate. The antiviral assay data were confirmed and/or confronted by RT-PCR.

2.6. RNA extraction

The material collected from the antiviral assay was used to extract RNA. RNAs were extracted by a modified silica method (Boom et al., 1990). Briefly, 60 μL of material was treated with 200 μL lysis buffer (60 g guanidine isothiocyanate, Invitrogen, 50 mL of 0.1 M Tris–HCl pH 6.4, Invitrogen, 11 mL of 0.2 M EDTA pH 8.0, Invitrogen, 1.3 g of Triton X-100, Packard Instrument Co.) and 50 μL sterilized silica solution (prepared according to Boom et al., 1990). After centrifugation, the silica was washed with a washing buffer (60 g guanidine isothiocyanate, 50 mL of 0.1 M Tris–HCl pH 6.4), followed by two washes with 70% ethanol (Merck) and acetone (Merck). The material was resuspended in water treated with diethyl pyrocarbonate 0.1% and after centrifugation the RNA was collected in the upper phase and maintained at −80 °C until required.

2.7. RT-PCR assay

(i) cDNA synthesis – the cDNA synthesis was conducted in a 20 μL reaction using Improm II™ Reverse Transcriptase (Promega). Firstly, 1 μg of viral RNA, 1 μM of each primer and 7% dimethyl sulfoxide, were incubated at 95 °C for 5 min and then chilled on ice for 5 min. After this incubation, 1× reaction buffer was added, 3 mM $MgCl_2$, 0.5 mM of each dNTP and 1 μL Improm II™ Reverse Transcriptase. The reaction was incubated for 5 min at 25 °C, 60 min at 42 °C, and the enzyme was inactivated for 15 min at 70 °C. The cDNAs were maintained at −80 °C until required.

(ii) PCR control – each reaction set was checked for contamination using negative control (all reagents included and water instead of DNA). In addition to this negative control, cells not infected were included. Each reaction set included the reference sample SA11 as a positive control.

(iii) Primers – the primers used for rotavirus VP6 region amplification were: Rota A – Fwd 1: 5′-GGATGTCCTGTACTCCTTGTCAAAA-3′ and Rota A – Rev 1: 5′-TCCAGTTTGGAACTCATTTCCA-3′, which amplify a 144-bp product (Logan et al., 2006).

(iv) PCR assay – the PCR using 5 μL of cDNA was conducted in reaction of 50 μL containing 22 mM Tris–HCl pH 8.4, 55 mM KCl, 1.65 mM $MgCl_2$, 220 μM each deoxynucleoside triphosphate (dNTP), 800 nM each primer and 0.5 U recombinant *Taq DNA Polymerase* (Invitrogen). The thermal cycle used for rotavirus amplification was 10 min at 95 °C and 30 cycles of 15 s at 95 °C, 1 min at 60 °C and 1 min at 72 °C.

(v) Detection of amplified DNA – 10 μL of the PCR amplified DNA underwent electrophoresis on a silver stained 8% poliacrylamide gel electrophoresis (PAGE).

2.8. Phytochemical screening

The extracts were tested for the presence of different classes of natural products by thin-layer chromatography (TLC) using methods described by Wagner and Bladt (2001). The analysis was performed on Merck silica gel 60 F254 aluminum plates, which were developed according to Table 2. Others tests described by Matos (1997) were carried out to determine the presence of tannins and saponins. The tannins were detected by proteins precipitation test while saponins were determined using the foam test.

3. Results

In this study, ethanolic crude extracts from 14 medicinal species of 8 different botanical families (Table 1) were studied to detect antiviral activity against rotavirus and were also submitted to phytochemical screening.

The cytotoxicity of the extracts was determined by optical microscopy examination of the morphology integrity of MA-104 cells after an incubation period of 48 h. Fig. 1 shows a cytotoxic effect characterized by typical morphological alterations, detachment of cells from the substrate and disruption of cell monolayer, compared with untreated cells. The maximum non-toxic concentration (MNTC) of each extract was determined. All extracts were toxic at a concentration of 5000 μg/mL but no extract showed cytotoxicity at 50 μg/mL. In addition, four extracts showed MNTC to 500 μg/mL, as shown in Table 3. The concentrations of the extracts

Table 2
TLC conditions to test the presence of different classes of natural products in the ethanolic extracts.

