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Inhibition of *Leishmania amazonensis* arginase by fucogalactan isolated from *Agrocybe aegerita* mushroom

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ABSTRACT

The inhibition of arginase from *Leishmania* spp. is considered a promising approach to the leishmaniasis treatment. In this study, the potential of a fucogalactan isolated from the medicinal mushroom *Agrocybe aegerita* was evaluated against arginase (ARG) from *Leishmania amazonensis*. The polysaccharide was obtained via aqueous extraction, and purified by freeze thawing and precipitation with Fehling solution. Its chemical structure was established by monosaccharide composition, methylation analysis, partial acid hydrolysis, and NMR spectroscopy. The data indicated that it is a fucogalactan (FG-*Aa*; M_w = 13.8 kDa), having a (1→6)-linked α-D-Gal*p* main-chain partially substituted in O-2 by non-reducing end-units of α-L-Fuc*p*. FG-*Aa* showed significant inhibitory activity on ARG with IC_{50}potency of 5.82 ± 0.57 μM. The mechanism of ARG inhibition by the heterogalactan was the competitive type, with $K_i$of 1.54 ± 0.15 μM. This is the first report of an inhibitory activity of arginase from *L. amazonensis* by biopolymers, which encourages us to investigate further polysaccharides as a new class of ARG inhibitors.

1. Introduction

Leishmaniasis is a parasitic infection that remains nowadays, affecting millions of people per year around the world (WHO, 2018). The chemotherapeutics currently used against leishmaniasis have several side effects and resistance issues (Rojo et al., 2015). In regarding to the need for new approaches for human leishmaniasis treatments, polysaccharides are macromolecules with a great structural diversity that have been shown leishmanicidal and antitumor activities related to immunomodulatory effects (Adriazola et al., 2014; Amaral et al., 2015; Kangussu-Marcolino et al., 2015; Moretão, Zampronio, Gorin, Iacomini, & Oliveira, 2004; Valadares et al., 2011).

The polysaccharides are considered relevant responsible agents for biological response modification related to medicinal usage of mushrooms (Meng, Liang, & Luo, 2016; Rathore, Prasad, & Sharma, 2017). *Agrocybe aegerita*, commonly known as "*black poplar*", "*Pioppino*" or "*Yanagi-matsutake*" mushroom, is used as a traditional Chinese herbal medicine and recognized for its potential health benefit. Several biological activities, such as antioxidant (Lo & Cheung, 2005; Petrović et al., 2015), anti-inflammatory (Diyabalanage, Mulabagal, Mills, DeWitt, & Nair, 2008), antimicrobial (Petrović et al., 2014) and antitumoral properties (Diyabalanage et al., 2008; Liang et al., 2011; Lin, Ching, Lam, & Cheung, 2017; Yang et al., 2009) have been described for this species, which were attributed to its secondary compounds (phenolic compounds, indole derivatives, among others), polysaccharides, and lectins. However, the polysaccharides related to biological activities from *A. aegerita* have not been chemically characterized up to now.

In attempt to improve antileishmanial efficacy for drug design, new enzymes have been explored as molecular targets for therapeutic intervention, such as arginase (ARG) from *Leishmania amazonensis* (da Silva, Maquiaveli, & Magalhães, 2012; da Silva, Zampieri, Muxel, Beverley, & Floeter-Winter, 2012; Robertson, 2005). ARG is a metalloenzyme that catalyzes the hydrolysis of L-arginine to L-ornithine and urea carrying out reactions for essential metabolites for *Leishmania*

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spp. development. TH_2 cytokine activation increases ARG expression leading to the establishment of *Leishmania* infection, thus arginase appears as a promising target against leishmaniasis (Blaña-Fouce et al., 2012; Colotti & Ilari, 2011; da Silva et al., 2008; da Silva, Maquiaveli et al., 2012).

Inhibitors of arginase have been searched in order to identify new antileishmanial leads (da Silva, Maquiaveli et al., 2012; da Silva, Zampieri et al., 2012; de Sousa, Ramalho, Burger et al., 2014, b; Maquiaveli et al., 2016). Among the natural products pointed as ARG inhibitors, glycoside compounds from plants have shown potency in decreasing arginase catalytic activity (da Silva, Maquiaveli et al., 2012; de Sousa, Ramalho, Burger et al., 2014; Maquiaveli et al., 2016). *In silico* studies have showed interaction between glucopyranose and the active site of the enzyme (Maquiaveli et al., 2016).

In a search for new arginase inhibitors, the heteropolysaccharide (FG-*Aa*) isolated from *A. aegerita* was structurally characterized and investigated by ARG enzymatic assay. FG-*Aa* was identified as potent ARG inhibitor and its mechanism of action was determined. Furthermore, the identified polysaccharide was revealed as a new class of natural products as ARG inhibitors.

