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Antioxidant, antibacterial, leishmanicidal and trypanocidal activities of extract and fractions of *Manilkara rufula* stem bark

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Abstract*—Manilkara rufula belongs to family Sapotaceae and has received little attention regarding its pharmacological properties and chemical composition. Therefore, our objective was to determine the farmacological activities and preliminary chemical profile of the stem bark from M. rufula. The plant stem bark of M. rufula was collected and ethanolic extract and fractions were prepared. The antioxidant activity was determined by •DPPH and ABTS•+ scavenger methods and inhibition of β-carotene oxidation. The minimum inhibitory and bactericidal concentrations were estimated through the broth microdilution method against gram positive and negative bacteria. Cytotoxicity, antioxidant and cytoprotection potential were investigated on culture macrophages. Antiproliferative activity was evaluated by Alamar Blue method. The in vitro arginase activity from Leishmania amazonensis was determined in presence of the samples. In addition, their effects on survival of L. amazonensis and Trypanosoma cruzi amastigotes in mice macrophages were assessed. The preliminary phytochemical profile was evaluated by qualitative methods of classical phytochemistry, infrared spectroscopy, LC-MS/MS and CG-MS. The samples presented antioxidant, bactericidal, leishmanicidal and trypanocidal properties without toxic characteristics on normal cells. Triterpenes were observed in the hexane fraction, while glycosides, aromatic rings, fatty esters, proanthocyanidin dimers and trimers, catechin and various terpenes were observed in the other fractions.*

Keywords*— *Sapotaceae, Manilkara rufula, pharmacological activities, chemical composition.

I. INTRODUCTION

Sapotaceae is a family with approximately 1000 trees and shrubs species, distributed in 50 genera, latex producers and ecologically important for their area of occupation. In addition, some species of this family are employed in folk medicine, ornamentation and animal feeding (Kuera et al., 2002; Araújo Neto, 2009). The most important genera of this family in Brazil are *Pouteria*, *Sideroxylon* and *Manilkara* (Gomes et al., 2003).

The genus *Manilkara* has 19 species identified in Brazil, distributed in the Atlantic Forest, sandbanks, coast al tableland forests, Caatinga, Cerrado and Amazonian Forest. In the Northeast region, twelve *Manilkara* species have already been confirmed in different ecosystems. The plants of this genus have been described to have anti-inflammatory, antiparasitic, antitumor, antibacterial and antioxidant properties, mainly due to the presence of various secondary metabolites, such as flavonoids, phenolic acids, saponins and triterpenes (Gomes et al., 2003; Ma et al. 2003; Eibond et al., 2004).

Among the species belonging to this genus, *M. rufula*, popularly known as "maçaranduba", stands out. This is a 5 to 10-meter-high tree that is very often used for logging, but with no references of popular use as medicinal plant.

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Regarding the pharmacological activity and chemical composition of *M. rufula*, a recentstudy showed the trichomonicidal effect of some compounds isolated from the plant stem, whereasits methanolic extract was demonstrated to have low activity against *S. aureus*, *E. coli* and *K. pneumonia* strains (de Souza, 2015; Vieira et al., 2016).

Therefore, the objective of this study was to determine the antioxidant, bactericidal, leishmanicidal, trypanocidal and antiproliferative (to cancer cells) activities, as well as the preliminary phytochemical profile, of the stem bark of *M. rufula*, a plant that has been poorly studied so far, in order to contribute to the discovery of bioactive molecules particularly in species present in the semi-arid region of Bahia State.

II. MATERIAL AND METHODS

A. Collection and boanical identification

Specimens of *M. rufula* were collected in the municipality of Maracás in Bahia State, Brazil Northeastern (13º26'27"S 40º25'51"O) in September 2015. Multiple plants were identified at the harvesting site and one representative voucher specime was deposited in the Herbarium at State University of the Southwest of Bahia under number 12455. Plant materials were formally identified by Guadalupe Edilma Licona de Macedo, PhD, Full Professor at the Department of Biological Sciences, State University of the Southwest of Bahia. The collection was approved by Brazilian Genetic Heritage Management Council (CGEN 010557/2015-6).

B. M, rufula sample and fractionation

The stem bark of *M. rufula* (50 g) was extracted three times with absolute ethanol for 72 h and filtered to obtain the ethanolic extract of *M. rufula* (EEMR = 23.3%). EEMR was fractionated via liquid-liquid partitioning with hexane, ethyl acetate, methanol and hydroalcoholic solvents in order of increasing polarity to obtain hexane (HFMR = 21.0%), ethyl acetate (EAFMR = 20.7%), methanol (MFMR = 45.1%) and hydroalcoholic fractions of *M. rufula* (HAFMR =6.3%)n (Kuroshima, 2002; Silva et al., 2014). Solvents were purchased from Synth (Sao Paulo, Brazil) from analytical grade or better.

C. Quantification of total phenols method

Total phenols in samples was determined by Folin-Ciocalteu method, with minor modifications (Singleton et al., 2002). The samples were diluted in ethanol to a final concentration of 1 mg/mL. In assay tubes, 125 µL of each sample were mixed with 125 µL of Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) and 1 mL of distilled water. After 3 min, 125 µL of a saturated solution of Na_2CO_3 were added and the samples were incubated at 37ºC. After 30 min, the absorbance of samples was measured at 750 nm. The amount of total phenols in samples was determined based on the standard curve of gallic acid (0.5 at 25 µg; Sigma-Aldrich, St. Louis, MO, USA) and expressed as equivalent µg of gallic acid per milligram of sample (µg GAE/mg). The calibration curve equation for gallic acid was y= 0.066x + 0.0651, R^2= 0.9805.

D. Qualitative phytochemical study

Extract and fractions of the stem bark of *M. rufula* were analyzed by thin layer chromatography for qualitative identification of alkaloids and flavonoids, with specific developers for each class. The samples were previously solubilized in methanol, except for the hexane fraction, which was solubilized in chloroform. They were then plated with silica gel (stationary phase) and eluted in a glass vat containing an ethyl acetate: methanol (8:2, Synth, Sao Paulo, Brazil) mixture. The plates were observed under UV light 254 nm and 365 nm and then sprayed with the NP-PEG (1% diphenylboryloxyethylamine in methanol, followed by 5% polyethylene glycol 4000 solution in ethanol) and Dragendorff reagents for discovery of flavonoids and alkaloids, respectively (Wagner and Blad, 2009).

The presence of triterpenes was evaluated by Liebermann-Burchard method. About 2 mg of extract and fractions were diluted in 2 mL of chloroform (Synth, Sao Paulo, Brazil). Then, 4 mL of acetic anhydride (Synth, Sao Paulo, Brazil) and 4 drops of sulfuric acid (Synth, Sao Paulo, Brazil) were added. The presence of triterpenes wasindicated by the change from blue to green (Matos, 1997).

