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Rhizoctonia solani fucomannogalactan: Chemical characterization and antiproliferative activity

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ABSTRACT

A fucomannogalactan from *Rhizoctonia solani* biomass was obtained after hot aqueous extraction and purified by freeze-thaw cycles and gel filtration chromatography on Sepharose CL-6B. The polysaccharide was homogeneous after HPSEC/RID analysis (M_w/M_n ~ 1.1), displaying an average molecular weight of 15.4×10^3 Da. Its chemical structure was determined by methylation analysis (GC/MS) and spectroscopy (FTIR, 1D and 2D NMR). The polysaccharide had a branched α-1,6-linked Gal*p* backbone with 66% linear residues, a number of which were at *O*-3 methylated. Side chains (34%) were always linked at *O*-2 positions of the main chain and consisted of single, non-reducing ends of α-D-Man*p* (6%) and α-L-Fuc*p* (28%). Analysis of its biological activity showed that the highly purified fucomannogalactan from *R. solani* inhibited the proliferation of colon cancer cells *in vitro*, but that it did not have the same activity against lung cancer cells.

1. Introduction

Fungi are renewable sources of biomolecules potentially useful for application in the food, cosmetic and pharmaceutical industries. Basidiomycetes are a group of fungi formed by mushrooms, jelly fungi, rust, and sooty molds [1], which have been thoroughly investigated to obtain bioactive molecules. Hot aqueous and alkaline extracts of fungal biomass are rich in polysaccharides, usually cell wall components, whose chemical and biological characterization requires prior purification.

Glucans (β and/or α, linear or branched) and heteropolysaccharides are the most studied polymers from basidiomycetes. Potentially active polysaccharides isolated from fungal biomass have shown immunostimulant [2], antitumor [3], anti-inflammatory [4], and antinociceptive activities [5]. Among fungal heteropolysaccharides, heterogalactans are the most common and usually have an α-D-Galp-(1 → 6) main chain with fucose or mannose side branches linked to *O*-2 [6,7].

Many reports describe the chemical structures and biological activities of cell wall or extracellular polysaccharides isolated from various species of basidiomycetes, mainly mushrooms. Little is known about polysaccharides obtained from phytopathogens. *Rhizoctonia solani* is a basidiomycete frequently found as a soil phytopathogen in cotton [8], potato [9] or tomato [10] crops. De Candolle first reported the genus *Rhizoctonia* in 1815, and later, in 1858, Kuhn described *R. solani* [11]. Vidhyasekaran et al. [12] and Yang et al. [13] isolated phytotoxin from *R. solani* whose bioactive component proved to be carbohydrate. According to the literature, there is few information on compounds isolated from *R. solani*, so the aim of the present investigation was to determine the chemical structure and antitumor activity of a polysaccharide isolated from *R. solani* biomass.

2. Materials and methods

2.1. Biological material and culture conditions

The fungus *Rhizoctonia solani*, originally isolated from melon, was provided by the URM Culture Collection, Universidade Federal de Pernambuco, UFPE-Brazil (registration number 4014). It was maintained at 4 °C on potato-dextrose-agar. The fungus was grown according to Steluti et al. [14], changing sucrose by glucose as carbon source. The biomass recovered by centrifugation was separated from the culture

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medium by exhaustive washing with distilled water until no sugars were detectable in the washings.

2.2. Analytical techniques

Total sugars were detected and quantified by the phenol sulfuric acid method [15], using a D-glucose solution as standard. Protein was determined by the Bradford method [16] using bovine serum albumin as standard.

2.3. Extraction and purification of cell wall polysaccharides

Extraction and purification of polysaccharides from *R. solani* biomass were done as shown in Fig. 1. Once dried and pulverized, 18 g of biomass powder were Soxhlet-extracted with ethanol (1:20 w/v, 78 °C, 12 h, 1×). The ethanolic extract was filtered and the resulting residue was dried and submitted to aqueous extraction (1:20 w/v, 100 °C, 4 h). The last procedure was repeated three times and the supernatant solutions were combined and concentrated. Three volumes of cold ethanol were added to precipitate polysaccharides (E_{H2O}) from the aqueous extract. The precipitate was solubilized in distilled water and subjected to eight successive freeze-thawing and centrifugation treatments (freezing overnight at −20 °C in freezer, thawing at room temperature) until no further precipitate appeared. The insoluble material ($E_{H2O\text{-}P}$) was lyophilized and stored. The soluble fractions were pooled together and the polysaccharides were precipitated with three volumes of ethanol ($E_{H2O\text{-}S}$) to be analyzed in this study. Aliquots of 12 mg of $E_{H2O\text{-}S}$ were resuspended in water (2 mL), centrifuged (5000 ×*g*, 5 min) and fractionated by gel filtration chromatography on Sepharose CL-6B using a 2.5 × 45 cm column and water as eluent (1 mL/min). Fractions (2.5 mL) were collected and monitored for carbohydrate by the phenol sulfuric acid method, as reported above. Fractions corresponding to each peak (P_I to P_{IV}) were pooled and freeze-dried. Gel filtration chromatography was repeated until sufficient material was obtained for further analysis.

