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Partially methylated galactans containing different proportions of 3-*O*-methyl-galactose from *Pleurotus citrinopileatus*

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ABSTRACT

Pleurotus citrinopileatus, popularly known as "golden oyster mushroom" have medicinal properties, which are attributed mainly to the presence of bioactive polysaccharides. In this work, two partially 3-*O*-methylated galactans were isolated from the fruiting bodies of this fungus, via successive aqueous extraction, followed by fractionation by freeze-thawing, and precipitation of soluble material with Fehling solution. The structural assignments were carried out using mono- and bidimensional NMR spectroscopy, monosaccharide composition, and methylation analyses. The polysaccharides were characterized as linear, partially 3-*O*-methylated (1 → 6)-linked α-galactopyranans, containing only Gal and 3-*O*-Me-Gal, in 2:1 and 1:1 molar ratios, with molar masses of 37.6×10^3 g/mol and 28.5×10^3 g/mol, respectively. Similar structures have been described for other *Pleurotus* spp., but showing a lower content of 3-*O*-Me-Gal.

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1. Introduction

Mushrooms have received great attention due mainly their nutritional value and medicinal properties. Although there are several studies about its chemical composition and health beneficial effects, they still comprise a large and untapped source of bioactive compounds [1], being the polysaccharides considered the most potent. These macromolecules have a broad spectrum of biological effects, such as antibiotic, antioxidant, antitumoral, anticoagulant, and immunostimulating activities, which are related to the wide structural diversity presented by these polymers.

Among mushrooms that are extensively known for their medicinal properties, mainly due to the presence of bioactive polysaccharides, one can find *Pleurotus citrinopileatus*, commonly called "golden oyster mushroom".

Preliminar data have shown that the extracts or polysaccharidic fractions of this fungus are effective in antioxidation [2,3], antigenotoxicity [4], immunomodulation [5,6], antihyperglycemic [7] and antitumor activities [8,9]. However, it is not possible to attribute a relation between structure and activity because most of the investigations were carried out with crude polysaccharide extracts or unpurified fractions. Moreover, many of these do not report the chemical structures of these polymers. Among these studies were isolated and partially chemically characterized from *P. citrinopileatus* four fractions containing β-glucans and a heteropolysaccharide constituted by mannose, galactose, arabinose and glucose, which presented potent antitumor activity against Sarcoma 180 implanted in mice [9], and one (1 → 3), (1 → 6)-linked β-glucan (M_w 450 kDa) with immunomodulatory properties [5,6]. Detailed studies of structure have been presented for a branched glucan (M_w 45 kDa) constituted by a backbone comprised of (1 → 6)-β-D-glucopyranosyl units, which were substituted at O-3 by 3-*O*-β-D-glucopyranosyl-D-glucopyranose [10] and for a linear α-galactopyranan partially 3-*O*-methylated (M_w 27.4 kDa) formed by (1 → 6)-linked α-galactose, (1 → 6)-linked 3-*O*-Me-α-galactose and (1 → 4)-linked α-glucose in a molar ratio of 3.0:1.0:0.6 [11].

We now describe the structural features of two linear (1 → 6)-

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linked α-galactans partially 3-*O*-methylated isolated from the fruiting bodies of *P. citrinopileatus*, distinct of the previously reported.

2. Experimental

2.1. General experimental procedure

GC-MS analysis was performed with an Agilent 7820A gas chromatograph interfaced to an Agilent 5975E quadrupole mass spectrometer, fitted with split/splitless capillary inlet system, an Agilent G4513A autosampler, and a capillary DB 225-MS column (30 m × 0.25 mm i.d.). Injections of 1 μL were made in the splitless mode at injection temperature of 250 °C and detector at 280 °C. The column oven temperature was initially held at 50 °C for 1 min, then it was programmed at 40 °C.min^{-1} to 220 °C or 210 °C (constant temperature) for quantitative analysis of alditol acetates and partially *O*-methylated alditol acetates, respectively. Helium was the carrier gas at a flow rate of 1 mL.min^{-1}. Electron impact (EI) analysis was performed with the ionisation energy set at 70 eV.