Class	Eluent	Spray
Tannins	Toluene:acetic acid:formic acid (70:167:14)	$K_3Fe(CN)_6$ 1%:$FeCl_3$ 2% (1:1)
Flavonoids	Ethylacetate:methanol:water (176:22:22)	$AlCl_3$ reagent (2%)
Anthraquinones	Toluene:acetone:chloroform (40:25:35)	KOH reagent (5%)
Terpenes	Hexane:ethylacetate (1:1)	Anisaldehyde-sulfuric acid reagent
Cardiotonic glycosides	Ethylacetate:methanol:water (20:3:2)	Kedde reagent
Alkaloids	Ethylacetate:formic acid:acetic acid:water:ethylmethylcetone (86:16:23:47:78)	Dragendorff reagent
Coumarins	Toluene:chloroform:acetone (145:53:53)	KOH reagent (5%)
Saponins	Chloroform:acetic acid:methanol:water (112:60:22:15)	Anisaldehyde-sulfuric acid reagent

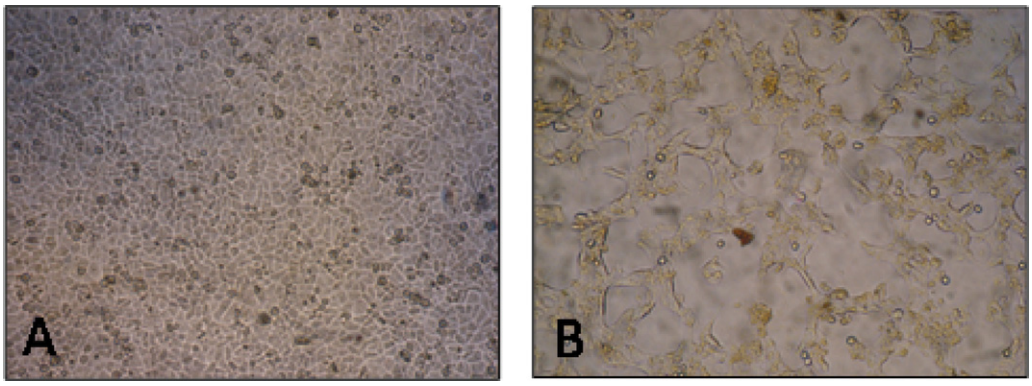

Fig. 1. The rhesus monkey kidney cell line MA-104. (A) Untreated control cells. (B) Typical toxic effect observed in cells after 48 h of incubation with toxic concentrations of plant extract (magnification, 20×).

showing cytotoxic effect on MA-104 cells were excluded from the antiviral assay.

The extracts that were able to inhibit the viral CPE on MA-104 cells and prevent viral replication at 10 and 20 $TCID_{50}$, were considered potentially active against rotavirus. Fig. 2A shows uninfected cells and Fig. 2B shows the typical CPE induced by the virus in MA-104 cells. The morphological data observed in optical microscopy were compared with the molecular data by RT-PCR. Table 4 presents the results of RT-PCR, which were consistent with previous CPE data. *Byrsonima verbascifolia*, *Eugenia dysenterica*, *Hymenaea courbaril* and *Myracrodruon urundeuva* showed CPE inhibition of the virus at 10 and 20 $TCID_{50}$ and *Astronium fraxinifolium* only at 10 $TCID_{50}$ when they were tested at a concentration of 500 μg/mL. No extract showed inhibition of the CPE at 50 μg/mL.

Table 3
Evaluation of the cytotoxicity of crude extracts at 5000, 500 and 50 μg/mL in MA-104 cells after 48 h of incubation.

