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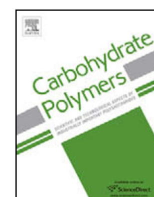

Chemical structure, antiproliferative and antioxidant activities of a cell wall α-D-mannan from yeast *Kluyveromyces marxianus*

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A B S T R A C T

Cell wall polysaccharides from filamentous fungi and yeasts have been reported as antioxidant and antiproliferative polymers. Thus, we evaluated these activities from cell wall polysaccharides from *Kluyveromyces marxianus* CCT7735. By using a centrifugal filter, a 203 kDa α-D-mannan (KMM-5) was obtained. KMM-5 exhibited no effect on HeLa cells and a weak antiproliferative activity against Hep-G2 cells. In addition, at higher concentrations, it presented a cytotoxicity to the normal cell line, 3T3. However, KMM-5 showed copper- and iron-chelating abilities, the latter of which presented improved activity. By using 2D-NMR COSY, HSQC edited and HMBC experiments, a structure arrangement was proposed. The main chain was formed by 6)-α-D-Man*p*-(1 → 6) units substituted at the 2-*O*-position by non-reducing terminals α-D-Man*p*-(1 → 2) and by a branched tetrasaccharide. The latter was formed by an internal 2)-α-D-Man*p*-(1 → 2) unit with linked to it a 2,3)-α-D-Man*p*-(1 → 2) unit substituted at the 2-*O*-position by a non-reducing terminal α-D-Man*p*-(1 → 2), and at the 3-*O*-position by a non-reducing terminal α-D-Man*p*-(1 → 3). In conclusion, we considered *K. marxianus* CCT7735 a source of natural and renewable polysaccharides with pharmacological properties.

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1. Introduction

Several yeast species commonly are grown in liquid media since a liquid medium enables the assimilation of nutrients more efficiently, which permits more-rapid cell growth compared to other microorganisms. For example, some strains of the yeast *Kluyveromyces marxianus* have the highest growth rate among eukaryotic cells (Groeneveld, Stouthamer, & Westerhoff, 2008) and tolerate temperatures up to 52 °C (Lachance, 1998). The genus *Kluyveromyces* is related to *Saccharomyces cerevisiae* (Lane & Morrissey, 2010) phylogenetically, and because they can assimilate lactose and use it for growing, they are frequently isolated from lactic environments. Added to the abovementioned characteristics, *K. marxianus* has a "Generally Recognized As Safe" (GRAS) status, which makes this yeast a natural source of bioactive molecules, attracting the interest of the scientific community for its biotechnological applications (Fonseca, Heinzle, Wittmann, & Gombert, 2008).

Yeasts are surrounded by a rigid cell wall that represents approximately 25–30% of the cell's dry weight. For example, in *Saccharomyces cerevisiae*, the cell wall has the following composition: 50–55% β-(1 → 3)-D-glucan, 5–10% β-(1 → 6)-D-glucan, 35–40% of mannoprotein complexes and 2% chitin (Kwiatkowski, 2009; Nguyen, Fleet, & Rogers, 1998). The ratio of cell wall components may vary among yeast species, growth media, pH, temperature, oxygen availability, and growth stage (Stewart & Russell, 1998). The cell wall polysaccharides extracted from yeasts have been described to possess important biological properties beyond their structural function, such as immunomodulatory (Mehdi & Hasan, 2012), antiproliferative (Salvador, Hansen, Cramer, Kong, & Jun,

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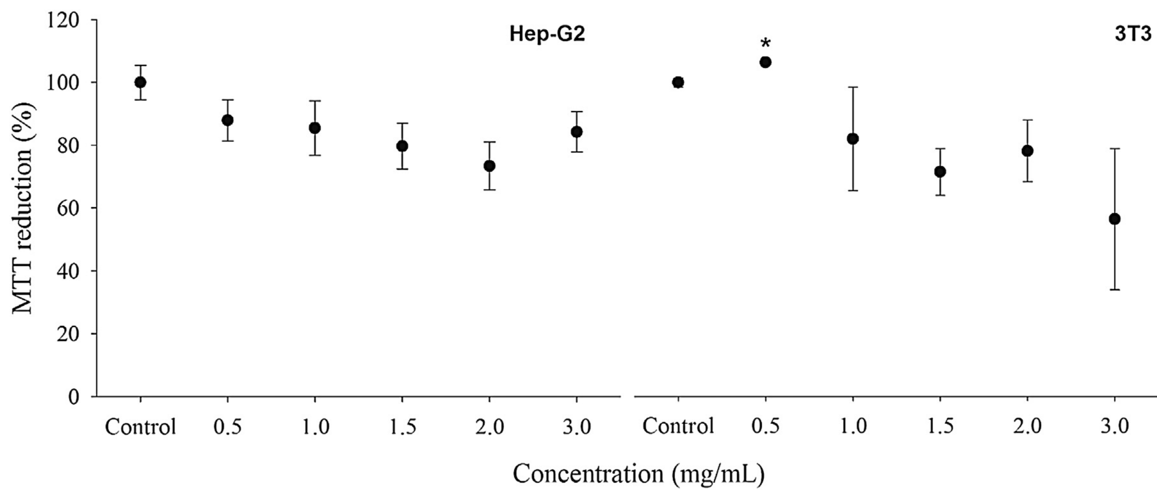

Fig. 1. Antiproliferative activity of cell wall α-mannan from *K. marxianus* CCT7735. Asterisk indicates significant differences ($p < 0.05$) between the concentrations in the respective cell line.