NMR spectra (^{1}H, ^{13}C, HSQC-DEPT, COSY, HMBC, 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. Chemical shifts are expressed in δ relative to the internal standard tetramethylsilane (TMS) (δ = 0.0 for ^{13}C and ^{1}H).

2.2. Macrofungus and culture conditions

Fresh *Pleurotus citrinopileatus* (950 g) was furnished by Yuki Cogumelos Company (Owner: José Francisco Ramos Fernandes Viana), located in Araçoiaba da Serra, State of São Paulo, Brazil, in December 2013. The fungus was grown on culture substrate constituted of eucalyptus sawdust, wheat bran, corn bran, and calcitic limestone in a mixture ratio by weight of 137.5:27.0:30.5:1.0, with 62% relative humidity. After homogenization, the substrate was filled into polyethylene bags (2 kg each), autoclaved for 90 min at 121 °C and 1 atm. After the substrates were cooled down to room temperature, each bag was inoculated with 20 g of mushroom spawn. The inoculated substrates were kept in a spawn running room at 23 °C for 20 days. When the mycelium fully covered the substrate bag (spawn run completed), bags were kept open in a cropping room in which the temperature was controlled at 19 °C, relative humidity at 85–90%.

2.3. Extraction and purification of galactans

The fresh fruiting bodies of *P. citrinopileatus* (950 g) were dried by lyophilization, pulverized and the polysaccharides extracted with water at 10 °C for 6 h (x 1, 1000 mL). The extract was filtered and after centrifugation at 9000 rpm at 20 °C for 20 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 10 °C for 20 min. The crude polysaccharide fraction was dissolved in H_2O, dialyzed against distilled water for 20 h to remove low-molecular-weight carbohydrates, and freeze-dried, giving rise to fraction CW-*Pc*. This fraction was then dissolved in distilled water and the solution submitted to freezing followed by mild thawing at 4 °C [12], giving cold water-soluble (SCW-*Pc*) and insoluble fractions (ICW-*Pc*), which were separated by centrifugation (9000 rpm at 10 °C for 20 min). SCW-*Pc* fraction was treated with Fehling's solution (15 mL) [13], and the precipitated material (FP-*Pc*) centrifuged off (9000 rpm at 10 °C for 20 min). Both FP-*Pc* (precipitate), after

