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Characterization and Antineoplasic Effect of Extracts Obtained from *Pleurotus sajor-caju* Fruiting Bodies

Nicole Dalonso · Rafaela Souza · Márcia Luciane Lange Silveira · Ângelo Adolfo Ruzza · Theodoro Marcel Wagner · Elisabeth Wisbeck · Sandra Aparecida Furlan

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Abstract Fungi of the *Pleurotus* genus present a great industrial interest due to their possibility of producing pharmacological compounds, pigments, aromas, organic acids, polysaccharides, enzymes, vitamins, amino acids, etc. Among the therapeutic products, we can highlight those with antineoplasic activity, attributed to the fungi cell wall components. Based on this, the objective of this work was to study the antineoplasic capacity of the polysaccharidic fractions obtained from *Pleurotus sajor-caju* fruiting bodies. Female Swiss mice were inoculated with the Ehrlich ascitic tumor (5×10^6 cells/animal) in ascitic form. The polysaccharidic fractions were administered intraperitoneally, during a 6-day period. Fractions FI and FII presented a lower volume of ascitic liquid (3.1 and 1.8 mL, respectively) and a higher reduction in the number of neoplasic cells present in the ascitic liquid (86.2% and 85%, respectively), when compared to the positive control (group inoculated with the tumor but without treatment). These fractions were characterized in terms of monosaccharide composition. Glucose was the major component detected, followed by galactose and mannose. The anomeric carbon configuration of the β-glucan was confirmed by the ^{13}C NMR (δ 103.7). Substituted and free C3 and C6 were also detected. Protein bands were confirmed through infrared analysis.

Keywords Antineoplasic activity · Bioactive molecules · Characterization · *Pleurotus sajor-caju* · Polysaccharidic fractions

Introduction

Cancer is a major health problem worldwide and one of the main causes of mortality in children and adults [1]. According to data from the National Cancer Institute [2], cancer is the second cause of death through disease in Brazil. Its treatment can be performed through surgery, radiotherapy, and chemotherapy or through a combination of these therapies [3, 4].

N. Dalonso · R. Souza · M. L. L. Silveira · Â. A. Ruzza · T. M. Wagner · E. Wisbeck · S. A. Furlan (✉)
University of the Region of Joinville—UNIVILLE, Campus Universitário,
s/n Bom Retiro, 89201-974 Joinville, Santa Catarina, Brazil
e-mail: sfurlan@univille.br

However, new therapy options, whether as a complement or not to the existing ones, for the purpose of establishing more precise prognoses in the treatment of this pathology have been studied [5, 6].

The discovery and identification of new safe drugs, without severe side effects, has become an important goal of research in the biomedical science [7]. Among these new possibilities, the medicinal properties of *Pleurotus* have attracted a great deal of interest from the scientific community for possessing the ability to modulate the immune system and hypoglycemic and antithrombotic activity and to reduce arterial pressure and blood cholesterol and for further possessing antitumor, antiinflammatory, and antimicrobial action [8, 9].

Studies involving the antineoplasic action of polysaccharides from the fruiting bodies and basidiomycete mycellium have been reported since 1957 [9], with various types of these fungi, such as *Pleurotus ostreatus* [10], *Pleurotus sajor-caju* [11], and *Pleurotus citrinopileatus* [8], presenting antineoplasic activities attributed to the polysaccharides of the cell wall components.

Based on the above, the objective of this work is to study the antineoplasic capacity of four fractions extracted from *P. sajor-caju* CCB 019 fruiting bodies.

Materials and Methods

Cultivation Conditions

The fungus used in this study was *P. sajor-caju* CCB 019, kept under refrigeration, in a wheat–dextrose–agar medium [12].

For fruiting body production, banana straw was packed in polypropylene bags, supplemented with rice bran, sterilized, and inoculated using 10% solid inoculum. The first step of the process, the mycelial growth, was carried out at 25 °C, 60% relative air humidity, with light, for 20 days. The second step, induction of the fruiting body formation, was achieved by perforating the plastic bags to increase air exchange, and by exposing them to light for a period of 12 h a day while increasing relative air humidity to 90% [13]. After 20 days, the fruiting bodies were harvested and frozen (−20 °C).

