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ORIGINAL ARTICLE

Arabinogalactan-Glycoconjugate Fractions from *Genipa americana* Leaves as a Source of Antichagasic Natural Products

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Abstract

The extract from leaves of *Genipa americana* L., Rubiaceae, has antichagasic effect. The major components of this extract, arabinogalactan-glycoconjugate fractions (PFI and PFII), have shown similar biological effects of the crude extract, becoming interesting to evaluate the antichagasic effect of PFI and PFII. This work aimed to access the trypanocidal effect of PFI and PFII against the benznidazole-resistant *Trypanosoma cruzi* Y strain and to investigate the mechanism of action. PFI and PFII were evaluated on epimastigote (24, 48, 72 h), trypomastigote (24 h), and amastigote (24 and 48 h) forms. The cytotoxicity on LLC-MK2 cells was made by MTT assay. Flow cytometry analysis with PE-annexin V (Ax), 7-AAD, and DCFH-DA staining and scanning electron microscopy (SEM) were performed in epimastigote forms for evaluation of the cell death pathway. PFI showed effect against epimastigote forms ($EC_{50/24\ h} = 580 \pm 0.17$ μg/ml; $EC_{50/48\ h} = 530 \pm 0.13$ μg/ml; $EC_{50/72\ h} = 500 \pm 0.14$ μg/ml), while PFII did not show effect on any tested concentrations. In trypomastigotes, the PFI and PFII showed effect with EC_{50} values of 100 ± 0.09 and 23 ± 0.06 μg/ml respectively. PFI and PFII were also able to decrease amastigotes/100 cells parameter. PFI and PFII were not cytotoxic in LLC-MK2 cells at the highest tested concentration, resulting in selectivity index (SI) higher than 15 for PFI and higher than 65 for PFII. The increase in labeling of 7-AAD and DCFH-DA fluorescence on cytometry flow assays suggested necrosis as the cell death pathway of PFI in epimastigotes of *T. cruzi*, being confirmed by pores in the membrane observed on SEM. PFI and PFII are partially responsible by the trypanocidal effect of the crude extract of *G. americana*, with PFI showing the same action mechanism of crude extract and PFII being the most selective and potent fraction, presenting the biotechnological potential for antichagasic natural products.

Keywords *Trypanosoma cruzi* · Fractions · Polysaccharide · Necrosis · Reactive oxygen species

Introduction

Genipa americana L., Rubiaceae, traditionally called "jenipapeiro," has a wide geographic distribution in Brazil (Corrêa 1984). It is used in folk medicine as an antimalarial plant by indigenous people in Amazonas (Kffuri et al. 2016) and extracts from leaves have demonstrated activity against protozoan *Plasmodium berghei* and *Plasmodium falciparum* (Deharo et al. 2001), showing antiparasitic potential.

Chagas disease, also known as American trypanosomiasis, a parasitic tropical disease caused by the protozoan *Trypanosoma cruzi*, affects approximately 7 million people worldwide. The complications related to this disease are related to approximately 12,000 deaths of people each year. The parasites are mainly transmitted to humans by the infected feces of blood-sucking triatomine bugs, known as the "kissing bug" (WHO 2020). The only two drugs to treat Chagas disease, benznidazole (BZ) and nifurtimox (NFX), have limited efficacy and several adverse effects, showing effectiveness only at the acute phase (Molina et al. 2015; WHO 2020). Thus, the search for new antichagasic substances is necessary and natural products are sources of compounds with trypanocidal activity and they may provide pharmacological

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tools against Chagas disease (Don and Ioset 2014; Newman and Cragg 2020; Morais et al. 2020).