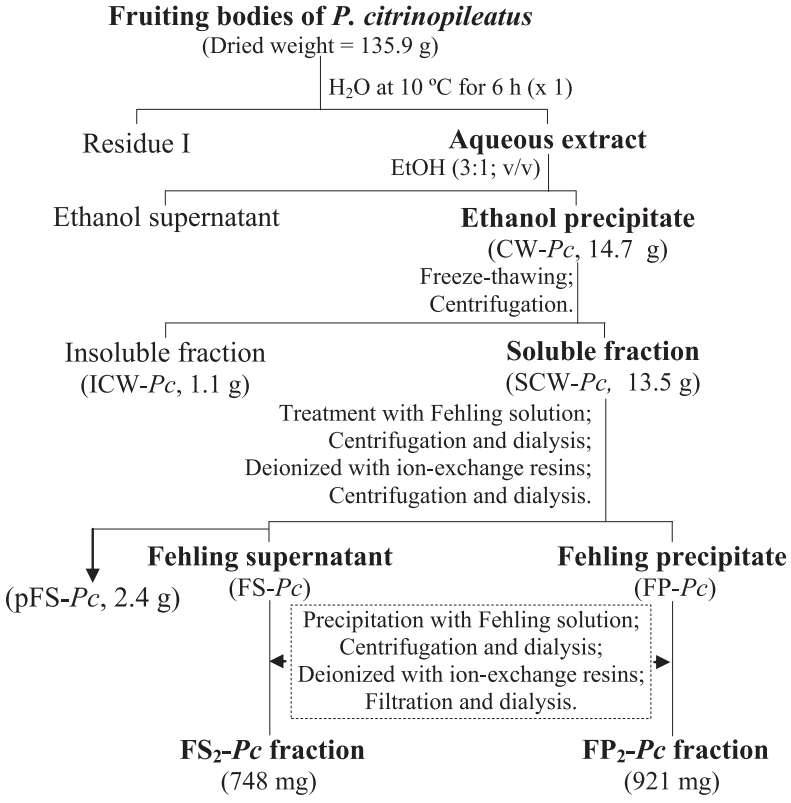

Fig. 1. Scheme of extraction and purification of the polysaccharides from *P. citrinopileatus*.

dissolution with destilled water, and FS-*Pc* (supernatant) fractions, were neutralized with HOAc, dialyzed against tap water and deionized with strongly acidic cation exchange resin (Dowex® 50WX2 hydrogen form), till total removal of the blue/green color characteristic of copper presence. During the treatment with ion-exchange resins, part of FS-*Pc* fraction became precipitated (pFS-*Pc* fraction). The resin was removed by filtration through a nylon woven filter and the filtrate centrifuged under same conditions above, giving fractions FS-*Pc* and pFS-*Pc*. Fehling treatment was repeated one more cycle under fractions FP-*Pc* and FS-*Pc*, giving the fractions FP_2-*Pc* and FS_2-*Pc*, respectively.

2.4. 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 $NaBH_4$ and/or $NaBD_4$ reduction, and acetylation with Ac_2O-pyridine (1:1, v/v) for 12 h at room temperature [14,15]. The resulting alditol acetates were analyzed by gas chromatography-mass spectrometry (GC-MS) as above cited (item 2.1) and identified by their typical retention times and electron impact profiles.

2.5. Determination of homogeneity of polysaccharides and their molecular weight

The homogeneity and molar mass (M_w) of the water-soluble fractions were determined by high performance steric exclusion chromatography (HPSEC), using a refractive index (RI) detector and a Wyatt Technology Dawn-F Multi-Angle Laser Light Scattering detector (MALLS). The eluent was 0.1 M $NaNO_3$, containing 0.5 g/L NaN_3. The polysaccharide solutions were filtered through a membrane, with pores of 0.22 μm diameter (Millipore). The specific refractive index increment (*dn/dc*) was determined. The samples were dissolved in 50 mM $NaNO_3$, and five increasing concentrations, ranging from 0.2 to 1.0 mg/mL, were used to determine the slope of the increment.

2.6. Methylation analysis of polysaccharides

Per-*O*-methylation of the purified fractions (5 mg each) was carried out using NaOH-Me_2SO-MeI [16]. The per-*O*-methylated derivatives (1 mg) were hydrolyzed with 45% v/v formic acid (HCO_2H, 250 μl) at 100 °C for 12 h, 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 [14,15] to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC-MS, and identified from *m/z* of their positive ions, by comparison with standards. The results were expressed as a relative percentage of each component.

3. Results and discussion

The basidiocarps (fruiting bodies) of *P. citrinopileatus* (950 g) were reduced to 14.3% of the original weight after desiccation in a freeze dryer. The powder was submitted to one extraction with water at 10 °C, and the extracted polysaccharides were recovered as