2. Experimental

2.1. Biological material

Fresh *Agrocybe aegerita* (3.0 kg) was donated by Yuki Cogumelos Company (Owner: José Francisco Ramos Fernandes Viana), located in Araçoiaba da Serra, State of São Paulo, Brazil, in September 2015. The fungus was grown on culture substrate constituted of eucalyptus sawdust (2%), wheat bran (8%), corn bran (5%), soybean bran (4%), and calcitic limestone (1%). After freeze-dried, the fruiting bodies of *A. aegerita* were reduced to 9.7% of the original weight, resulting in ~300 g of dry matter.

2.2. Extraction and purification procedures for polysaccharides from A. aegerita

The dried fruiting bodies of *Agrocybe aegerita* (~300 *g*), were pulverized and extracted with water at ~10 °C for 8 h (x 5, 1 L). The extract was filtered and after centrifugation at 9000 rpm at 20 °C for 15 min a clear solution was obtained. The polysaccharides were precipitated by addition of excess EtOH (3:1; v/v) to the concentrated supernatant, and then recovered by centrifugation at 9000 rpm at 15 °C for 10 min. The crude polysaccharide fraction was dissolved in H_2O, dialyzed against distilled water for 48 h to remove low-molecular-weight carbohydrates, and freeze-dried, giving rise to fraction CW-*Aa*. This fraction was then dissolved in distilled water and the solution submitted to freezing followed by mild thawing at 4 °C (Gorin & Iacomini, 1984), giving cold water-soluble (SCW-*Aa*) and insoluble fractions (ICW-*Aa*), which were separated by centrifugation (9000 rpm at 10 °C for 10 min). SCW-*Aa* fraction was treated with Fehling's solution (Jones & Stoodley, 1965), and precipitated Cu^{++} complex (FPCW-*Aa*) was removed by centrifugation at 9000 rpm for 10 min, at 15 °C. The precipitate was neutralized with HOAc, dialyzed against tap water (48 h), deionized with mixed ion exchange resins, and then freeze-dried. Fehling treatment was repeated one more cycle on fraction FPCW-*Aa*, giving the further purified fraction FP_2CW-*Aa* that was denominate FG-*Aa*.

2.3. Monosaccharide composition

Monosaccharide components of the polysaccharides were identified and their ratios were determined following hydrolysis with 1 M TFA for 8 h at 100 °C, and conversion to alditol acetates (GC–MS) by successive $NaBH_4$ reduction, and acetylation with Ac_2O-pyridine (1:1, v/v) for 12 h at room temperature (Wolfrom & Thompson, 1963a, 1963b). The resulting alditol acetates were analyzed by gas chromatography-mass spectrometry (GC–MS) and identified by their typical retention times and electron impact profiles. GC–MS analysis was performed with an Agilent 7820 A gas chromatograph interfaced to an Agilent 5975E quadrupole mass spectrometer, fitted with split/splitless capillary inlet system, an Agilent G4513 A autosampler, and a capillary HP5-MS column (30 m × 0.25 mm i.d.). Injections of 1 u L were made in the splitless mode at injection temperature of 250 °C and detector at 280 °C. The column oven temperature was initially hold at 75 °C for 1 min, programmed at 35 °C min^{-1} to 100 °C (5 min), then 45 °C min^{-1} to 150 °C, hold for 5 min, 55 °C min^{-1} to 200 °C (15 min), and 65 °C min^{-1} to 240 °C (2 min.) for quantitative analysis of the alditol acetates. Helium was the carrier gas at a flow rate of 1 mL.min^{-1}. Electron impact (EI) analysis was performed with the ionization energy set at 70 eV.

2.4. Methylation analysis of polysaccharides

Per-*O*-methylation of the purified fraction (FP_2CW-*Aa*; 12 mg) was carried out using NaOH-Me_2SO-MeI (Ciucanu & Kerek, 1984). The per-*O*-methylated derivatives (1 mg) were hydrolyzed with 1 M TFA (250 μL) for 10 h at 100 °C, followed by evaporation to dryness. The resulting mixture of *O*-methylaldoses was reduced with $NaBD_4$ and acetylated with Ac_2O-pyridine (1:1, v/v) for 12 h at room temperature (Wolfrom & Thompson, 1963a, 1963b) to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS as above cited (item 2.3). For quantitative analysis of the partially *O*-methylated alditol acetates (PMAAs) was used a DB-225 capillary column (30 m × 0.25 mm i.d.) held at 50 °C during injection and later programmed to 220 °C (constant temperature) at 40 °C min^{-1}. PMAAs were identified from *m/z* of their positive ions, by comparison with standards (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005). The relative percentage of the resulting PMAAs was calculated by determination of the each peak area using the Agilent ChemStation software.

2.5. Determination of polysaccharide homogeneity and molecular weight

The homogeneity of FP_2CW-*Aa* was determined by high performance steric exclusion chromatography (HPSEC) coupled to a refractive index (RI) detector model RID 10 A. The chromatography system consisted of an HPLC pump (Model Shimadzu-10 AD), a manual injection valve (Shimadzu) fitted with a 200-μL loop and an Ultrahydrogel column (7.8 × 300 mm) system (Waters) with exclusion limit of 7×10^6, 4×10^5, 8×10^4 and 5×10^3 Da arranged in series. The mobile phase was 0.1 M $NaNO_3$ with sodium azide (0.03%), and a flow rate 0.6 mL/min. Data analysis was performed using LC solution software (Shimadzu Corporation). To determine the average molecular weight of FP_2CW-*Aa* the standard curve of dextran with molecular weights of 670, 410, 266, 150, 72.2, 60.0, 40.2, 22.8, and 9.4 kDa was made.