Species	5000 μg/mL	500 μg/mL	50 μg/mL
Anacardium humile	+	–	–
Annona crassiflora	+	+	–
Astronium fraxinifolium	+	–	–
Byrsonima coccolobifolia	+	+	–
Byrsonima verbascifolia	+	–	–
Campomanesia pubescens	+	–	–
Curatella americana	+	–	–
Eugenia dysenterica	+	–	–
Eugenia uniflora	+	+	–
Hymenaea courbaril	+	–	–
Luehea paniculata	+	–	–
Myracrodruon urundeuva	+	–	–
Psidium guajava	+	–	–
Stryphnodendron adstringens	+	+	–

+, presence of cytotoxicity; –, absence of cytotoxicity.

3.1. Phytochemical screening

The phytochemical screening revealed that tannins, flavonoids, terpenes and saponins were the major classes of natural products found in the leaf extracts of the medicinal species studied (Table 5), while anthraquinones, cardiotonic glycosides and alkaloids were not detected.

4. Discussion

Viral infections are the cause of many human and animal diseases that have a significant economic impact. The limited availability of antiviral drugs could be partially attributed to the

Table 4
Conventional RT-PCR analysis of material collected after incubation for 48 h of MA-104 cells with rotavirus at 10 or 20 $TCID_{50}$ and crude extracts at concentrations of 50 or 500 μg/mL.

Species	500 μg/mL		50 μg/mL	
	10 $TCID_{50}$	20 $TCID_{50}$	10 $TCID_{50}$	20 $TCID_{50}$
Anacardium humile	+	+	+	+
Annona crassiflora	ND	ND	+	+
Astronium fraxinifolium	–	+	+	+
Byrsonima coccolobifolia	ND	ND	+	+
Byrsonima verbascifolia	–	–	+	+
Campomanesia pubescens	+	+	+	+
Curatella americana	+	+	+	+
Eugenia dysenterica	–	–	+	+
Eugenia uniflora	ND	ND	+	+
Hymenaea courbaril	–	–	+	+
Luehea paniculata	+	+	+	+
Myracrodruon urundeuva	–	–	+	+
Psidium guajava	+	+	+	+
Stryphnodendron adstringens	ND	ND	+	+

–, no amplification; +, specific rotavirus amplification; ND, not done (the concentrations of the extracts that showed cytotoxic effect on MA-104 cells were excluded from testing).

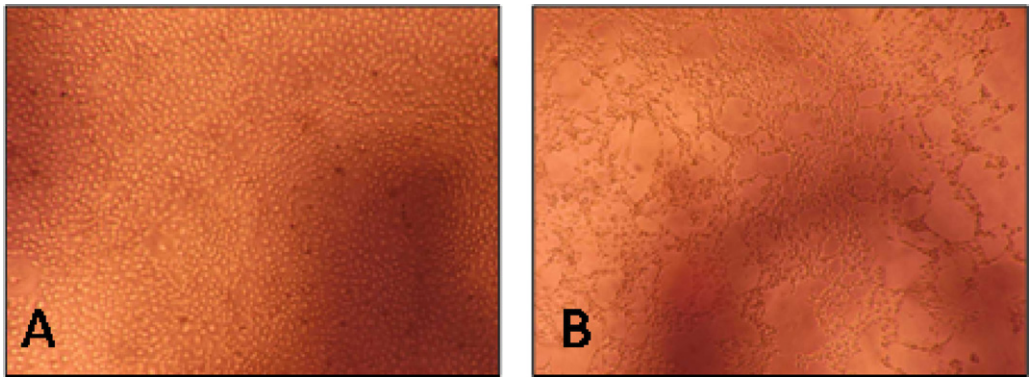

Fig. 2. The rhesus monkey kidney cell line MA-104. (A) Intact uninfected cells monolayer. (B) Typical cytopathic effect observed in cells infected with the simian rotavirus SA11, after 48 h of incubation (magnification, 20×).

difficulty of finding targets for drugs that eliminate the virus without harming the host cells (Cock and Kalt, 2010). Natural products from higher plants and marine organisms represent an almost inexhaustible resource of antiviral drug leads (Palombo, 2006; Sagar et al., 2010; Yasuhara-Bell et al., 2010). Several natural compounds including polysaccharides, flavonoids, terpenes, alkaloids, phenolics and amino acids showing antiviral activity have been isolated from plants used in traditional medicine (Vlietinck and Vanden Berghe, 1991; Longanga et al., 2000; Palombo, 2006). These compounds may be involved in the defense of the plants against invading pathogens such as insects, bacteria, fungi, and viruses (Friedman, 2007).

