Carbohydrate Polymers 85 (2011) 514–521

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Sulfation of the extracellular polysaccharide produced by the edible mushroom *Pleurotus sajor-caju* alters its antioxidant, anticoagulant and antiproliferative properties *in vitro*

Cinthia B.S. Telles[a], Diego A. Sabry[a], Jailma Almeida-Lima[a], Mariana S.S.P. Costa[a], Raniere F. Melo-Silveira[a], Edvaldo S. Trindade[b], Guilherme L. Sassaki[c], Elisabeth Wisbeck[d], Sandra A. Furlan[d], Edda L. Leite[a], Hugo A.O. Rocha[a,*]

[a] *Laboratório de Biotecnologia de Polímeros Naturais-BIOPOL, Departamento de Bioquímica, Universidade Federal do Rio Grande do Norte - UFRN, Natal, RN, Brazil*
[b] *Departamento de Biologia Celular, Universidade Federal do Paraná – UFPR, Curitiba, PR, Brazil*
[c] *Departamento de Bioquímica, Universidade Federal do Paraná – UFPR, Curitiba, PR, Brazil*
[d] *Universidade da Região de Joinville–UNIVILLE, Joinville, SC, Brazil*

ARTICLE INFO

Article history:
Received 25 October 2010
Received in revised form 21 February 2011
Accepted 22 February 2011
Available online 1 March 2011

Keywords:
Mannogalactoglucan
Sulfated polysaccharide
Biological activities
HeLa cells

ABSTRACT

An extracellular polysaccharide (PN) composed of mannose:3-O-methyl-galactose:galactose:glucose (44.9:16.3:19.8:19) was obtained from *Pleurotus sajor-caju*. This polymer showed specific antioxidant activities such as total antioxidant capacity, superoxide radical scavenging, reducing power and ferric chelating, but it did not possess anticoagulant or antiproliferative activities. Through chemical modification of PN, the hydroxyl radical scavenging and reducing power activities of PN increased. A derived sulfated polysaccharide of PN, with a DS of 0.17 and named PS, was obtained using a sulfuric acid/n-propanol method. The sulfation was confirmed by electrophoresis, NMR and FT-IR. In addition, PS prolonged the time of coagulation in a concentration-dependent manner in aPTT test. The antiproliferative effect against HeLa cells was time dependent; after 72 h, the inhibition rate of PS (1.0 mg/mL) was approximately 60%. PS demonstrated promising antioxidant, antiproliferative and anticoagulant properties and has been selected for further studies.

1. Introduction

In recent decades, much attention has been focused on the biological properties of polysaccharides and their chemical derivatives, especially sulfated derivatives (Yang, Gao, Han, & Tan, 2005). Sulfated polysaccharides have a broad range of important bioactivities, including anticoagulant, antioxidant, antiproliferative, antitumor, anti-complement, anti-inflammatory, antiviral, antipeptic and antiadhesive activities (Costa et al., 2010; Damonte, Matulewicz, & Cerezo, 2004; Rocha et al., 2005). Water-insoluble polysaccharides show little bioactivity, whereas sulfated derivatives exhibit higher water solubility along with enhanced bioactivities (Wang, Zhang, Li, Hou, & Zeng, 2004).

Edible mushrooms have been consumed to maintain health and promote longevity (Manzi & Pizzoferrato, 2000). Mushrooms which have demonstrated medicinal or functional properties include species of the genera *Lentinus*, *Hericium*, *Grifola*, *Flammulina*, *Pleurotus* and *Tremella* (Kues & Liu, 2000). *Pleurotus* is an important genus of edible basidiomycetes and it is a good source of molecules including polysaccharides that can act as antiproliferative, antifungal, antioxidant and antiviral agents (Croan, 2004; Kashangura, Hallsworth, & Mswaka, 2006).

Besides studies of solid culture for production of fruiting bodies, the submerged culture of the *Pleurotus* genus has also been studied for the production of several molecules, including polysaccharides. Polysaccharide polymers represent the major constituent that determines the rigidity and morphological properties of the fungal cell wall. Depending on the culture conditions, they can be secreted into the culture medium. Thus, these polysaccharides are usually classified as extracellular polysaccharides (Sutherland, 1998).

The species *Pleurotus sajor-caju*, known in Japan as “Houbitake”, is a mushroom originally found in India, where it grows naturally on a succulent plant (*Euphorbia royleans*) in the foothills of the Himalayas (Pramanik, Chakraborty, Mondal, & Islam, 2007). Polysaccharides from the fruiting body of *P. sajor-caju* have been shown to possess antineoplastic and antimicrobial activities

* Corresponding author at: Universidade Federal do Rio Grande do Norte, Centro de Biociências, Departamento de Bioquímica, Laboratório de Biotecnologia de Polímeros Naturais-BIOPOL, Av. Salgado Filho S/N, Natal, RN, Brazil.
Tel.: +55 84 32119208; fax: +55 84 32119208.
E-mail address: hugo@cb.ufrn.br (H.A.O. Rocha).

0144-8617
doi:10.1016/j.carbpol.2011.02.038

(Zhuang et al., 1993). However, the biological activities, such as antioxidant, anticoagulant and antiproliferative activities, of the extracellular polysaccharides from this species have not been studied.