2.6. Partial acid hydrolysis of heterogalactan

FP_2CW-*Aa* (88 mg) was partially hydrolyzed with 0.2 M TFA (2 mL) for 3 h at 100 °C. After neutralization with NaOH, the material was dialyzed (2 kDa *cut-off* membrane) against distilled water. The retained fraction was lyophilized (HPFP_2-*Aa*, 59 mg) and analyzed by ^{13}C NMR.

2.7. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra (^1H, ^{13}C, HSQC-DEPT, HSQC-TOCSY and HSQC-NOESY) were obtained using a 500 MHz Bruker Avance spectrometer incorporating Fourier transform. Analyses were performed at 50 or 70 °C on samples dissolved in D_2O or Me_2SO-d_6. Chemical shifts are expressed in δ relative to the internal standard tetramethylsilane (TMS) (δ = 0.0 for ^{13}C and ^1H) or Me_2SO-d_6 (δ = 39.70 and 2.50 for ^{13}C and ^1H signals, respectively).

2.8. Arginase activity measurements

The expression and purification of recombinant ARG of *L. amazonensis* was performed as previously described (de Sousa, Ramalho, Fernandes et al., 2014), as well detailed conditions of assay are the same previously established by de Sousa, Ramalho, Burger et al. (2014), de Sousa, Ramalho, Fernandes et al. (2014).

The samples and negative control were briefly diluted in MilliQ water. The polysaccharide FG-*Aa* (10 μM) was incubated with arginase solution (CHES buffer solution at pH 9.6; Sigma-Aldrich) for 10 min at 37 °C. Then, the substrate L-arginine (Sigma-Aldrich) was added to the reaction (50 mM of CHES buffer and 50 mM of L-arginine at pH 9.6), incubating similarly for 10 min at 37 °C. Thereafter, the second reaction takes place (Urea kit - Bioclin, Brazil) and urease catalysis allowed to determine the indirect ARG activity by measuring the absorbance of indophenol blue at 600 nm using a Varian Cary UV/Visible spectrophotometer. Indophenol was generated by reaction of ammonia and Berthelot´s reagent, from which, 10 μL of arginase reaction mixture were added to 500 μL of a solution (100 mM phosphate buffer, pH 6.8, 300 mM salicylate, 5.0 mM sodium nitroprusside, and 10,000 IU urease) and incubated for 10 min at 37 °C and 500 μL of a second solution (10 mM NaOCl and 1.5 M NaOH) was added and incubated similarly. Additionally, uncoupled assay was performed to ensure ARG inhibitory activity.

IC_{50} of FG-*Aa* was determined by rate measurements with inhibitor concentrations ranging from 0.7 to 355 μM. The enzymatic assay was performed in duplicate and titration of inhibitor was reproduced three times in independent experiments. Data fitting for IC_{50} were processed using 4 parameters logistic equation.

The kinetics experiments were performed by increasing substrate concentrations (6.25–100 mM) with 1.4, 3.5 and 7.0 μM of inhibitor. A control was used without the addition of inhibitor. The type of inhibition was determined analyzing kinetics data by Lineweaver-Burk, Dixon and Cornish-Bowden plots. The K_i was calculated by using the Dixon equations (Cortés, Cascante, Cárdenas, & Cornish-Bowden, 2001; de Sousa et al., 2015; Dixon, 1953):

$v_0/v_i = 1 + ([I]/ K_i^{app})$, and

$K_i = K_i^{app}/(1 + [L\text{-}Arg]/K_m)$, with [L-Arg] = 50.0 mM and K_m = 18.3 ± 1.4 mM

The experimental data were analyzed with the program GraFit® (Erithacus Software Ltd, Horley, Surrey, UK, 2006).

3. Results and discussion

The crude polysaccharide fraction (CW-*Aa*, 13.2 g) was isolated from the freeze-dried fruiting bodies (300 g) of the mushroom *Agrocybe aegerita*, via cold water extraction (Fig. 1). It showed to be composed of galactose (Gal, 48.9%) as its main component, in addition to fucose (Fuc, 25.0%), glucose (Glc, 26.0%), and traces of mannose (Man), according to GC–MS results of its derived alditol acetates. Fractionation of CW-*Aa* by freeze/thawing process gave water-soluble (SCW-*Aa*, 11.5 g) and insoluble (ICW-*Aa*, 1.4 g) polysaccharidic fractions, which were separated by centrifugation.

Fraction SCW-*Aa*, composed of Fuc (16.9%), Man (5.7%), Gal (52.8%), and Glc (24.6%), was treated with Fehling solution twice, sequentially, giving rise to a precipitate (FP_2CW-*Aa*, 924 mg), which was homogeneous (M_w/M_n = 1.18) on HPSEC (Fig. 2), and had M_w 13.8 kDa.