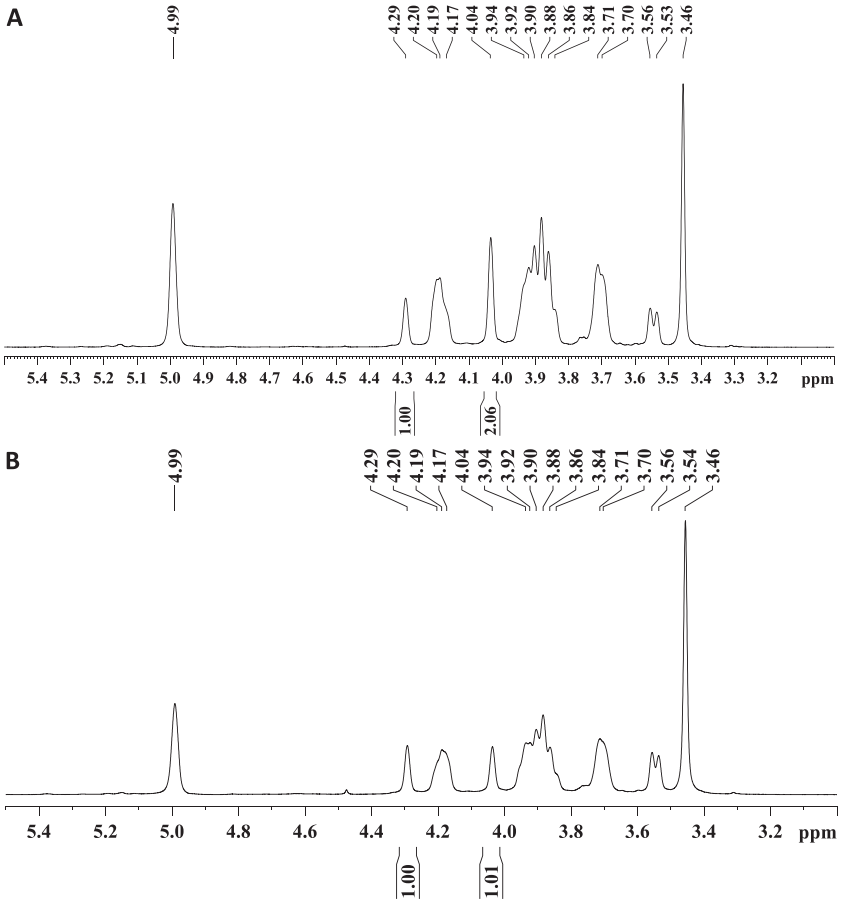

Fig. 2. ^1H NMR spectra of FP_2-*Pc* (A) and FS_2-*Pc* (B) fractions, in D_2O at 50 °C (500 MHz).

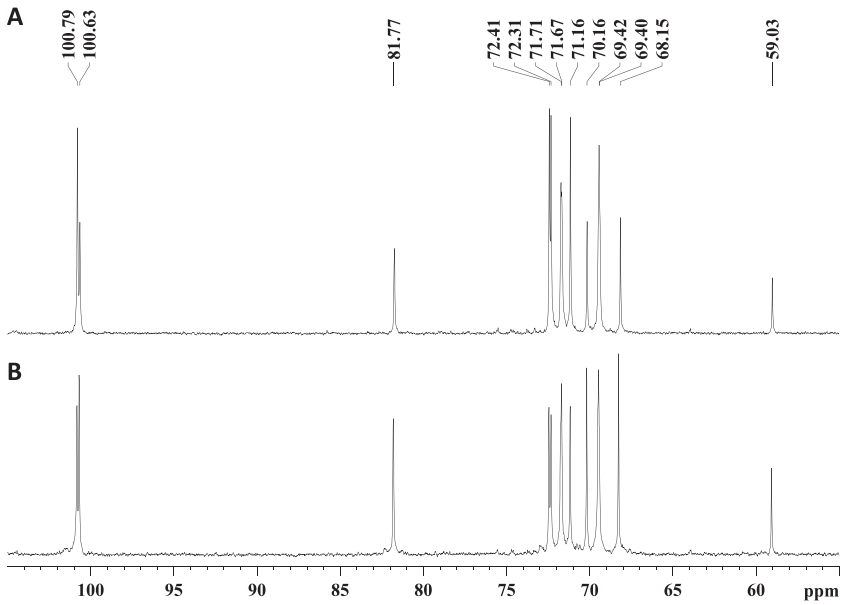

Fig. 3. ^{13}C NMR spectra of galactans (**A**: FP_2-*Pc*; **B**:FS_2-*Pc*) from *P. citrinopileatus,* in D_2O at 70 °C (125 MHz).

ethanol precipitate, dialysis against tap water, and freeze-dried (fraction CW-*Pc*, 14.7 g) (Fig. 1).