2.4. Monosaccharide composition

Each polysaccharide fraction (0.05 mg total sugar) was hydrolyzed with 4 M TFA (0.3 mL) in a sealed tube for 2 h at 100 °C [17]. After hydrolysis, the acid was removed and the residue dissolved in 0.5 mL water and dried. The dissolution-evaporation cycle was repeated twice. The final residue was dissolved in 0.2 mL water, and 0.025 mL aliquots analyzed by high performance anion exchange chromatography/

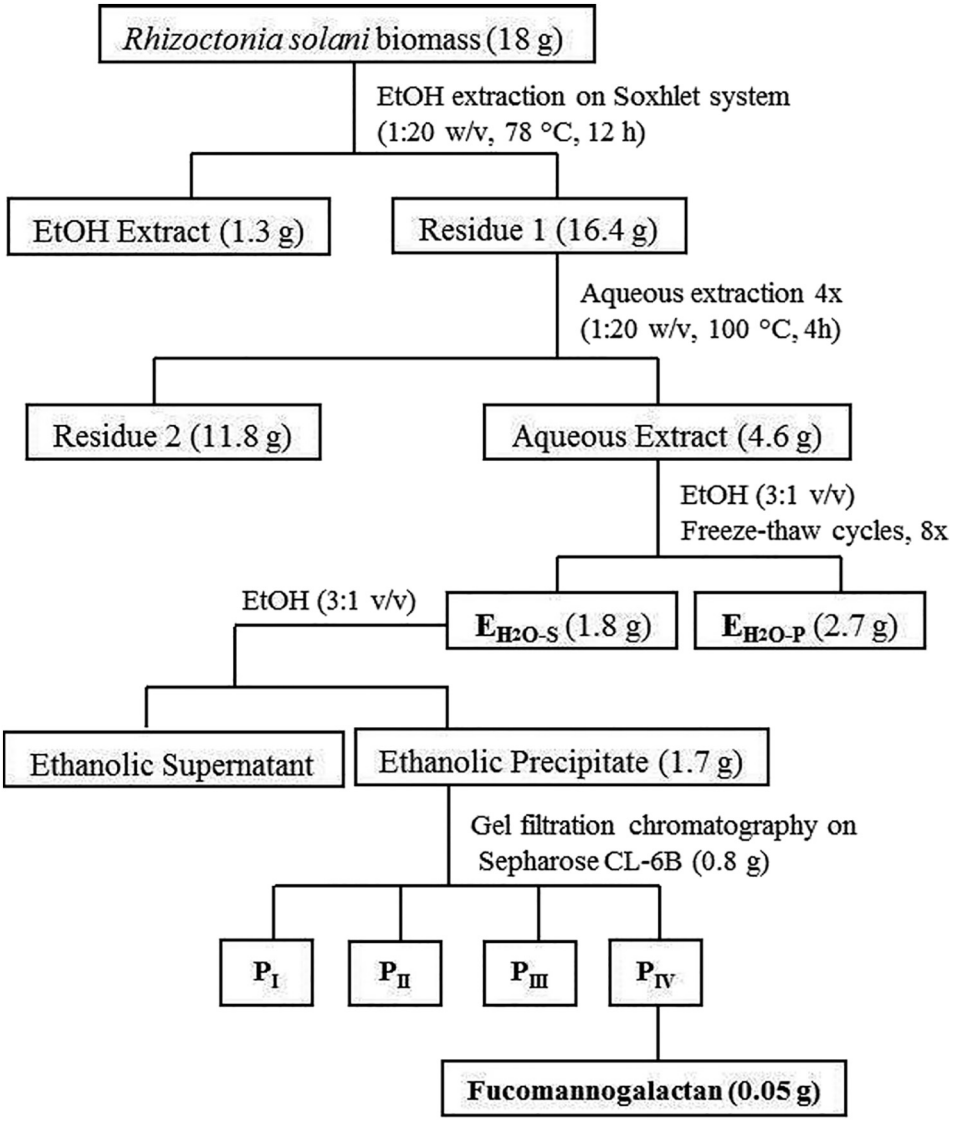

Fig. 1. Scheme outlining the protocol for extraction and purification of polysaccharides from *Rhizoctonia solani* biomass.

pulsed amperometric detection (HPAEC/PAD) on a Dionex Chromatograph DX 500 equipped with a CarboPac-PA-1 (Dionex Chromatography) column (4 × 250 mm) and a PA-1 guard column. The system was equilibrated at a flow rate of 1 mL/min with 0.014 M NaOH, prepared from water (solvent 1) and 0.2 M NaOH (solvent 2). Neutral monosaccharides were separated isocratically using the same flow and eluent. The column was regenerated after a 20 min run with 100% solvent 2 for 15 min, and then re-equilibrated to 0.014 M NaOH. Quantification was carried out from the peaks areas of sample components, using the response factors calculated for commercial standards of neutral monosaccharides.

2.5. Determination of polysaccharide homogeneity and molecular weight (M_w)

The homogeneity of each polysaccharidic fraction (1 mg/mL water solutions) was determined by high performance size exclusion chromatography (HPSEC) coupled to a refractive index (RI) detector model RID 10A. The chromatography system consisted of an HPLC pump (Shimadzu-10AD), a manual injection valve (Shimadzu) fitted with a 200 μL loop and four Ultrahydrogel columns (7.8 × 300 mm) (Waters) with exclusion limits 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%) at a flow rate of 0.6 mL/min. Data analysis was performed using the LC solution software (Shimadzu Corporation). The average molecular weight of P_{IV} was estimated by 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.