E. Infrared spectroscopy

Infrared analyzes were performed using the Perkin Elmer spectrophotometer model Spectrum Two ATR-FTIR, with horizontal attenuated total reflectance accessory employing a zinc selenide crystal. The spectra were obtained by spreading the sample onto the crystal surface of ATR. For each analysis, the cell was cleaned with acetone (Synth, Sao Paulo, Brazil). All spectra were obtained in the region of 4000 to 500 cm^{-1}, with resolution of 4 cm^{-1} and 32 scans (Ruschel et al., 2014).

F. LC-MS/MS

Chromatographic analyses of samples were performed using a UPLC Acquity chromatograph coupled with a TQD Acquity mass spectrometer (Micromass-Waters), with an electrospray ionization (ESI) source in the negative mode. The column was a Phenomenex Luna C-18 (250x4.6 mm, 5 µm). The mobile phases were foram water/formic acid 0.1% (phase A) e acetonitrile/formic acid 0.1% (phase B). The flow rate was 1 mL/min with a

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linear gradient starting at 0% B and increasing to up 100% in 60 min, before holding until 5 min, and then returning to initial conditions, followed by column re-equilibration. The ESI conditions were: capillary = 4.5 kV, cone = 30 V, source temperature = 300 °C, desolvation temperature = 300 °C, and collision energy = 30 V, with data acquisition between *m/z* 50 and 1000. The components of *M. rufula* samples were putatively identified by comparing their *m/z* values and fragmentation patterns with previous reports.

G. CG-MS

The identification of the compounds in the hexane fraction from *M. rufula* stem bark was performed after separation by high resolution gas chromatography with the capillary column "Rtx – 5MS" – 30 m (length) x 0.25 mm (internal diameter) x 0.25 µm (film thickness) nominal, helium gas as drag gas coupled to a mass detector (CG-MS Model QP2020, Shimadzu, Kyoto, Japan). The identification of compounds was performed by comparing the mass spectra of the samples with those found in NIST version 14.

H. Determination of antioxidant activity

The antioxidant activity of *M. rufula* samples were evaluated using three well-known methods: •DPPH (2,2–diphenyl-1-picrylhydrazyl) free radical scavenging, *ABTS•+(*2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid cation radical*) decolorization and* β*-carotene-linoleic acid* cooxidation assays (Marco, 1968, Miller, 1971).

In the DPPH assay, the samples (1.25-150 µg mL^{-1}) were incubated to 2 mL of an ethanolic solution of •DPPH (Sigma-Aldrich, St. Louis, MO, USA) (70 µM). After 20min at 25°C, the absorbance was measured at 517 nm.In the *ABTS•+ assay, we first prepared* ABTS radical cation by reacting 7 mM ABTS salt (Sigma-Aldrich, St. Louis, MO, USA) with 2.45 mM potassium persulfate (Synth, Sao Paulo, Brazil) and allowing the mixture to stand in the dark at room temperature for 16 h before use. The stock *ABTS•+*solution was diluted with ethanol (absorbance of 0.7 at 734 nm) and incubated to samples (1.25-150 µg mL^{-1}) in a final volume of 2 mL. The absorbance was measured at 734 nm after 7 min of incubation.

In the β*-carotene-linoleic acid*cooxidation assay, a stock solution of β-carotene/linoleic acidwas initially prepared by dissolving 2 mg of β-carotene (Sigma-Aldrich, St. Louis, MO, USA) in 100 µL of chloroform (Synth, Sao Paulo, Brazil). Ten microliters of β-carotene solution were mixed to 40 mg of linoleic acid and 530 µL of Tween 40 (Sigma-Aldrich, St. Louis, MO, USA). The chloroform was rotaevaporated and aerated distilled water was added to the mixture until an initial absorbance of 0.65 at 470 nm. Two milliliters of β-carotene/linoleic acid emulsion were mixed with the samples (1.25-150 µg mL^{-1}). The reactions were incubated at 50 °C for 2 h before taking another absorbance reading.

In all experiments, the values were expressed as the concentration of sample necessary to reduce 50% the free radicals or the β-carotene bleaching (IC_{50}). Gallic acid was used as control in •DPPH and ABTS•+*methods*, while Trolox, in the β-carotene bleaching assay.

I. Cell culture assays

A monocyte cell line THP-1 (human leucemia monocytes) was obtained from Instituto de Química, Universidade de São Paulo (São Paulo, Brazil). THP-1 was grown in DMEM (Gibco-BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin and 100 µg mL^{-1} β-marcaptoethanol (all from Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in a 5% CO_2 atmosphere. THP-1 monocytes ($2x10^6$ mL^{-1}) were differentiated into macrophages by medium supplementation with phorbol-12-myristate-13-acetate (PMA, 5 ng mL^{-1}, Sigma-Aldrich, St. Louis, MO, USA) and incubated for 2 days at 37 ºC under 5% CO_2. The adhered cells were collected by trypsinization (Park et al., 2007).

The cell viability was first determined by lactate dehydrogenase assay.[17] The cells ($1x10^5$ $well^{-1}$) were dropped into 96-well plates and pre-incubated with the extract and fractions of *M. rufula* at concentration of 10, 50 and 100 µg mL^{-1} at 37 °C for 24 h. Afterwards, the supernatant was collected and the lactate dehydrogenase was determined by using a biochemical kit (Labtest). As positive control, the cells were incubated with Triton X-100 (1%) (Sigma-Aldrich, St. Louis, MO, USA).

J. Citoprotective and antioxidant activities

Macrophages ($1x10^5$ $well^{-1}$) derived from THP-1 cell line were placed into a 96-well plate and pre-incubated at 37 °C with extract and fractions of *M. rufula* (50 µg mL^{-1}). For the cytoprotective assay, cells were washed twice in phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) 24 h later. The medium was replaced, and H_2O_2 (Synth, Sao Paulo, Brazil) (1 mM) was added, following incubation for 18 h. The residual cell viability was determined as described above (Facundo et al., 2007).

To determine the antioxidant activity of isolated extract and fractions in cells, the same procedure was performed. After incubation with H_2O_2, the cells were washed in PBS, incubated in 2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA) at 10 µM for 1 h at 37°C in a dark chamber. The extracellular DCFH-DA was removed after washing the cells twice in PBS. The oxidation of DCFH was determined by fluorescence (λ_{ex}=485 nm; λ_{ex}=520 nm) using a microplate reader, since the fluorescent signal indicates the intracellular redox state. In both assays, ethanol (5%) and tempol (10 µM)

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were selected as negative and positive controls, respectively (Jeong et al., 2009).