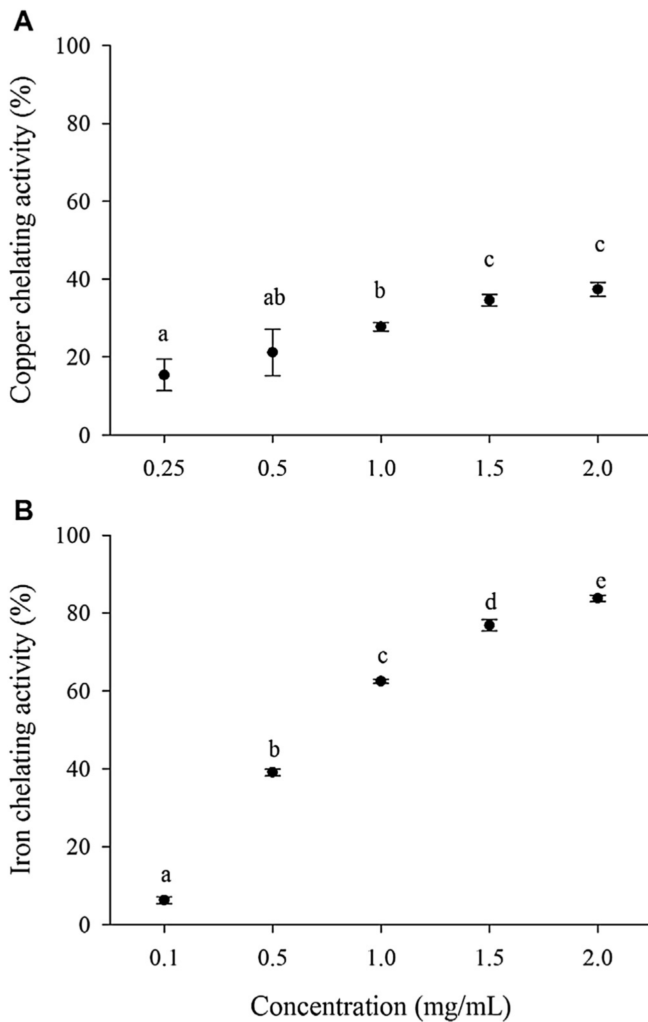

Fig. 2. (A) Copper- and (B) iron-chelating abilities of KMM-5 from *K. marxianus* CCT7735. The percentages of the respective activity are related to the polysaccharide KMM-5. Different lowercase letters indicate significant differences ($p < 0.05$) between concentrations in the same test.

2008) and antioxidant (Jaehrig, Rohn, Kroh, Fleischer, & Kurz, 2007; Machová & Bystrický, 2013).

Our research group has isolated a *K. marxianus* strain and has studied it for more than a decade for biotechnological purposes. Considering the potential pharmacological properties described for yeast polysaccharides and the intention to acquire new information about the yeast *K. marxianus* CCT7735, an α-mannan-rich fraction was obtained, and its antiproliferative activity and ability in chelating copper and iron ions was evaluated. In addition, a structural α-mannan model was suggested based on a nuclear magnetic resonance (NMR) analysis.

2. Material and methods

2.1. Materials

Monosaccharide standards (D-glucose, D-galactose, D-fructose, L-rhamnose, D-mannose, D-arabinose, L-fucose, D-xylose, D-glucuronic acid), gallic acid, L-ascorbic acid, Folin-Ciocalteu reagent and dextran standards were purchased from Sigma-Aldrich® (São Paulo, São Paulo, Brazil). Other solvents and chemicals used in this study were of analytical grade.

2.2. Microorganism and maintenance

The yeast *Kluyveromyces marxianus*, was isolated by our research group and identified by the Institute of Yeasts Identification, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (Silveira, Passos, Mantovani, & Passos, 2005). It was deposited at the André Tosello Foundation under the code, CCT7735. The culture was maintained in Erlenmeyer flasks with malt extract (2% w/v) and glucose (2% w/v) broth at 4 °C. Before each polysaccharide extraction, the culture's purity was confirmed using microscopic observation.

2.3. Chemical and structural characterization

2.3.1. Production, extraction and purification of cell wall polysaccharides

Kluyveromyces marxianus was grown in 7.5 L of malt extract (20 g/L) and glucose (20 g/L) broth in a 10-l reactor (Biostat® B. Braun, Melsungen, Germany) for 72 h at 37 °C, with stirring at 200 rpm and aeration (3 L/min). After growth, cells were harvested by centrifugation at 9,800 × *g*/10 min at 25 °C. The cell mass pel-