In the present study, *in vitro* assays were established and employed to detect antiviral activity of extracts obtained from plants traditionally used to treat intestinal disorders as well as other diseases in accordance with an ethnopharmacological approach. As our main objective was to perform a rapid screening of the potential activity of these extracts against rotavirus, we decided to work with rhesus monkey kidney MA-104 cells and simian rotavirus SA11. MA-104 cells are the most used cells in experiments with SA11 due to their susceptibility to this virus, and their ability to show typical cytopathic and cytotoxic effects easily identified by optical microscopy within 48 h. Simian rotavirus SA11 was chosen because it is well characterized and is the most widely used reference strains in laboratories throughout the world (Malherbe and Strickland-Cholmley, 1967; Estes et al., 1979; López and Arias, 1992; Goncalves et al., 2005; Westerman et al., 2006). The leaf crude extracts were resuspended in DMSO to a concentration of 50 mg/mL and we checked the cytotoxicity of the extracts for MA-104 cells. In general, the extracts showed low cytotoxicity, which was expected, since the plant species chosen are already used by the population to treat some diseases. The toxicity at a concentration of 5000 μg/mL observed in all extracts, may be due to the 10% DMSO present in these dilutions of the extract, since the control (10% DMSO, without plant extract) was also toxic to cells. However, most of the extracts were not able to inhibit viral replication and the formation of CPE in infected cells. These results suggest that the antidiarrhoeal effects can occur in different situations other than those caused by rotavirus, such as by other pathogens or pathophysiological diarrhoeal condition. However, the results do not discard the role of these extracts for the treatment of diarrhoea. In addition, the symptoms of viral infection can be alleviated by compounds present in the selected plants, without necessarily requiring a direct action against the virus. For example, Roner et al. (2010) have suggested that saponins will 'coat' the epithelium of the host's small intestine and prevent attachment of rotavirus. On the other hand, some saponins have also been shown to exhibit direct virucidal mechanisms of action, including destruction of viral envelopes and interaction with host cell membranes, leading to the loss of viral binding sites (Arstila, 1974; Abe et al., 1981).

The species *Byrsonima verbascifolia*, *Myracrodruon urundeuva*, *Eugenia dysenterica* and *Hymenaea courbaril* exhibited the strongest *in vitro* activity against rotavirus. Their extracts prevented the formation of CPE, and RT-PCR analysis detected no amplification of genetic material from rotavirus. The RT-PCR data suggest that in the leaves of these plants there are bioactive molecules capable of inhibiting viral replication. Tannins, flavonoids, saponins, coumarins and terpenes were the major

Table 5
Phytochemical screening and yields of ethanolic crude extracts of leaves.

Species	Extract yield (%)	Natural products classes							
		Tan	Fla	Ant	Ter	Car Gly	Alk	Cou	Sap
Anacardium humile	26.7	+	+	–	+	–	–	+	+
Annona crassiflora	40.9	+	+	–	+	–	–	–	+
Astronium fraxinifolium	29.0	+	+	–	+	–	–	+	–
Byrsonima coccolobifolia	22.1	+	+	–	+	–	–	+	+
Byrsonima verbascifolia	28.9	+	+	–	+	–	–	+	+
Campomanesia pubescens	23.6	+	+	–	–	–	–	–	+
Curatella americana	16.1	+	+	–	+	–	–	–	+
Eugenia dysenterica	21.0	+	+	–	+	–	–	–	+
Eugenia uniflora	26.2	+	+	–	+	–	–	+	+
Hymenaea courbaril	16.9	+	+	–	+	–	–	+	–
Luehea paniculata	18.5	+	+	–	+	–	–	–	–
Myracroduon urundeuva	19.4	+	+	–	+	–	–	+	+
Psidium guajava	15.6	+	+	–	+	–	–	+	+
Stryphnodendron adstringens	31.6	+	+	–	+	–	–	–	+

Tan, tannins; Fla, flavonoids; Ant, anthraquinones; Ter, terpenes; Car Gly, cardiotonic glycosides; Alk, alkaloids; Cou, coumarins; Sap, saponins; +, presence; –, absence.

classes of natural products found in the leaf extracts that showed antiviral activity. Most of these classes have been reported in plants that have shown antidiarrhoeal activity (Lima et al., 2011).