Bioactive Compound Extraction

Extraction of bioactive compounds of the fruiting bodies was based on the methodology proposed by Zhang et al. [8] and modified in our laboratories as follows (Fig. 1): 2.4 kg of frozen mushrooms was chopped into small pieces and then extracted with two volumes of 96% ethanol at 80 °C for 3 h. This procedure was repeated four times to remove the low molecular weight components. Residue “S” was extracted with boiling water for 3 h, and this extraction was repeated four times. FI fraction was then obtained from filtered I with five volumes of 96% ethanol and freeze-dried. Residue I was treated four times with 1% NH_4 oxalate at 100 °C for 3 h. After the liquid was separated, five volumes of 96% ethanol were added to the filtered II, which after precipitation and freeze-drying formed the FII fraction. Five percent NaOH and 0.05% $NaBH_4$ were added to residue II and maintained for 24 h. This extraction was performed twice and the resulting filtered III was precipitated by the addition of AcOH up to pH 6–7. The resulting precipitate was then dialyzed with distilled water and freeze-dried, forming the FIII-I fraction. Two volumes of 96% ethanol were added to the supernatant, and the formed precipitate was dialyzed

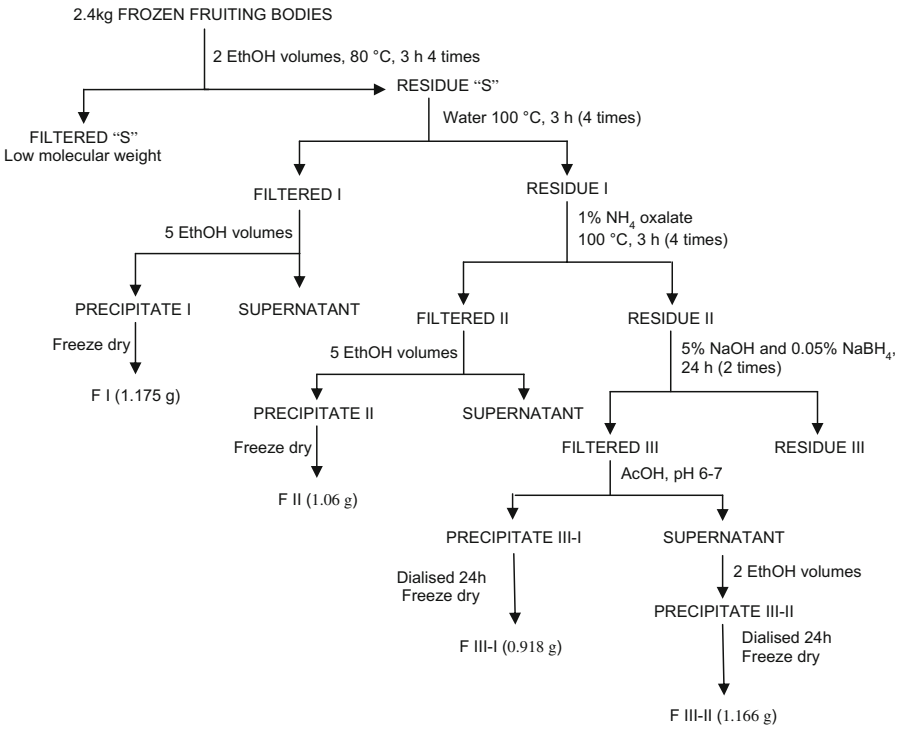

Fig. 1 Methodology flow chart for extracting the FI, FII, FIII-I, and FIII-II fractions from *P. sajor-caju* fruiting bodies

with distilled water and freeze-dried, forming the FIII-II fraction. All filtered, after the addition of the precipitating agent (ethanol or AcOH), were kept under refrigeration (24 h), and the precipitate formed was centrifuged at 5,000 rpm for 5 min (Sigma 4K, rotor 12254).

Tumor, Animals, and Treatments

The tumor used in this study was the Ehrlich Ascitic Tumor, obtained in vivo and maintained through weekly intraperitoneal injections in mice [14].

FI, FII, FIII-I, and FIII-II fraction solutions were elaborated separately, in a 1 g/L phosphate-buffered saline (PBS solution), as described by Zhang and Cheung [15].