Recently, we demonstrated trypanocidal action of a polysaccharide extract from *G. americana* leaves against a benznidazole-resistant strain of *T. cruzi* (Souza et al. 2018). Two arabinogalactan-glycoconjugates from this extract were separated and characterized, being mainly composed of arabinose, galactose, and uronic acid. They are denominated as PFI and PFII, having high and low molecular weight respectively (Madeira et al. 2018). These two arabinogalactan-glycoconjugate fractions are partially responsible for some biological effects of the polysaccharide extract from *G. americana* leaves (Madeira et al. 2018, 2020). Thus, considering the ethnopharmacological uses against protozoan, this work aimed to evaluate the trypanocidal activity of arabinogalactan-glycoconjugate fractions of *G. americana* leaves against *T. cruzi* Y strain and to assess the action mechanism.

Material and methods

Chemicals

Benznidazole (BZ) was kindly donated by the Lafepe (Laboratório Farmacêutico do Estado de Pernambuco, Brazil). DEAE-cellulose and DMEM were obtained from Vitrocell, São Paulo, Brazil. Fetal bovine serum (FBS) was obtained from Invitrogen (Grand Island, NY, USA). PE conjugated to annexin V/7-AAD was obtained from BD Pharmingen, CA, USA, and DCFH-DA from Sigma-Aldrich™, St. Louis, USA. LIT medium was obtained from HiMedia Laboratories (Mumbai, Ind), sodium dodecyl sulfate (SDS) was obtained from Vetec (São Paulo, Brazil), and 3-[4,5-dimethylthiazol-2-yl]2,5-diphenytetrazolium bromide formazan (MTT) was obtained from Amresco (OH, USA). All other reagents were of analytical grade.

Obtaining of polysaccharides from *Genipa americana* leaves

Leaves of *Genipa americana* L., Rubiaceae, were obtained in the district of Custodio-Quixadá, state of Ceará, Brazil (5° 0′ 56.498″ S, 39° 6′ 58.889″ W) and identified by an expert of State University of Ceará. A plant exsiccate was deposited at Prisco Bezerra Herbarium of the Federal University of Ceará (No. 4683). The leaves were washed, dried at 40 °C, grounded to powder, and suspended in methanol (1:50 w/v, 76 °C, 2 h). After that, this suspension was filtered twice and the insoluble material was extracted with 0.1 M NaOH (1:50 w/v, 97 °C) three times, resulting in alkaline extracts. These extracts were pooled, neutralized, precipitated with ethanol, and centrifuged. The supernatant was dialyzed for 72 h and centrifuged and the final supernatant was lyophilized, obtaining the extract of *G. americana*—GaEPL (Souza et al. 2015).

Extract of *G. americana* was dissolved in distilled water (2:1, w/v) and fractionated by ion-exchange chromatography (DEAE-cellulose). The column was equilibrated, washed, and acidic polysaccharides eluted (60 ml/h) in a stepwise method with 0.1–1.0 M NaCl. The major fractions of the acidic arabinogalactan-glycoconjugate were eluted with NaCl 0.1 mol/l (PFI) and 0.25 mol/l (PFII), pooled, dialyzed, and lyophilized. The chemical analysis of fractions revealed higher content of carbohydrate (PFI: 36% (9% uronic acid), PFII: 23% (30% uronic acid)) than of proteins (PFI: 4%, PFII: 5%). The dosage of polyphenols revealed the presence of these compounds (PFI: 0.77 mg/g; PFII: 0.81 mg/g) in fractions. PFI is composed mainly by 27% of arabinose, 23% of galactose, 15% of glucose, 16% of fucose, 7% of xylose, 7% of rhamnose, and 5% of mannose, while PFII is composed mainly by 48% of arabinose, 23% of galactose, 4.5% of glucose, 2% of fucose, 10% of xylose, 12% of rhamnose, and 0.5% of mannose (Madeira et al. 2018).

