Revista Brasileira de Farmacognosia 26 (2016) 197–202

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Antiviral activity of *Myracrodruon urundeuva* against rotavirus

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ARTICLE INFO

Article history:
Received 26 December 2014
Accepted 7 October 2015
Available online 2 December 2015

Keywords:
Medicinal plant
Myracrodruon urundeuva
Crude extract
Leaves
Antiviral activity rotavirus

ABSTRACT

Myracrodruon urundeuva Allemão, Anacardiaceae, is a medicinal plant widely found in Brazil, especially in the northern region. In our previous study, the ethanolic extract from leaves of *M. urundeuva* showed antiviral activity against simian rotavirus SA-11. Here, the crude extract was subjected to fractionations in order to subsequently work with more concentrated and pure bioactive compounds, which were analyzed by TLC and HPLC methods to support a better understanding of their virucidal effect. The antiviral activity was evaluated using a rotavirus infection model in MA-104 cells treated with the maximum non-cytotoxic concentration of the crude extract and its fractions. Data were expressed as the percentage inhibition of viral replication calculated by the inhibition of cytopathic effect in the treated cells compared to untreated controls after 48 h of incubation. First, we conducted a fractionation, generating five fractions (F1–F5) which were submitted to antiviral assay. Then, the fraction that showed the highest virucidal effect (F3, PI = 75%) was subjected to a larger partition, yielding eighteen subfractions, which were submitted to new antiviral assays. Terpenes, flavonoids and tannins were the major secondary metabolites detected by TLC analysis in F3. SF1, a flavonoid-enriched fraction, showed the strongest *in vitro* activity against rotavirus (PI = 92%), preventing cytopathic effect. Chromatographic profiles were obtained by HPLC for the crude extract and SF1, the most potent subfraction. Overall, our data point to the potential anti-rotavirus activity of flavonoid-enriched fraction (SF1) of *M. urundeuva* leaves, corroborating the traditional use of this species to treat diarrhea and broadening our perspectives on *in vivo* assays in mice with SF1 isolated or associated with other fractions.

Introduction

Rotaviruses are recognized as a major cause of non-bacterial gastroenteritis especially in infants and young children worldwide (Parashar et al., 2006; Junaid et al., 2011). It is an unpredictable disease highly contagious and may lead to severe dehydration and even death (Offit and Clark, 2000). Its transmission is person to person through the fecal-oral route. Control and prevention of this infection are difficult due to the lack of any effective treatment other than palliative measures and the presence of asymptomatic children shedding virus (Dennehy, 2000). Despite two live oral rotavirus vaccines that have already been licensed, a monovalent human rotavirus vaccine (Rotarix, GlaxoSmithKline Biologicals) and a pentavalent bovine-human reassortant rotavirus vaccine (RotaTeq, Merck), their effectiveness in developing countries has shown a pooled efficacy of about 51% requiring efforts to optimize (Jiang et al., 2010).

Nevertheless, in several cases, where the host is suffering from prolonged diarrhea and fever, virus-specific treatment will be necessary if possible (Takahashi et al., 2001). Natural products have been the source of most of the active ingredients of medicines and more than 80% of drug substances were natural products or inspired by a natural compound (Harvey, 2008). In this context, *in vitro* assays have been established and used by our research group to screen antiviral activities of extracts, fractions and natural substances with potential therapeutical action.

In previous work, using similar methodology employed here, we evaluated ethanolic crude extracts of different Brazilian medicinal plants and we observed an *in vitro* anti-rotavirus activity of *Myracrodruon urundeuva* Allemão, Anacardiaceae (Cecílio et al., 2012), a medicinal plant widely used in Brazil, mainly due to its anti-inflammatory, antimicrobial and wound healing properties (Monteiro et al., 2006; Souza et al., 2007; Sá et al., 2009a). This plant is a representative species of the Anacardiaceae family occurring in

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http://dx.doi.org/10.1016/j.bjp.2015.10.005
0102-695X/

Brazil and other South American countries, notably in the Cerrado region (Leite, 2002).