2.6. FT-IR and NMR spectroscopy

FT-IR spectroscopy was performed using an IRAffinitty-1 Shimadzu spectrometer, 0.8 mg of freeze-dried sample (P_{IV}) in 250 mg KBr discs, and scans conducted within 4000–500 cm^{-1} at a resolution of 4 cm^{-1}.

For ^1H and ^{13}C NMR experiments, fraction P_{IV} was deuterium-exchanged by successive lyophilization steps in D_2O and the spectra were obtained on a Bruker 500 MHz (500/125 MHz, H/C) equipped with 5-mm wide bore probe, operating at 50 °C. The experiments were carried out using the pulse programs supplied with the Bruker manual. Proton chemical shifts refer to residual HDO at δ 4.61 ppm (50 °C) and carbon chemical shifts to internal acetone at δ 31.07 ppm [18]. 2D NMR experiments were performed using standard Bruker software, and the 2D NMR spectra included $^1H/^1H$ homonuclear correlation spectroscopy (COSY), heteronuclear single-quantum correlation spectroscopy (HSQC), heteronuclear multiple-bond correlation spectroscopy (HMBC), nuclear Overhauser enhancement spectroscopy (NOESY) and rotating frame nuclear Overhauser effect spectroscopy (ROESY).

2.7. Methylation analysis

The P_{IV} sample (3 mg) was methylated (3 times) using the procedure described by Ciucanu and Kerek [19]. The methylated products were isolated by partitioning in a CH_2Cl_2 and H_2O mixture (4 times), and the organic phase containing the methylated sugars was washed three times with water and dried. The methylated products were hydrolyzed using 3 M TFA (1 mL) at 120 °C for 1 h, then reduced with sodium borodeuteride and acetylated with acetic anhydride-pyridine (1:1). The products were analyzed in an Agilent 7980A-5975C gas chromatography–mass spectrometry (GC–MS) system with He as the carrier gas at a flow rate of 1 mL/min. A HP5-MS capillary column (30 m × 0.25 i.d. × 0.25 μm) was used applying a temperature gradient of 160 to 210 °C at 2 °C/min.

2.8. Tumor cell culture

Human colon cancer (HCT-116 and HT-29) and murine lung cancer (3LL) cell lines were cultured in complete culture medium (RPMI 1640 liquid culture medium supplemented with 10% fetal bovine serum, 200 μM L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, 25 mM HEPES, and 40 mg/L antibiotic/antimycotic mix) at 37 °C, under 5% constant tension of CO_2. The cell monolayer was detached from the bottle surface by treatment with 0.05% trypsin solution and washed with phosphate buffered salt solution (PBS), followed by washing with complete culture medium.

2.9. Assay of antiproliferative activity

Direct antiproliferative activity of fraction P_{IV} was evaluated against two tumor colorectal cell lines (HT-29, HCT-116), one of murine lung cancer (3LL) and one non-tumor mouse fibroblast (L929) cells using the MTT assay [20] modified for this study. Briefly, target tumor cells and fibroblasts were adjusted for 2×10^5 and 1×10^4 cells/mL, respectively and seeded into 96 flat-bottomed-well culture plates (100 μL/mL). Eighteen hours later, tumor cells and fibroblasts monolayers were challenged with 100 μg of fucomannogalactan solutions at 100, 50, 25 or 12.5 μg/mL (tumor cells) and 50, 25, 12.5 or 6.25 μg/mL (fibroblast cells), prepared with complete culture medium. After 24 h, culture supernatants were carefully harvested and changed by 100 μL of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (1 mg/mL). After an additional 2 h culture, the supernatant was removed and reduced. Formazan crystals were dissolved by adding 100 μL of dimethylsulphoxide (DMSO). The plate was kept at room temperature for 5 min and further read in an ELISA reader under a 540 nm wavelength. Optic density (O.D.) of test wells were compared with spontaneously grown culture wells in order to calculate the percentage of living cells. Cytotoxic agent MMS (final concentration of 150 mM) was used as positive control for tumor cells (HT-29 and HCT-116).

2.10. Statistical analysis

The results of the MTT assay were expressed as the mean ± standard deviation of antiproliferative effect. The data were compared using one-way ANOVA and Tukey's test [21] using OriginLab Program version 8.0.

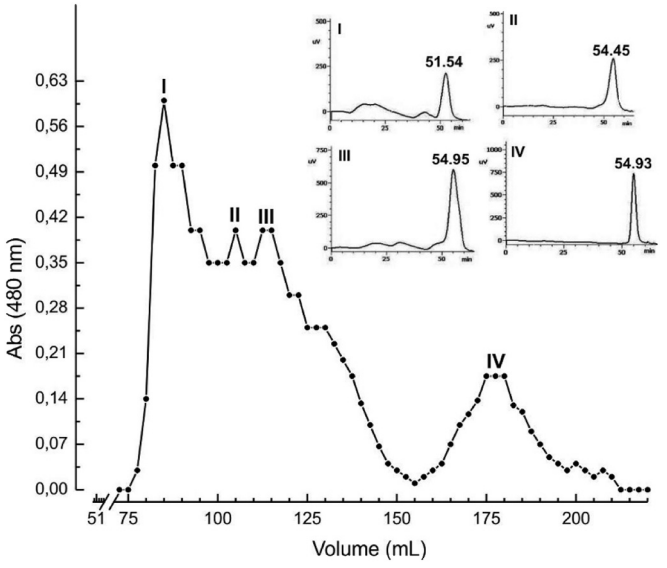

Fig. 2. Gel filtration chromatography on Sepharose CL-6B of fraction E_{H2O-S}. HPSEC/RID (inset) of P_I, P_{II}, P_{III} and P_{IV}.