CW-*Pc* fraction showed to be composed by galactose (49.2%) as main monosaccharide, besides of mannose (15.7%), 3-*O*-Me-galactose (16.6%) [confirmed by the presence of the ions at *m/z* 130 and 190, after reduction ($NaBD_4$) and acetylation], and glucose (11.6%), according to GC-MS of derived alditol acetates.

Fractionation and purification of CW-*Pc* extract was carried out by a freeze-thawing procedure [12], which resulted in a respective cold water-soluble (SCW-*Pc*, 13.5 g) and an -insoluble (ICW-*Pc*, 1.1 g) fraction, which were separated by centrifugation. SCW-*Pc* contained galactose (47.9%), 3-*O*-methylgalactose (19.9%), mannose (24.8%), and

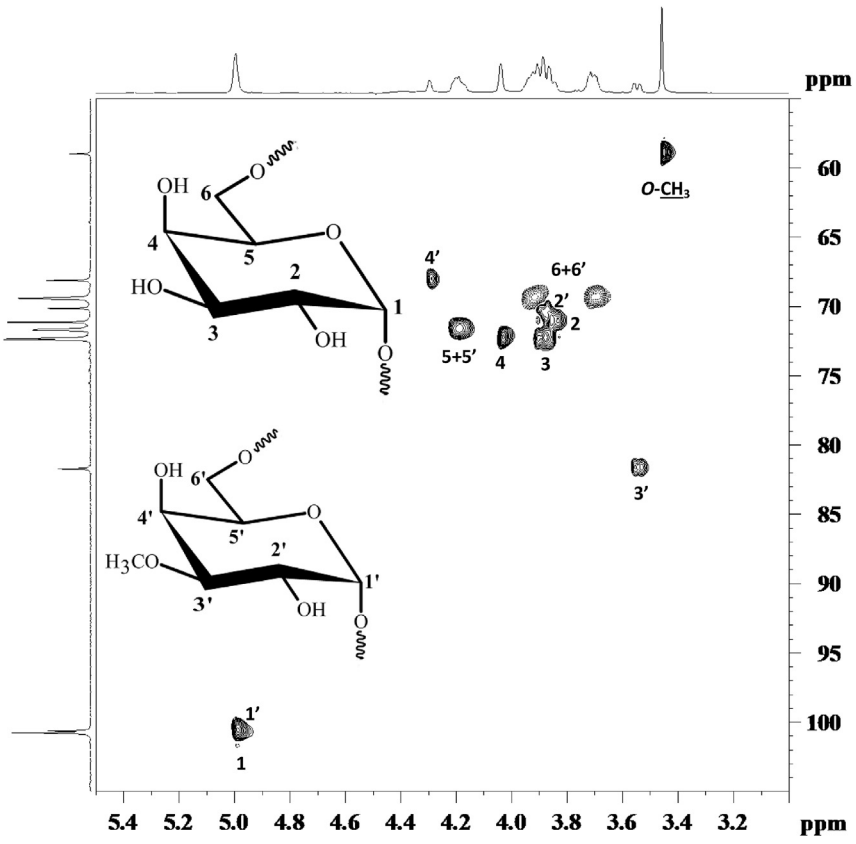

Fig. 4. HSQC-DEPT spectrum of FP_2-*Pc* fraction obtained from *P. citrinopileatus*, in D_2O at 50 °C.

Table 1
^{1}H and ^{13}C NMR chemical shifts of galactans from *P. citrinopileatus*.[a,b]

Units		1	2	3	4	5	6		-*O*-$\underline{CH_3}$
							6a	6b	
→6)-α-Gal*p*-(1→	^{13}C	100.79	71.14	72.41	72.31	71.71	69.39	-	–
	^{1}H	4.998	3.84	3.86	4.04	4.20	3.70	3.90	–
→6)-3-*O*-Me-α-Gal*p*-(1→	^{13}C	100.63	70.16	81.77	68.15	71.67	69.40	–	59.03
	^{1}H	4.986	3.89	3.56	4.29	4.18	3.71	3.92	3.46

[a] Assignments are based on ^{1}H,^{13}C, HSQC-DEPT, HSQC-TOCSY, and COSY examination.
[b] The values of chemical shifts were recorded with reference to TMS as internal standard.