Analgesic, anti-inflammatory, antioxidant, antifungal, and antibacterial, among other activities, have been reported from preparations made with *M. urundeuva* extracts. Viana et al. (1997, 2003) have observed analgesic and anti-inflammatory effects of the tannin and chalcones fractions isolated from *M. urundeuva* barks in studies conducted in mice. Souza et al. (2007) have also shown that tannin-enriched fraction from stem bark of this plant presents anti-inflammatory and antiulcer effects in mice, partly due to its antioxidant action, known to be present in polyphenols, including tannins. Interestingly, De Mendonça Albuquerque et al. (2011) demonstrated inhibition of myeloperoxidase activity and antioxidant effects of chalcones from *M. urundeuva* stem barks on an allergic conjunctivitis model in guinea pig, indicating them as candidates for the treatment of allergic conjunctivitis and other inflammatory conditions.

According to Sá et al. (2009a), lectins isolated from *M. urundeuva* heartwood showed antimicrobial activity against bacteria and fungi that attack plants, including woods. In addition, stem bark hydroethanolic extract was active against *Staphylococcus aureus*, *Klebsiella pneumonia*, *Enterococcus faecalis* and *Candida* spp. (Alves et al., 2009; Gomes et al., 2013), and these activities were attributed to the presence of bioactive compounds such as tannins, flavonoids and alkaloids.

Furthermore, experiments in rats that point to anti-diarrheal (Chaves et al., 1998) and neuroprotective activities (Nobre-Júnior et al., 2009), colonic anastomotic healing (Goes et al., 2005), and even larvicidal effect against *Aedes aegypti* (Napoleão et al., 2012) and termite repellent action (Sá et al., 2009b) show the biological potential of this plant, which hides a rich source of compounds that could be employed to therapeutic and biotechnological applications, among other purposes.

Considering the pharmacological potential of *M. urundeuva* and our previous data of anti-rotavirus activity (Cecílio et al., 2012), the crude extract from the leaves of this plant was fractionated and subjected to antiviral assay, being the active fractions characterized by TLC method in an attempt to identify the bioactive compounds involved in the virucidal effect.

Material and methods

Plant material

Myracrodruon urundeuva Allemão, Anacardiaceae, leaves were collected from adult plants in the "cerrado" area of Santana do Pirapama, in the State of Minas Gerais, Brazil, between September 2006 and February 2007. The plant was identified as a voucher specimen and was deposited at the Herbário PAMG da Empresa de Pesquisa Agropecuária de Minas Gerais under the number PAMG 53312.

Preparation of extract and fractionation

The crude extract (MUL) was prepared by percolation of the dried and powdered material with ethanol 95 GL (Vetec Química Fina) until exhaustion at room temperature and evaporated under reduced pressure at 40 °C. The ethanolic extract (250 g) was fractioned by filtration chromatography in silica gel (silica gel 60, 0.040–0.063 mm, Merck), giving five fractions after elution with hexane (F1, 30 g), dichloromethane (F2, 21 g), dichloromethane-ethyl acetate (1:1) (F3, 14.7 g), ethyl acetate (F4, 45.5 g) and methanol (F5, 120 g). A portion of the most potent fraction, F3 (10 g), was further fractioned in a silica gel column (silica gel 60, 0.040–0.063 mm, Merck), with solvents of increasing polarities (dichloromethane, ethyl acetate, ethyl acetate, methanol, water and formic acid 1% and mixtures of these), yielding eighteen subfractions. SF1 (47 mg), obtained with DCM:EtOAc (1:1), showed the best antiviral activity and its chemical composition was evaluated by TLC and HPLC profiles. The low amount obtained prevented further fractionation.

Phytochemical screening

MUL, fractions (F1–F5) and the subfraction SF1 were subjected to phytochemical screening to determine the presence of different classes of natural products using methods described by Wagner and Bladt (2001). The analysis was performed by thin-layer chromatography (TLC) on Merck silica gel 60 F254 aluminum plates, which were developed according to Table 1. The presence of tannins was determined using a protein precipitation test (Matos, 1997).