K. Hemeoxigenase activuty

Cells ($5x10^5$ $well^{-1}$) were pre-incubated for 24 h with fractions at 50 µg mL^{-1} or tempol (10 µM). Afterwards, the harvested cells were subjected to three cycles of freeze-thawing before addition to a reaction mixture consisting of phosphate buffer (1 ml final volume, pH 7.4) containing magnesium chloride (2 mM), NADPH (0.8 mM), glucose-6- phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 U), rat liver cytosol as a source of biliverdin reductase, and the substrate hemin (20 µM). The reaction mixture was incubated in the dark at 37 °C for 1 h and was terminated by the addition of 1 mL of chloroform. After being vigorously vortexed and centrifuged, the extracted bilirubin in the chloroform layer was measured by the difference in absorbance between 464 and 530 nm ($\varepsilon = 40$ $mM^{-1}\cdot cm^{-1}$) (Jeong et al., 2009).

L. Suscetibility testing

The antibacterial activity of EEMR and fractions were examined by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), according to the Institute of Clinical and Laboratory Standards (Santos et al., 2017). To determine the MIC, 5×10^5 CFU mL^{-1} diluted in brain heart infusion medium (Difco, USA) were incubated with EEMR and fractions (1–1000 µg mL^{-1}) in 96-well microplates for 24 h at 37 °C. The vehicle control was ethanol (5%). MIC was defined as the lowest concentration of EEMR or fraction that allowed no visible growth after incubation with 0.01% resazurin dye (Sigma-Aldrich, St. Louis, MO, USA) for 60 min at room temperature. MBC was determined by sub-culturing 10 µL of each incubated well that had a concentration higher than the MIC on Müller-Hinton agar. The MBC was then treated as the lowest concentration of each sample with no visible colony growth on agar plates.

The bacterial strains used in this study were *S. aureus* (ATCC 29213), *K. pneumoniae* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922) and *P. mirabilis* (ATCC 17407) and were kindly provided by Prof Dr Milena Soares dos Santos, Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia.

M. Antiproliferatuve activity to cancer cells

The antiproliferative effect of EEMR was determined in cancercell lines HepG2 (human hepatocellular carcinoma) and HL-60 (human promyelocytic leukemia). These cell lines were kindly provided by Hospital A.C. Camargo (São Paulo, SP, Brazil) and maintained in Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco-BRL, Gaithersburg, MD, USA) medium supplemented with 10% FBS(Cultilab, Campinas, Sao Paulo, Brazil), 2mML-glutamine (Vetec Química Fina, Duque de Caxias, Brazil), and 50 µg/mL gentamycin (Novafarma, Anapolis, Brazil). All cell lines were cultured in cell culture flasks at 37 °C in 5% CO_2 and subcultured every 3–4 days to maintain exponential growth (.The cells were plated in 96-well plates ($3x10^5$ cells mL^{-1} for HL-60 and $7x10^4$ cells mL^{-1} for HepG2) and, after 24 h, samples solubilized in DMSO (50 µg mL^{-1},Synth, Sao Paulo, Brazil) were added into each well and incubated for 72 h. Four hours before the end of the incubation period, resazurin (0.312 mg mL^{-1}, Sigma-Aldrich, St. Louis, MO, USA) was added into each well and the absorbance was measured at wavelengths of 570 nm (reduced) and 595 nm (oxidized) using a plate reader (Ahmed et al., 1994; Rodrigues et al., 2015).

Doxorubicin (5 µg mL^{-1}) and DMSO (0.5%) were used as positive and negative controls, respectively. The results were expressed as percentage of inhibition of cell proliferation in cancer cell lines.

N. Inhibition of Leishmania arginase

Recombinant arginase from *Leishmania amazonensis*(ARG-L) was preparedaccording previously described (da Silva et al., 2008; da Silva et al., 2012). The inhibition test for ARG-L were performed at pH 9.5 in CHES (2-(Cyclohexylamino)ethanesulfonic acid,50 mM, Sigma-Aldrich, St. Louis, MO, USA) buffer with L-arginine (50 mM; Sigma-Aldrich, St. Louis, MO, USA), ARG-L andsamples. The reaction mixtures were incubated in a 37 °C water bath for 15 min. The quantity of the enzyme used was adjusted to 10% of the maximum consumption of L-arginine substrate. Quantification of urea production was performed by the method described by Berthelot (Fawcett and Scott, 1960; Manjolin et al., 2013). Briefly, arginase catalytic capacity was stopped by transferring 10 µL of the reaction mixture into 750 µL of reagent A (20 mM phosphate buffer pH 7, containing 60 mM salicylate, 1 mM sodium nitroprusside and 500 IU of uréase; Labclin, Sao Paulo, Brazil). This mixture was incubated at 37 °C for 5 min. Next, 750 µL of reagent B (10 mM sodium hypochlorite and 150 mM NaOH; Labclin) was added, and the samples were incubated at 37 °C for 10 min. The absorbance was measured at at 600 nm using a GEHAKA 340G spectrophotometer. The positive and negative controls were performed under the same conditions in the absence of inhibitor. The experiments were performed in triplicate in at least two independent experiments.

O. Leishmanicidal activity

Peritoneal exudate macrophages were obtained by injection ofthioglycolate (3%) into the peritoneal cavity of

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Male BALB/c mice (n=2; 2 months; 20-25 g) to induce inflammation and migration of macrophages into the region. After 4 days, mice were euthanized by exposure to a carbon dioxide chamber and the macrophages were collected from the peritoneal cavity by using a serynge and the cells were cultured (Gomes et al., 2003). The animals were obtained from Gonçalo Moniz Institute/Fiocruz vivarium, housed at eight per cage, kept at 24±2 °C with a 12/12-h light/dark cycleand provided with water and chow *ad libitum*. This procedure was approved by Ethics Committee on the Use of Animals (CEUA) of the Gonçalo Moniz Institute/Fiocruz under the number 1126 e performed at Laboratório de Engenharia Tecidual e Imunofarmacologia (Fiocruz).

Briefly, the exudate cells were recovered by peritoneal lavage with sterile saline. Cell suspension was then centrifuged at 1500 rpm for 10 min at 4 °C and the pellet was resuspended in DMEM medium (Sigma-Aldrich, St. Louis, MO, USA). After counting in a Newbauer camera, cells were plated in 96-well plates ($5x10^4$/well) in DMEM medium and incubated for 16 h at 37 °C under CO_2 atmosphere (5%). Plates were then washed three times with warm saline to remove non-adherent cells. The macrophages were infected with stationary growth phase promastigotes of *L. amazonensis* at a ratio of 5:1 macrophage. The co-culture was then incubated at 35°C under CO_2 atmosphere (5%) during 24 h, followed by washing to remove the non-internalized parasites.