Table 1
Linkage types and their proportions (%), deduced from GC–MS analysis of the partially *O*-methylated alditol acetates obtained from the fucomannogalactan (P_{IV}) from *R. solani*.

R_T (min)	Components	Linkage	Molar ratio (%)	Mass fragments (*m*/*z*)
5.51	2,3,4-Me_3-Fuc*p*	Fuc*p*-(1→	28	72, 89, 102, 118, 131, 162, 175, 207
7.53	2,3,4,6-Me_4-Man*p*	Man*p*-(1→	06	71, 87, 102, 118, 129, 145, 161, 162, 205
11.33	2,3,4-Me_3-Gal*p*	6→)-Gal*p*-(1→	34	71, 87, 99, 102, 118, 129, 162, 189, 233
14.05	3,4-Me_2-Gal*p*	2,6→)-Gal*p*-(1→	32	71, 87, 99, 129, 189, 234

The differences were considered significant when the error probability was <5% ($p < 0.05$).

3. Results and discussion

3.1. Extraction, purification, monomeric composition and molecular weight of polysaccharide

Fungal biomass was thoroughly washed to eliminate free monosaccharides, salts, secondary metabolites and exopolysaccharides. Complete elimination of soluble sugars from the biomass preparation was confirmed when washings were negative for sugars as determined by the phenol sulfuric acid method [15]. The sequence of extractions and the procedures used to obtain the polysaccharide are shown in Fig. 1.

Prior to hot aqueous extraction, the powdered biomass was subjected to ethanol extraction to remove pigments and fats, as previously reported for the extraction of polysaccharides from the fruiting bodies of other basidiomycetes, such as *Pleurotus geesteranus* [7] and *Coprinus comatus* [22]. The polysaccharides contained in the hot water extracts of defatted fungal biomass (E_{H2O}) were subjected to repeated freeze-thaw cycles. The soluble fraction ($E_{H2O\text{-}S}$) contained carbohydrate (86%) and protein (14%). After acid hydrolysis, glucose (79%), galactose (14%), fucose (7%) and mannose (<1%) were identified as the monosaccharide components of the carbohydrate portion. HPSEC/RID analysis of $E_{H2O\text{-}S}$ showed a single peak at 54.5 min, asymmetric and with an enlarged base, characteristic of a polydisperse substance.

Despite finding a single peak in the previous analysis, and taking into account that aqueous extracts of fungi usually consist of a mixture of polysaccharides, preparative gel filtration chromatography on Sepharose CL-6B was used in a trial to obtain pure polysaccharides from $E_{H2O\text{-}S}$. Four fractions (P_I to P_{IV}) were separated according to their molecular weights (Fig. 2), but only the small peak that eluted at 54.93 min (P_{IV}) was homogeneous after HPSEC/RID analysis (M_w/M_n ~ 1.1), displaying an average molecular weight of 15.4×10^3 Da,