Cells and parasites culture conditions

LLC-MK2 cells (ATCC CCL-7), epimastigote and trypomastigote forms of *T. cruzi* Y strain were donated by the Chemistry Laboratory of the Biochemistry Institute (University of São Paulo, São Paulo, Brazil). LLC-MK2 were maintained in DMEM (Vitrocell, São Paulo, Brazil) supplemented with 10% of fetal bovine serum (FBS) and 1% antibiotics (penicillin 100 UI/ml and streptomycin 100 μg/ml) at 37 °C and 5% CO_2 atmosphere.

Epimastigote forms of *T. cruzi* Y strain were maintained at 28 °C in liver infusion tryptose (LIT) with 10% FBS and antibiotics (penicillin 100 UI/ml and streptomycin 100 μg/ml). Trypomastigote forms were obtained by the infection of LLC-MK2 cells in DMEM with 2% FBS and antibiotics at 37 °C in a 5% CO_2 atmosphere.

Effects against *Trypanosoma cruzi* epimastigote and trypomastigote forms

Epimastigote and trypomastigote forms of *T. cruzi* (10^6 cells/ml) were plated on their respective culture conditions and incubated PFI or PFII (2.92–1500 μg/ml), positive control BZ (1.56–200 μg/ml), or negative control (PBS) at 24, 48, and 72 h for epimastigote forms and 24 h for trypomastigote forms. After incubation, parasite viability was determined in a Neubauer chamber. The concentration correspondent to 50% of viability (EC_{50}) was calculated by nonlinear regression, being 100% of viability the value of negative control (da Rodrigues et al. 2014).

Cytotoxicity to LLC-MK2 cells

LLC-MK2 cells (10^5 cells/ml) were plated in a 96-well microplate and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. After that, they are treated with different concentrations of PFI or PFII (2.92–1500 μg/ml), BZ (1.56–200 μg/ml), or PBS and incubated for 24 h. MTT assays were performed and reading at 570 nm by a microplate reader (Biochrom® Asys Expert Plus). The cell viability was calculated considering negative control as 100% of viability. The CC_{50} was determined by nonlinear regression (Vanden Berghe et al. 2013). The selectivity index (SI) was determined by the formula: CC_{50} of LLC-MK2/EC_{50} of trypomastigote forms (Nwaka and Hudson 2006).

Effects against *Trypanosoma cruzi* amastigote forms

LLC-MK2 cells were seeded in 24-well plates containing glass coverslips (13 mm) and incubated for 24 h in DMEM medium 10% FBS and 1% antibiotics (penicillin 100 UI/ml and streptomycin 100 μg/ml) at 37 °C in a 5% CO_2 atmosphere. Subsequently, the cells were infected with trypomastigote forms (20:1) in DMEM 2% FBS and incubated for 48 h. After internalization, LLC-MK2 cells were washed with PBS to remove non-internalized parasites and subsequently treated with EC_{50} and $EC_{50}/2$ from trypomastigote forms (50 and 100 μg/ml to PFI and 11.5–23 μg/ml to PFII), while to positive control BZ was used EC_{50} and 2× EC_{50} (67 and 134 μg/ml). Infected cells treated with PBS were considered negative control. After incubation for 24 or 48 h at 37 °C, the coverslips were removed, washed, fixed in Bouin's solution, and submitted to Giemsa coloration (Lima et al. 2016). The number of amastigotes per 100 cells and the percentage of infected cells were determined by counting 300 cells in triplicate.

Cell death pathway assay

Epimastigote forms (10^6 cell/ml) were treated with PFI at 580 μg/ml, half minimal effective concentration (EC_{50}), to observe the process of death or with PBS to observe normal conditions and incubated for 24 h at 28 °C. Subsequently, cells were washed and labeled with PE-annexin V (Ax) and/or 7AAD (BD Pharmigen, CA, USA) or DCFH-DA (Sigma-Aldrich™, St. Louis, USA), according to the manufacturer's instructions. Epimastigotes treated with PBS were used as negative controls and flow cytometry assays were performed in triplicate in a FACS Calibur system and analyzed using the Cell Quest software (Becton-Dickinson, CA, USA).