The animals used were female Swiss albino mice (*Mus musculus*), weighing between 25 and 45 g, on a diet of water and dry pellets ad libitum, and temperature was controlled at 22± 1 °C, with 12 h light per day. The animals were divided into 20 cages, with six animals per cage, according to the treatment. For each fraction tested, one test group (with tumor induction and treatment) with 18 animals, one negative control group (without tumor induction or treatment) with six animals, and one positive control group (with tumor induction and without treatment) with six animals were used.

Tumor induction was performed by intraperitoneal injection of 0.2 mL of a cell suspension in a concentration of 2.5×10^7 cells/mL, in each mouse (5×10^6 cells/animal) from the test group as well as from the positive control group [16]. The negative control received a 0.2 mL intraperitoneal injection of PBS.

Treatment of the test group and negative control group involved the intraperitoneal application of previously prepared solutions for six consecutive days, commencing 24 h after tumor inoculation, administered in a daily dose of 10 mg/kg body weight [17]. PBS solution was applied to the negative control group.

Tumor development was assessed through the determination of the ascitic liquid volume in the peritoneal cavity and through counting the number of neoplasic cells present in the ascitic liquid [16]. Weight gain was also evaluated.

In order to measure the volume of ascitic liquid, 4 mL of 0.9% saline solution was inserted into the peritoneal cavity, and after careful massaging, the liquid was suctioned out and the volume measured. To determine the number of neoplasic cells present in the ascitic liquid, the trypan blue exclusion method was used: 20 μL of ascitic liquid was added to 180 μL of trypan blue solution and 200 μL of PBS 0.01 M, and the cells were later counted in a Neubauer hemocytometer.

Statistical Analysis

Weight gain, the number of neoplasic cells, and volume of ascitic liquid were analyzed using the Dixon "Q" Test with 90% reliability [18]. The results were expressed as the mean value of replicates ± standard error. Variance analysis of the average values was determined through the ANOVA test, with a 5% significance level.

Monosaccharide Identification

Monosaccharide identification was based on the methodology proposed by Vinogradov and Wasser [19] with some adaptations. One milligram of the selected fractions was hydrolyzed (0.5 mL of 5 M TFA, 100 °C, overnight), evaporated to dryness under a nitrogen stream, dissolved in water (1 mL), reduced with $NaBH_4$ (5 mg, 30 min), treated with AcOH (0.5 mL), and dried. The resulting material was added to methanol (1 mL), and the mixture was dried twice. The residue was acetylated with Ac_2O (0.5 mL, 100 °C, 30 min), dried, added to water (1 mL), and extracted with chloroform (1 mL). The fractions were analyzed by CG (model 7890B) with a mass spectral detector (model MSD-5975C), using HP-5 capillary with a temperature gradient (180–240 °C) at 2 °C/min.

Total Protein

Approximately 5 mg of the selected fractions was used for elementary composition analysis in a CHNS/O Elementary Analysis CE Instruments EA 1100. The percentage of total protein was obtained by multiplying the nitrogen content (percent) by the conversion factor 4.38 [20].

^{13}C Nuclear Magnetic Resonance Analysis

The ^{13}C nuclear magnetic resonance (NMR) spectrum of the isolated sample was performed in a Varian Mercury Plus model apparatus at 400 MHz and 80 °C, in a 5 mm tube. A 45º pulse (6.4 ms) was used with a relaxation delay equal to 1 s and acquisition time of 1.3 s. The samples were prepared at a concentration of 12 mg/mL, in dimethyl sulfoxide (DMSO)-d_6, and the analysis was performed over a period of 63 h (nt=96,000) with 64K data point numbers. Chemical shifts are expressed in parts per million (δ), relative to resonance of DMSO-d_6 at δ 39.50 (^{13}C).

Fourier Transform Infrared (FTIR) Spectroscopy Analysis

The infrared analysis was performed in an ABB, BOMEM Inc. spectrometer (FTLA 2000 model). The spectra were made in KBr pastilles, with around 3.0 mg sample, conducted in transmittance mode.

Results and Discussion

As shown in Fig. 1, four fractions were obtained through extractions of *P. sajor-caju* fruiting bodies which were tested for antitumor activities.