FP_2CW-*Aa* contained mainly fucose (29.0%), and galactose (65.8%) as monosaccharide components, suggesting the presence of a fucogalactan (named as FG-*Aa*).

The glycosidic linkages pattern of FG-*Aa* was determined by methylation procedure.Analysis by GC–MS of the partially *O*-methylated alditol acetates showed a highly branched structure, containing non-

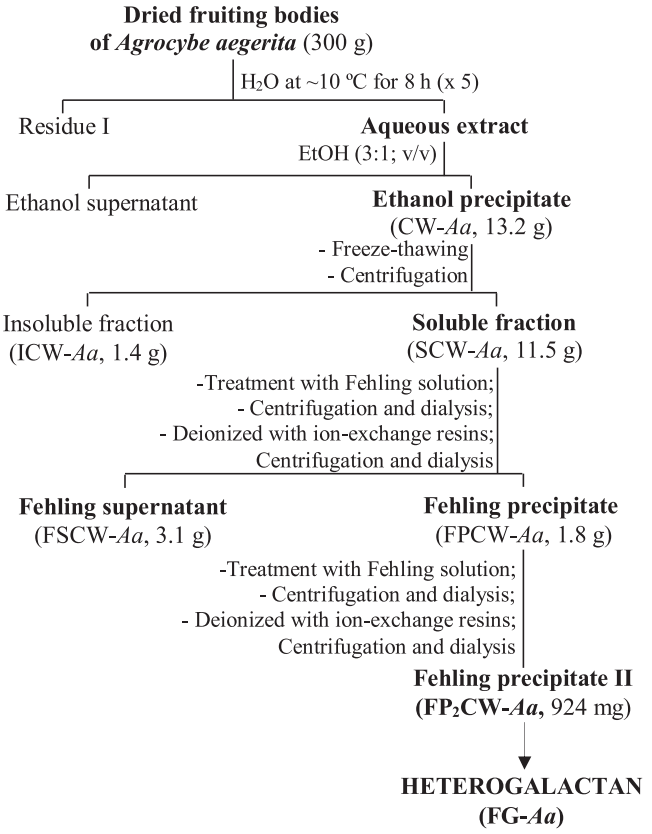

Fig. 1. Scheme of extraction and purification of the heteropolysaccharide from *A. aegerita*.

reducing end units of Fuc*p* (2,3,4-Me_3-Fuc; 31.3%), 6-*O*-substituted (2,3,4-Me_3-Gal; 33.0%) and 2,6-di-*O*-substituted (3,4-Me_2-Gal; 33.7%) of Gal*p* units, together with minor amounts of non-reducing end units of Gal*p* (2,3,4,6-Me_4-Gal, 2.0%).

The anomeric region of the ^{1}H NMR spectrum of the polysaccharide FG-*Aa* (FP_2CW-*Aa* fraction) contained three signals of H-1 at δ 5.09, 5.04, and 4.99 (Fig. 3), which were assigned as residue A, residue B, and residue C, respectively, and accordingly in the anomeric region of the ^{13}C-NMR, three carbon resonances appeared at δ 104.10, 100.87, and 101.02 (Fig. 4). The relative areas of peak A, B, and C in the ^{1}H-NMR spectrum were 1:1:1 (Fig. 3).

All units showed an α-configuration by high-frequency H-1 (δ 5.09, 5.04, and 4.99) and low-frequency C-1 signals (δ 104.10, 100.87, and 101.02) (Figs. 3–5) (Agrawal, 1992).

Further NMR experiments, HSQC-DEPT (Fig. 5) and HSQC-TOCSY (Fig. 6), were performed in order to elucidate the structure of this heteropolysaccharide (FG-*Aa*). The connectivities observed in the HSQC-TOCSY spectrum allowed to assign all carbons and protons of the each unit (Table 1S). Once the protons had been identified, the chemical shifts of their corresponding carbons were confirmed by HSQC-DEPT analysis (Fig. 5; Table 1).

On the basis of NMR analyses, the identities of the monosaccharide residues A, B, and C were established (Tables 1 and 1S). Residue A was assigned as α-L-fucopyranosyl unit. This was strongly supported by the presence of characteristics ^{1}H (δ 1.25) and ^{13}C (δ 18.42) signals for a CH_3 group, besides typical carbon chemical shifts (C-1 to C-6) corresponding to the standard values of methyl glycosides (Agrawal, 1992). The downfield shifts of the C-2 and C-6 at δ 80.53 and 70.03, respectively, indicated that residue B was a 2,6-di-*O*-substituted α-D-galactopyranose unit. In residue C, the downfield shift of the C-6 at δ 69.48 (C-6) confirmed the presence of 6-*O*-substituted α-D-galactopyranose.