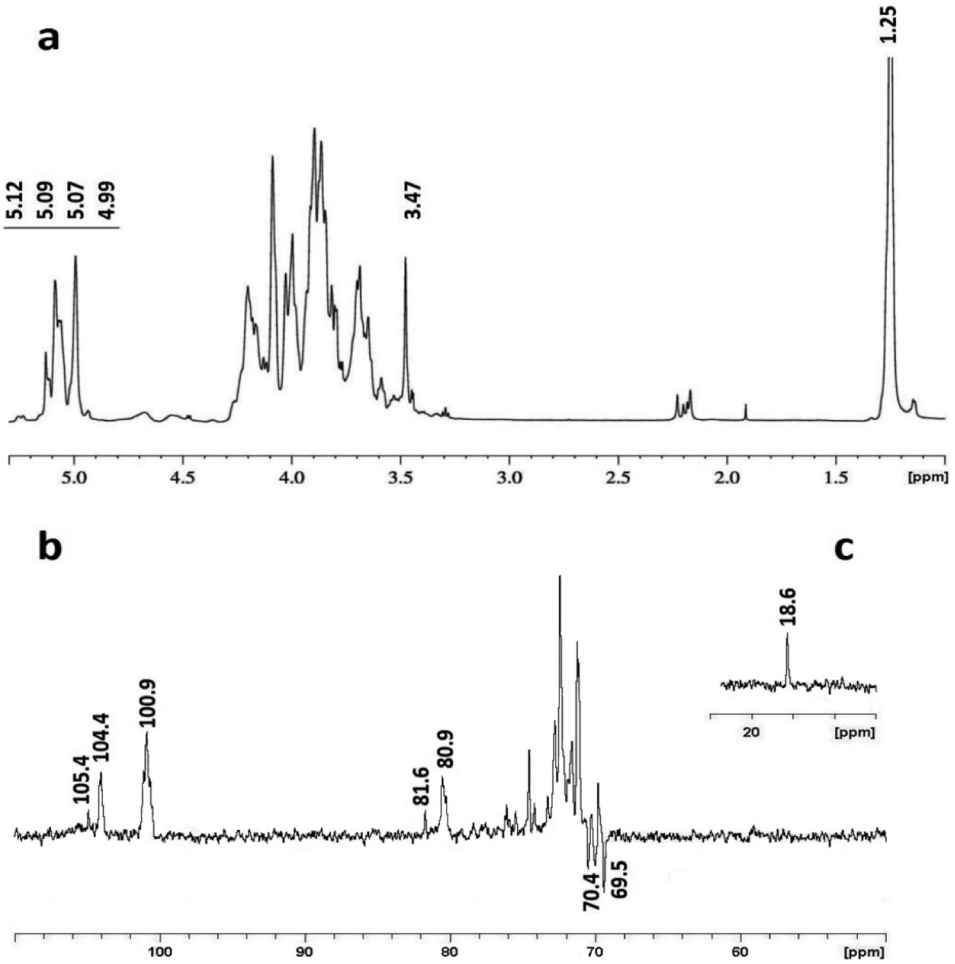

Fig. 3. a) ^{1}H NMR and b) ^{13}C NMR DEPT135 spectra of P_{IV} in D_2O at 50 °C. c) Inset of CH_3-6 region from Fuc*p* units.

according to a dextran standard curve. This fraction, which exhibited 99%carbohydrate content, was then selected for further characterization. Its monosaccharide composition, determined after acid hydrolysis by HPAEC/PAD, was galactose (64%), fucose (30%), mannose (6%) and traces of glucose (<1%).

3.2. Structural analyses of polysaccharide: methylation analysis, FT-IR and NMR spectroscopy

For investigation of linkage types, P_{IV} was methylated, and the resulting *O*-methylalditol acetates were analyzed by GC–MS, revealing the presence of a branched structure (Table 1) with non-reducing end-units of Fuc*p* (2,3,4-Me_3-Fuc) and Man*p* (2,3,4,6-Me_4-Man), besides the 6-*O*-(2,3,4-Me_3-Gal) and 2.6-di-*O*-substituted units (3,4-Me_2-Gal) of galactopyranose. The presence of a single dimethylated derivative suggested that the ramifications (Fuc*p* and Man*p*) occupied the same position (*O*-2) at the galactopyranosyl residues, obviously not at the same time. Methylation results indicated that the heteropolysaccharide (fraction P_{IV}) was a fucomannogalactan.

The FTIR spectrum (not shown) of P_{IV} showed a band at 856 cm^{-1}, typical of α-anomers, and no band at 890 cm^{-1}, which is characteristic of the β configuration. According to this analysis, all glycosidic linkages were in the α configuration [23]. The values of the coupling constants $J_{C\text{-}1,\ H\text{-}1} = 170 \pm 1$ Hz (observed in coupled HSQC spectrum) confirmed that all residues were in the α configuration. Such values agreed with the low-field H-1 signals (δ 5.12 to δ 4.99 ppm) in the ^{1}H NMR spectrum (Fig. 3a), which also showed an intense signal at δ 1.25 ppm, associated with the hydrogen (H-6) connected to the C-6 of the deoxysugar which was probably derived from fucose. In addition, the signal at δ 3.47 ppm was related to the protons from methyl groups, suggesting the presence of *O*-methylated units in the heteropolysaccharide [6,24].

1D NMR (^{1}H, ^{13}C and DEPT135) and 2D NMR (^{1}H-^{1}H COSY, NOESY, ROESY, HSQC and HMBC) analyses, together, provided enough information to assign the majority of protons and carbons of the heteropolysaccharide.

In the ^{1}H NMR spectrum, the chemical shift at δ 5.12 ppm (Fig. 3a) was assigned to H-1 from residue A and the C-1 for the same residue was obtained by HSQC at 105.4 ppm (Fig. 3b). Through the ^{1}H-^{1}H COSY (Fig. 4a), ROESY (Fig. 4b) and NOESY (Fig. 4c) spectra, the chemical shifts of H-2, H-3, H-4 and H-5 were determined at δ 4.08, δ 3.67, δ 3.99 and δ 3.78 ppm, respectively, and H-6a/b was obtained by C-6/H-6 coupling from the HSQC spectrum (Fig. 5c). The other carbons from residue A were also obtained from the HSQC spectrum, and they are shown in Fig. 5c and Table 2. Residue A was identified as α-Man*p*-(1→, and the results were in accordance with the results from GC–MS analysis.

The anomeric proton from residue B was assigned at δ 5.09 ppm (Fig. 3a) and the coupled carbon signal was at δ 104.4 ppm in the HSQC spectrum (Fig. 5b). The assignments to H-2, H-3, H-4 and H-5 were made from the ^{1}H-^{1}H COSY (Fig. 4a), ROESY (Fig. 4b), NOESY (Fig. 4c) spectra, and the signals from the corresponding carbons were identified from the HSQC spectrum (Fig. 5c). The cross peak in HSQC with the chemical shift of δ 1.25 ppm and δ 18.6 ppm (Fig. 5a) suggested that the —CH_3 group was probably derived from residue B. According to the chemical shifts, it was proposed that residue B was α-Fuc*p*-(1→, also consistent with the results from hydrolysis and GC–MS analysis.