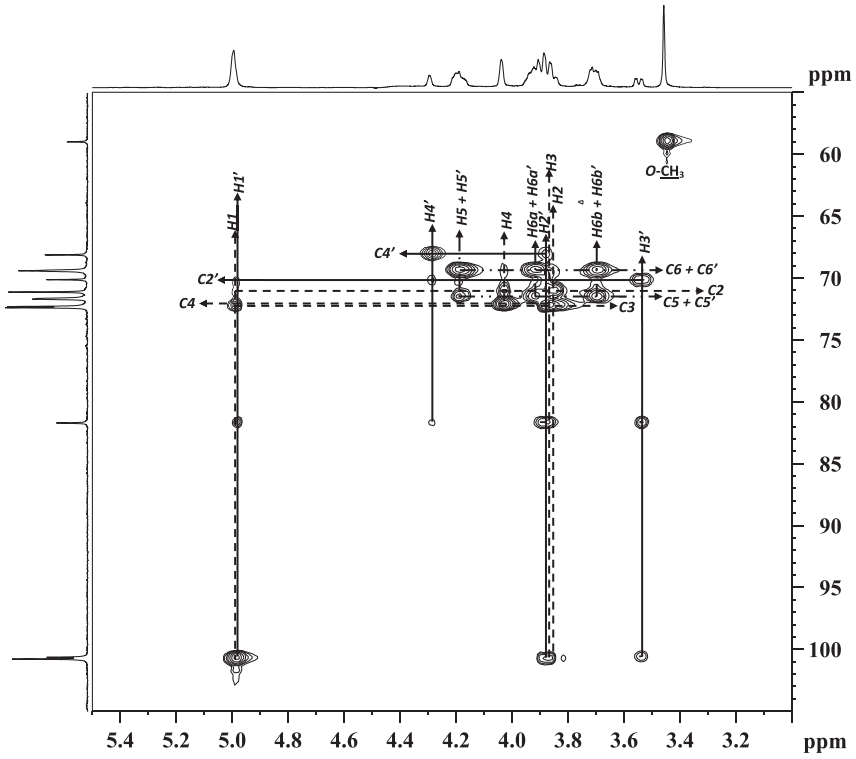

Fig. 5. HSQC-TOCSY spectrum of FP_2-*Pc* fraction obtained from *P. citrinopileatus*, in D_2O at 50 °C.

glucose (7.4%), and HPSEC–MALLS analysis showed heterogeneity. This fraction was then treated with Fehling solution two times sequentially, giving rise to a precipitate (FP_2-*Pc*; 921 mg) and a supernatant (FS_2-*Pc*; 748 mg). Part of the latter fraction becaming insoluble during treatment with ion-exchange resins (pFS-*Pc*; 2.4 g) (Fig. 1).

After treatment with Cu^{+2} solution, FP_2-*Pc* and FS_2-*Pc* fractions gave homogeneous HPSEC elution profiles, and had $M_{W=}37.6 \times 10^3$ g mol^{-1} and 28.5×10^3 g/mol ($dn/dc = 0.150$ mL g^{-1} for both fractions), respectively. The monosaccharide compositions

Table 2
The significant connectivities observed in HSQC-TOCSY spectrum of the galactans from the *P. citrinopileatus*.

