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)