Figure 2 presents the increase in weight gain of the animals, the accumulation of ascitic liquid in the peritoneal cavity, and the number of neoplasic cells in the ascitic liquid of the animals treated with the FI, FII, FIII-I, and FIII-II fractions obtained from *P. sajor-caju* fruiting bodies, in addition to the negative and positive control groups.

Figure 2a shows that the animals treated with the FII and FIII-I fractions did not show any significant difference from each other and obtained the highest weight gain; however, this value was less than that of the positive control. The FI fraction had a similar weight increase to that of the negative control and the FIII-II presented a slight weight increase, which could be associated to an intensive diarrhea presented by this animal group after the second day treatment.

Analyzing the results referring to ascitic liquid accumulation (Fig. 2b), it could be observed that all fractions presented significant differences in relation to the controls. The FII fraction provided the least accumulation of ascitic liquid in the peritoneal cavity (1.8±0.5 mL), and the FIII-I and FIII-II fractions presented greater value, even exceeding that of the positive control.

For the number of neoplasic cells (Fig. 2c), the FIII-I and FIII-II did not present significant differences among themselves, with the same being true for the FI and FII fractions. The latter presented lower mean values in the number of neoplasic cells ($14.2\times10^6\pm3.3$ cells/mL for FI and $15.4\times10^6\pm4.3$ cells/mL for FII).

Figure 3 presents the reduction percentage in the number of neoplasic cells for each fraction tested, in relation to the positive control. FI and FII fractions were those providing the highest percentage of neoplasic cell reduction in relation to the positive control, with 86% and 85%, respectively. The FIII-I and FIII-II fractions were less effective, providing a 54% and 52% reduction, respectively, in the number of neoplasic cells.

Zhang et al. [8] extracted the polysaccharides from *P. citrinopileatus* fruiting bodies and tested their antitumor activity on the Sarcoma 180 (solid), applying a daily dose of 10 mg/kg to animals for 10 days. After 3 weeks, the water-soluble fraction FI showed a 23% tumor inhibition, with water-insoluble fractions FII, FIII-I, and FIII-II showing 24.1%, 79.5%, and 93.5%, respectively.

The results obtained in this work were clearly distinct from those presented by Zhang et al. [8] which can be justified by the following factors: in this study, the Ehrlich tumor was used in ascitic form, while the authors used the Sarcoma 180 in solid form; the fractions tested were obtained from the *Pleurotus* genus fungi, but in this study, the fractions were from distinct species (*P. sajor-caju* and *P. citrinopileatus*).

Zhang et al. [17] report that a water-insoluble fraction of α-(1→3)-D-glucan from *Ganoderma lucidum* was tested on the Ehrlich ascitic tumor, presenting a 41.5% inhibition using a daily dose of 29.7 mg/kg and 34.8% inhibition using a daily dose of 14.8 mg/kg, during a 7-day period. These results are much lower than those obtained in the present study, in lower doses, on the same tumor.