Scanning electron microscopy

To observe the cell death process, epimastigote forms were treated with PFI ($EC_{50/2}$—290 and EC_{50}—580 μg/ml) and incubated for 24 h. Subsequently, the parasites were fixed for 2 h with 2.5% glutaraldehyde solution (Electron Microscopy Sciences, Hatfield, PA), washed, and dehydrated with progressive concentrations of ethanol. After, samples were dried with CO_2 5%, coated with gold, and analyzed in a FEG Quanta 450 scanning electron microscope (FEI, OR, USA). Digital images were acquired and stored in a computer (Lima et al. 2018).

Statistical analysis

The statistical analysis was done using the GraphPad Prism 5 program (GraphPad Software, San Diego, CA, USA). The results were expressed as mean ± S.E.M. and analyzed by ANOVA and Bonferroni's post-test. Significance was defined as $^*p < 0.05$.

Results and discussion

The polysaccharide extract of *G. americana* showed activity against all main forms of a benznidazole-resistant *T. cruzi* strain, suggesting a necrotic pathway with ROS involvement. Therefore, GaEPL can be a source of substances against Chagas disease (Souza et al. 2018). In this work, we evaluated two arabinogalactan-glycoconjugate fractions of this extract.

Fractions obtained from natural extracts with trypanocidal action, such as the leaves of *Byrsonima crassifolia*, the barks of *Gliricidia sepium*, and leaves from *Lygodium venustum*, did not show any effect on epimastigote forms (Berger et al. 1998; Morais-Braga et al. 2013). In this work, one of the fractions of the polysaccharide extract from *G. americana* leaves showed a higher effect than the crude extract (GaEPL) in epimastigote forms; the fraction PFI showed effect after 24, 48, and 72 h of exposure, showing better activity at 72 h ($EC_{50} = 500 \pm 0.14$ μg/ml). However, PFII did not present effect in any tested concentrations or times of exposure (Table 1).

About twelve fractions obtained from the ethanolic extract of *Neurolaena lobata*, only butanol fraction showed a 90% inhibition at concentration 25.6 μg/ml of trypomastigotes, while the others were able only to inhibit at a concentration above 200 μg/ml (Berger et al. 1998). Mafezoli et al. (2000) evaluated the activity of twenty-five fractions isolated from plants of the Rutaceae family against the trypomastigote form of *T. cruzi*. Among them, only leaves of *Almeidea coerulea* (butanol fraction) and *Conchocarpus inopinatus* (fraction dichloromethane) were found active with an EC_{50} of 940 and 1290 μg/ml respectively. Izumi et al. (2011) studied forty-

Table 1 Antiparasitic activity of *Genipa americana* polysaccharide fractions on *Trypanosoma cruzi* and host cell cytotoxicity

	Epimastigote			Trypomastigote			LLC-MK$_2$[a]			SI		
	PFI EC_{50} ± S.D. (μg/ml)	PFII EC_{50} ± S.D. (μg/ml)	BZ EC_{50} ± S.D. (μg/ml)	PFI EC_{50} ± S.D. (μg/ml)	PFII EC_{50} ± S.D. (μg/ml)	BZ EC_{50} ± S.D. (μg/ml)	PFI CC_{50} ± S.D. (μg/ml)	PFII CC_{50} ± S.D. (μg/ml)	BZ CC_{50} ± S.D. (μg/ml)	PFI	PFII	BZ
24 h	580 ± 0.17	> 1500	56.76 ± 15	100 ± 0.09	23 ± 0.06	67 ± 20	> 1500	> 1500	160 ± 75.09	> 15	> 65	2.3
48 h	530 ± 0.13	> 1500	15.91 ± 3	-		-	-		-			
72 h	500 ± 0.14	> 1500	16.5 ± 1	-		-	-		-			