In the present study, the compounds that may be responsible for the antiviral activity have not been identified. Unlike those observed for *Byrsonima verbascifolia* and *Myracrodruon urundeuva*, which showed all classes of metabolites previously mentioned, the extract of *Eugenia dysenterica* did not show the presence of coumarins, while saponins were not found in *Hymenaea courbaril*. However, extracts of both plants still showed inhibitory activity on the replication of rotavirus, according to data from the RT-PCR. *Astronium fraxinifolium* showed partial antiviral activity, because it was not able to inhibit viral replication when the amount of virus increased from 10 to 20 $TCID_{50}$. According to the phytochemical screening, the same classes of metabolites mentioned for those species that showed the strongest antiviral activity against rotavirus were also detected in the extract of *Astronium fraxinifolium* with the exception of saponins.

Lopez et al. (2001) also observed antiviral activity of *Byrsonima verbascifolia* against herpes simplex virus. Phytochemical studies carried out previously also described the presence of triterpenes in the leaves of this species (Gottlieb et al., 1975).

Some phytochemical and biological studies with these four more active plants have been reported in literature. Essential oil from the leaves of *Eugenia dysenterica* were showed to inhibit the growth of *Candida* species and *Cryptococcus neofarmans* isolated from HIV infected patients (Costa et al., 2000). In agreement with our results, the presence of tannins in the leaves was also reported by Palhares (2003). Many sesquiterpenes and diterpenes (Nogueira et al., 2001) have been isolated from *Hymenaea courbaril*, and some of them showed cytotoxicity for tumour cells (Abdel-Kader et al., 2002).

Pre-clinical and clinical studies with the bark of *Myracrodruon urundeuva* showed anti-inflammatory, analgesic, wound healing and antiulcer activity (Goes et al., 2005). From its seeds were isolated quercetin, aromadendrinol and biflavonoid (Goes et al., 2005). Flavonoids are dietary components and the most ubiquitous phenolic compounds found in nature, showing a range of pharmacological activities, including antiviral action (Bae et al., 2000; Cushnie and Lamb, 2005; Friedman, 2007; Li et al., 2008). Previous studies have demonstrated that some flavonoids (diosmin and hesperidin) were able to inhibit rotavirus propagation, as well as protect the cell cultures against virus invasion (Bae et al., 2000).

It is important to mention that the *in vitro* activity against simian rotavirus SA11 reported here are only the preliminary results. However, they encourage further studies to evaluate the leaf extracts from *Byrsonima verbascifolia*, *Myracrodruon urundeuva*, *Eugenia dysenterica* and *Hymenaea courbaril* using other antiviral assays against rotavirus, such as tests with other cell lines, and *in vivo* assay with murine rotavirus strains in mice (Ward et al., 1990; McNeal et al., 2004; Goncalves et al., 2005; Westerman et al., 2006). These will confirm the results from the present work as well as to investigate possible mechanisms of action. It will be interesting to obtain the active plant extracts by the bioassay-guided fractionation and isolation of bioactive compounds. The crude extracts can contain other compounds which were not detected by the phytochemical screening that can be active to inhibit or kill the virus. Ongoing experiments are being performed by our research group aiming to identify the possible anti-rotavirus fraction(s) or compound(s) bioactive(s), and the results will be reported in due course.

The present study corroborates ethnopharmacological data as a valuable source in the selection of plants with antiviral activity and to some extent validates their traditional uses.