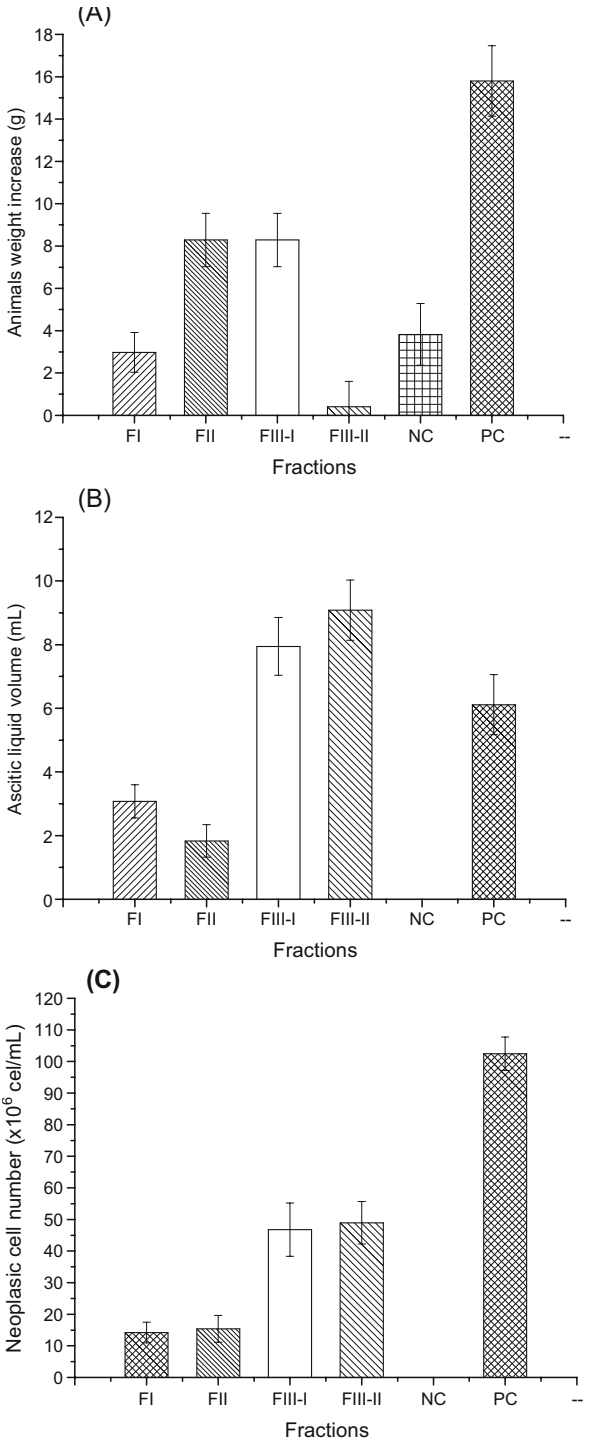

Fig. 2 Average animal weight increase (**a**), average accumulation of ascitic liquid (**b**), and average number of neoplasic cells in the ascitic liquid (**c**) in animal groups treated with the FI, FII, FIII-I, and FIII-II fractions from *P. sajor-caju* fruiting bodies and in the negative (*NC*) and positive (*PC*) control groups

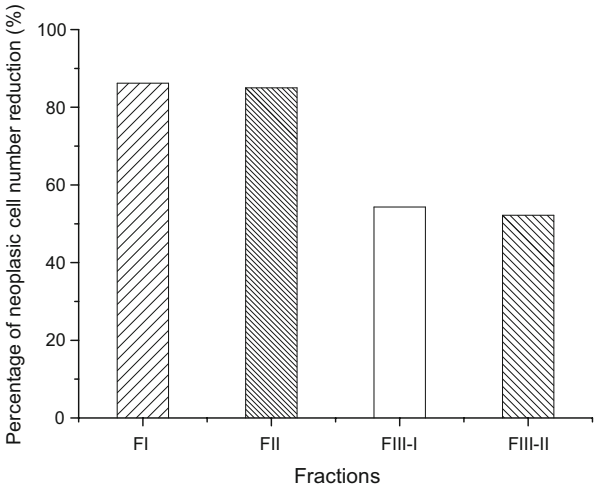

Fig. 3 Reduction percentage in the number of neoplasic cells in animals treated with the FI, FII, FIII-I, and FIII-II fractions in relation to the positive control group (PC)

Pagno et al. [15] tested intraperitoneally (5 mg/kg for 5 days) an extract obtained from *Vernonia scorpioides* with dichloromethane solvent, on the Ehrlich ascitic tumor. The authors observed no ascitic liquid accumulation in the peritoneal cavity, or the presence of neoplasic cells in this liquid, characterizing an antineoplasic action on this extract and providing the best results found in literature for Ehrlich ascitic tumor inhibition.

Fractions showing the best antitumor activity (FI and FII) were characterized in terms of their monosaccharide composition, total protein, RMN (^{13}C), and FTIR. Monosaccharide composition analysis showed that FI is composed of glucose (63.9%), galactose (23.9%), and mannose (12.0%), while FII is composed of different levels of the following sugars: glucose (71.4%), galactose (15.5%), and mannose (9.5%). The total protein content of the fractions was 11.3% and 4.2% for FI and FII, respectively.