PFI, polysaccharide fraction from *G. americana* leaves; *PFII*, polysaccharide fraction from *G. americana* leaves; *BZ*, benznidazole; EC_{50}, inhibitory concentration at 50%; CC_{50}, cytotoxic concentration. [a] Cell viability of LLC-MK2 determined 24 h after treatment

three fractions of plants of the families Meliaceae and Rutaceae. They found fractions of *C. heterophyllus* and *Galipea carinata* were the most active, causing 100% lysis of trypomastigotes with a concentration of 2000 μg/ml. Our results show that PFI and PFII from *G. americana* have an antiparasitic effect on trypomastigote forms of *T. cruzi* being able to kill 50% of parasites at 100 and 23 μg/ml concentrations, respectively (Table 1).

In vitro cytotoxicity tests are fundamental because they provide information that guides the experimental design such as to define the studied concentrations of the compound and the time of treatment. Two neolignans obtained from Brazilian northeastern plants were effective against trypomastigotes and did not cause significant death of peritoneal macrophages (Cabral et al. 2010). In contrast, the ethyl acetate fraction of *Lygodium venustum* leaves at concentrations of 500 and 100 μg/ml showed fibroblasts toxicity of 67 and 30%, respectively, and the methanolic fraction (500 μg/ml) caused toxicity of 53% (Morais-Braga et al. 2013). In the other studies, it was observed that galactomannan of lichen *Ramalina celastri* did not affect the viability of host cells in concentrations 500 to 2000 μg/ml during 2 h of incubation, while it affected the viability of these cells by approximately 20% for both incubation times at concentrations of 250 to 750 μg/ml (24 h) and