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References

Abe, H., Konishi, H., Komiya, H., Arichi, S., 1981. Effects of saikosaponins on biological membranes. 3. Ultrastructural studies on effects of saikosaponins on the cell surface. Planta Medica 42, 356–363.
Abdel-Kader, M., Berger, J.M., Slebodnick, C., Hoch, J., Malone, S., Wisse, J.H., Werkhoven, M.C.M., Mamber, S., Kingston, D.G., 2002. Isolation and absolute configuration of ent-Halimane diterpenoids from *Hymenaea courbaril* from the Suriname rain forest. Journal of Natural Products 65, 11–15.
Agra, M.F., Freitas, P.F., Barbosa-Filho, J.M., 2007. Synopsis of the plants known as medicinal and poisonous in Northeast of Brazil. Revista Brasileira de Farmacognosia 17, 114–140.
Alanís, A.D., Calzada, F., Cervantes, J.A., Torres, J., Cebalhos, G.M., 2005. Antibacterial properties of some plants used in Mexican traditional medicine for the treatment of gastrointestinal disorders. Journal of Ethnopharmacology 100, 153–157.
Almeida, S.P., Proença, C.E.B., Sano, S.M., Ribeiro, J.F., 1998. Cerrado: Espécies Vegetais Úteis. EMBRAPA - CPAC, Planaltina.
Arstila, P., 1974. Characteristics of vesicular stomatitis virus envelopes released with saponin. Journal of General Virology 24, 319–326.
Atherly, D., Dreibelbis, R., Parashar, U.D., Levin, C., Wecker, J., Rheingans, R.D., 2009. Rotavirus vaccination: cost-effectiveness and impact on child mortality in developing countries. Brazilian Journal of Infectious Diseases 200, S28–S38.
Bae, E.A., Han, M.J., Lee, M., Kim, D.H., 2000. In vitro inhibitory effect of some flavonoids on rotavirus infectivity. Biological & Pharmaceutical Bulletin 23, 1122–1124.
Black, R.E., Cousens, S., Johnson, H.L., Lawn, J.E., Rudan, I., Bassani, D.G., Jha, P., Campbell, H., Walker, C.F., Cibulskis, R., Eisele, T., Liu, L., Mathers, C., 2010. Global, regional, and national causes of child mortality in 2008: a systematic analysis. The Lancet 375, 1969–1987.
Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertherim-Van Dillen, P.M.E., Van Der Noorda, J., 1990. Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology 28, 495–503.
Brandão, M., Carvalho, P.G.S., Jesué, G., 1992. Guia Ilustrado de Plantas do Cerrado de Minas Gerais. CEMIG, Governo de Minas Gerais, Belo Horizonte.
Cock, I., Kalt, F.R., 2010. A modified MS2 bacteriophage plaque reduction assay for the rapid screening of antiviral plant extracts. Pharmacognosy Research 2, 221–228.
Cos, P., Hermans, N., De Bruyne, T., Sindambiwe, J.B., Vanden Berghe, D., Pieters, L., Vlietinck, A.J., 2002. Further evaluation of Rwandan medicinal plant extracts for their antimicrobial and antiviral activities. Journal of Ethnopharmacology 79, 155–163.
Cos, P., Vlietinck, A.J., Vanden Berghe, D., Maes, L., 2006. Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'. Journal of Ethnopharmacology 106, 290–302.
Costa, T.R., Fernandes, O.F.L., Santos, S.C., Oliveira, C.M.A., Lião, L.M., et al., 2000. Antifungal activity of volatile constituents of *Eugenia dysenterica* leaf oil. Journal of Ethnopharmacology 72, 111–117.
Cragg, G.A., Newman, D.J., 2005. Biodiversity: a continuing source of novel drug leads. Pure and Applied Chemistry 77, 7–24.
Cushnie, T.P., Lamb, A.J., 2005. Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents 26, 343–356.
D'Agostino, J., 2006. Considerations in assessing the clinical course and severity of rotavirus gastroenteritis. Clinical Pediatrics (Phila) 45, 203–212.
De Clercq, E., 2005. Recent highlights in the development of new antiviral drugs. Current Opinion in Microbiology 8, 552–560.
Dennehy, P.H., 2008. Rotavirus vaccines: an overview. Clinical Microbiology Reviews 21, 198–208.
Estes, M.K., Graham, D.Y., Gerba, C.P., Smith, E.M., 1979. Simian rotavirus SA11 replication in cell cultures. Journal of Virology 31, 810.
Farthing, M.J., 2001. Treatment of gastrointestinal viruses. Novartis Foundation Symposium 238, 289–300.
Friedman, M., 2007. Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. Molecular Nutrition & Food Research 51, 116–134.
Gilbert, B., Ferreira, J.L.P., Alves, L.F., 2005. Monografias de Plantas Medicinais Brasileiras e Aclimatadas. Abifito, Curitiba.
Goes, A.C., Rodrigues, L.V., Menezes, D.B., Grangeiro, M.P., Cavalcante, A.R., 2005. Histologic analysis of colonic anastomotic healing, in rats, under the action of 10% Aroeira-do-sertao (*Myracrodruon urundeuva* fr. all.) enema. Acta Cirúrgica Brasileira 20, 144–151.
Goncalves, J.L., Lopes, R.C., Oliveira, D.B., Costa, S.S., Miranda, M.M., Romanos, M.T., Santos, N.S., Wigg, M.D., 2005. In vitro anti-rotavirus activity of some medicinal plants used in Brazil against diarrhoea. Journal of Ethnopharmacology 99, 403–407.
Gottlieb, O.R., Henriques Mendes, P., Taveira Magalhães, M., 1975. Triterpenoids from *Byrsonima verbascifolia*. Phytochemistry 14, 1456–1456.
Li, N., Liu, J.H., Zhang, J., Yu, B.Y., 2008. Comparative evaluation of cytotoxicity and antioxidative activity of 20 flavonoids. Journal of Agricultural and Food Chemistry 56, 3876–3883.