HPLC analysis

MUL and SF1 were prepared at 10 mg/ml and at 5 mg/ml in MeOH HPLC grade, respectively (Vetec Química Fina). After centrifugation at 9300 × *g* (Eppendorf, model, 5415D) the solution was injected in an Agilent Technologies 1200 series HPLC system equipped with DAD detector. Chromatographic analysis was obtained in a Zorbax XDB C18 column (50 × 4.6 mm, 1.8 μm), at 40 °C, flow rate of 0.3 ml/min and detection at λ 210 nm. The profiles were obtained in linear gradient elution of water (A) and acetonitrile (B), from 15 to 90% of B from 0 to 50 min, and from 90 to 95% of B between 50 and 60 min. The UV spectra were recorded online in the 190–400 nm range for all retention times.

Biological assays

Sample preparation

For the bioassays, each sample was solubilized in dimethylsulfoxide (DMSO, Sigma–Aldrich) at 50 mg/ml and centrifuged at 9300 × *g* (Eppendorf, model, 5415D). The samples were diluted to work concentration using culture media.

Cells and viruses

MA-104 cells (a rhesus monkey kidney cell line) were cultivated in Dulbecco's modified Eagle media (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml of streptomycin (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 the 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) (Reed and Muench, 1938).

Cytotoxicity

The cytotoxicity of the samples (MUL, fractions and subfractions) was determined using the method described by Miranda et al. (1999) based on cellular morphologic alterations. Several concentrations (5000, 500, 50, 5 and 0.5 μg/ml) of samples were placed in contact with confluent MA-104 cells monolayers prepared in 12-well microplates and were 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)

Table 1
TLC conditions to test the presence of different classes of natural products in the ethanolic extract.

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

and was confirmed using Promega's CytoTox 96® Non-Radioactive Cytotoxicity Assay, according to the manufacturer's instructions.

Antiviral assay

Antiviral activity was evaluated based on the ability of samples to diminish the multiplication of the virus, including inhibiting the cytopathic effect (CPE) of rotavirus on treated MA-104 cells monolayers (4×10^4 cells/well) cultivated in 96-well microplates. Thus, MUL and fractions (at MNTC) were added to the cells, followed by the addition of 10 $TCID_{50}$ of activated rotaviruses, and the plates were incubated in a 5% CO_2 atmosphere at 37 °C for 48 h. The antiviral activity was expressed as a percentage of inhibition (PI) (Miranda et al., 1999) using antilogarithm values of $TCID_{50}$ for rotavirus, as follows: PI = [1 − (antilogarithm T/antilogarithm C)] × 100 being "T" viral titer of treated cells and "C" viral titer of untreated cells (positive control). Experiments were carried out in octoplicate. The presence of rotavirus was also verified by RT-PCR.

RNA extraction

Supernatants from the antiviral assays were submitted to RNA extraction by a modified silica method (Boom et al., 1990). Briefly, 60 μl of each sample 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 of sterilized silica solution (prepared according to Boom et al., 1990). After being centrifuged, the silica was washed with a washing buffer (60 g guanidine isothiocyanate) (Invitrogen), 50 ml of 0.1 M Tris–HCl pH 6.4 (Promega), 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 being centrifuged, the RNA was collected in the upper phase and maintained at −80 °C until required.

cDNA synthesis

The cDNA synthesis was conducted in a 20 μl reaction containing 7% dimethyl sulfoxide, 7 μl of viral RNA and 1 μM of the primers Rota A – Fwd 1: 5′GGATGTCCTGTACTCCTTGTCAAAA3′ and Rota A – Rev 1: 5′TCCAGTTTGGAACTCATTTCCA3′, which amplify a 144-bp product (Logan et al., 2006) from rotavirus VP6 region. The reaction was incubated at 95 °C for 5 min and then chilled on ice for 5 min. Next, 1× reaction buffer was added, 3 mM $MgCl_2$, 0.5 mM of each dNTP and 1 μl Improm II™ Reverse Transcriptase (Promega). Finally, 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.