For these reasons, we prepared the sulfated derivative of the extracellular polysaccharide isolated from the edible mushroom *P. sajor-caju* and tested its potential antioxidant, anticoagulant and antiproliferative activities *in vitro*.

2. Materials and methods

2.1. Materials

Potassium ferricyanide, ferrous sulfate (II), n-propanol, acetic acid, ethanol and sulfuric acid were obtained from Merck (Darmstadt, Germany). 1,3-Diaminopropane and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Clexane (enoxaparin) was purchased from Sanofi-Aventis Farmacêutica Co. (São Paulo, Brazil). Agarose low-MR was purchased from Bio-Rad (Richmond, CA). HeLa cells (ATCC CCL-2) were a gift from Prof. Silvia R. B. Medeiros, Department of Genetic and Cell Biology, UFRN. Cell culture media components [minimum essential medium (MEM), L-glutamine, sodium bicarbonate, non-essential amino acids, sodium pyruvate, fetal bovine serum (FBS) and phosphate buffered saline (PBS)] were purchased from Invitrogen Corporation (Burlington, ON). All other solvents and chemicals were of analytical grade.

2.2. Microorganism and maintenance

P. sajor-caju was obtained from the "Centro de Cultivo de Basidiomicetos da Universidade de São Paulo" under the code CCB 019. The culture was maintained in Petri dishes containing WDA medium (1 L wheat extract, 20.0 g dextrose and 15.0 g agar) at 4 °C.

2.3. Production of extracellular polysaccharide

After 14 days of growth, the *P. sajor-caju* culture was submerged in POL medium (5.0 g L^{-1} $(NH_4)_2SO_4$; 0.2 g L^{-1} $MgSO_4{\cdot}7H_2O$; 1.0 g L^{-1} K_2HPO_4; 2.0 g L^{-1} yeast extract; 1.0 g L^{-1} peptone and 20 g L^{-1} glucose at pH 6.5–7.0) in Erlenmeyer flasks at 30 °C for 120 min^{-1}. The culture broth was separated from the biomass and treated with cool acetone at 8 °C using a ratio of acetone/culture broth of 3:1. After 24 h of refrigeration at 4 °C for extracellular polysaccharide precipitation, the mixture was centrifuged at 4500 × *g* for 5 min and washed twice with an acetone/ethanol/distilled water (3:1:1) solution (Wolff et al., 2008). The extract obtained was lyophilized and used in tests.

2.4. Preparation of sulfated extracellular polysaccharide

Sulfation of the extracellular polysaccharide from *P. sajor-caju* was performed using a heterogeneous reaction system composed of sulfuric acid and n-propanol at a molar ratio of 1:4, according to Wang, Yao, Guana, Wu, and Kennedy (2005), in which polysaccharides are sulfated with a reaction solution of sulfuric acid/n-propanol. N-propanol controls the concentration of sulfuric acid to prevent adverse reactions related to a high concentration of sulfuric acid, such as hydrolysis and carbonization. The product obtained by sulfation was referred to as PS. To evaluate the effectiveness of the sulfation procedure, the sulfate content of polysaccharides (native – PN and chemically sulfated – PS) was determined after acid hydrolysis (4 N HCl, 100 °C, 6 h) as described previously (Dodgson & Price, 1962). The degree of substitution (DS), which is the average number of sulfate groups on each sugar residue, was calculated from the sulfur content using the following formula (Whistler & Spencer, 1964), in which S = % sulfur:

$$S(\%) = \frac{SO_4\,(\mu g) \times 0.1374 \times 100}{\text{sample}\,(\mu g)}$$

$$DS = \frac{162 \times S}{3200 - 102 \times S}$$

Confirmation of the attachment of sulfate groups to the extracellular polysaccharide was shown by electrophoretic analysis on 0.6% agarose gels in 0.05 M 1,3-diaminopropane acetate buffer (PDA) (Dietrich & Dietrich, 1976) as well as by infrared spectroscopy and NMR.

2.5. Chemical analyses

Total sugars were estimated with the phenol–H_2SO_4 reaction (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using D-mannose as a standard. Protein was measured with the Bradford method (Spector, 1978) using bovine serum albumin as a standard. Total phenolic compounds were determined using the colorimetric method of Folin–Ciocalteu, using gallic acid as a standard (Athukorala, Kim, & Jeon, 2006).

2.6. Structural analysis

2.6.1. Monosaccharide composition

Each fraction (2 mg) was hydrolyzed in 2 M trifluoroacetic acid at 100 °C for 8 h, followed evaporation to dryness. The residue was successively reduced with excess $NaBH_4$ and acetylated with Ac_2O–pyridine (1:1, v/v; 2 mL) at room temperature for 12 h (Wolfrom & Thompson, 1963a, 1963b). The resulting alditol acetates were analyzed by GC–MS as described above and identified by their typical retention times and electron impact profiles (Jansson, Kenne, Liedgren, Lindberg, & Lönngren, 1976).

2.6.2. Agarose gel electrophoresis

Agarose gel electrophoresis of the polysaccharides was performed with 0.6% agarose gels (7.5 cm × 10 cm × 0.2 cm thick) prepared in 0.05 M 1,3-diaminopropane acetate buffer, pH 9.0, as previously described (Dietrich & Dietrich, 1976). Polysaccharide aliquots (about 50 μg) were applied to the gel and subjected to electrophoresis. The gel was fixed with 0.1% cetyltrimethylammonium bromide solution for 2 h, dried and stained for 15 min with 0.1% toluidine blue in 1% acetic acid in 50% ethanol. The gel was then destained with a solution of 1% acetic acid in 50% ethanol.