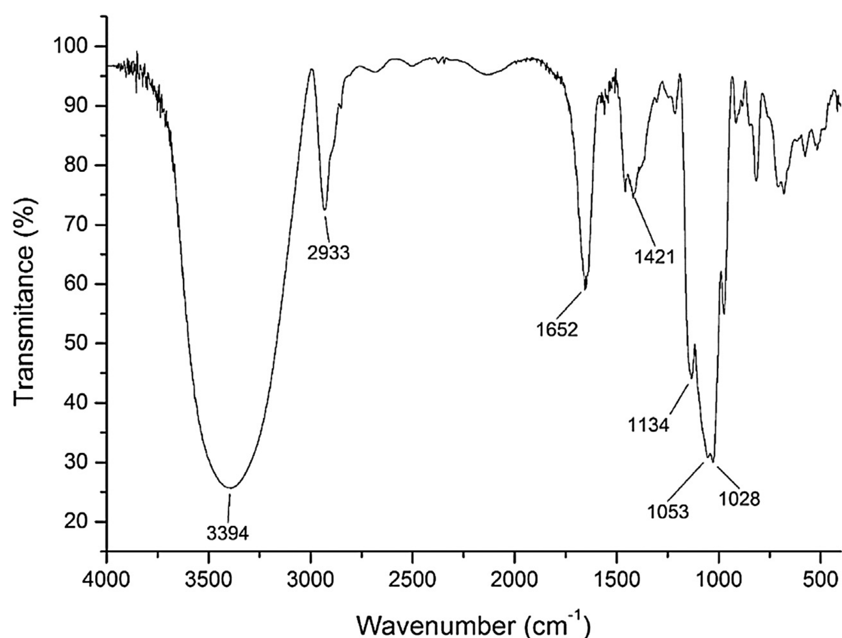

Fig. 3. Fourier transform infrared spectrum of cell wall mannan from *K. marxianus* CCT7735. Signals of chemical groups found in polysaccharides are assigned.

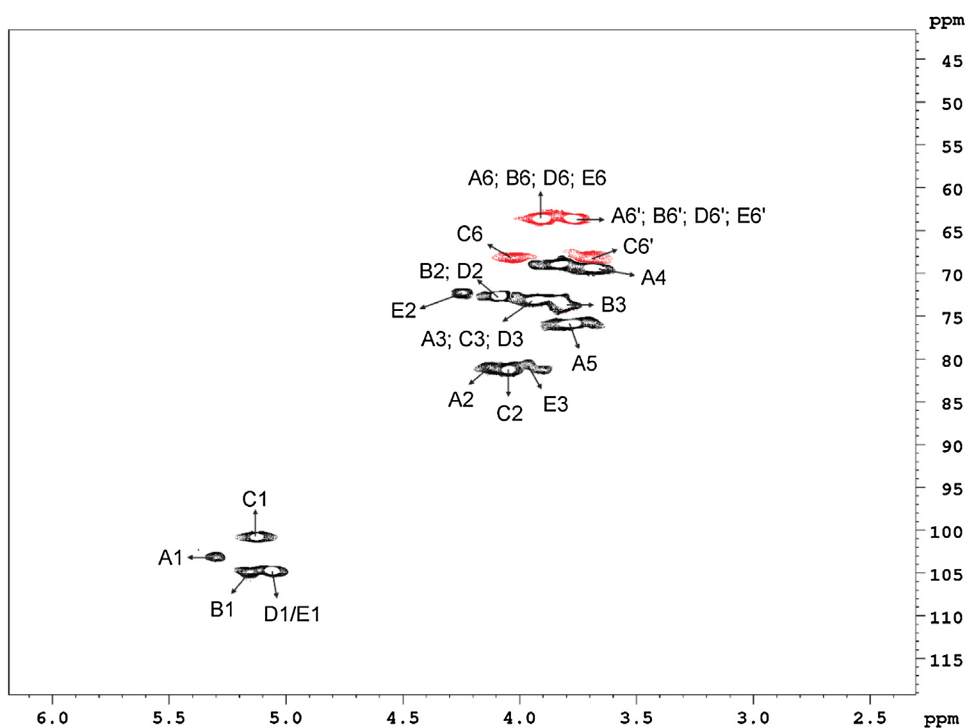

Fig. 4. HSQCed spectra of the cell wall α-mannan obtained from *K. marxianus* CCT7735 in D_2O at 30 °C. Cross peaks from H1/C1 to H6′/C6 of each mannopyranose unit are respectively numbered. Chemical shifts are expressed in ppm referred to internal standard trimethylsilyl propionic acid (0 ppm).

let was washed 5 times with distilled water and then freeze-dried. The dried cell mass was crushed to powder, and cell wall polysaccharides were extracted 3 times with 200 mL 3% NaOH at 80 °C in water bath for 6 h. The mixture was cooled at room temperature and centrifuged at 9,800 × *g*/10 min at 4 °C. The pH of the supernatant containing the water-soluble polysaccharides was neutralized with 4 M HCl, followed by addition of 2 vols of methanol in order to precipitate the polysaccharides. After 12 h, the total polysaccharides were recovered by centrifugation at 9,800 × *g*/20 min at 4 °C. The pellet was added to 200 mL of distilled water, and the residual insoluble polysaccharides were separated from water-soluble polysaccharides by centrifugation at 15,300 × *g*/30 min at 4 °C.

The water-soluble polysaccharide was submitted to centrifugation with a 100 kDa cut-off centrifugal filter (EMD Millipore, Billerica, Massachusetts, USA). The polysaccharide was retained in the top of this filter. Then, it was dialyzed (*Mw* cutoff 6 kDa) against distilled water for 5 days, followed by lyophilization. It was named KMM-5 and used in all experiments described below.