According to the literature [21], the different types of monosaccharides present in these antitumor polysaccharides are glucose, galactose, mannose, xylose, arabinose, fucose, ribose, and glucuronic acid. In some mushroom species, polysaccharides are bound with proteins or peptides as a polysaccharide–protein or peptide complex which show higher potent antitumor activity. It has been suggested that mushroom polysaccharides containing glucose and mannose may have some antitumor action as a polysaccharide receptor was found in human macrophages, which demonstrated a high specificity for these monosaccharides. Structural features such as (1→3)-β-linkages in the main chain of the glucan and additional (1→6)-β-branch points have been indicated as important factors in antitumor action. However, antitumor polysaccharides may have other chemical structures, such as hetero-β-glucans, heteroglycan, β-glucan-protein, α-manno-β-glucan, α-glucan-protein, and heteroglycan–protein complexes.

Figure 4 presents the ^{13}C nuclear magnetic resonance spectra for FI and FII (Fig. 4a, b). The chemical shifts are expressed in parts per million (δ) for FI and FII fractions. In the ^{13}C NMR spectra, the anomeric carbon signal of both α and β configurations were detected close to δ 100 and 104, respectively [22]. The region from δ 87.74 to 86.53 refers to substituted C3, and other shifts (δ 80.041 to 54.239) observed could be attributed to C2, C3, C4, C5, and C6 of the glycoside ring [23]. The additional presence of signals from δ 40 to 20 may suggest the presence of the glucan–protein structure [22]. Chemical shift could be observed in δ 128.6 to 132.6, concerning the double bonds of unsaturated lipids present in FI fraction and small quantities in the FII fraction. A small signal in the region of δ 174.5 (Fig. 4a) corresponds to