2.6.3. Fourier transform infrared spectra (FT-IR)

The polysaccharides (PN and PS) were mixed with dry potassium bromide. A pellet was prepared and the infrared spectrum was measured on a Thermo Nicolet Nexus spectrometer instrument.

2.6.4. NMR analyses

^{13}C NMR spectroscopy analyses were carried out using a 400 MHz Bruker model DRX Avance spectrometer that incorporated Fourier transform. Samples were dissolved in D_2O and examined at 70 °C. Chemical shifts are expressed in ppm (δ) relative to the resonance of acetone at δ 30.20 (^{13}C) in D_2O.

2.7. Biological activities

2.7.1. Antioxidant activity

2.7.1.1. Determination of total antioxidant capacity. The assay for total antioxidant capacity is based on the reduction of Mo (VI) to Mo (V) by the sulfated polysaccharide and subsequent formation of a

Table 1
Chemical contents and monosaccharide composition of PN and PS polysaccharides of the edible mushroom *Pleurotus sajor-caju*.

Polysaccharides	Sugar content (%)	Protein content (%)	Fenolic compounds (%)	Sulfate content (%)	Monosaccharide composition (%)[a]			
					Mannose	3-O-methyl-galactose[b]	Galactose	Glucose
PN	22.6	5.0	0.4	0.0	44.9	16.3	19.8	19.0
PS	33.4	0.0	0.8	22.5	49.7	14.4	17.7	18.2

[a] Analyzed by GC–MS, after total acid hydrolysis, reduction with $Na_2B^2H_4$ and acetylation.
[b] Confirmed by GC–MS ions at *m*/*z* 130 and 190 after reduction with $Na_2B^2H_4$ and acetylation.

green phosphate/Mo (V) complex at acidic pH (Smirnoff & Cumbes, 1989).

2.7.1.2. Hydroxyl radical scavenging activity assay. The hydroxyl radical scavenging activity of seaweed polysaccharides (from 0.01 to 0.5 mg/mL) was investigated using Fenton's reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet$). These results were expressed as the rate of inhibition. Hydroxyl radicals were generated using a method described previously (Dasguptaa & De, 2007).

2.7.1.3. Superoxide radical scavenging activity assay. The superoxide radical scavenging assay was based on the capacity of polysaccharides (from 0.01 to 0.5 mg/mL) to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in the riboflavin-light-NBT system.

2.7.1.4. Ferric chelating. The ferrous ion chelating ability of the samples (from 0.1 to 2.0 mg/mL) was investigated according to Wang, Zhang, Zhang, and Li (2008).

2.7.1.5. Reducing power. The reducing power of the samples (from 0.2 to 1.0 mg/mL) was quantified as previously described (Wang et al., 2008).

2.7.2. Anticoagulant activity

The prothrombin time (PT) and activated partial thromboplastin time (aPTT) coagulation assays were performed with a coagulometer and measured using citrate-treated normal human plasma (Albuquerque et al., 2004). All assays were performed in duplicate and repeated at least three times on different days ($n = 6$).

2.7.3. Antiproliferative activity

HeLa cells were grown in 75 cm^2 flasks in DMEM medium. Cells were seeded into 96-well plates at a density of 5×10^3 cell/well and allowed to attach overnight in 100 μL of medium at 37 °C and 5% CO_2. The polysaccharides PN and PS were added at final concentrations of 0.5, 1.0, 1.5 and 2.0 mg/mL for 24, 48 and 72 h at 37 °C and 5% CO_2. At the end of the incubation period, cells were incubated with 100 μL of a MTT solution (1 mg/mL) for 4 h at 37 °C and 5% CO_2. To solubilize the product of MTT cleavage, 100 μL of isopropanol con-