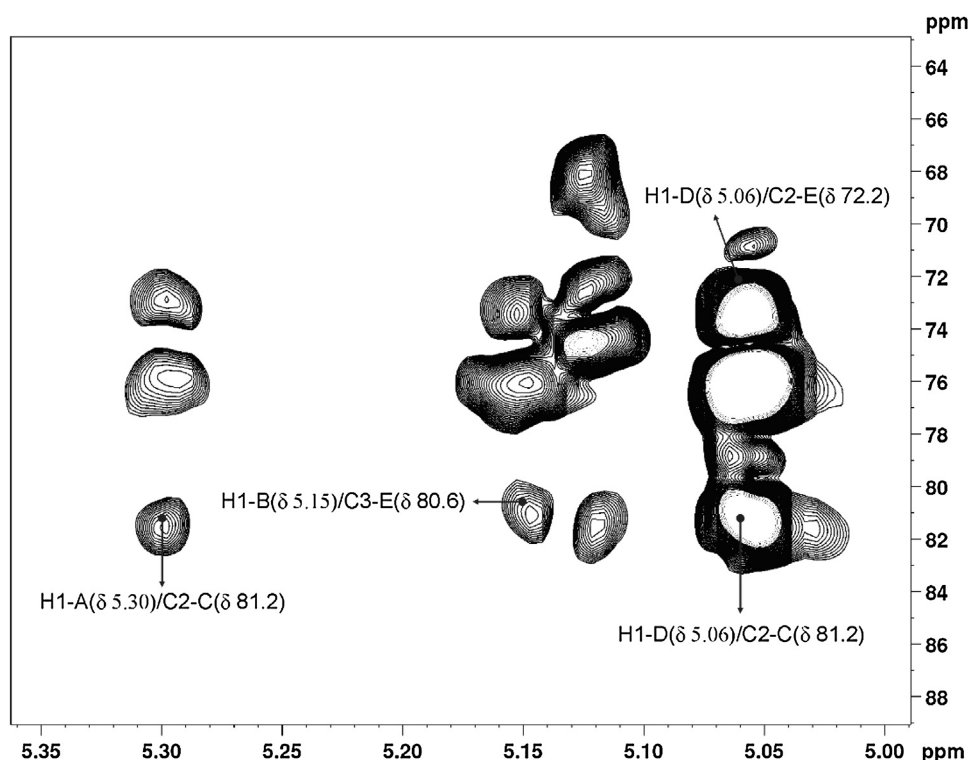

Fig. 5. HMBC spectra exhibiting the branch linkage of mannopyranose units. Signals (δ) represent the long-range correlation of H1 from an anomeric carbon of a mannose unit and the respective carbon of another mannose unit.

2.3.2. Chemical composition

Total sugars were estimated using the phenol-sulfuric acid method according to Dubois, Gilles, Hamilton, Rebers, & Smith (1956), using D-mannose as the standard. Total proteins were measured employing the Bradford (1976) method, using bovine serum albumin as the standard. Total phenolic compounds were quantified by the colorimetric technique of Folin-Ciocalteu, applying gallic acid as the standard (Costa et al., 2010).

2.3.3. Molecular-weight determination

High-pressure size-exclusion chromatography (HPSEC) was applied to determine the molecular weight (Mw) of KMM-5. Ultrahydrogel columns 500 and 250, which were 7.8 × 300 mm in size (Waters Corp., Milford, Massachusetts, USA) and connected in series, were coupled to an Accela® HPLC with a refractive index detector (Thermo Scientific, Waltham, Massachusetts, USA). The eluent was filtered (0.22 μm membrane) in pure water with 0.1 M $NaNO_2$ in a flow rate of 0.6 mL/min at 30 °C. A set of dextran standards (M_r ~6, 10, 40, 72.1, 147 and 270 kDa) was used to build the standard curve and allow us to determine the *Mw* of the polysaccharide.

2.3.4. Fourier transform infrared spectra (FT-IR)

KMM-5 was mixed with dry potassium bromide, and a pellet was prepared. The infrared spectrum was obtained on a FT-IR 8400S spectrometer (Shimadzu Corp., Kyoto, Japan).

2.3.5. Monosaccharide composition

The polysaccharide (1 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The sample was successively reduced with $NaBH_4$ (1 mg) and acetylated with 200 μL Ac_2O-pyridine (1:1, v/v) at 100 °C/30 min, as described by Sassaki et al. (2008). The alditol acetates were analyzed with a Varian Saturn 2000R-3800 gas chromatograph (Agilent, Santa Clara, California, USA) coupled to a Varian Ion-trap 2000R mass spectrometer with He as the carrier gas. The GC was equipped with a DB-225 capillary column (30 m × 0.25 mm i.d.) programmed from 50 to 220 °C at a rate of 40 °C/min. The products were identified by their specific retention times.

2.3.6. NMR spectroscopy

NMR spectra were obtained with a 600 MHz Bruker spectrometer (Avance III HD, Bruker, Billerica, Massachusetts, USA). Analyses of 2D-NMR experiments – COSY (homonuclear ^1H–^1H correlation spectroscopy), HSQCed (Heteronuclear Single Quantum Coherence edited spectroscopy) and HMBC (Heteronuclear Multiple Bond Correlation spectroscopy) – were performed at 30 °C in deuterium oxide (D_2O). Chemical shifts of the polysaccharide were expressed in δ (ppm) relative to trimethylsilyl propionic acid (TMSP). The ratio of the mannan units was calculated by integrating the area of specific anomeric signals corresponding to each cross-peak assignment on the 2D-HSQCed spectra. The structural model was designed using the ChemSketch v. 12.0 software (ACD/Labs, Toronto, Ontario, Canada).