PCR assay

The PCR assay was conducted in a reaction of 50 μl containing 5 μl of cDNA, 22 mM Tris–HCl pH 8.4, 55 mM KCl, 1.65 mM $MgCl_2$, 220 μM each deoxynucleoside triphosphate (dNTP), 800 nM of the same primers used in the cDNA synthesis and 0.5 U recombinant *Taq DNA Polymerase* (Invitrogen). The cycling program consisted of an initial denaturation at 95 °C for 10 min, followed by 30 cycles of 95 °C for 15 s, 60 °C for 1 min and 70 °C for 1 min. Each reaction set contained the reference sample SA11 as a positive control. Negative control consisted of a reaction with all reagents included and water instead of cDNA. Then, 10 μl of the PCR amplified product was analyzed on a silver-stained 8% polyacrylamide gel electrophoresis (PAGE).

Results

Determining the maximum non-cytotoxic concentration

To carry out the *in vitro* antiviral assays, we first determined the concentration of MUL and fractionated samples that could be used without causing damage to the cell, based on cellular morphologic alterations and Promega's CytoTox Assay data. The maximum non-toxic concentrations (MNTC) observed were 500 μg/ml for MUL, and 50 μg/ml for most of the generated fractions, except for F2 and SF3, 4, 6 and 15, which showed MNTC at 5 μg/ml (Table 2).

Fig. 1A shows a cytotoxic effect characterized by typical morphological alterations including detachment of cells from their substratum and disruption of cell monolayer, compared with healthy control cells (Fig. 1C).

Table 2
Maximum non-toxic concentration (MNTC) and percent inhibition of rotavirus (PI) in MA-104 cells treated with crude extract (MUL) from *Myracrodruon urundeuva* leaf and its fractions (F) and subfractions (SF).

Selected materials	MNTC (μg/ml)	PI (%)
Crude extract		
MUL	500	100
Fractions		
F1	50	0
F2	5	21
F3	50	75
F4	50	21
F5	50	68
Subfractions		
SF1	50	92.06
SF2	50	0
SF3	5	80.05
SF4	5	0
SF5	50	68.38
SF6	5	36.90
SF7	50	20.57
SF8	50	20.57
SF9	50	0
SF10	50	0
SF11	50	0
SF12	50	20.57
SF13	50	0
SF14	50	0
SF15	5	0
SF16	50	0
SF17	50	60.19
SF18	50	0

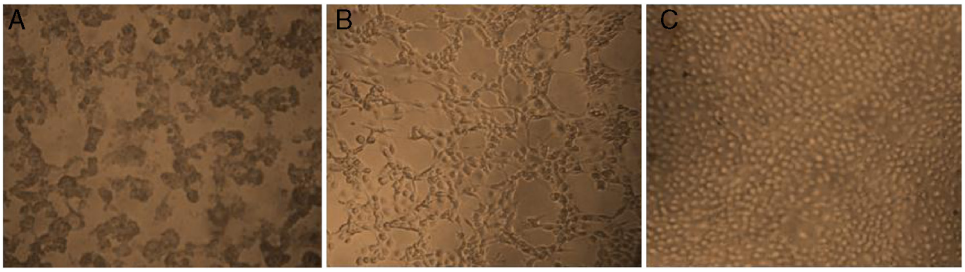

Fig. 1. The rhesus monkey kidney cell line MA-104. (A) Typical cytotoxic effect induced by toxic concentration of extract. (B) Typical cytopathic effect observed in cells infected with the simian rotavirus SA11. (C) Uninfected and untreated control cells (magnification, 20×).