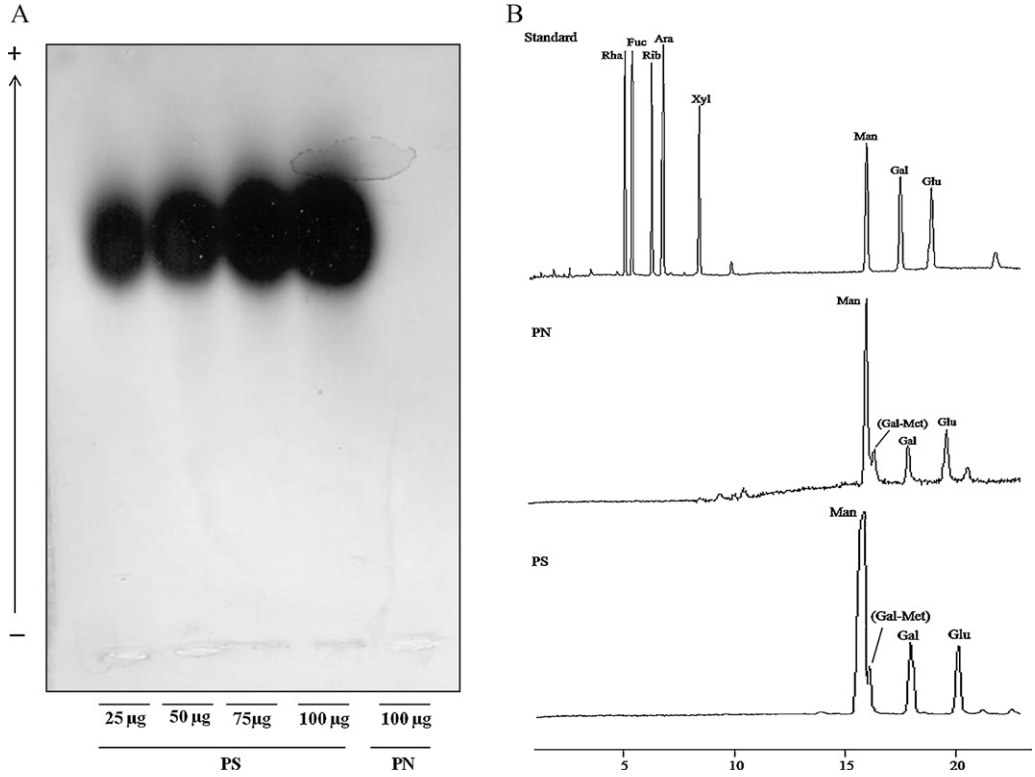

Fig. 1. Electrophoresis of polysaccharides from edible mushroom *Pleurotus sajor-caju* (A). GC–MS of alditol acetates of PN and PS polysaccharides and sugar standards (B). Sugar standards: Rha, rhaminose; Fuc, fucose; Rib, ribose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Gal-Met, 3-O-methyl-galactose; Glu, glucose. PN, native polysaccharide; PS, sulfated polysaccharide.

taining 0.04 N HCl was added to each well and thoroughly mixed using a multichannel pipettor. Within 1 h of HCl–isopropanol addition, the absorbance at 570 nm was read using a Multiskan Ascent Microplate Reader (Thermo Labsystems, Franklin, MA). The percent inhibition of cell proliferation was calculated as follows:

$$\%\,\text{inhibition} = \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \times 100$$

2.8. Statistical analysis

All data are expressed as means ± standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA). Student–Newmans–Keuls post-tests were performed for multiple group comparison. In all cases, statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Physicochemical properties and chemical analysis of PN and PS

The polysaccharide extracted from *P. sajor-caju* (PN) was sulfated as described in Section 2, which was then referred to as PS. The chemical compositions of all samples are given in Table 1. Relative to PS, the total sugars and phenolic compounds increased after modification, whereas protein was not detected. After sulfation, the sulfate content in PS was about 22%, indicating that the degree of substitution (DS) was 0.17.

To confirm that the sulfate groups were really linked to the polymer, PS and PN were submitted to agarose gel electrophoresis (Fig. 1A). Diamine forms different complexes with sulfated polysaccharides due to the spacing between the negative charges, which is caused mainly by sulfation substitutions in a specific sugar residue conformation. Thus, the diamine separates compounds with different sulfate group distributions along their chain, even if they have the same DS and/or the same sulfate content as well as the same proportion and sequence of the sugar residues (Dietrich & Dietrich, 1976). The presence of a purple band stained by toluidine blue confirms that PS is a sulfated polymer. In addition, even using a high amount of PS (100 μg), only a single and homogeneous component was obtained, which indicates that we obtained a unique sulfated polysaccharide population.

In comparison with PN, two characteristic absorption bands appeared in the FT-IR spectrum of PS (data not shown), one at 1232 cm^{-1} for an asymmetric S=O stretching vibration and the other at 822 cm^{-1} for a symmetric C–O–S vibration associated with a C–O–SO_3 group, which are consistent with incorporation of a sulfate group. These results indicated that the sulfation reaction had occurred.

Fig. 1B shows the GC–MS analysis of the native and chemically sulfated polysaccharides from *P. sajor-caju*. The chromatogram indicates that the main monosaccharide components of both polymers were mannose, 3-O-methyl-galactose, galactose and glucose. The monosaccharide percentages are shown in Table 1; mannose was the major monosaccharide in the structures of PS and PN, followed by glucose, galactose and 3-O-methyl-galactose.

These data agree with the monosaccharide composition of extracellular polysaccharides synthesized by several species of *Pleurotus* (Gutiérrez, Prieto, & Martínez, 1996; Pramanik, Mondal, Chakraborty, Rout, & Islam, 2005). However, in *P. sajor-caju*, a different proportion of man:gal:glu was found previously (31:12:30). In addition, Gutiérrez et al. (1996) showed a polysaccharide contained a high amount of protein (about 27%), and the presence of methyl-galactose was not shown. These differences in the proportions of monosaccharides are probably due to several factors, including the method of polysaccharide extraction and purification. We found a similar situation when we compared data from the monosaccharide composition of polysaccharides extracted from *P. sajor-caju* fruiting bodies. Pramanik et al. (2005) obtained a polysaccharide from the fruiting body that consisted of mannose, glucose and galactose in an equimolar ratio, while Roy, Maiti, Mondal, Das, and Islam (2008) extracted a polysaccharide from the fruiting body of the same species that consisted of glucose and galactose in a molar ratio of 3:1.