The residues C, D and E showed signs of anomeric carbons at δ 100.9 ppm (Fig. 3b), and the anomeric protons at δ 5.07(C) and δ 4.99 (D and E) ppm (Fig. 3a). The assignments for H-2, H-3, H-4 and H-5 were obtained from the two-dimensional spectra (^{1}H-^{1}H) COSY, ROESY and NOESY (Fig. 4a, b and c). The correlation between C/H was also made from the HSQC spectrum (Fig. 5c). The coupling between C-6/H-6 from 6-*O*-substituted Gal*p* units, without and with *O*-methyl groups, occurred at δ 69.5/3.93/3.70 ppm (Fig. 5c). Similarly, the coupling at δ 70.4/3.99/3.66 ppm was attributed to C-6/H-6 of 2.6-di-*O*-substituted Gal*p* unit. These chemical shift values have been confirmed by inverted signals in the ^{13}C NMR DEPT135 (Fig. 3b) and HSQC-DEPT135 spectra (Fig. 5c). The HSQC-DEPT135 spectrum (Fig. 5c) also

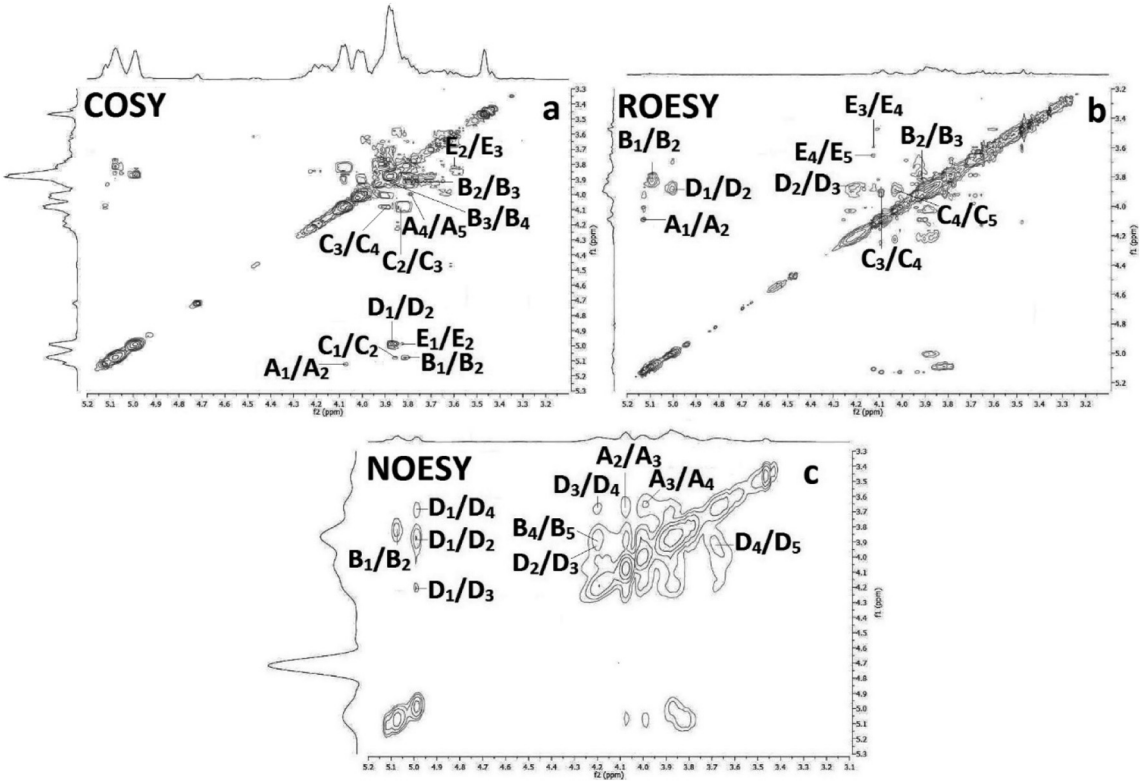

Fig. 4. a) ^{1}H-^{1}H COSY, b) ROESY and c) NOESY spectra of P_{IV} in D_2O at 50 °C.

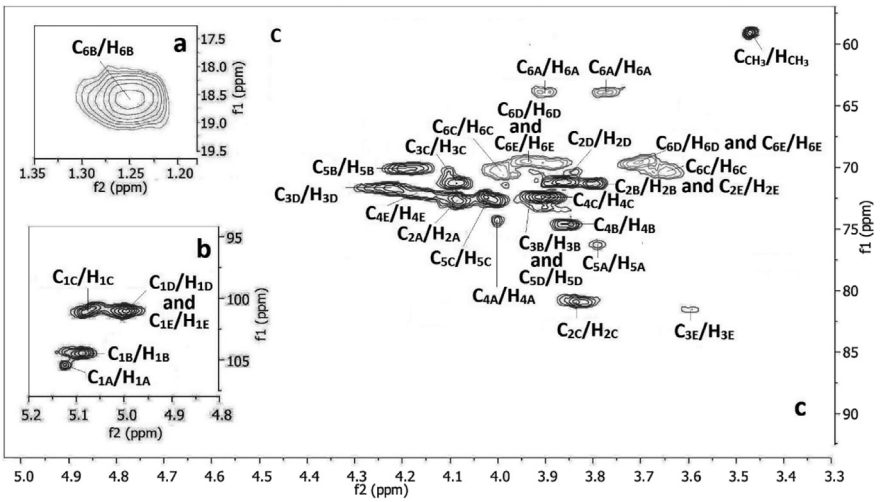

Fig. 5. HSQC-DEPT135 spectrum of P_{IV} in D_2O at 50 °C: a) CH_3-6 region from Fuc*p* units, b) anomeric region and c) other signals.

allowed us to assign the couplings between free C-6/H-6 from Man*p* units at δ 63.9/3.91/3.78 ppm and C-6/H-6 of Fuc*p* units (δ 18.6/1.25 ppm), already mentioned above.