Infected macrophages were incubated with the plant extract and fractions (20 µg mL^{-1}) in DMEM medium for 72 h at 37 °C. The wells were then washed with saline solution, fixed with paraformaldehyde solution (4%; Sigma-Aldrich, St. Louis, MO, USA) and stained with Draq5 (5µM, Biostatus, United Kingdom) to label the cellular DNA. Then, the amount of amastigotes and macrophages in each well was counted in the Operetta High-Content Imaging System (Perkin Elmer, Massachusetts, USA) confocal microscope. Amphotericin B (1 µM; Gibco-BRL, Gaithersburg, MD, USA) was used as a positive control (Chaves et al., 2009). The infectivity index of the parasite was determined by multiplying the mean number of amastigotes per cell by the percentage of infection (Tanaka et al., 2007). The percentage inhibition of this index was then calculated.

P. Anti-T. cruzi activity

Murine macrophages adhered in plaque ($6x10^3$ $well^{-1}$) were infected with *T. cruzi* Y strain trypomastigotes ($6x10^4$ trypomastigotes $well^{-1}$) for 24 h. The wells were then washed three times with sterile saline and cells were incubated with the extract and fractions (100 µg mL^{-1}) for 72 h at 37 °C under CO_2 atmosphere (5%) in DMEM medium. The wells were then washed with saline, fixed with 4% paraformaldehyde solution and stained with Draq5 (4 µM). Quantification of amastigotes and macrophages was performed on the Operetta High-Content Imaging System confocal microscope and the data were expressed as infectivity index. Benzonidazole (5 µM) was used as positive control (Bastos, 2013).

This procedure was also approved by Ethics Committee on the Use of Animals (CEUA) of the Gonçalo Moniz Institute /Fiocruz under the number 1126.

Q. Statical analisys

Data were expressedas the mean ± standard deviation or IC_{50} values based on three independent experiments. Significant differences ($p< 0,05$) were detected by one-way ANOVA with Dunnet post-test using GRAPHPAD Prisma (5.0), using significance of 5%. The correlation between antioxidant, trypanocidal and leishmanicidal activity and phenolic content were determined by the Pearson correlation test with significance of 5%.

III. RESULTS

R. Determination of total phenolic and antioxidant activities

The concentration of phenolics in the extract and fractions of the stem bark of *M. rufula* is shown in Table 1. The AEFMR fraction had the highest phenolic concentration in the samples (143 µg EAG mg^{-1}), whereas HFMR contained about 5 times less phenolic compounds (29 µg EAG mg^{-1}).

Extracts and fractions of *M. rufula* were evaluated by three different antioxidant methods: •DPPH and $ABTS^{•+}$ free radical scavenging and inhibition of lipid peroxidation. All samples were able to deactivate •DPPH and $ABTS^{•+}$ radicals and inhibit β-carotene oxidation by lipid radicals (Table 1). The MFMR fraction was the most potent in reducing the •DPPH (IC_{50} = 3 µg mL^{-1}) radical compared to the gallic acid standard (IC_{50} = 1.5 µg mL^{-1}). In addition, it was also the most effective fraction in inhibiting the β-carotene decay, with 20 times more potency than trolox standard (IC_{50} = 21µg mL^{-1}). In the case of the $ABTS^{•+}$ method, the hexane fraction unexpectedly reduced the radical with IC_{50} of 0.12 µg mL^{-1}, but reacted with •DPPH at higher concentrations (IC_{50} = 60 µg mL^{-1}).

In the comparison between the content of reducing compounds, among them phenolics, with the antioxidant activity, the results of the •DPPH and $ABTS^{•+}$ assays were not correlated with the concentration of these compounds (r = 0.643 and p = 0.241 and r = 0.645 and p = 0.239, respectively). On the other hand, there was a correlation between these compounds and the β-carotene co-oxidation

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assay (r = -0.929 and p = 0.022), which suggests that the reducing compounds of the Folin-Ciacalteou reagent are also important in the protection against oxidation of β-carotene.

S. Cell viability, cytoprotection and antioxidant acitivity in cell model

The extract and fractions (50 µg mL^{-1}) of the stem bark of *M. rufula* were effective in protecting macrophage-differentiated THP-1 against H_2O_2-induced cell death when pre-incubated for 24 h with the cells before oxidant addition (Fig. 1A). In particular, the ethyl acetate fraction protected the cells in approximately 83%. Furthermore, this fraction inhibited the intracellular oxidation of DCFH mediated by H_2O_2 in these cells exposed to samples (50 µg mL^{-1}) was also greater for the ethyl acetate fraction (Fig. 1B). The activity of an important antioxidant and cytoprotective enzyme, hemeoxygenase, increased almost twice when the cells were pre-incubated with the same fraction (Fig. 1C).

It is important to note that, even under concentrations of up to 100 µg mL^{-1} of the extract or fraction of *M. rufula* stem bark, THP-1-derived macrophages remained viable (> 90%).

Table 1. Total phenolics and antioxidant activities of the M. rufula stem bark and fractions.

Sample	Total Phenolics µg GAE mg^{-1}	•DPPH IC_{50} (µg mL^{-1})	$ABTS^{\bullet+}$ IC_{50} (µg mL^{-1})	β-carotene-linoleic acid cooxidation (µg mL^{-1})
EEMR	42.6±0.1	11±1	1.68±0.04*	18±1
EAFMR	143±3	3.30±0.02	0.87±0.03	23±1
HAFMR	112.0±0.5	3.11±0.04	1.72±0.02	29±2
MFMR	96.9±0.4	3.05±0.01	1.67±0.03	1.1±0.3*
HFMR	29.2±0.3	59±1*	0.12±0.01*	16±1
Gallic acid	-	1.45±0.01	1.08±0.04	-
Trolox	-	-	-	21±2

EEMR: Ethanolic extract of *M. rufula*stem bark. EAFMR: Ethyl acetate fraction of *M. rufula* stem bark. HAFMR: Hydroalcoholic fraction of *M. rufula* stem bark. MFMR: Methanolic fraction of*M. rufula* stem bark. HFMR: Hexanic fraction of *M. rufula* stem bark.Values represent the mean ± standard deviation based on triplicate measurements. *p<0.05, when compared with gallic acid (•DPPH e $ABTS^{\bullet+}$) and trolox (β-carotene/linoleic acid) by ANOVA and Dunnett's post-test.