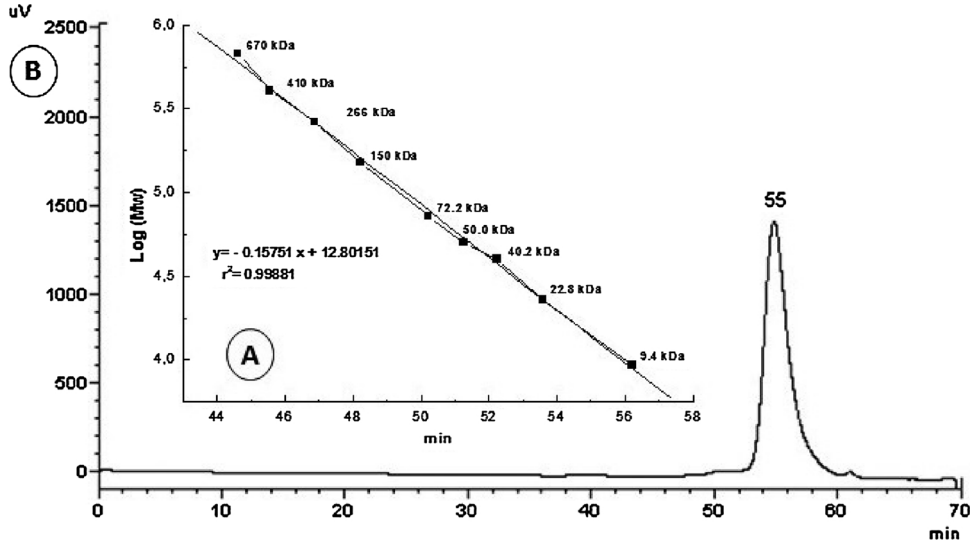

Fig. 2. The standard curve of dextran (A) and HPSEC chromatogram (B) of FP_2CW-*Aa* fraction.

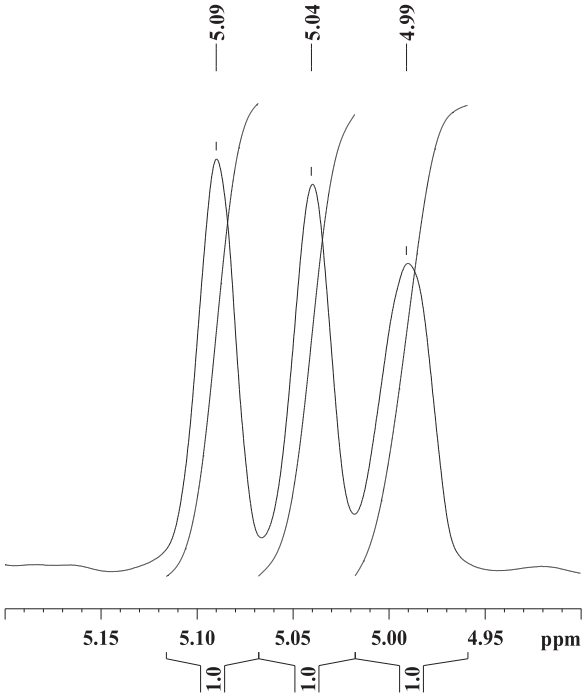

Fig. 3. Anomeric region of ^{1}H NMR spectrum of fucogalactan (FG-*Aa*) from *A. aegerita*, analyzed in D_2O at 50 °C (chemical shifts are expressed in δ ppm).

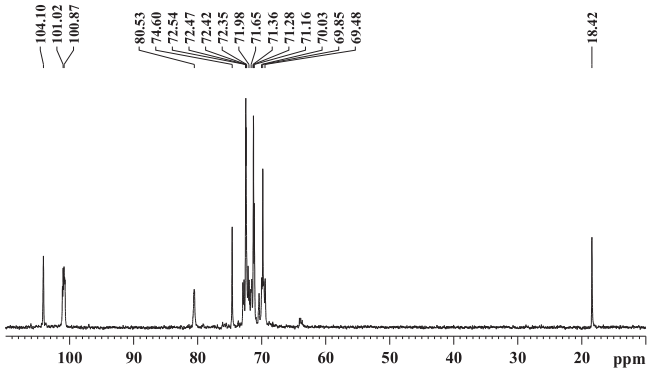

Fig. 4. ^{13}C NMR spectrum of fucogalactan (FG-*Aa*) from *A. aegerita*, analyzed in D_2O at 70 °C.

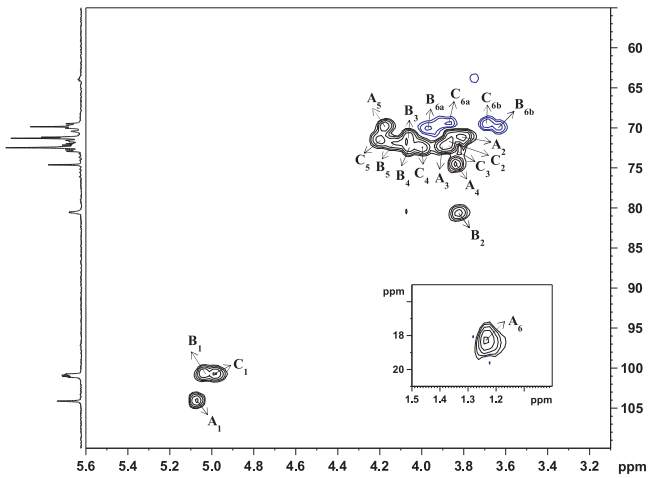

Fig. 5. HSQC-DEPT spectrum of fucogalactan (FG-*Aa*) from *A. aegerita*, with amplified insert of the C-6 region of Fuc*p*, analyzed in D_2O at 50 °C. **A** = non reducing ends α-Fuc*p*; **B** = 2,6-di-*O*-substituted α-Gal*p* units; **C** = 6-*O*-substituted α-Gal*p* units.