The chemical shifts at δ 80.9/3.84 ppm (Fig. 5c) were attributed to C-2/H-2 from residue C, and the coupling H-1/H-2 (δ 5.07/3.84 ppm) observed in the COSY spectrum (Fig. 4a) confirmed the assignment C-1/H-1 (δ 100.9/5.07 ppm) given to unit C.

As presented earlier, the fucomannogalactan from *R. solani* had some Gal*p* units naturally methylated. The signal that corresponds to the C/H coupling of —OCH_3 group was detected at δ 59.1/3.47 ppm in the HSQC-DEPT135 spectrum (Fig. 5c, top right corner). The methyl group position was confirmed by coupling of the signals from C-3 of naturally methylated Gal*p* units with protons from —OCH_3 group at δ 81.6/3.47 ppm, observed in the HMBC spectrum (Fig. 6b). In addition, the ROESY spectrum (Fig. 6c) showed the coupling of proton from —OCH_3 group with H-3 of naturally methylated Gal*p* units at δ 3.47/3.60 ppm. The coupling between C-3/H-3 from *O*-Me-Gal*p* units is observed at δ 81.6/3.60 ppm in the HSQC-DEPT135 spectrum (Figs. 5c and 6d).

All the chemical shifts attributed to the carbons and hydrogens of the fucomannogalactan are shown in Table 2.

Fucogalactans obtained by aqueous extraction of fungal biomass have been described as having a main chain of (1 → 6)-α-galactopyranose residues, substituted at *O*-2 by single α-L-fucopyranosyl units [22]. Nevertheless, additional types of side chains have also been reported, such as *O*-2 substitutions with β-D-Gal*p* residues [25], 3-*O*-D-mannopyranosyl-L-fucopyranosyl groups [26], or β-D-Man*p* [5]. Alquini et al. [26] found a polysaccharide composed of a main chain of (1 → 6)-linked α-D-galactopyranosyl residues, partially substituted at *O*-2 by 3-*O*-D-mannopyranosyl-L-fucopyranosyl disaccharides (18%) or α-D-mannopyranosyl residues (10%) and in minor proportion by α-L-fucopyranosyl units (2%). The fucomannogalactan from *R. solani* studied in this work had a small proportion of α-D-mannopyranosyl residues (~6%) and ~28% of α-L-fucopyranosyl units. These differences in the type and/or the percentage of the substituents could be attributable to slight variations during the synthesis of these polymers in different genera of basidiomycetes. It should be recalled that polysaccharides are not a direct product of gene expression but formed from the incorporation of monosaccharidic units in a mechanism catalyzed by a complex enzymatic machinery. This fact causes in most cases an uneven distribution and proportion of substituents even for the polysaccharides produced by a same microorganism [27].

3.3. Antiproliferative activity

Medicinal properties attributed to polysaccharides isolated from various basidiomycetes, mainly glucans, include immunomodulatory [28], antitumor [29], prebiotic [30] and antioxidant [31] activities. Heterogalactans, especially those containing fucose, have also been reported to be antitumor [25], antiinflammatory and antinociceptive [4,24] agents.

Aiming to evaluate the potential therapeutic applications of the *R. solani* polysaccharide, we investigated the effect of the fucomannogalactan on tumor cells. Different concentrations (12.5; 25.0; 50.0; 100.0 μg/mL) of fucomannogalactan on the antiproliferative activity of HT-29, HCT-116 and 3LL cells were determined by estimating the viable cell quantities through MTT assay [20]. Fucomannogalactan reduced the number of HT-29 viable cells in a dose-dependent manner.

Table 2
^1H and ^{13}C NMR chemical shifts expressed as δ (ppm) of the fucomannogalactan from *Rhizoctonia solani*.

Units		1	2	3	4	5	6	—OCH_3
α-Man*p*-(1→	^{13}C	105.4	72.8	nd*	74.5	76.2	63.9	–
(A)	^1H	5.12	4.08	3.67	3.99	3.78	3.78/3.91	
α-Fuc*p*-(1→	^{13}C	104.4	71.4	72.4	74.7	70.1	18.6	–
(B)	^1H	5.09	3.82	3.92	3.86	4.19	1.25	
→2,6)-α-D-Gal*p*-(1→	^{13}C	100.9	80.9	71.4	72.5	72.4	70.4	–
(C)	^1H	5.07	3.84	4.09	3.89	4.02	3.66/3.99	
→6)-α-D-Gal*p*-(1→	^{13}C	100.9	71.2	71.8	nd*	72.4	69.5	–
(D)	^1H	4.99	3.88	4.23	3.68	3.92	3.70/3.93	
→6)-3-*O*-Me-α-D-Gal*p*-(1→	^{13}C	100.9	71.4	81.6	72.3	nd*	69.5	59.1
(E)	^1H	4.99	3.82	3.60	4.13	3.65	3.70/3.93	3.47