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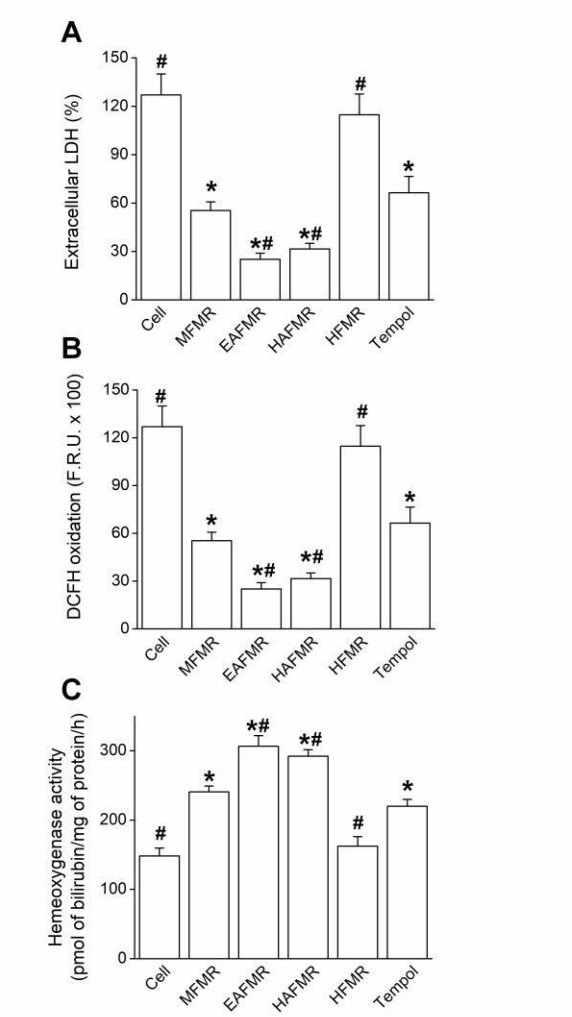

*Fig. 1 Effects of M. rufula fractions on H_2O_2-induced death (A), antioxidant profile (B) and hemeoxygenase activity (C) in THP-1-derived macrophages. Ethyl acetate fraction of the stem bark of M. rufula (EAFMR), hydroalcoholic fraction of the stem bark of M. rufula (HAFMR), methanolic fraction of the stem bark of M. rufula (MFMR) and hexane fraction of the stem bark of M. rufula (HFMR) at 50 µg/mL were pre-incubated with cells for 24 h. The cell viability was determined by releasing of lactate dehydrogenase to supernatant after H_2O_2 (1 mM) incubation, where Triton X-100 (1%) was used as control of a complete cell death (100%) (A). The intracellular antioxidant status was followed by DCFH oxidation in presence of H_2O_2 (100 µM) (B). The bilirubin levels were indicative of hemeoxygenase activity in the cells (C). Tempol (10 µM) was used as positive control in all assays. *p < 0.05 and #p<0.05, when compared to cell and methanolic fraction, respectively, by ANOVA and Dunnett's post-test.*

T. Antibacterial activity

Extract and fractions of the stem bark of *M. rufula* were evaluated for antibacterial potential against gram positive (*S. aureus*) and negative (*P. mirabilis, P. aeruginosa, E. coli* and *K. pneumoniae*) bacteria (Table 2). EEMR and HFMR did not present any antibacterial activity against the bacteria tested at concentrations up to 1 mg mL^{-1}. EAFMR was active against *K. pneumoniae* (MIC = 100 and CBM = 500 µg mL^{-1}), *S. aureus* (MIC = CBM = 500 µg mL^{-1}) and *E. coli* (MIC = 500 = CBM = 500 µg mL^{-1}). On the other hand, MFMR acted similarly on *K. pneumoniae* and *E. coli* (MIC = CBM = 50 µg mL^{-1}), being the most active fraction and still with bactericidal profile (CBM/MIC ≤ 4), according to stablished by French et al. (2006).

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Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the M. rufula stem bark and fractions.

Bacterial strain	EEMR		EAFMR		HAFMR		MFMR		HFMR	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	a	a	500	500	a	a	a	a	a	a
K. pneumoniae	a	a	100	500	a	a	50	50	a	a
P. aeruginosa	a	a	a	a	a	a	a	a	a	a
E. coli			500	500			50	50		
P. mirabilis	a	a	a	a	a	a	a	a	a	a

EEMR: Ethanolic extract of *M. rufula* stem bark. EAFMR: Ethyl acetate fraction of *M. rufula* stem bark. HAFMR: Hydroalcoholic fraction of *M. rufula* stem bark. MFMR: Methanolic fraction of *M. rufula* stem bark. HFMR: Hexanic fraction of *M. rufula* stem bark. (a): Values ≥ 1000 µg/mL.

U. Antiproliferative activity to cancer cells

The antiproliferative activity of the ethanolic extract of *M. rufula* on HepG2 (human hepatocellular carcinoma) and HL-60 (human promyelocytic leukemia) cancercells was investigated. The extract at the concentration of 50 µg/mL showed weak antiproliferative activity on both cell lines (14.2 and 17.9%, respectively), when compared to doxorubicin (90.3 and 95.0%, respectively).

V. In vitro inhibition of L. amazonensis arginase

Extract and fractions of *M. rufula* were also evaluated for the ability to inhibit the enzyme arginase of *Leishmania amazonensis*. The ethanolic extract and the ethyl acetate fraction had the lowest IC_{50} values (15.7 and 15.6 µg mL^{-1}, respectively) (Table 3). Quercetin, used as a positive control, inhibited the enzyme with an IC_{50} of 1.0 µg mL^{-1}. Furthermore, there were no correlation between phenolic compounds and *L. amazonensis* arginase inhibition (r = -0.446 and p = 0.451).

Table 3. Inhibition of L. amazonensis arginase by M. rufula stem bark and fractions.

Samples	IC_{50} (µg mL^{-1})
EEMR	15.7±1.0*
EAFMR	15.6±1.1*
HAFMR	29.4±1.4*
MFMR	26.±1.*
HFMR	>100.0*
Quercetin	1.0±0.1

EEMR: Ethanolic extract of *M. rufula* stem bark. EAFMR: Ethyl acetate fraction of *M. rufula* stem bark. HAFMR: Hydroalcoholic fraction of *M. rufula* stem bark. MFMR: Methanolic fraction of *M. rufula* stem bark. HFMR: Hexanic fraction of *M. rufula* stem bark.Values represent the mean ± standard deviation based on triplicate measurements. *p<0.05, when compared with quercetin by ANOVA and Dunnett's post-test.

W. Anti-L. amazonensis and T. cruzi activities

In macrophages infected with *L. amazonensis* amastigotes, the ethanolic extract and the hexanic fraction reduced the infectivity index of the parasite to 4.1% and 6.9%, respectively (Table 4). MFMR, HAFMR and EAFMR, however, reduced the infectivity of *L. amazonensis* amastigotes to 21.4, 24.4 and 19.5%, respectively. Additionally, the extract and fractions of *M. rufula* were evaluated for *in vitro* trypanocidal potential. Murine macrophages infected with *T. cruzi* and treated with EAFMR, MFMR, HAFMR and HFMR had the infectivity index reduced by 78.1, 53.9, 54.9, and 10.5%, respectively (Table 4). There was no association between the effect observed on *L. amazonensis* amastigotes and the phenolic concentration in the samples (r = 0.862 and p = 0.060). On the other hand, a positive correlation between the concentration of reducing compounds in the samples with the trypanocidal activity was found (r = 0.972 and p = 0.006).

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Table 4. Leishmanicidal and trypanocidal activities of the M. rufula stem bark and fractions.