2.4. Antiproliferative activity of KMM-5

The antiproliferative activity of KMM-5 was evaluated *in vitro* according to the method described by Mosmann (1983). Tumor-cell lines from human uterus and liver cells (HeLa ATCC CCL-2 and Hep-G2 ATCC HB-8065, respectively) and a non-tumor mouse fibroblast (3T3 ATCC CCL-92) were cultivated in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Briefly, 5×10^3 cells/well (1×10^4 for Hep-G2) were plated in a 96-well plate. After 24 h incubation (95% air, 5% CO_2, 37 °C), the medium was replaced by a DMEM without FBS, followed by incubation for another 24 h in order to stimulate cells to enter in G0 phase. The medium was replaced by a DMEM with 10% FBS added to KMM-5 at concentrations 0.5, 1.0, 1.5, 2.0 or 3.0 mg/mL. At the end of incubation period (24 h), the medium was replaced by a new DMEM

without FBS added to 1.0 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), followed by incubation for 4 h at 37 °C. The medium was removed and formazan crystals were dissolved with 100 μL of 95% ethanol. After 15 min shaking in a rocking shaker, absorbance was read (570 nm) in a microplate spectrophotometer (Biotek, Winooski, Vermont, USA). As a negative control, cells were cultivated only with DMEM with 10% FBS. Results were expressed in the percentage of MTT reduction, as in Eq. (1).

$$\text{Percentage MTT Reduction} = (\text{Absorbance of sample}/\text{Absorbance of control}) \times 100 \quad (1)$$

2.5. Copper- and iron-chelating activities of KMM-5

The copper-chelating activity of KMM-5 was determined as described by Melo et al. (2013), using the pyrocatechol violet method. Briefly, the reaction mixture contained KMM-5 (from 0.25 to 2 mg/mL), pyrocatechol violet (4 mM), and copper II sulfate pentahydrate (50 μg/mL). The solution was homogenized and the absorbance was measured at 632 nm in a microplate reader. EDTA was used as the positive control.

Iron-chelating activity of KMM-5 was determined according to Melo-Silveira et al. (2012). Briefly, the reaction mixture contained KMM-5 (from 0.1 to 2.0 mg/mL), $FeCl_2$ (2 mM) and ferrozine (5 mM). The mixture was homogenized. After 10-min incubating at 37 °C the absorbance was read (562 nm) in a microplate reader. A reaction mixture without polysaccharide was used as a blank. EDTA was used as the positive control.

2.6. Statistical analysis

The results of antiproliferative activity and copper- and iron-chelating abilities were expressed as the means ± standard deviations (n = 3) of three independent determinations. One-way analysis of variance (ANOVA) was performed by using SigmaPlot® 12.0 (Systat software, San Jose, CA, USA) and Student-Newman-Keuls, and results were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Characterization of KMM-5

Yeasts were grown in culture media composed only of malt extract and glucose, producing 3.55 g of dried cell/L of medium. The total cell wall polysaccharide extracted yielded 214 mg/g of cell. When the insoluble polysaccharides were removed by centrifugation, 164 mg water-soluble polysaccharide/g of cell were obtained. After filter centrifugation, we obtained 133 mg of KMM-5/g of cell. As far as we know, this is the first work about polysaccharides extraction of *K. marxianus* CCT7735. On the other hand, the amount collected, 214 mg/g of cell, was higher than those achieved with other *K. marxianus* strains, like as *K. marxianus* R157 (113 mg/g of cell) (Nguyen et al., 1998) and *K. marxianus* FII 510700 (200 mg/g of cell) (Lukondeh, Ashbolt, & Rogers 2003). This variability in polysaccharide amount might be related to different yeast strains, culture medium composition and growth conditions used in the mentioned studies.

KMM-5 has an Mw around 203 kDa, which was higher than the Mw described for other *Kluyveromyces* strains. Lukondeh et al. (2003) reported polysaccharides from *K. marxianus* FII 510700 with Mws ranging from 66 to 97 kDa. The lower polysaccharide Mws obtained by these authors probably are due to the high heat used in the extraction step (121 °C for 30–120 min). According to the proposition of Jeantet, Croguenec, Schuck, & Brulé (2016), higher temperatures may cause glycosidic-bonds disruption, producing small polysaccharides. As moderate temperature was used to obtain KMM-5, we suppose that this is why this fraction had a higher Mw (203 kDa). We expect that future studies will obtain polysaccharides from *K. marxianus* with Mws closer to 200 kDa.

The monosaccharide analyses revealed that KMM-5 was composed of 10% D-glucose and 90% D-mannose. These monosaccharides also were found in the mannans cell wall of *K. marxianus* R157 and *S. cerevisiae* 1117, which were made of 1% and 5% D-glucose and 95% and 93% D-mannose, respectively (Nguyen et al., 1998). Based on these data, we suggest that KMM-5 is a 203 kDa mannan.

The total content of sugar, protein and phenolic in KMM-5 were 94.22 ± 4.07%, 2.68 ± 0.12% and 0.16 ± 0.01%, respectively. The amount of protein present in KMM-5 was 23.4 and 10.6% lower than the protein content verified in cell wall polysaccharides from *K. marxianus* R157 and *S. cerevisiae* 1117, respectively (Nguyen et al., 1998). Moreover, the protein concentration in cell wall polysaccharide extracts from *K. marxianus* FII 510700 varied from 4.2 to 7% (Lukondeh et al., 2003). Both studies applied different polysaccharide extraction methods, indicating that the extraction method adopted here was more efficient in acquiring polysaccharides with low protein contamination. In addition, the amount of phenolic compound was very low. These are relevant pieces of information, since both proteins and phenolic compounds are antioxidant molecules in diverse biological systems, which could create doubts regarding whether the possible activities might be caused by the polysaccharides.