Lima, T.B., Silva, O.N., Silva, L.P., Rocha, T.L., Grossi-de Sá, M.F., Franco, O.L., Leonardecz, E., 2011. In vivo effects of cagaita (Eugenia dysenterica, DC.) leaf extracts on diarrhoea treatment. Evidence-Based Complementary and Alternative Medicine 2011, 1–11.

Logan, C., O'Leary, J.J., Sullivan N., O', 2006. Real-time reverse transcription-PCR for detection of rotavirus and adenovirus as causative agents of acute viral gastroenteritis in children. Journal of Clinical Microbiology 44, 3189–3195.

Longanga, O.A., Vercruysse, A., Foriers, A., 2000. Contribution to the ethnobotanical, phytochemical and pharmacological studies of traditionally used medicinal plants in the treatment of dysentery and diarrhoea in Lomela area, Democratic Republic of Congo (DRC). Journal of Ethnopharmacology 71, 411–423.

Lopez, A., Hudson, J.B., Towers, G.H., 2001. Antiviral and antimicrobial activities of Colombian medicinal plants. Journal of Ethnopharmacology 77, 189–196.

López, S., Arias, C.F., 1992. Simian rotavirus SA11 strains. Journal of Virology 66, 1832.

Lorenzi, H., 2002. Árvores brasileiras: manual de identificação e cultivo de plantas arbóreas do Brasil, vol. 2., 2a ed. Instituto Plantarum, Nova Odessa.

Lorenzi, H., Matos, F.J.A., 2002. Plantas Medicinais no Brasil: nativas e exóticas. Editora Plantarum, Nova Odessa.

Malek, M.A., Curns, A.T., Holman, R.C., Fischer, T.K., Bresee, J.S., Glass, R.I., Steiner, C.A., Parashar, U.D., 2006. Diarrhoea- and rotavirus-associated hospitalizations among children less than 5 years of age: United States, 1997 and 2000. Pediatrics 117, 1887–1892.

Malherbe, H.H., Strickland-Cholmley, M., 1967. Simian virus SA11 and the related O agent. Archives of Virology 22, 235–245.

Matos, F.J.A., 1997. Introdução à Fitoquímica Experimental, vol. 1., 2a ed. Edições UFC, Fortaleza.

Matos, F.J.A., 2000. Plantas medicinais: guia de seleção e emprego de plantas usadas em fitoterapia no Nordeste do Brasil, 2a ed. Imprensa universitária da UFC, Fortaleza.