We found an extracellular polysaccharide that contained 3-O-methyl-galactose in *P. sajor-caju*. A 3-O-methyl mannogalactan has been isolated from *P. ostreatus* (Jakovljevic, Miljkovic-Stojanovic, Radulovic, & Hranisavljevic-Jakovljevic, 1998) and O-methyl galactans were in the main chain of *P. pulmonarius*, *P. eryngii* and *P. ostreatoroseus* polysaccharides (Carbonero et al., 2008; Smiderle, Carbonero, Sassaki, Gorin, & Iacomini, 2008). These findings show that O-methyl-polysaccharides could be characteristic of the genus *Pleurotus*.

The ^{13}C NMR spectrum of PS is presented in Fig. 2B, where the chemical shifts were compared to that of PN (Fig. 2A). The anomeric region near 102–98 ppm showed four main signals at 98.2 ppm, 100.5 ppm, 101.6 ppm and 102 ppm in PN, which were assigned as C-1 of glucose, galactose, O-methyl-galactose and mannose, respectively. We did not obtain signals between 55 and 59 ppm; therefore, we ruled out the presence of 2-O-, 6-O and 4-O-methyl-galactose. In addition, the high signal near 61 ppm indicated the presence of 3-O-methyl-galactose. After sulfation, the ^{13}C NMR spectra became more complicated because the carbon directly attached to the electron withdrawing sulfate group shifted to a lower field position, while the carbon indirectly attached to sulfate group shifted to a higher field position. PS showed a split in the signals at 98–100 ppm for C-1. It is known that the signal of C-1 splits when an OH group on C-2 is functionalized, and this splitting of the C-1 signal correlated well with the extent of substitution of C-2. In addition, the disappearance of the signal near 68.4 ppm indicated that C-2 of one of the monosaccharides was completely sulfated. The new peaks in the 78 ppm region of PS were assigned to the O-4 substituted carbons, suggesting that O-4 was sulfated.

In view of the intensities of the signals of the O-substituted carbons, we conclude that nonselective sulfation of the extracellular polysaccharide has occurred; in particular, C-2 and C-4 were partially substituted, whereas C-6 was not affected. A similar pattern of sulfation was also obtained when polysaccharides from rice were chemically sulfated (Wang, Mcghee, Gibas, Tsuneda, & Currah, 2009).

3.2. Biological activities

3.2.1. Antioxidant activity

The antioxidant activities of the PN and PS polysaccharides were compared *in vitro* by several antioxidant assay methods, including total antioxidant capacity (TAC), hydroxyl and superoxide radical scavenging activities, reducing power and ferric chelating ability.

In the TAC assay (expressed as ascorbic acid equivalents) both polysaccharides showed low activity as reducing agents. Moreover, no significant difference in activity was observed between PN and PS, both polysaccharides showed an activity equivalent to approximately 8.0 mg/g of acid ascorbic equivalents.

The addition of electron-withdrawing groups to the polysaccharide enhances antioxidant activity (Yanagimoto, Lee, Ochi, & Shibamoto, 2002). Thus, the presence of a sulfate group could increase the radical scavenging activity. However, both PS and PN did not exhibit superoxide radical scavenging ability at concentrations below 0.5 mg/mL (Table 2). This data indicate that the electron density of the carbon atoms on a heterocyclic ring of PS is not a determinate factor for its superoxide radical scavenging.

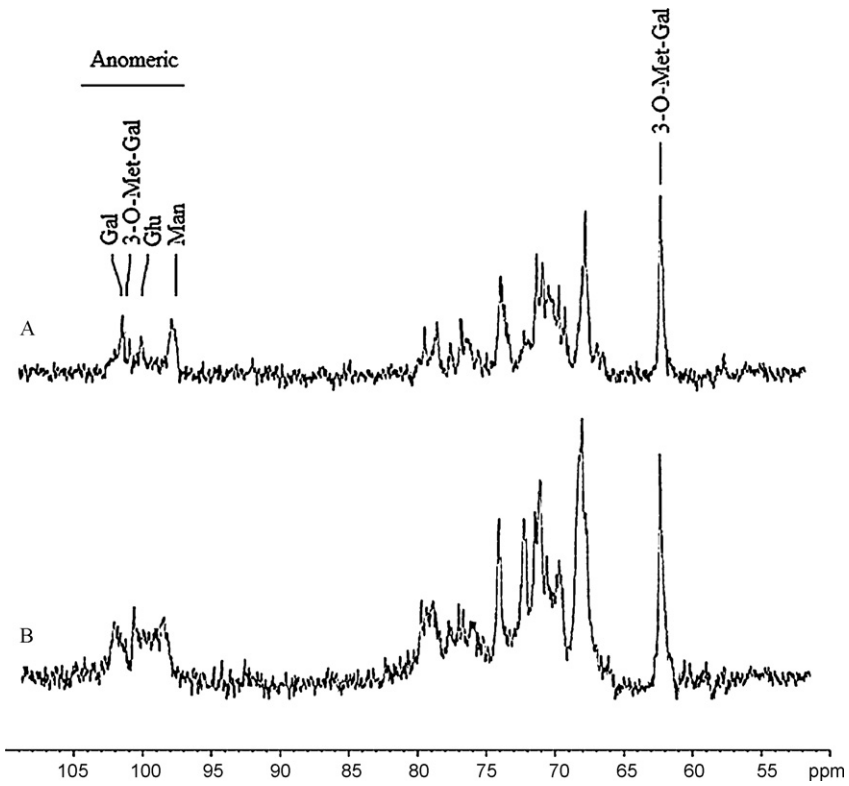

Fig. 2. ^{13}C NMR spectroscopies of the native polysaccharide (A) and the sulfated polysaccharide (B). Gal, galactose; Gal-Met, 3-O-methyl-galactose; Glu, glucose; Man, mannose.