Antiviral assays

The MNTC of the samples were used to conduct the antiviral assays to assess their potential activity against rotavirus. Based on cytopathic effect, we determined the percent inhibition (PI) of the samples on the replication of rotavirus. There were no cytopathic effects (CPE) in cells treated with the MUL. However, we observed CPE (data not shown) and different PI (ranging from 0 to 75%) in the cells treated with the fractions (Table 2). Thus, the fraction with the highest PI (F3, PI = 75%) was selected for further fractionation. The tests with the subfractions revealed that none of them were able to inhibit 100% of viral replication, and most of them showed no antiviral effect. Interestingly, SF1 (a subfraction from F3) showed the highest PI (92%), even higher than F3 (Table 2). Fig. 1B shows the typical CPE induced by the virus in MA-104 cells. The morphological data observed in optical microscopy were consistent with the molecular data assayed by RT-PCR. The results of the amplifications were visualized in a silver-stained 8% polyacrylamide gel showing the band corresponding to the 144-bp from rotavirus VP6 region.

Phytochemical and HPLC analysis

MUL, F1–F5 and SF1 were subjected to phytochemical analysis to determine the main classes of natural products which could be involved in the antiviral activity. Tannins, flavonoids and terpenes were detected in MUL. F1 and F2 showed terpenes, F4 and F5 showed flavonoids and tannins, and F3 showed terpenes, flavonoids and tannins (Table 3). Because F3 showed the highest PI, it was selected for an additional fractionation, yielding eighteen subfractions. Then, SF1, the subfraction with the highest PI, was also analyzed, revealing flavonoids and terpenes. Finally, chromatographic profiles of MUL and SF1 were obtained by HPLC. MUL showed a predominance of compounds of low polarity as shown in Fig. 2A. SF1, which had the highest antiviral activity, showed peaks distributed throughout the chromatogram, indicating the presence of compounds with different polarities. Most of the compounds were eluted with retention time lower than 20 min. In this time range, we observed two major peaks (r.t. of 17.16 and 18.84 min) with UV profile characteristic of flavonoids (Fig. 2B). This fraction also showed peaks with retention time higher than 40 min, which had low UV absorption, with maximum absorption at *ca.* 210 nm.

Discussion

Our tests were conducted using a simple but very effective model for detection of bioactive samples. Our strategy was to determine the working concentration of crude and fractionated extract, and then determine the percentage inhibition of rotavirus replication in MA104 cells. The Simian rotavirus strain SA11 was chosen because this strain is well characterized as a group A rotavirus and is one of the most widely used reference strains (Gutiérrez et al., 2010). Rotaviruses are differentiated into seven groups (A–G). Groups A, B, and C are associated with acute gastroenteritis in humans and animals while groups D, E, F, and G only infect animals (Saif and Jiang, 1994; Westerman et al., 2006; Estes and Greenberg, 2013). Besides, group A rotaviruses are the most common viral agents causing diarrheal infections in children younger than three years (Parashar et al., 2006; Junaid et al., 2011). These viruses are internalized into MA104 cells showing typical cytopathic effects (Malherbe and Strickland-Cholmley, 1967; López and Arias, 1992; Gonçalves et al., 2005; Logan et al., 2006; Gutiérrez et al., 2010; Wolf et al., 2011) which are essential features for the success of our test.

Rotaviruses are nonenveloped viruses composed of a triple-layered protein capsid that surrounds the viral genome. Nonstructural glycoprotein 4 (NSP4) encoded by rotavirus is the only viral protein currently believed to function as an enterotoxin. Identification of NSP4 as the first viral enterotoxin is of interest because it shows this protein has pleiotropic properties besides its intracellular role in viral replication. The outermost layer is composed of the protein VP7 and protruding spikes of trimeric VP4 (López and Arias, 2006; Estes and Greenberg, 2013). The VP4 cleavage by trypsin promotes rearrangements in the viral particles that rigidify the spikes and is required for receptor binding and cell penetration (López et al., 1985; Arias et al., 1996).

In our work, the chemical fractionation of the crude extract resulted in five fractions, and one of them (F3) showed potential antiviral activity with CPE inhibition at 50 μg/ml (data not shown) and a satisfactory PI (75%), although lower than the activity found to the crude extract (PI = 100%). As expected, the fractionation produced samples with higher cytotoxicity due to the concentration of some compound classes, forcing the evaluation of the antiviral activity at lower concentrations than that used for the crude extract.