A partial acid hydrolysis eliminated almost all α-Fuc*p* units from FP_2CW-*Aa*, as shown by ^{13}C NMR spectrum (Fig. 7), which contained main signals characteristics of a linear (1→6)-linked α-galactopyranan (C-1, 98.82; C-2, 68.55; C-3, 69.59; C-4, 69.02; C-5, 68.96; C-6, 66.51) (Oliveira et al., 2018). An interresidue cross peak AH-1/BC-2 in the HSQC-NOESY experiment further confirmed the substitution at O-2 of the residue B by non-reducing ends of α-Fuc*p* (residue A).

According to the molar ratio of the residues, determined by ^{1}H NMR and methylation analysis, and partial acid hydrolysis results, it could be concluded that the fucogalactan from *A. aegerita* (FG-*Aa*) consist of a (1→6)-linked α-D-galactopyranosyl main-chain, substituted at O-2 by non-reducing end units of α-L-Fuc*p*, on the average of one to every second residues of the backbone (Fig. 8), which has been supported by previous studies about similar heteropolysaccharides (Li et al., 2016; Ruthes, Rattmann, Carbonero, Gorin, & Iacomini, 2012; Ye et al., 2008).

Bioactive fucogalactans have been found from several macrofungi (Li et al., 2016; Ruthes et al., 2012; Ye et al., 2008). However, despite the general structure in common, FG-*Aa* had higher fucose content

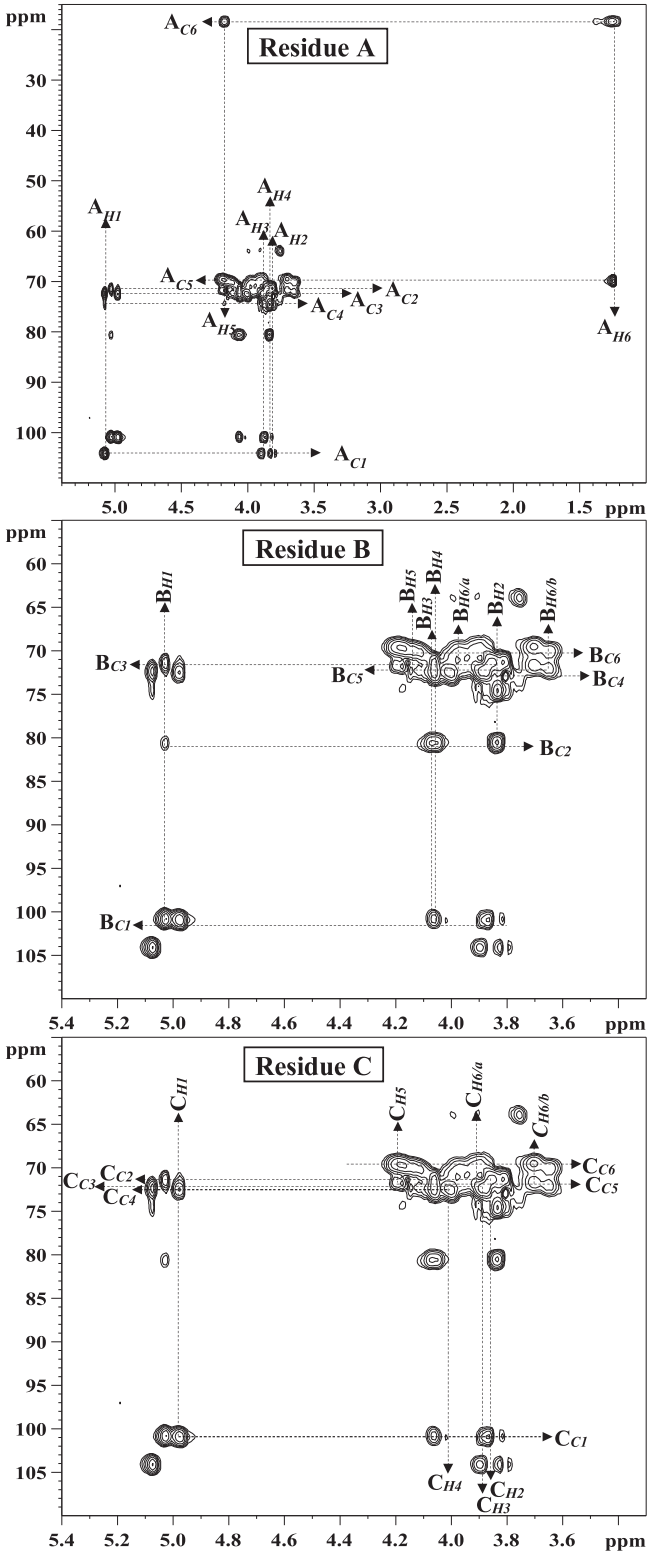

Fig. 6. HSQC-TOCSY spectrum of fucogalactan (FG-*Aa*) from *A. aegerita*, analyzed in D_2O at 50 °C. **A** = non reducing ends α-Fuc*p*; **B** = 2,6-di-*O*- substituted α-Gal*p* units; **C** = 6-*O*- substituted α-Gal*p* units.

among the fucogalactans already described.