Samples	Inhibition of infectivity index (%)	
	L. amazonensis	*T. cruzi*
EEMR	4.1 0.1-8.1	n.a.
EAFMR	19.5 18.5-20.4	78.1* 76.2-77.6
MFMR	21.4 21.0-21.7	53.9* 46.9-60.2
HAFMR	24.4* 17.7-31.0	54.9* 51.3-60.7
HFMR	6.9 4.6-9.1	10.5 6.8-13.0
Amphotericin B	1.67 1.65-1.70	-
Benznidazole	-	0.55 0.60 – 0.51

EEMR: Ethanolic extract of *M. rufula* stem bark. EAFMR: Ethyl acetate fraction of *M. rufula* stem bark. HAFMR: Hydroalcoholic fraction of *M. rufula* stem bark. MFMR: Methanolic fraction of *M. rufula* stem bark. HFMR: Hexanic fraction of *M. rufula* stem bark. (n.a.):No activity. The values represent the mean and the values of the replicates of the experiment. The data were expressed as a function of the reduction of the infectivity index of the parasite in comparison of control. The concentration of the sample was 20 or 100 µg/mL for the tests with *L. amazonensis* or *T. Cruzi* amastigotes, respectively.

X. Qualitative analysis of phytochemicals

The phytochemical profile of the stem bark of *M. rufula* was initially analyzed for the presence of triterpenes, alkaloids and flavonoids by some classical phytochemical methods. The assay did not demonstrate the presence of these compounds in the samples, except for HFMR, whose qualitative Liebermann-Burchard test indicated the presence of triterpenes. Recognizing the limitations of these methods (Simões et al., 1999), plant extract and fractions were also analyzed by infrared technique to identify which functional groups were present and to correlate them with groups common to the Sapotaceae family, followed by LC-MS/MS and GC-MS analysis.

Y. Infrared spectroscopy

The IR spectrum (Table 5 and Fig. 2) of the HFMR fraction showed a profile of CH_3- and CH_2-rich compounds which along with C=O and C-O bands of esters indicate the presence of fatty esters and/or terpenes, common compounds in low polarity fractions of plant extracts. The infrared analysis does not allow the distinction between the groups above mentioned. However, considering the qualitative analysis, it can be stated that HFMR has triterpenes. In the EAFMR fraction, a relative decrease in the intensity of the bands related to fatty esters was observed. In contrast, there was a strong band of O-H groups present in phenols or alcohols, besides the presence of numerous C-O binding bands at 1020 to 1200 cm^{-1}, indicating the presence of glycosidic groups. On the other hand, the analysis of the spectra allowed to conclude that the chemical profile of the constituents present in the HAFMR fraction was similar to the constituents of the MFMR fraction, being observed an intense band corresponding to O-H groups, which is in agreement with the greater polarity of these fractions, but absence of compounds with C=O groups of esters and ketones. Furthermore, it was found that both fractions had a large number of C-O stretching bands, indicating the presence of glycosidic groups, as well as a band of carbonyl conjugated to aromatic rings, common in classes of compounds such as flavones or aromatic acids (Barbosa, 2013).

Table 5. Infrared analysis of theM. rufulastem bark and fractions.

Wave n° (cm^{-1})	Functional group	EEMR	EAFMR	HAFMR	HFMR	MFMR
3328,8	νO-H (Alcohol orPhenol)	XXX	XXX	XXX	-	XXX

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3000-2800	νC_{sp3}-H	XXX	XX	XX	XXX	XX
1733.9	νC=O (ester)	X	X	-	XX	-
1606.3	νC=O conjugatedwith aromatic	XX	XX	XXX	-	XX
1516	νC=C (alkene or aromatic ring)	X	X	X	-	X
1440 – 1480	δCH_2	X	XX	XX	XX	XX
1370 – 1390	δCH_3	X	-	X	XX	X
1243.3	νC-O (phenol)	X	XX	X	XX	X
1020 – 1200	νC-O (alchool)	XX	XX	XX	X	XX
700 – 850	C-H (aromatic ring)	-	X	X	-	X

EEMR: Ethanolic extract of *M. rufula* stem bark. EAFMR: Ethyl acetate fraction of *M. rufula* stem bark. HAFMR: Hydroalcoholic fraction of *M. rufula* stem bark. MFMR: Methanolic fraction of *M. rufula* stem bark. HFMR: Hexanic fraction of *M. rufula* stem bark. (-): Absence of band in the spectrum. (X): Smallband intensity in the spectrum. (XX): Meduim band intensity in the spectrum. (XXX): High band intensity in the spectrum.

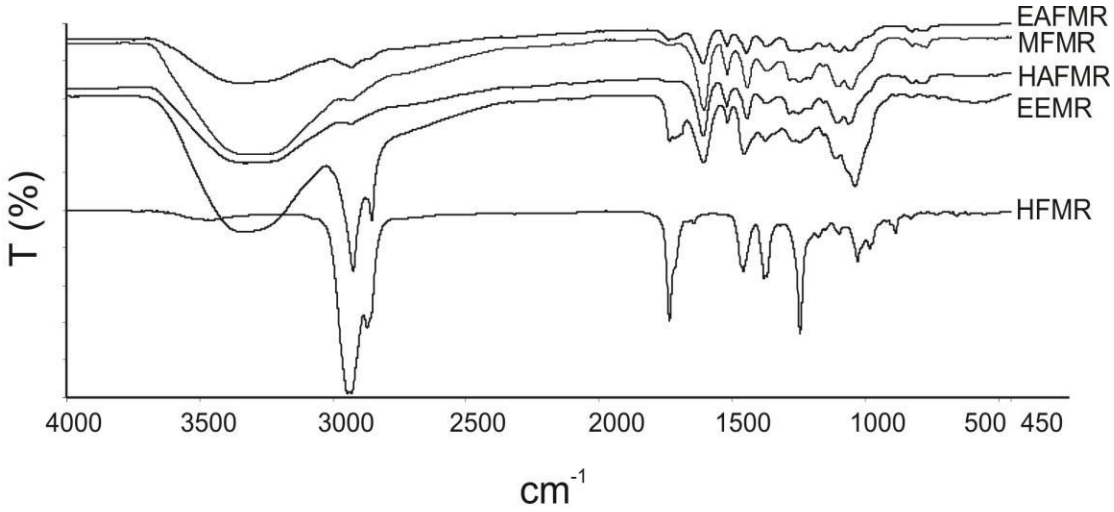

Fig. 2 Infrared spectrum of extract and fractions of the stem bark of M. rufula. EEMR: Ethanolic extract from the stem bark of M. rufula. EAFMR: Ethyl acetate fraction of the stem bark of M. rufula. HAFMR: Hydroalcoholic fraction of the stem bark of M. rufula. MFMR: Methanolic fraction of the stem bark of M. rufula. HFMR: Hexanic fraction of the stem bark of M. rufula.