McNeal, M.M., Belli, J., Basu, M., Choi, A.H., Ward, R.I., 2004. Discovery of a new strain of murine rotavirus that is consistently shed in large quantities after oral inoculation of adult mice. Virology 320, 1–11.

Miranda, M.M.F.S., Almeida, A.P., Costa, S.S., Santos, M.G.M., Lagrota, M.H.C., Wigg, M.D., 1997. In vitro activity of extracts of *Persea americana* leaves on acyclovir-resistant and phosphonoacetic resistant Herpes simplex virus. Phytomedicine 4, 347–352.

Nogueira, R.T., Shepherd, G.J., Laverde, A., Marsaioli, A.J., Imamura, P.M., 2001. Clerodane-type diterpenes from the seed pods of *Hymenaea courbaril* var. stilbocarpa. Phytochemistry 58, 1153–1157.

Palhares, D., 2003. Characterization pharmacognostic leaves from *Eugenia dysenterica* D. C. (Myrtaceae Jussieu). Revista Lecta 21, 29–36.

Palombo, E.A., 2006. Phytochemicals from traditional medicinal plants used in the treatment of diarrhoea: modes of action and effects on intestinal function. Phytotherapy Research 20, 717–724.

Panizza, S., 1997. Plantas que curam: cheiro de mato, 28 ed. IBRASA, São Paulo.

Rajbhandari, M., Wegner, U., Jülich, M., Schöpke, T., Mentel, R., 2001. Screening of Nepalese medicinal plants for antiviral activity. Journal of Ethnopharmacology 74, 251–255.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. American Journal of Epidemiology 27, 493–497.

Rodrigues, V.E.G., Carvalho, D.A., 2001. Plantas Medicinais no Domínio dos Cerrados. UFLA, Lavras.

Roner, M.R., Tam, K.I., Kiesling-Barrager, M., 2010. Prevention of rotavirus infections in vitro with aqueous extracts of *Quillaja Saponaria* Molina. Future Medicinal Chemistry 2, 1083–1097.

Sagar, S., Kaur, M., Minneman, K.P., 2010. Antiviral lead compounds from marine sponges. Marine Drugs 8, 2619–2638.

Simões, C.M., Falkenberg, M., Mentz, L.A., Schenkel, E.P., Amoros, M., Girre, L., 1999. Antiviral activity of south Brazilian medicinal plant extracts. Phytomedicine 6, 205–214.

Tallett, S., MacKenzie, C., Middleton, P., Kerzner, B., Hamilton, R., 1977. Clinical, laboratory, and epidemiologic features of a viral gastroenteritis in infants and children. Pediatrics 60, 217–222.

Viana, G.S.B., Bandeira, M.A.M., Matos, F.J.A., 2003. Analgesic and antiinflammatory effects of chalcones isolated from *Myracrodruon urundeuva* Allemão. Phytomedicine 10, 189–195.

Vlietinck, A.J., Vanden Berghe, D.A., 1991. Can ethnopharmacology contribute to the development of antiviral drugs. Journal of Ethnopharmacology 32, 141–153.

Wagner, H., Bladt, S., 2001. Plant Drug Analysis. A Thin Layer Chromatography Atlas, 2nd ed. Springer, Berlin.

Ward, R.L., McNeal, M.M., Sheridan, J.F., 1990. Development of an adult mouse model for studies on protection against rotavirus. Journal of Virology 64, 5070–5075.

Westerman, L.E., Jiang, B., McClure, H.M., Snipes-Magaldi, L.J., Griffin, D.D., Shin, G., Gentsch, J.R., Glass, R.I., 2006. Isolation and characterization of a new simian rotavirus, YK-1. Virology Journal 3, 40.

Walker, C.L.F., Friberg, I.K., Binkin, N., Young, M., Walker, N., Fontaine, O., Weissman, E., Gupta, A., Black, R.E., 2011. Scaling up diarrhoea prevention and treatment interventions: a lives saved tool analysis. PLoS Medicine 8, e1000428.

Yasuhara-Bell, J., Yang, Y., Barlow, R., Trapido-Rosenthal, H., Lu, Y., 2010. In vitro evaluation of marine-microorganism extracts for anti-viral activity. Virology Journal 7, 182.