Table 3
Phytochemical screening and yield of crude ethanolic extract (MUL), fractions (F) and subfractions (SF) from *Myracrodruon urundeuva* leaf.

Selected materials	Extract yield (%, w/w)	Tan	Fla	Ant	Ter	Car Gly	Alk
MUL	19.4	+	+	–	+	–	–
F1	12.0	–	–	ND	+	ND	ND
F2	8.4	–	–	ND	+	ND	ND
F3	5.9	+	+	ND	+	ND	ND
F4	18.2	+	+	ND	–	ND	ND
F5	48.0	+	+	ND	–	ND	ND
SF1	0.5	–	+	ND	+	ND	ND

Tan: tannins; Fla: flavonoids; Ant: anthraquinones; Ter: terpenes; Car Gly: cardiotonic glycosides; Alk: alkaloids; (+): presence; (–): absence; ND: not done.

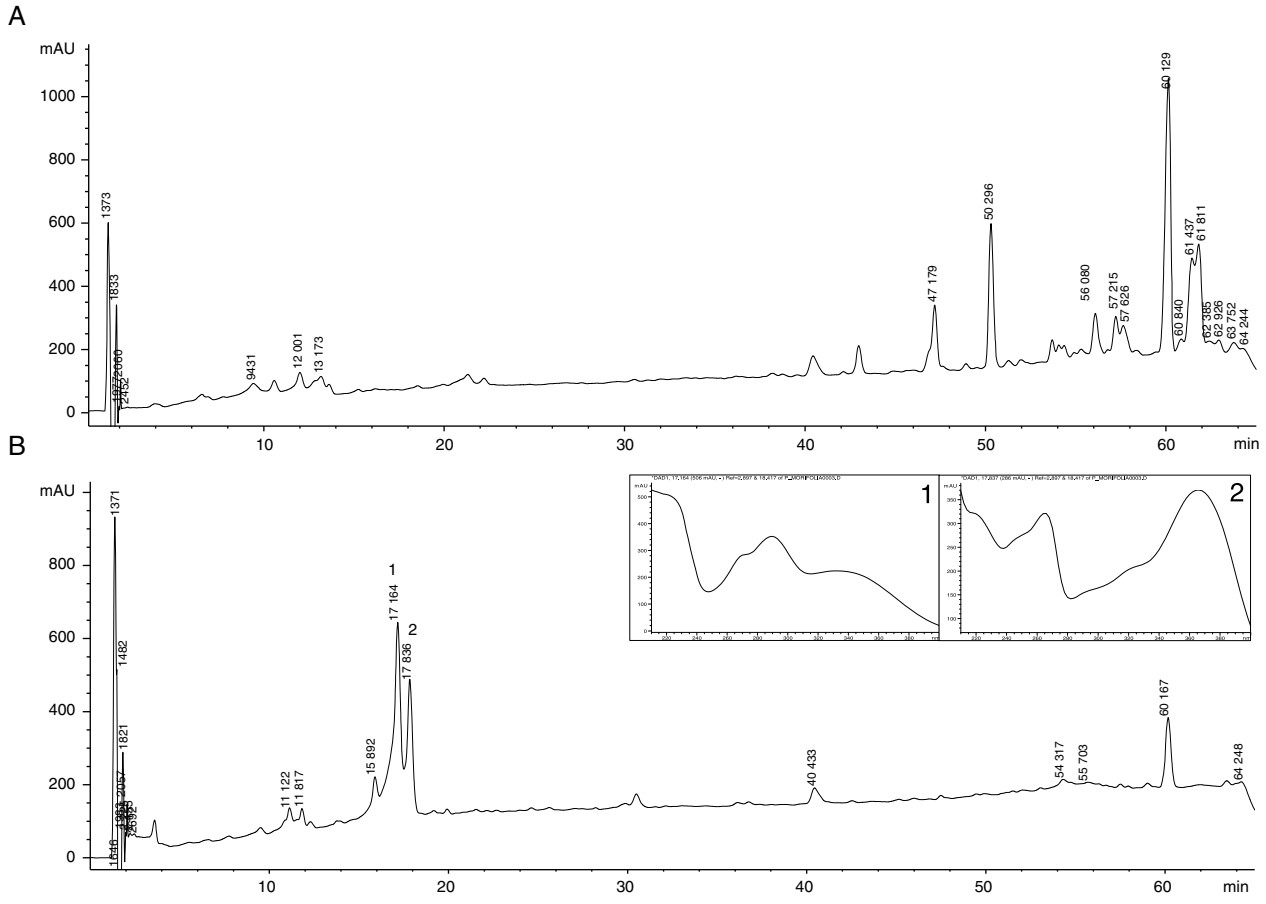

Fig. 2. Chromatographic profile of crude extract MUL (A) and fraction SF1 (B) with UV spectra obtained online for peaks 1 and 2.

Interestingly, a further F3 fractionation yielded two active subfractions (SF1 and SF3) with PI values greater than F3 (92% and 80%, respectively). In SF1, which showed the highest activity, flavonoids were the major class of compounds found in addition to small amounts of terpenes, as evidenced by TLC (data not shown) and HPLC profiles. The TLC analysis of this fraction showed predominance of spots with yellow fluorescence under UV_{365nm} light, which was intensified with the use of NP/PEG spray reagent. This finding was corroborated by the HPLC profile, which showed peaks associated with UV spectra characteristic of flavonoids and maximum absorption at *ca.* 275 and 350 nm (Fig. 2). Terpenes were evidenced by the presence of spots of purple color when the TLC plate was sprayed with anysaldehyde/sulfuric acid and these compounds may be the responsible for the peaks observed in the chromatogram of SF1 with retention times in the 40–65 min range. The final purification of the chemical constituents of SF1 was not performed due to the low amount obtained for this sample (47 mg).

The antiviral activities of flavonoids have been extensively reported. Mass spectrometric data have demonstrated the presence of gallotannins in *M. urundeuva* (Da Silva et al., 2011). Interestingly, gallotannins are potent calcium-activated Cl^- channel inhibitors whose biological activity provides antisecretory benefits (Namkung et al., 2010). On the other hands, there are evidences that NSP4, a nonstructural glycoprotein released from rotavirus-infected cells, induces the release of Ca^{2+} from the endoplasmic reticulum, resulting in increased paracellular permeability in enterocytes, as well as increased secretion in the crypt cells mediated by activation of Cl^- transporter (Ramig, 2004). Thus, these findings point to a possible course of action for the anti-rotavirus activity of *M. urundeuva*.