Z. LC-MS/MS analysis

The methanolic, hydroalcoholic and ethyl acetate fractions of the *M. rufula* stem bark were analyzed in the sequence, by negative ionization mass spectrometry. The ions *m/z* 577 and 865 were found in the EAFMR and HAFMR fractions (Table 6). The comparison of *m/z* values and their respective fragments with the literature indicated the presence of proanthocyanidin dimers (Bystrom et al., 2008). Proanthocyanidin trimers (*m/z* 865.28 and 865.33) were also potentially found in the EAFMR and HAFMR fractions (Simões et al., 1999, Barbosa, 2013). Ions 289 and 577 also suggested the presence of proanthocyanidin subtypes as catechin in ethyl acetate and hydroalcoholic fractions, whereas the 341 ion was possibly the glycoside caffeoyl glucose present in the MFMR fraction (Bastos et al., 2007).

The EAFMR, HAFMR and MFMR fractions presented the ions 757 and 367, whose fragmentation did not allow the identification of the potential compound, but the literature suggests them as quercetin-3-O-triglycoside and feruloylquinic acid (Chen et al., 2012). Besides, the nature of several other ions and their fragments has not been suggested, which demonstrates the importance of further studies in order to reliably isolate and identify these compounds.

Table 6. Retention times and fragments of the major ions found in the M. rufula stem bark by LC-MS/MS in the negative mode.

Sample	Retention time (min)	$[M-H]^-$	Fragments	Putative substances

https://dx.doi.org/10.22161/ijaers.6.6.78 *ISSN: 2349-6495(P) | 2456-1908(O)*

EAFMR	14.4	577	289; 425	Proantocyanidin dimer
	15.5	865	287;695;739	Proantocyanidin trimer
	15.5	289	205; 245	Catechin
	18.1	577	289;425	Proantocyanidin dimer
HAFMR	14.4	577	289;425	Proantocyanidin dimer
	15.7	865	695;739	Proantocyanidin trimer
MFMR	3.8	341	179	Caffeoil glucose

EAFMR: Ethyl acetate fraction of *M. rufula* stem bark. HAFMR: Hydroalcoholic fraction of *M. rufula* stem bark. MFMR: Methanolic fraction of *M. rufula* stem bark.

AA. CG-MS analysis

Finally, the HFMR fraction was analyzed by gas chromatography coupled to mass spectrometry, which detected the presence of olean-12-en-3-one, lanosta-8,24-dien-3-one, lupenone, lupeol acetate, cycloheucalenol acetate, cyclolanostan-3-ol, cis-3,14-clerodadien-13-ol, by comparison with the NIST library and similarity of 92, 76, 89, 87, 76, 85 and 79%. The chemical structure of the compounds identified in the HFMR is shown in Fig. 3.

IV. DISCUSSION

Species of the genus *Manilkara* are known to contain flavonoids, phenolic acids, saponins and triterpenes, which are associated with pharmacological properties, including anti-inflammatory, antiparasitic, antitumor, antibacterial and antioxidant properties (Ma et al., 2003; Eibonde et al., 2004; Eskander et al., 2014). Among the different metabolites, phenolic compounds are important due to their inherent antioxidant potential, being able to reduce damage to the host tissue during inflammatory and/or oxidative processes typical of several chronic diseases such as cancer, arthritis, diabetes, atherosclerosis, among others (Fernandes, 2010; Parick and Patel, 2016). Thus, concentrations of phenolics and other reducers in all samples of the stem bark of *M. rufula* were determined, and the concentrations found here are higher than those in the study of Parikh and Patel (2016) from the methanolic extract of the fruit of *Manilkara hexandra* (8.1 µg EAG/mg) . It is noteworthy that the AEFMR fraction presented higher phenolic concentration in relation to the HFMR fraction likely because most of reducing compounds are more polar (Andreo and Jorge, 2006).

As for the antioxidant activity, it was possible to observe that all the samples were able to deactivate the •DPPH and $ABTS^{\bullet+}$ radicals and to inhibit the oxidation of β-carotene by the lipid radicals. Although studies demonstrating the antioxidant activity of *M. rufula* are missing, investigations with *Sideroxylon obtusifolium* from same family as *M. rufula* have shown that the ethanolic extract of this plant reduces the •DPPH radical with IC_{50} of 9.5 µg/mL, indicating a similar antioxidant activity between these two species (Leite et al., 2015). The fractions of *M. rufula* probably contain molecules with cytoprotective activity, particularly the ethyl acetate fraction that protected the cells against death induced by H_2O_2 in approximately 83%. This same fraction also inhibited intracellular redox stress more significantly than tempol, a well recognized antioxidant in several *in vitro* and *in vivo* models (Soule et al., 2007). The ethyl acetate fraction may protect the macrophages against H_2O_2-induced damage through Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway, which is an important transcription factor for many antioxidant and cytoprotective enzymes, including hemeoxygenase. A recent study with *Madhuca indica* from Sapotaceae family showed that the compound 3,5,7,3',4'-pentahydroxyflavone was cytoprotective by inducing the expression of antioxidant enzymes related to Nrf2-Keap1 system (Wang et al., 2010). Indeed, Nrf2 signaling pathway may be activated by several phytochemicals, including polyphenols and triterpenoids, which could explain the effects mediated by *M. rufula* on macrophages (Park et al., 2007; Facundo et al., 2005). An intriguing point is that all the samples of *M. rufula* showed considerable toxicity against THP-1-derived macrophages up to the concentration of 100 µg/mL. In the literature, the ethyl acetate fraction of *Pouteria venosa* (Sapotaceae) plant showed no toxicity on macrophages of the J774 lineage at the concentration of 200 µg/mL, which is in agreement with our results (Santos et al., 2015). Therefore, these preliminary data suggest that this plant possibly has low toxicity for application in *in vivo* models.