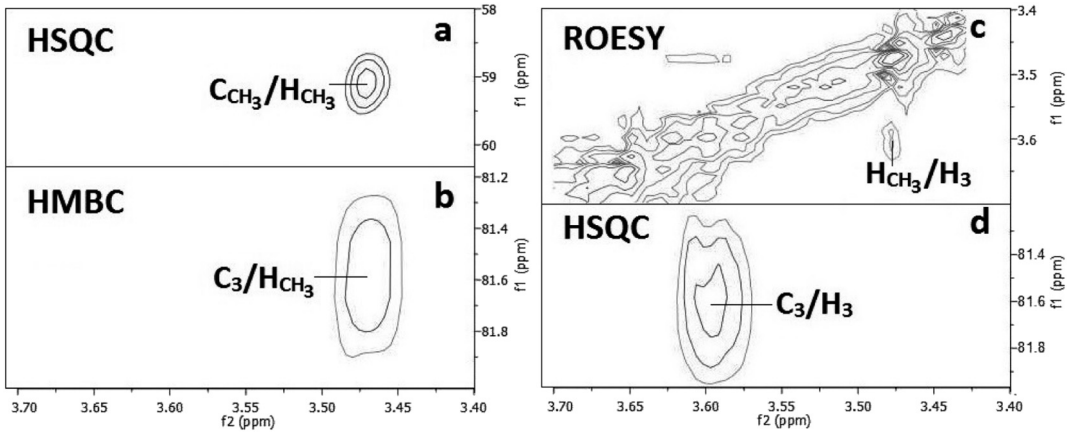

Fig. 6. a) Coupling C/H of the —OCH_3 group; b) coupling of C-3 from naturally methylated Gal*p* units with proton of —OCH_3 group; c) coupling of proton of —OCH_3 group with H-3 of naturally methylated Gal*p* units; and d) coupling between C-3/H-3 the *O*-Me-Gal*p* units.

Fig. 7a shows the reduction of 39.3 ± 3.5% of such cells 24 h after the addition of 100 μg/mL of the polysaccharide. Fucomannogalactan also inhibited the growth of HCT-116 cells; the antiproliferative activity was slightly higher compared to that of HT-29 cells in all polysaccharide concentrations, and, particularly, at 100 μg/mL, the activity was estimated to be 44.6 ± 1.9% (Fig. 7a). MMS (methyl methanesulfonate) is a direct-acting DNA alkylating agent known to cause cell death and it was used as positive control to two tumor colorectal cell lines. Using the same experimental conditions the MMS inhibited 79.5 ± 2.1% of HT-29 and 68.2 ± 1.4 of HCT-116 cells growths (Fig. 7a).

A similar assay was conducted with mouse fibroblasts L929 cells in order to determine whether the fucomannogalactan exerts toxic effects on normal cells. Up to 50 μg/mL fucomannogalactan concentration, no significant effect was observed against the viability of fibroblasts cell (Fig. 7b).

In contrast, the fucomannogalactan promoted the growth of 3LL murine lung cancer cells. Li et al. [32] reported that fucogalactans bind to cells targeting an elastin peptide receptor. Although there are no reports on the expression of such receptors on human lung cells, Wasano, Hirakawa and Nakamura [33] observed its occurrence in rat lung cells. Therefore, it is possible that 3LL cells, derived from the malignant transformation of murine lung cells, preserve the expression of this receptor, and that its interaction with fucomannogalactan stimulates the proliferation of these cancer cells. A similar phenomenon is observed when other kinds of polysaccharides bind to pattern recognition receptors (PRR), for instance, the interaction between β-glucans and dectin-1 [34].

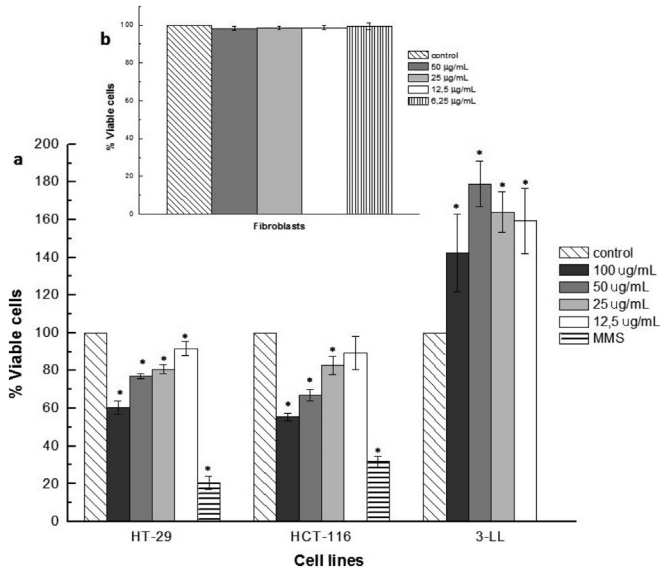

Fig. 7. Effect of fucomannogalactan on growth of human colorectal cancer cell lines (HT-29 and HCT-116) and murine lung cancer cell line (3LL) (a) and on growth of non-tumor mouse fibroblast L929 cells (b), after 24 h. *The cell viability (%) was significant when compared to the control group according to the Tukey's test ($p < 0.05$).

To the best of our knowledge, elastin peptide receptors are not expressed in intestinal cells. This leads us to think that the proliferative activity triggered by fucomannogalactan depended on the expression of such receptors, explaining the different behavior of colon- and lung-derived cancer cells.

4. Conclusions

We can conclude on the basis of the above results that fraction P_{IV} contains a novel fucomannogalactan, whose structure is characterized by a branched α-1,6-linked Gal*p* backbone with 66% linear residues, a number of which are methylated at HO-3. Side chains (34%) were always linked at *O*-2 positions of the main chain and consisted of single, non-reducing ends of α-Man*p* (6%) and α-L-Fuc*p* (28%). Taken together, our data show that the highly purified fucomannogalactan from *R. solani* biomass is able to kill colon cancer cells *in vitro* but not lung cancer cells.

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