Moreover, when the antioxidant activities of sulfated galactans with different DS values were analyzed for scavenging of the 1,1-diphenyl-2-picry-hydrazyl (DPPH) radical, the data showed that high DS galactans (DS = 2.13) had low activity compared to low DS galactans (DS = 0.08) (Yanagimoto et al., 2002).

Both polysaccharides showed hydroxyl radical scavenging activity. PN showed dose dependent activity up to a concentration of 0.1 mg/mL and reached about 13.14 ± 0.07% inhibition; however, at higher concentrations, the activity of this polysaccharide started to decrease and disappeared at 0.5 mg/mL. With the sulfated polymer, we observed a different pattern. PS did not show detectable hydroxyl radical scavenging activity at low concentrations; however, when the amount of PS increased, its activity became higher than the PN hydroxyl radical scavenging activity. PS at 0.5 mg/mL showed the greatest hydroxyl radical scavenging activity (31.29 ± 0.17%), which is approximately 3.12-fold lower than gallic acid at 0.5 mg/mL (Table 2).

Fig. 3 shows the chelating effects of PN and PS and the standard, EDTA, which acts as a chelating agent and forms complexes readily with diverse metallic ions. The results revealed that both polysaccharides presented low ferric chelating capacity when compared with EDTA, and the activity was not dose dependent. PN reached its maximum activity (17.8%) in the lowest tested concentration; this activity did not change even when a large amount of PN was tested. PS in low concentrations showed a weaker chelating effect than PN. At a concentration of 1.5 mg/mL, PS reached a similar level

Table 2
Hydroxyl and superoxide radical scavenging activity of PN and PS polysaccharides of the edible mushroom *Pleurotus sajor-caju*.

Polysaccharides	Concentration (mg/mL)	Inhibition (%)	
		$OH^{\bullet}$	$O2^{\bullet -}$
PN	0.01	nd	nd
	0.05	10.05 ± 0.15	nd
	0.1	13.14 ± 0.07	nd
	0.25	0.56 ± 0.10	nd
	0.5	nd	nd
PS	0.01	nd	nd
	0.05	nd	nd
	0.1	18.73 ± 0.06	nd
	0.25	23.56 ± 0.12	nd
	0.5	31.29 ± 0.17	nd
Gallic acid	0.05	11.6 ± 1.7	28.9 ± 3.8
	0.1	43.6 ± 2.4	41.8 ± 4.7
	0.25	64.3 ± 3.0	72.1 ± 2.9
	0.5	93.7 ± 3.7	86.3 ± 3.1

nd, not detected. Results are presented as a mean ± standard deviation of three different experiments.

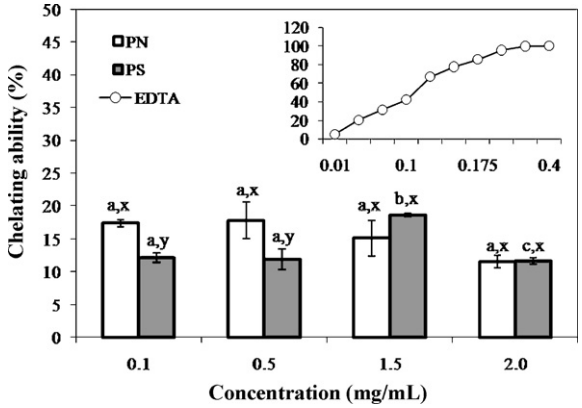

Fig. 3. Chelating ability of PN and PS polysaccharides from *Pleurotus sajor-caju*. Data are expressed as means ± standard deviation. [a,b,c,d,e]Different letters indicate significant difference between the concentrations of the same polysaccharide. [x,y]Different letters indicate a significant difference between different polysaccharide in the same concentration. $p < 0.05$.

of activity to the native polysaccharide, with an activity of around 18%.

Thus, in inhibiting hydroxyl radical production from Fenton's reaction, PS was more effective than PN. In the Fenton system, the hydroxyl radical is generated by the reaction of the ferrous ion and hydrogen peroxide (Zhang et al., 2010). PS exhibited weak ferric chelating ability, and this activity was not dose dependent (Fig. 3), indicating that the hydroxyl radical scavenging activity of PS was directly related to its affinity for the radical in the assay. Similar data were found with a sulfated glucan derivative obtained from the marine filamentous fungus *Phoma herbarum*. This sulfated polysaccharide had hydroxyl radical scavenging activity due to its affinity for the OH radical and did not show ferric chelating activity (Yang et al., 2005).