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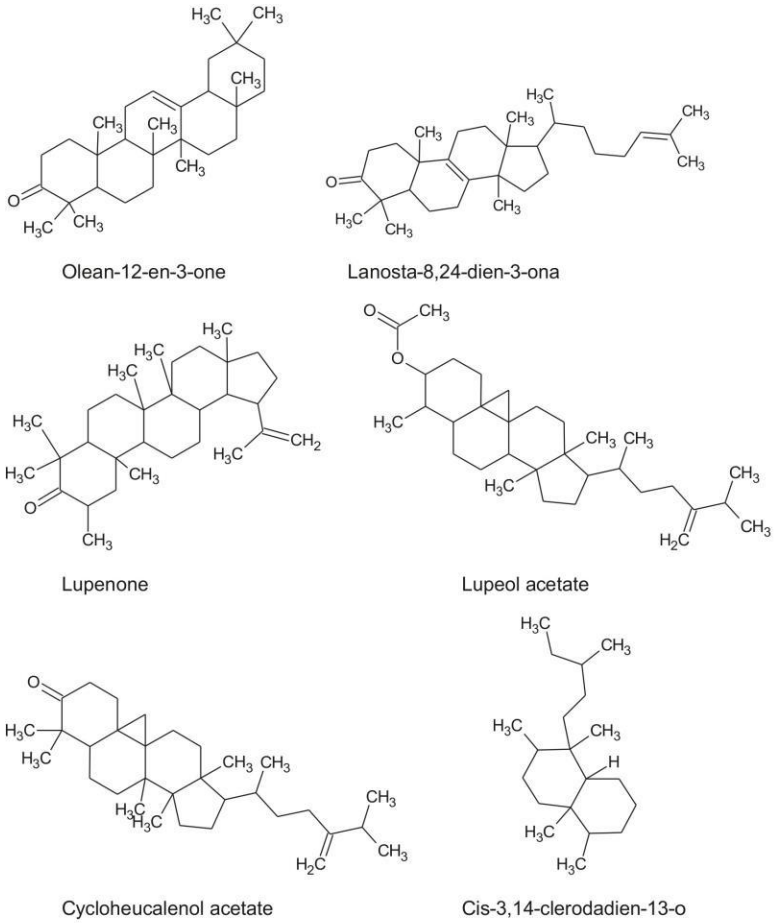

Fig. 3 Chemical structure of the compounds found in the hexane fraction of the stem bark of M. rufula by CG-MS.

Scientific and ethnopharmacological studies have also reported the use of Sapotaceae plants in the treatment of cancer (Bhaumik et al., 2015). Nevertheless, the EEMR showed only weak antiproliferative activity on HepG2 and HL-60 cell lines. The ability of some constuints from this plant in activating the Nrf2 pathway may justify, at least in part, its low toxicity against cancer cells (Chen et al., 2015).

Sapotaceae also contains plant species to which antiprotozoal activity has been reported, including against *Trypanosoma*, *Plasmodium* and *Trichomonas vaginalis* (Vieira et al., 2016; Silva et al., 2013; Inoccente et al.,2014). Arginase stands out among the possible targets in trypanosomatidsbecause thist enzyme catalyzes the hydrolysis of L-arginine to L-ornithine and urea in trypanosomatids like *Leishmania* sp. and *T. brucei*, but not in *T. cruzi*. Ornithine is a precursor of the polyamines pathway and therefore involved in mechanisms related to cell proliferation and survival, constituting an important target for the treatment of parasites such as leishmaniasis (Birkholtz et al., 2011; Cruz et al., 2013). Mammals also have two arginase isoforms but they contain two non-conserved amino acids that creates distinct channel like domainsin comparison to trypanossomatids, which allows the development of specific inhibitors (Riley et al., 2011).

Extract and fractions of *M. rufula* were evaluated for the ability to inhibit the arginase of *Leishmania amazonensis*. EEMR and EAFMR presented the lowest IC_{50} values (15.7 and 15.6 µg mL^{-1}, respectively). A study that aimed the anti-arginase activity by extract of the medicinal plant *Cecropia pachystachya* had IC_{50} 17µg mL^{-1} (Cruz et al., 2013). Isolated polyphenols as (+)-catechin and (-)-epicatechin that are momeric unit of proanthocianidin present in the fractions EAFMR, HAFMR, showed a great arginase inhibition (dos Reis et al., 2013; de Souza et al., 2014). The caffeoil glucose present at MFMR fraction contributed to the *L. amazonensis* arginase inhibition as observed by activity of verbascoside, a compound that containing caffeoil moiety as pharmacophoric group (Maquiaveli et al., 2016). In fact, antileishmanial verbascoside target selectively parasite arginase (Maquiaveli et al., 2017). Verbascoside also is a major compound present in *Stachytarpheta caynnensis*a traditional medicinal plant used to treat leismaniasis (Maquiaveli et al., 2016).

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In the case of macrophages infected with *L. amazonensis* amastigotes, the MFMR, HAFMR and EAFMR fractions reduced the infectivity of *L. amazonensis* amastigotes more sharply, which leads us to infer that these fractions act on other targets associated with the survival of *L. amazonensis* (Singh et al., 2012). In contrast, it was observed that EEMR inhibited arginase *in vitro*, but did not reduce the infectivity of *Leishmania* in a cellular model, which could be due to lower permeability of phytochemicals through the membranes or to metabolism of these compounds by the parasite/macrophage (Silva et al., 2012). Moreover, there was no association between the effect observed on *L. amazonensis* amastigotes and the phenolic concentration in the samples, which indicates that other compounds must be responsible for the leishmanicidal activity. The IC_{50} of arginase inhibition by fraction EAFMR, MFMR, HAFMR are closed related to the reduction of infectivity index of *L. amazonensis* amastigotes showed in this study. These data are an evidence of the extract and fractions kill *L. amazonensis* targeting parasite arginase. Nevertheless, the development of therapeutic strategies based on antioxidants sources should take into account the potential risk of altering host resistance to parasite infection and worsering the infection (Silva and Castilhos, 2015).

Regarding the *in vitro* trypanocidal activity, all fractions significantly reduced *T. cruzi* infection (10.5 to 78.1%). There are no specific studies on the leishmanicidal and trypanocidal activities of *M. rufula* in the literature. However, a study demonstrated the trichomonicidal effect of the dichloromethane extract of leaves and branches of this plant (Vieira et al., 2016). The aqueous extract from the leaves of *P. ramiflora* (Sapotaceae) was able to induce the death of promastigote forms of *Leishmania amazonensisin vitro* (Linares et al., 2008). Cruz et al. (2010) found that a flavonoid glycoside present in leafs of *Cecropia pachystachya* (Cecropiaceae) altered the mitochondrial DNA of *L. amazonensis*, besides inhibiting the enzyme arginase, and thus preventing the development of this parasite. In addition, the triterpenoids isolated from the dichloromethane extract of the fruit pericarp of *Omphalocarpum procerum* (Sapotaceae) led to the death of *T. cruzi, L. donovani, P. falciparum* and *T. brucei rhodesiense* in different cell models (Ngamgweet al., 2014).

The present study indicated the presence of several types of terpenes, such as proanthocyanidins, in *M. rufula* from Maracás, Bahia, Brazil. Previous studies with *M. zapota* and *M. rufula* had isolated several types of proanthocyanidins, which is in agreement with the present findings (Wang et al., 2010). Based on these preliminary results, the pharmacological activities described for *M. rufula* can be explained by the presence of proanthocyanidins, as they are widely known in the literature because of their biological activities such as anti-inflammatory, anticancer, antibacterial, antifungal, antiviral, antiparasitic activity, inhibition of platelet aggregation and cytoprotective (Augustin et al., 2011; Trentin et al., 2013). However, this can only be affirmed after isolation of *M. rufula* metabolites for reliable identification of plant components.

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