Units	H/C δ_H/δ_C	*Observed cross peaks*
		δ_H/δ_C (Atom)
→6)-α-D-Gal*p*-(1→	100.79 (C1)	4.998 (H1); 3.86 (H3); 3.84 (H2)
	71.14 (C2)	4.998 (H1); 4.04 (H4); 3.86 (H3); 3.84 (H2)
	72.41 (C3)	
	72.31(C4)	
	71.71 (C5)	4.20 (H5); 3.90 (H6a); 3.70 (H6b)
	69.39 (C6)	4.20 (H5); 4.04 (H4); 3.90 (H6a); 3.70 (H6b)
→6)-3-*O*-Me-α-D-Gal*p*-(1→	100.63 (C1)	4.986 (H1); 3.89 (H2); 3.56 (H3)
	70.16 (C2)	4.986 (H1); 4.29 (H4); 4.18 (H5); 3.89 (H2); 3.56 (H3)
	81.77 (C3)	4.986 (H1); 4.29 (H4); 3.89 (H2); 3.56 (H3)
	68.15 (C4)	4.29 (H4); 3.89 (H2)
	71.67 (C5)	4.18 (H5); 3.92 (H6a); 3.71 (H6b)
	69.39 (C6)	
	59.03 (-*O*-$\underline{CH_3}$)	3.46 (-*O*-$\underline{CH_3}$)

of FP_2-*Pc* and FS_2-*Pc* fractions were similar. 3-*O*-methylgalactose (3-*O*-Me-Gal) and galactose (Gal) were present in a 1:2 and 1:1 molar ratio, respectively, suggesting the presence of partially methylated galactans. The molar ratios of 3-*O*-Me-Gal and Gal determined by GC-MS as alditol acetates were confirmed by integration area of well-resolved H-4 signals (δ 4.29 and 4.04 for 3-*O*-Me-Gal and Gal units, respectively) in ^{1}H NMR spectrum (Fig. 2).

The ^{13}C NMR spectra of the galactans from the two fractions of *P. citrinopileatus* were similar (Fig. 3A and B), with variations in the intensity of signals corresponding of the 3-*O*-methylgalactose units.

In order to elucidate the structures of the galactans, further NMR spectroscopy analyses (HSQC-DEPT, COSY, and HSQC-TOCSY) were carried out for both fractions, but due to their great similarity only the data obtained for FP_2-*Pc* fraction were illustrated. Since coupling of protons (H-1 to H-4 and H-5/H-6) of each of the units observed in COSY spectrum, the assignments of respective ^{13}C signals were established using HSQC-DEPT analysis (Fig. 4; Table 1), which were confirmed by correlations in HSQC-TOCSY spectrum (Fig. 5; Table 2).

The α-configuration was shown by high frequency H-1 signals (δ 4.998 and 4.986) and low frequency C-1 signals (δ 100.79 and 100.63), and the O-6 substitution indicated by the inversion of C-6 signals of Gal*p* (δ 69.39/3.90; 3.70), and 3-*O*-Me-Gal*p* (δ 69.40/3.92; 3.71) units, in the HSQC-DEPT spectrum (Fig. 4; Table 1).

HMBC and HSQC-NOESY experiments were carried out, but it was not possible to determine the sequence of units in this polymer due to overlapping signals.

In order to confirm the linkage type of this polymer, both fractions were submitted to methylation analysis, which showed only the alditol acetates of 2,3,4-Me_3Gal, and traces of 2,3,4,6-Me_4Gal.

In summary, the above results show that the purified polysaccharides (FP_2-*Pc* and FS_2-*Pc* fractions) consisted of linear (1 → 6)-linked α-galactopyranans partially 3-*O*-methylated, with differences in the molar mass and levels of methyl groups (Gal and 3-*O*-Me-Gal, in 2:1 and 1:1 molar ratios, respectively). Similar structures have been isolated from fruiting bodies of *Pleurotus citrinopileatus* [11], *P. eryngii*, and *P. ostreatoroseus* [17], but containing Gal and 3-*O*-Me-Gal, in a 3:1 molar ratio.

Partially 3-*O*-methylated (1 → 6)-linked α-galactopyranans have been described only for this genus, suggesting to be characteristic of the “oyster mushroom”. However, it has been observed that the amounts of 3-*O*-Me-Gal seems to be influenced probably by some factor related to cultivation, fungal strain, among others.

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