3.2. Antiproliferative activity of KMM-5

KMM-5 had no inhibitory effect on HeLa cells. On the other hand, it presented a low antiproliferative activity against Hep-G2 cells in all concentrations evaluated (Fig. 1), reaching the lowest MTT reduction (73.4 ± 7.57%) at a concentration of 2.0 mg/mL, which indicates an inhibition of about 26.6%. There was no difference in inhibition among concentrations tested ($p > 0.05$). The cytotoxicity of KMM-5 also was estimated in a normal cell line (3T3). Except at 0.5 mg/mL ($p < 0.05$), which showed a 6.4 ± 0.28% MTT reduction above the control, all other concentrations were cytotoxic for the 3T3 cell line. These results indicate that 0.5 mg/mL is the only concentration that can be used for KMM-5 antiproliferative evaluation in vivo without compromising the health of the model. People with cancer expect the development of compounds with minor or no collateral effects in comparison to synthetic drugs. Keeping this in mind, it is fundamental to search for new compounds that present antiproliferative activity, especially those derived from natural and renewable sources. In this way, polysaccharides from corn cobs (Melo-Silveira et al., 2012), filamentous fungi (Telles et al., 2011; Yang, Jin, Ren, Lu, & Meng, 2014) and yeast (Kogan et al., 2008) have shown antiproliferative activity. Despite *K. marxianus* CCT7735 having low antiproliferative activity, it is important to continue searching for different growth conditions and other yeast strains in order to obtain a variety of polysaccharides with improved antiproliferative activities.

3.3. Copper- and iron-chelating ability of KMM-5

Copper is an important mineral in human nutrition because it is necessary for the proper function of metalloenzymes, such as cytochrome c oxidase and galactose oxidase (Stern et al., 2007). However, high levels of copper in organisms caused by accidental ingestion (acute intoxication) or hereditary diseases, such as in Wilson's disease (Dong & Wu, 2012) and Menkes syndrome, can lead to mitochondria dysfunction increasing the production of ROS,

causing cell damage (Møller et al., 2012). In order to maintain the levels of copper in individuals with Wilson's disease, chelating compounds are administered and decreased intakes of foods rich in copper are recommended (Brewer, 2006).

As the administration of copper chelators is one of the treatment options for health disorders like Wilson's disease, prospection of alternative copper-chelating compounds is desirable, particularly if those compounds come from natural and renewable sources. In this fashion, the KMM-5 exhibited a copper-chelating activity (Fig. 2A), reaching the highest effect (37.34 ± 1.8%) at 2.0 mg/mL. There was no difference ($p > 0.05$) in activity between 2.0 and 1.5 mg/mL concentrations, since the latter had chelated 34.57 ± 1.46% of copper ions. To our knowledge, this is the first report that discusses yeast mannan with copper-chelating ability. This information makes us optimistic and opens precedents for new studies be carried out with other yeast species to obtain copper-chelating polysaccharides with higher activity.

The ferrous chelating effect of KMM-5 is shown in Fig. 2B. Iron is another important mineral for humans and is found complexed to proteins such as hemoglobin (involved in oxygen transport), myoglobin in muscles and heme enzymes (Kremastinos & Farmakis, 2011). In order to avoid intoxication, the concentration of iron is strongly controlled in healthy people but is relatively uncontrolled in people with hereditary hemochromatosis (De Domenico, Ward, & Kaplan, 2007). There is no excretory pathway for this metal, and its accumulation above safe levels can lead to the formation of ROS, which are the molecules primarily responsible for lipid peroxidation, and damage to other cell structures (Ayala, Muñoz, & Argüelles, 2014). Additionally, excessive iron levels may cause diabetes (Hansen, Moen, & Mandrup-Poulsen, 2014), liver cancer, and osteoporosis among other disorders (Dixon & Stockwell, 2014). To date, there is no cure for patients with iron overloads. Thus, iron levels are controlled by phlebotomy or administration of iron chelators (Hansen et al., 2014).

Polysaccharides from different natural sources have shown iron-chelating activity (Machová & Bystrický, 2013; Melo-Silveira et al., 2012; Telles et al., 2011). KMM-5 exhibited an iron-chelating activity in a concentration-dependent manner, reaching the maximum effect (84.65 ± 0.86%) at 2.0 mg/mL (Fig. 2B). The Fe^{2+} chelation of KMM-5 at 1.0 mg/mL was at least 5 times higher than that observed by Machová and Bystrický (2013) for cell wall mannans extracted from *S. cerevisiae*, *Candida dubliniensis*, *Candida tropicalis* and *Candida albicans* ser A and B. Additionally, KMM-5 presented 5 times more iron-chelating activity, with half concentration evaluated (2.0 mg/mL), than the carboxymethylated mannans from *S. cerevisiae*, *C. dubliniensis*, *C. tropicalis* and *C. albicans* ser A (Machová, Cízová, & Bystrický, 2014). Moreover, Telles et al. (2011) verified that cell wall polysaccharides from filamentous fungi *Pleurotus sajor-caju* exhibited 4 times less iron chelation at 0.5 mg/mL than KMM-5 at this concentration. Considering these results, we suggest that KMM-5 is a promising natural and renewable iron chelator for testing in in vivo models.

3.4. Determination of structural features of KMM-5

The FT-IR of KMM-5 was made in order to confirm that the sample contained a carbohydrate, and its spectra is shown in Fig. 3. Accordingly to Liu et al. (2008), some vibrations in the FT-IR analysis might indicate the features of a polysaccharide in the sample. The *K. marxianus* cell wall polysaccharide showed a strong and broad signal at 3394 cm^{-1} for O—H stretching vibrations; a signal at 2933 cm^{-1} for C—H stretching of CH_2 groups; and an intense band in the region of 900–1200 cm^{-1} corresponding to C—O, C—C stretching and C—OH bending vibrations, commonly present in sugars. Furthermore, the band in 1652 cm^{-1} are related to C=O, and the band in 1421 cm^{-1} correspond to the absorbance of the CH_2 functional group (Silverstein, Webster, & Kiemle, 2005). We did not found signals indicating the presence of amines, which could correspond to the low protein contamination of KMM-5.