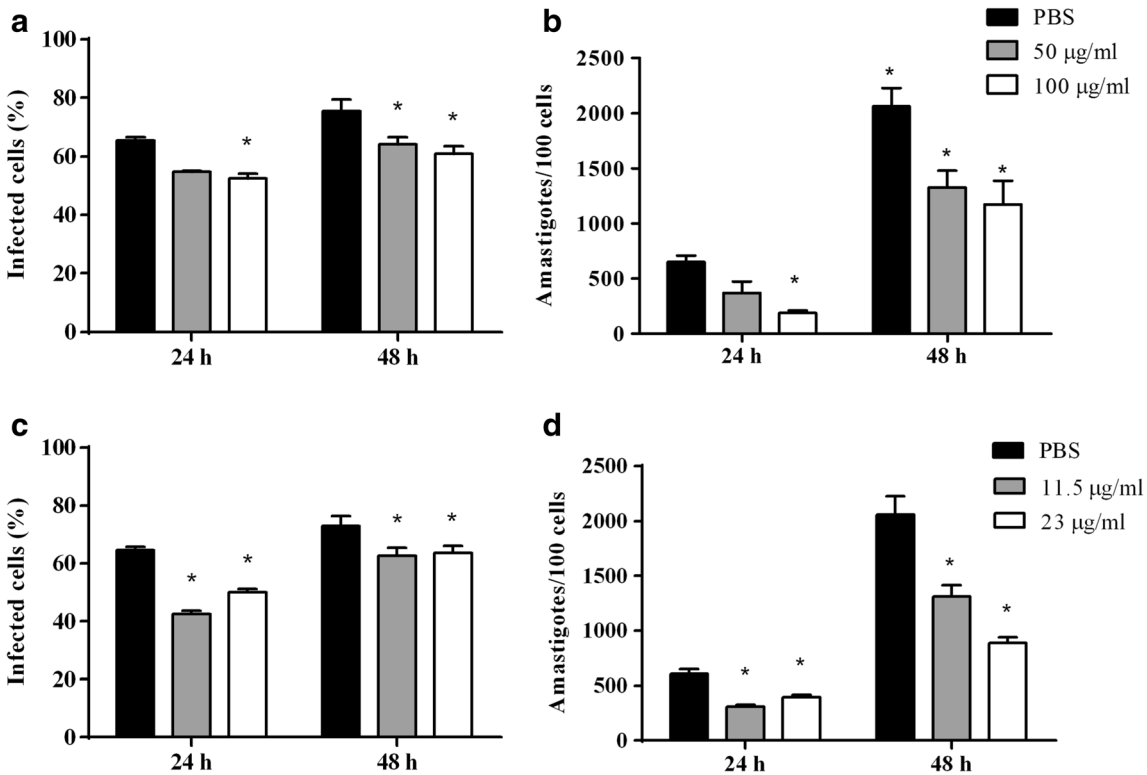

Fig. 1 Effects of PFI (50 or 100 μg/ml) and PFII (11.5 or 23 μg/ml) on *Trypanosoma cruzi* amastigote forms treated for 24 and 48 h. The percentage of infected cells treated with PFI (**a**), amastigotes per 100 cells treated with PFI (**b**), the percentage of infected cells treated with PFII (**c**), and amastigotes per 100 cells treated with PFII (**d**). Mean ± S.E.M. of three independent experiments. ANOVA and Bonferroni post-test, $^*p < 0.001$, the data were compared to control, where PBS was added

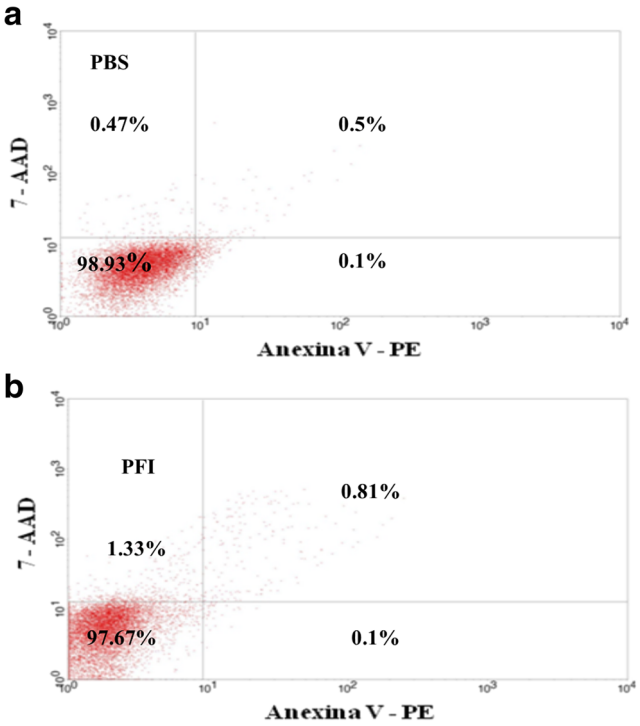

Fig. 2 Flow cytometry analysis of epimastigotes treated with 580 μg/ml of PFI for 24 h. Percentage of 7AAD/Ax labeling. Control epimastigotes treated with PBS (**a**). Epimastigotes treated with 580 μg/ml (**b**). Mean ± S.E.M. of three independent experiments. ANOVA and Bonferroni post-test, $*p < 0.001$, compared to PBS

2000 μg/ml (48 h) (Noleto 2002). PFI and PFII in the 24-h exposure period did not show any cytotoxicity at the tested concentrations on LLC-MK2 cells, being considered higher than 1500 μg/ml.

After the evaluation of non-toxic concentrations, the SI of the PFI and PFII were calculated. This evaluation is considered important since a substance is considered promising when it exhibits selectivity on the target cells without significantly damaging the host cell (Nwaka and Hudson 2006). Our results showed SI higher than 15 for PFI and higher than 65 for PFII (Table 1), being PFII the most promising on this parameter. The composition of PFII could be related to its best performance, which shows a higher uronic acid and arabinose content than PFI.