Some antioxidant compounds show the capacity to reduce ferrous ions from Fe^{3+} to Fe^{2+}. This reducing capacity *in vitro* was evaluated by the formation of a complex between Fe^{2+}/ferric chloride, and the sample activity was measured as the increase in the absorbance of this complex. Fig. 4 shows the results of the reducing power assay for both polysaccharides, and the results are expressed as the percentage of ascorbic acid activity (0.2 mg/mL). Both polysaccharides showed dose-dependent activity. For PN, the greatest activity was observed at 0.5 mg/mL, which was 36% compared to ascorbic acid, and was constant at higher concentrations. On the other hand, as shown in Fig. 4, PS at all doses had greater reducing power. In addition, at the tested maximum concentration (1.0 mg/mL), PS had an activity of around 48%.

These data agrees with the data of other groups, who have shown that sulfate groups increase the reducing power of polysaccharides (Xing et al., 2005; Zhang, Jin, & Shi, 2008). Sulfated fucans from *Laminaria japonica* (Wang et al., 2008) and sulfated polysaccharide from *Ulva pertusa* (Kaeffer, Benard, Lahaye, Blottiere, & Cherbut, 1999) have been shown to have reducing power; however, their effects were weaker than that of vitamin C. The reducing activities were usually related to the development of reductones, which terminate free radical chain reactions by donating a hydrogen atom. In most cases, irrespective of the stage in the oxidative chain at which the antioxidant action is assessed, most non-enzymatic antioxidative activity is mediated by redox reactions (Bohn & BeMiller, 1995). Our data suggest that the reducing power of polysaccharides contribute to the observed antioxidant effect. In addition, PS had a low DS (0.17) and showed the same potency in reducing power as a high DS (2.13) sulfated glucan (Zhang et al., 2010) which indicates that this activity also depends on the spatial patterns of the sulfate groups rather than merely the charge density.

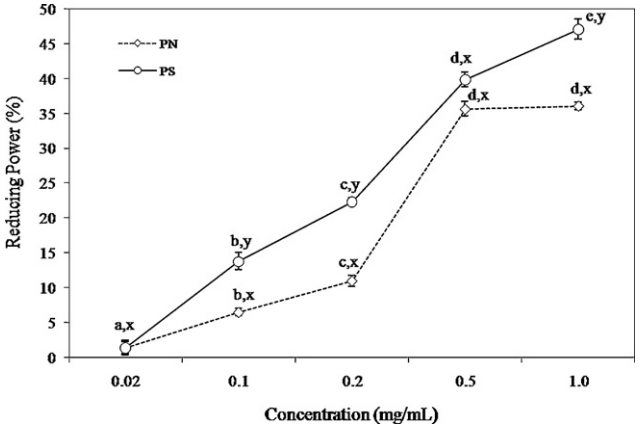

Fig. 4. Reducing power of PN and PS polysaccharides from *Pleurotus sajor-caju*. Data are expressed as means ± standard deviation. Reducing power is expressed as a percentage of activity shown for 0.2 mg/mL of ascorbic acid. [a,b,c,d,e]Different letters indicate significant difference between the concentrations of the same polysaccharide. [x,y]Different letters indicate a significant difference between different polysaccharide in the same concentration. $p < 0.05$.

Table 3
Anticoagulant activity of human plasma in the presence of PN and PS fractions as determined by APTT (activated partial thromboplastin time) and PT (prothrombin time) assays.

Polysaccharides	Amount (μg/μL)	Clotting times (s)	
		PT	APTT
PN	Control	15.2s ± 0.3	33.2s ± 3.9
	0.1	16.9s ± 0.5	33.7s ± 4.0
	0.5	16.5s ± 3.0	35.4s ± 0.4
	1.0	15.4s ± 0.7	36.9s ± 0.1
	2.0	15.4s ± 0.4	37.6s ± 0.5
PS	Control	15.2s ± 0.3	33.2s ± 3.9
	0.1	15.0 ± 0.8	35.9s ± 0.7
	0.5	15.7 ± 0.3	#46.4s ± 1.5[a]
	1.0	15.4 ± 0.3	#83.5 ± 2.8[a,b]
	2.0	15.4 ± 1.2	#101.0s ± 0.8[b]
LMWH	0.1	*	65.2s ± 0.4

APTT, activated partial thromboplastin time; PT, protrombin time; *, it was not determined; $n = 3$; s, seconds; LMWH, low molecular weight heparin (Clexane). Results are presented as a mean ± standard deviation of three different experiments. Student–Newman Keus, [a]$p < 0.001$ (0.5 vs 1.0 μg/μL); [b]$p < 0.01$ (1.0 vs 2.0 μg/μL); #$p < 0.05$ vs. control.

Overall, the antioxidant analysis showed that the addition of sulfate groups to the extracellular polysaccharide of *P. sajor-caju* did not increase its activities in the TAC, superoxide anion scavenging and ferrous chelating tests. On the other hand, sulfation increased the hydroxyl radical scavenging activity and reducing power. This supports the idea that the degree of sulfation (DS) and the sulfate group distribution in polymer chains are important characteristics that influence the specific antioxidant activity of a sulfated polysaccharide.

3.2.2. Anticoagulant activity

The anticoagulant activities of the PN and PS fractions were investigated using the classical aPTT and PT coagulation assays using low molecular weight heparin (Clexane®) as a reference. In both assays, PN did not show anticoagulant activity, indicating an absence of blood coagulation inhibitory activity in all tested pathways. In contrast to the native polysaccharide, PS exhibited distinct anticoagulant effects in the aPTT test, which evaluates the intrinsic coagulation pathway. PS prolonged the time of coagulation in a concentration-dependent manner, reaching a 3.0-times higher time compared to the baseline value in the aPTT (2.0 mg/mL). However, in the PT test, which evaluates the extrinsic coagulation pathway, no inhibition was observed with all concentrations tested (Table 3), such as heparin and several other sulfated polysaccharides (Mourão et al., 2001).