Using a 2D-NMR COSY experiment, the hydrogen–hydrogen correlations in KMM-5 were obtained, and by applying a 2D-NMR HSQCed experiment the directly bonded $^{1}H/^{13}C$ interactions in KMM-5 were assigned (Table 1). Five mannopyranose forms (named A to E units) have been identified. These mannopyranose units were confirmed to have glycosidic bonds in the α-configuration, by comparing the anomeric carbons and their respective hydrogen signals to those reported on literature (Kobayashi et al., 1997; Kobayashi, Kawakami, Ogawa, Shibata, & Suzuki, 2013; Shibata, Suzuki, Kobayashi, & Okawa, 2007; Zarnowski et al., 2014). No signals corresponding to glucose units were identified. Most likely, these glucoses are randomly distributed throughout the mannan structure or they are from a contaminant polysaccharide.

The molar ratio of each mannopyranose form (A:B:C:D:E – 2:2:5:5:2) was obtained by integrating the anomeric area of the cross peaks in HSQCed (Fig. 4). The *K. marxianus* CCT7735 cell wall α-D-mannan is a highly branched structure, with predominant non-reducing terminal α-D-(1 → 2)-linked mannopyranose (Unit D). This type of residue was present in other yeast cell wall polysaccharides, such as *Candida catenulata* (Kobayashi et al., 1997) and *C. albicans* (Shibata et al., 2007), and in a biofilm from *C. albicans* (Zarnowski et al., 2014). These cell wall and biofilm polysaccharides also were reported to be branched structures, as seen in our study. Moreover, non-reducing terminal α-D-(1 → 3)-linked mannopyranose (Unit B) with H1/H2 signals (δ 5.15/4.09) were recorded. Similar residues were found in cell wall mannans from other yeast species, such as *C. catenulata* (Kobayashi et al., 1997), *Candida zeylanoides* (Kobayashi et al., 2013) and *C. albicans* (Shibata et al., 2007). These two α-D-mannopyranose non-reducing terminal units (B and D) differed only in anomeric $^{1}H/^{13}C$ cross peaks, indicating that these units were linked in different positions in the α-mannan backbone.

The major polysaccharide was consistent with a central core of α-D-(1 → 6)-linked mannopyranose units (Unit C), which had a substitution in the 2-*O*-position, as verified in other fungal mannan cell walls (Ahrazem et al., 2003; Kobayashi et al., 2013) and in mannans from yeast biofilm (Zarnowski et al., 2014). The 2-*O*-substitution was confirmed by an HMBC experiment (Fig. 5) in which H1 from the anomeric carbon of unit D had a correlation to carbon 2 from unit C (H1-D: δ 5.06/C2-C: δ 81.2). Furthermore, internal α-D-(1 → 2)-linked mannopyranose units (Unit A) were identified in the side chain. The HMBC experiment confirmed that these units were linked at the 2-*O*-position in the main backbone, since H1 from its anomeric carbon presented correlation to carbon 2 of unit C (H1-A: δ 5.30/C2-C: δ 81.2).

The HSQCed spectra exhibited only 4 anomeric signals in KMM-5, with 2 well-defined non-reducing terminal α-D-(1 → 2)- (Unit D) and α-D-(1 → 3)-linked mannopyranose (Unit B). However, unit B required a linkage point (i.e., a 3-*O*-substituted unit in an equivalent molar ratio). Therewith, by analysis of the COSY spectra, we determined that there was an overlap of H1 signals (δ 5.06) from the anomeric of Unit D and another unit with an H2-correlated signal at δ 4.23. Comparing the signals of H1/H2 (δ 5.06/4.23) to those described in the literature, a 5th mannopyranose unit (Unit E) was confirmed to be an 3)-α-D-Man*p*-(1 → 2) unit. This type of residue also was observed in *C. zeylanoides* (Kobayashi et al., 2013) and *C. albicans* mannan cell walls (Shibata et al., 2007). Moreover, considering the molar ratios of mannose units obtained by integrating the area of the cross peaks in HSQCed, two other linkage points must be present in the mannan structure to link Unit D. In this way, the HMBC experiment confirmed that unit E had a branching point with a 2-*O*-substitution where unit D was linked (H1-D: δ 5.06/C2-

Table 1
Assignment of hydrogen and carbon chemical shifts based on 2D-NMR experiments (COSY and HSQCed) for cell wall α-mannan from *K. marxianus* CCT7735.

Unit		Molar Ratio	Chemical shifts (ppm)[a]						Ref.[b]
			H1	H2	H3	H4	H5	H6/H6′	
			C1	C2	C3	C4	C5	C6	
A	2)-α-D-Manp-(1→2)	2	5.3	4.13	3.93	3.69	3.77	3.90/3.77	
			103.9	81	73.1	69.2	75.8	63.6	
B	α-D-Manp-(1→3) (NRT)	2	5.15	4.09	3.82			3.90/3.77	
			104.8	72.6	73.1			63.6	
C	6)-α-D-Manp(1→6)	5	5.12	4.05	3.93			4.03/3.71	
	2↑		100.7	81.2	73.1			68.1	
D	α-D-Manp-(1→2) (NRT)	5	5.06	4.09	3.82			3.90/3.77	
			104.7	72.6	73.1			63.6	
E	3)-α-D-Manp-(1→2)	2	5.06	4.23	3.96			3.90/3.77	
	2↑		104.7	72.2	80.6			63.6	
	2)-α-D-Manp-(1→2)		5.25	4.1	3.94	3.72	3.74	3.89/3.77	−1
			103.6	81.3	72.6	69.6	75.7	63.5	
	α-D-Manp-(1→3) (NRT)		5.14	4.06					(2, 3)
			5.14	4.07					−4
	6)-α-D-Manp(1→6)		5.07	3.99	3.93	3.79	3.7		−1
	2↑		101.1	81.2	72.6	69.6	75.7		
	α-D-Manp-(1→2) (NRT)		5.04	4.05	3.82	3.73			−1
			104.6	72.6	73.6	69.6			
			5.05	4.06					(3, 4)
	3)-α-D-Manp-(1→2)		5.04	4.21					(2, 4)