Amastigote forms correspond to the intracellular multiplicative evolutionary stage found in the vertebrate host and intrinsically linked to the development of tissue parasitism, being of great importance for the development of the chronic form of the disease and their eradication is aimed at prospection of a new chemotherapeutic agent (Clayton 2010). Substances against amastigote forms were obtained from plants and they were similarly able to reduce parasite proliferation (Uchiyama et al. 2002). The fractionation of the methanolic extract of the leaves of *Alchornea glandulosa* resulted in an alkaloid of guanidine that exhibited antiprotozoal activity against amastigote forms of strain Y of *T. cruzi*, presenting an EC_{50} of 27 μg/ml (Barrosa et al. 2014). The antiamastigote effect of PFI and PFII was evaluated at 24 and 48 h. The fractions were tested on this form in two concentrations, EC_{50} and $EC_{50/2}$, from trypomastigote forms. Our results demonstrated that in amastigote forms, the fractions of *G. americana* at a concentration of 100 μg/ml for PFI and 11.5 μg/ml and 23 μg/ml for PFII were able to decrease the number of infected cells, after 24 and 48 h of incubation. The same concentrations of the two fractions also can decrease the number of amastigotes/100 cells in both periods. PFI and PFII fractions showed better effects at 24 h of incubation. Besides, as noted, FII was more potent than FI, corroborating the effect of this fraction on trypomastigote forms (Fig. 1).

In this study, leading to the identification of the cell death mechanism induced by PFI of *G. americana* leaves, we initially analyzed the classical necrosis/apoptosis processes using fluorescent dyes 7-AAD and annexin V/PE (Kumar et al. 2015). This methodology allows the observation of cells in different populations: viable cells, which are not marked by any of the fluorochromes; necrotic cells, labeled only with 7-AAD; apoptotic cells, labeled only with annexin V/PE;

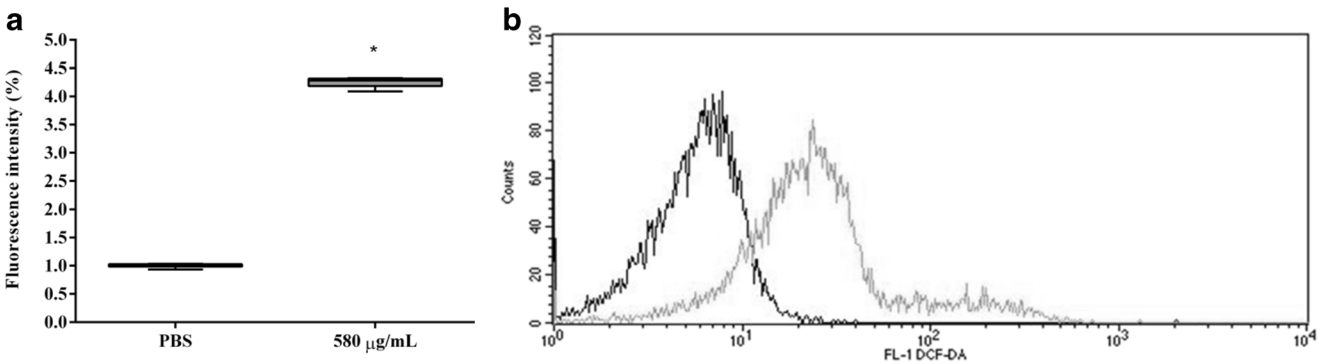

Fig. 3 ROS production in *Trypanosoma cruzi* epimastigotes treated with 580 μg/ml of FI for 24 h. Relative fluorescence of H_2DCFDA staining in flow cytometry analysis. Mean ± S.E.M. of three independent experiments. ANOVA and Bonferroni post-test, $*p < 0.05$, compared to PBS