In the literature, DS values of ≥0.80 have been reported to be necessary for anticoagulant activity (Barroso et al., 2008). However, despite the low DS value (0.17) for the sulfated derivative of the *P. sajor-caju* polysaccharide, a pronounced anticoagulant activity was observed. Furthermore, although it seems obvious that the addition of sulfate groups should promote the anticoagulant activity of polysaccharides, the results showed that sulfation does not always convey this effect to the sulfated polysaccharides. Fucans, polysaccharides rich in sulfated fucose, extracted from various marine algae did not possess anticoagulant activity (Bilan & Usov, 2008; Mourão, 2007; Rocha et al., 2005). A similar observation was reported for a variety of sulfated galactans from marine invertebrates (Mourão, 2007). These data suggest that the anticoagulant action of PS in the APTT test *in vitro* is not merely a consequence of the charge density produced by sulfate groups and is probably due to the position of the sulfate. The result obtained in this work

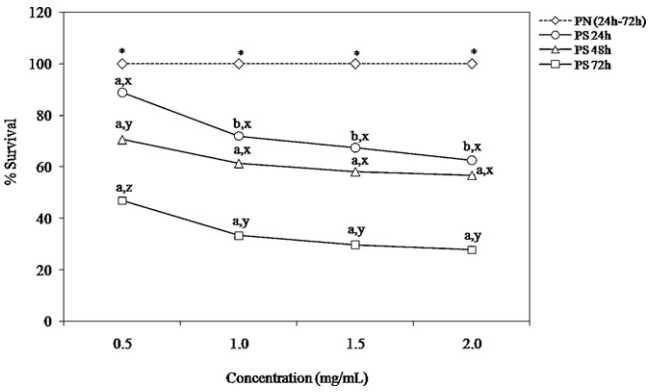

Fig. 5. Survival percentage of HeLa cells treated with the polysaccharides PN and PS at different concentrations for 24, 48 and 72 h. Data are expressed as means ± standard deviation. [a,b,c,d,e]Different letters indicate significant difference between the concentrations of PS at the same time of treatment. [x,y]Different letters indicate a significant difference of PS at different polysaccharide in the same concentration. $p < 0.05$.

agrees with several works that clearly show that the anticoagulant effect of sulfated polysaccharides is stereo-specific (Li, Lu, Wei, & Zhao, 2008).

3.2.3. Antiproliferative activity

In the present study, the growth inhibitory effects of the chemically sulfated polysaccharide against HeLa cells were examined. As shown in Fig. 5, PS presented a significantly high antiproliferative activity at dosages from 0.5 to 2.0 mg/mL. We did not find a dose dependent effect; however, the inhibition was time dependent. After 72 h, the inhibition rate of PS (1.0 mg/mL) on the HeLa cells was about 60%. No antiproliferative activity was observed with PN at any dosage tested. These results indicated that PS was more effective than PN in inhibiting HeLa cell proliferation.

Gamal-Eldeen, Amer, Helmy, Ragab, and Talaat (2007) reported similar results when sulfate groups were added to polysaccharides extracted from *Leucaena leucocephala*. This modification intensified the antiproliferative effect against three tumor cell lines, with a maximum effect of approximately 50% inhibition. The literature has suggested that the introduction of ionic groups to polysaccharides, such as sulfate groups, may promote changes in the physical and chemical characteristics and conformation of the chain (Bao, Liu, Fang, & Li, 2001; Franz & Alban, 1995; Lu et al., 2000). This change in the polysaccharide chain may be the result of intermolecular forces, such as dipole–dipole interactions, Van der Waals interactions, and hydrogen bonds, present between sulfates groups of polysaccharide and water molecules. These increases in rigidity of the chain and water solubility could promote the antitumoral effect of the polymer (Lin, Zhang, Chen, & Jin, 2004). In addition, the presence of sulfate groups in the polysaccharide chain can increase the strength of interactions between the polysaccharide and proteins, which could explain the addition or enhancement of the biological activities of chemically modified polysaccharides (Mulloy, 2005).

4. Conclusions

In conclusion, we obtained an extracellular polysaccharide (mannogalactoglucan) named PN from *P. sajor-caju*. This polymer showed specific antioxidant activities in the total antioxidant capacity, superoxide radical scavenging, reducing power and ferric chelating assays; however, it did not possess anticoagulant or antiproliferative activities. PS, the sulfated derivative of PN with a DS of 0.17, was obtained using a sulfuric acid/n-propanol method. The substituted positions of PS were C-2 and C-4. Through chemical modification of PN, the hydroxyl radical scavenging and reducing power activities of the extracellular polysaccharide increased. In addition, the sulfated extracellular polysaccharide also showed anticoagulant and antiproliferative activities. PS has exhibited potent biological properties and further research is required to investigate its role as a potential adjuvant against several diseases in food and pharmaceuticals.

Acknowledgements

This study was supported by grants and fellowship from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), MCT (Ministério de Ciência e Tecnologia) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

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