In order to investigate the biological action of the fucogalactan now isolated, it was evaluated for probable arginase inhibitory effect. The characterized heteropolysaccharide

isolated showed 60.5% of inhibition of ARG catalytic activity, when

Table 1
^{1}H and ^{13}C NMR chemical shifts of heterogalactan from *A. aegerita* [a,b].

Units		1	2	3	4	5	6	
							a	b
α-Fuc*p*-(1→	13C	104.10	71.28	72.42	74.60	69.85	18.42	
(Residue A)	1H	5.09	3.82	3.90	3.85	4.18	1.25	
→2,6)-α-Gal*p*-(1→	13C	100.87	80.53	71.28	72.47	72.00	70.03	
(Residue B)	1H	5.04	3.83	4.08	4.07	4.14	3.98	3.64
→6)-α-Gal*p*-(1→	13C	101.02	71.16	72.35	72.54	71.65	69.48	
(Residue C)	1H	4.99	3.87	3.89	4.01	4.20	3.89	3.70

[a] Assignments were based on ^{1}H, ^{13}C, HSQC-DEPT, and HSQC-TOCSY examinations.

[b] The values of chemical shifts were recorded with reference to TMS as internal standard.

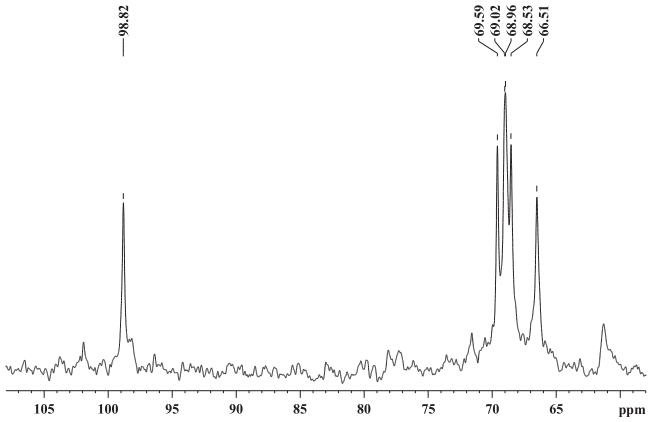

Fig. 7. ^{13}C NMR spectrum of partially degraded fucogalactan (FG-*Aa*) from *A. aegerita*, analyzed in Me_2SO-d_6 at 70 °C, chemical shifts are expressed in ppm.

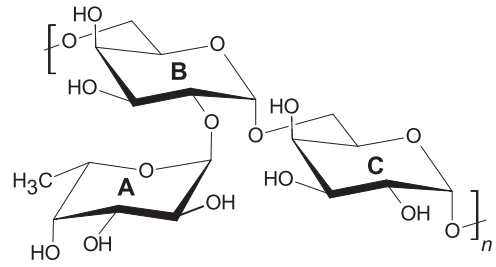

Fig. 8. Proposed structure for fucogalactan from *A. aegerita* (FG-*Aa*).

evaluated at 10 μM, with

IC_{50} potency of 5.82 ± 0.57 μM. Previously, glycoflavones and a phenylethanoid glycoside (verbascoside) were found as inhibitors of ARG with potency ranging from 2.0 to 12.2 μM (da Silva, Maquiaveli et al., 2012; da Silva, Zampieri et al., 2012; de Sousa, Ramalho, Burger et al., 2014; Maquiaveli et al., 2016).

Verbascoside is a competitive inhibitor of ARG and among the interactions showed by docking in the active site; H-bonds were established between glucopyranose and different ARG side chains (Maquiaveli et al., 2016). By the present kinetics experiments carried out with FG-*Aa* and ARG, a competitive behavior was observed (Fig. 9) supporting the findings that monosaccharide units interact in the active site.