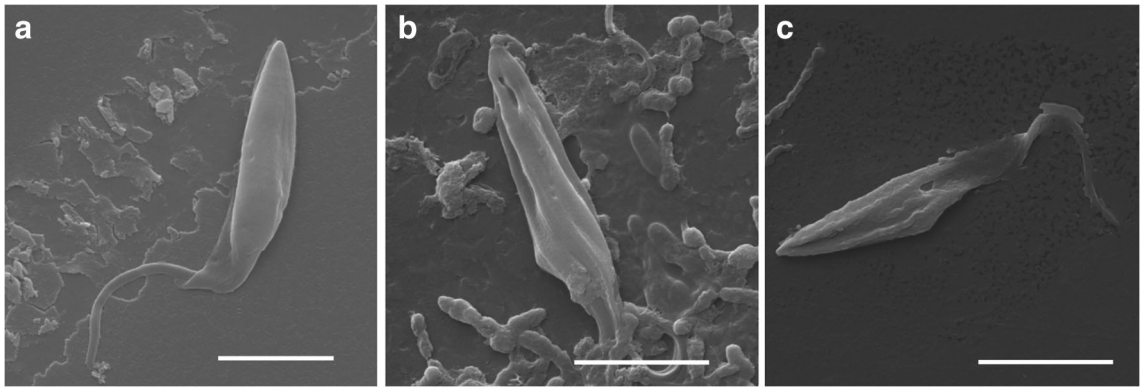

Fig. 4 Scanning electron microscopy of *Trypanossoma cruzi*. Control epimastigotes—PBS (**a**) and epimastigotes treated with 290 (**b**) and 580 μg/ml (**c**) concentrations of PFI. Scale bar = 5 μm

double-labeled cells, which are considered in late apoptosis or secondary necrosis (Vanden Berghe et al. 2013). In this study, PFI treatment caused an increase in necrotic and secondary necrotic cells after 24 h of treatment compared to the untreated parasites, indicating alteration of cell membrane integrity (Fig. 2). PFII did not show any effect on epimastigote forms and cytometry analyses were not performed.

Necrosis involves changes in mitochondria including mitochondrial depolarization, ATP depletion, ROS generation, cytosolic vacuolization, loss of calcium homeostasis, and especially plasma membrane rupture (Menna-Barreto 2019). *T. cruzi* needs to deal with different redox microenvironments because they inhabit different biological compartments. Thus, this parasite has an extensive and differentiated mechanism of detoxification, which acts to protect it from the attack of oxidizing molecules (Irigoín et al. 2008). To verify if the polysaccharides studied are able to induce the production of ROS, DCFH-DA (a reagent that is converted into a fluorescent product in the presence of ROS in the cytoplasm) was used. Its mechanism allows us to observe the intracellular production of ROS, such as singlet oxygen, hydrogen peroxide, and superoxide anion (Chen et al. 2010). Our data showed that PFI of *G. americana* induces an increase of intracellular ROS, which may be associated with its trypanocidal effect (Fig. 3). Morphological alterations were also evaluated by SEM, in which ultrastructural changes in the typical format were observed, such as flattening and shortening of the parasite and degradation of the cell membrane with pores confirming necrosis induction (Fig. 4).

In conclusion, the arabinogalactan-glycoconjugate fractions are partially responsible by the trypanocidal effect of the crude extract of *G. americana*, with PFI showing the same action mechanism of crude extract (necrosis with an increase on ROS) and PFII being the most selective and potent fraction. The results showed PFI and PFII as promising substances for further experiments and development of new natural products to be a potential candidate for the treatment of Chagas disease.

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Authors' contributions ROSS: writing, review and editing, methodology, investigation; PLS, RRPPBM, TLS: methodology, investigation; DBL: writing, review, and editing; MGP: methodology, investigation, resources; AMCM: writing, review and editing, funding acquisition, conceptualization, resources. All the co-authors read and approved the manuscript. Furthermore, each author certifies that this material has not been and will not be submitted to or published in any other publication before its appearance in *Brazilian Journal of Pharmacognosy*.

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Compliance with ethical standards

Competing interests The authors declare that they have no conflict of interest.

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