Another interesting finding in literature, which corroborates with the potent antiviral effect of flavonoid-enriched fraction SF1, is the inhibitory activity related to several flavonoids on reverse transcriptases and proteases even at low concentrations (Ko et al., 2009). Trypsin inhibitory activities have also recently been identified in flavonoids extracted from orange peel and green tea leaves (Shahwar et al., 2013). Since the infectivity of rotaviruses is increased by trypsin and the viruses replicate primarily in intestinal enterocytes during a natural infection, it is possible that the flavonoids present in SF1, may be disrupting viral entry by a protease inhibitor mechanism. Therefore, our data provide insights for further studies with this flavonoid-enriched subfraction, focusing on the suppressive effect of viral amplification in the early phase of infection, and on an inhibitory activity on a possible mechanism of action involving the NSP4 enterotoxin.

Finally, it is important to consider that a single compound could not be responsible for the full antiviral activity and the combination of compounds with balanced proportions in crude extract could be acting in an additive or synergistic mode resulting in more effective virucidal activity. These considerations encourage us to perform additional studies with SF1 and MUL using other complementary tests *in vitro* and *in vivo* assay with murine rotavirus strains in mice.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors contributions

ABC, coordination of the research and writing of the manuscript; PCO, biological assays; SC, PCR assay and analysis; PRVC, phytochemical screening and fractionation; FLF, preparation of extract; MGRD and VLA, HPLC analysis; LAMM, preparation of extract.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors thank the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) for financial support (N° EDT-3253/06) of this research and the Fundação Ezequiel Dias (Funed).

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