The plot of velocity as a function of substrate (Michaelis ± Menten equation) (Fig. 9A) showed that V_{max} values were kept constant at all inhibitor concentrations, otherwise apparent K_m values increased with increasing inhibitor concentration referring to a competitive inhibition type. The experimental data was processed using other approaches as well, Lineweaver-Burk, Dixon and Cornish-Bowden plots (Fig. 9B–D,

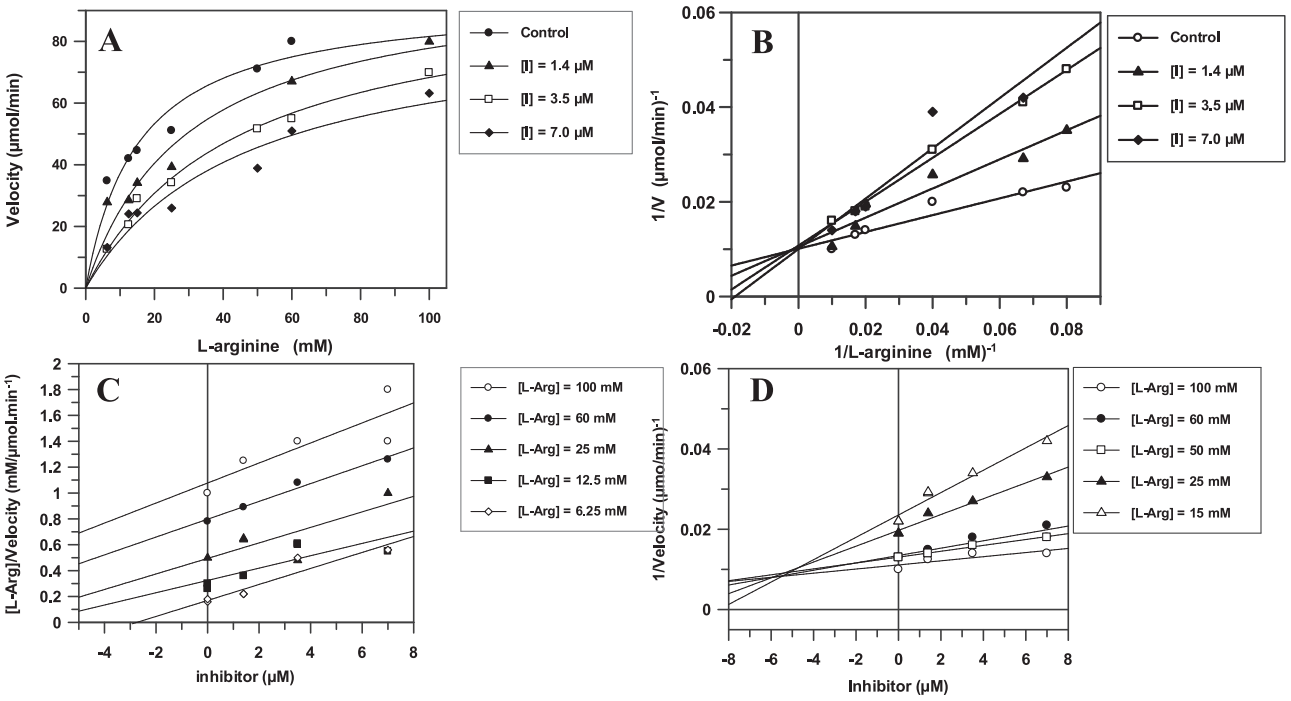

Fig. 9. Competitive inhibition of FG-*Aa* (K_i = 1.54 ± 0.15 µM). (**A**) Direct plot of velocity as a function of substrate; (**B**) Lineweaver-Burk plot; (**C**) Cornish-Bowden plot; and (**D**) Dixon plot. Data were expressed as the mean of independent assays.

respectively). In combination, the plots showed the characteristics of the ARG inhibition manner by FG-*Aa*, which compete with the substrate for the pool of free enzyme molecules with K_i of 1.54 ± 0.15 µM.

This is the first report of an inhibitory activity of arginase from *L. amazonensis* by a biopolymer with high molecular weight. This result encourages us to investigate further polysaccharides as new class of ARG inhibitors.

Additionally, polysaccharides have already been described as metabolites with leishmanicidal activity (2012, Adriazola et al., 2014; Amaral et al., 2015; Kangussu-Marcolino et al., 2015; Valadares et al., 2011). The usage of a drug conjugated of Amphotericin B and arabinogalactan reduced toxicity and showed better efficacy against *Leishmania* sp. as effect of the polysaccharides on the immune response (Ehrenfreund-Kleinman, Domb, Jaffe, Benni Leshem, & Golenser, 2005; Nishi et al., 2007).

The immune response showed by polysaccharides previously related is due to their interaction with macrophages cells inducing pathways and processes to prevent leishmania infection. Among the host cell responses stimulated by polysaccharides, are the increase of reactive oxygen intermediates (ROI) and nitric oxide (NO) (Amaral et al., 2015; Kangussu-Marcolino et al., 2015; Schepetkin & Quinn, 2006). The metabolic pathway of nitric oxide synthase (NOS) that generate reactive oxygen species is regulated by TH_1 and TH_2 cytokines as microbicidal response. However, *Leishmania* trick the immune response activating TH_2 cytokine and increasing arginase expression for their growth and survival (Blaña-Fouce et al., 2012; Colotti & Ilari, 2011; Kropf et al., 2005; Roberts et al., 2004). In this view, arginase inhibition by FG-*Aa* could be an additional clue on how polysaccharides act as immunomodulators.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2018.08.109.

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