(NRT): non-reducing terminal.
[a] Chemical shifts are referred to internal standard trimethylsilyl propionic acid (0 ppm).
[b] Signals reported in literature: (1) Zarnowski et al. (2014); (2) Kobayashi et al. (2013); (3) Kobayashi et al. (1997); (4) Shibata et al. (2007).

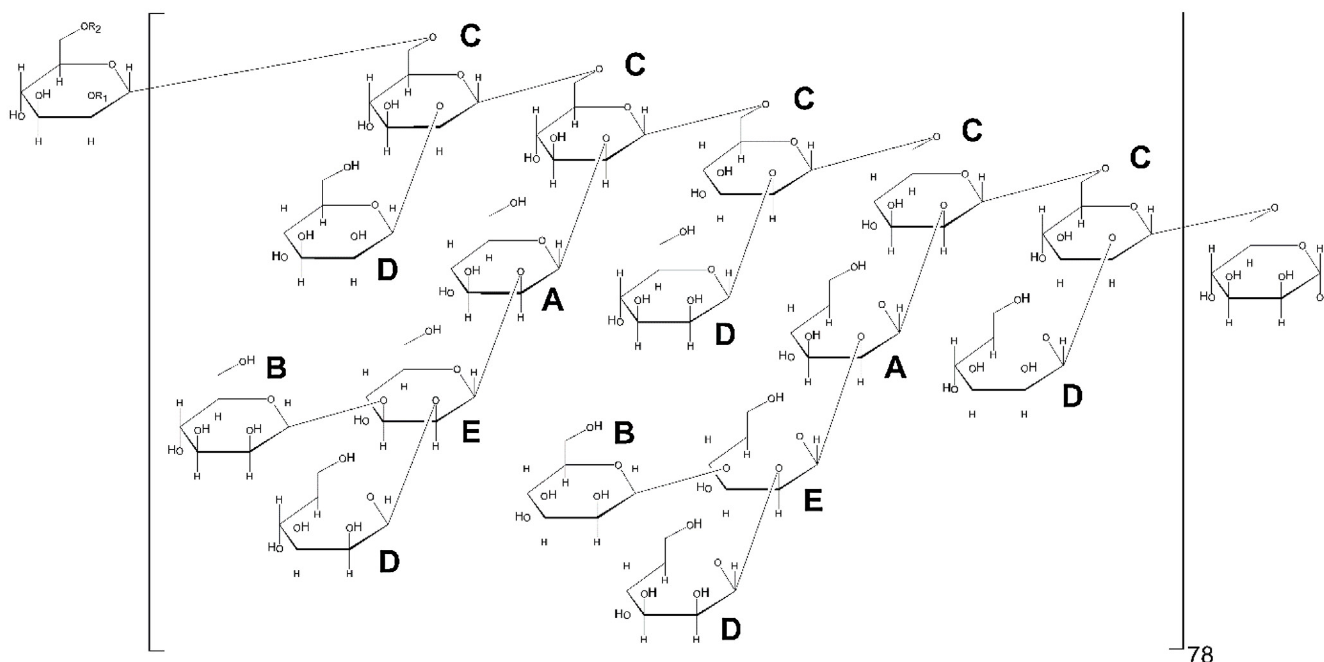

Fig. 6. The possible fragment structure of the cell wall α-mannan from *K. marxianus* CCT7735. A: 2)-α-D-Man*p*-(1 → 2); B: α-D-Man*p*-(1 → 3) non-reducing terminal; C: 2,6)-α-D-Man*p*-(1 → 6); D: α-D-Man*p*-(1 → 2) non-reducing terminal; E: 2,3)-α-D-Man*p*-(1 → 2). Each radical (R1 and/or R2) indicated in the non-reducing terminal outside the brackets might place a hydrogen atom or a mannopyranose unit.

E: δ 72.2). Faced with NMR spectral information obtained about the constituent units from KMM-5 α-mannan, a possible structure arrangement was proposed (Fig. 6).

4. Conclusions

The KMM-5 α-mannan cell wall from yeast *K. marxianus* is a highly branched structure consisting of 5 α-D-mannopyranose units, with the main chain formed by 6)-α-D-Man*p*-(1 → 6) units substituted in the 2-*O*-position by a non-reducing terminal α-D-Man*p*-(1 → 2) or by a branched tetrasaccharide. This polysaccharide had no cytotoxicity against HeLa cells and presented low antiproliferative activity against the Hep-G2 cell line. Additionally, it was not cytotoxic to normal cells but only at low concentrations, indicating this high-Mw polysaccharide is not suitable for cancer treatment. The major feature of this 203 kDa α-mannan was the antioxidant ability by chelating copper and iron ions, the latter being the best activity. Since *K. marxianus* α-mannan proved to be a promising iron chelator, in the future, we intend to explore this polysaccharide as an iron chelator in *in vivo* models.

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