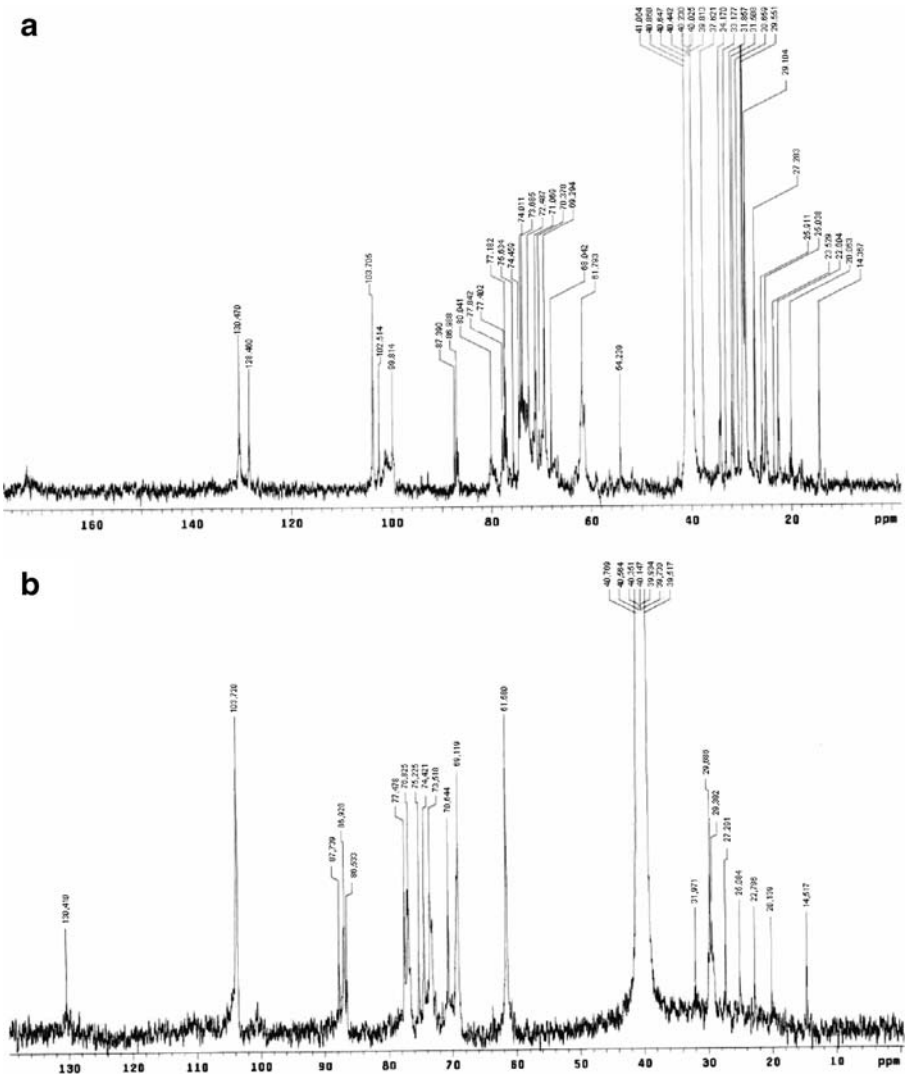

Fig. 4 ^{13}C NMR spectra of the FI (**a**) and FII (**b**) fractions obtained from *P. sajor-caju* in DMSO-d_6 at 80 °C (chemical shifts are expressed in parts per million)

an ester carbonyl group [24]. FII fraction shows a more intense anomeric signal in the β configuration region of the spectrum (δ 103.7) than FI fraction (Fig. 4).

Gorin [25] observed a chemical shift attributed to a linear β-D-glucan with (1→3) type bonds in 102.9 (C1), 86.1 (C3), 76.3 (C5), 72.8 (C2), 68.4 (C4), and 60.9 (C6). Gutiérrez et al. [23] isolated a β-D-glucan with principal chain (1→3) and ramification (1→6), observing shifts in 103.1 (C1); 73.9, 73, and 72.9 (C2); 87.3, 86.3, and 85.3 (substituted C3); 74.8 (C3); 79.6 (substituted C4); 71 and 68.8 (C4); 76.9 and 76.3 (C5); 70.1 and 69 (substituted C6); and 61.1 and 59.9 (C6).

In the infrared spectrum (Fig. 5), it is possible to observe the O–H axial absorption bands, with intermolecular hydrogen bonds between 3,600 and 3,000 cm^{-1}. In 1,639 cm^{-1},

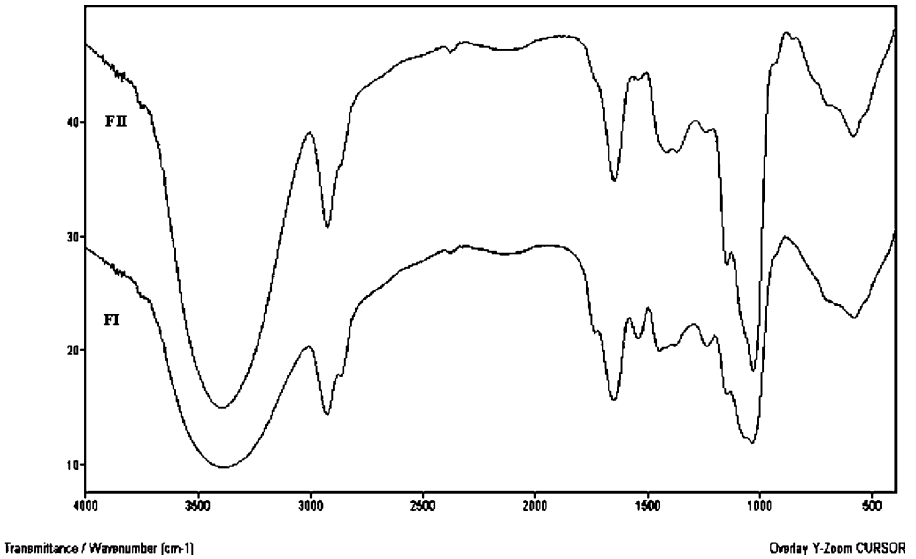

Fig. 5 FTIR spectra of *P. sajor-caju* fractions (FI and FII) obtained from solid samples using the KBr pastille method in an ABD, BOMEM Inc. spectrometer (4,000 to 400 cm^{-1})

there is an angular deformation in the bond. Asymmetrical stretch bands of CH_2 are represented in 2,900 to 2,950 cm^{-1}. The characteristic carbohydrate band appears between 900 and 1,200 cm^{-1}, confirming the C–O–C bond, due to the bond between the ether and the polymer [22, 26, 27]. The bands in 1,664 and 1,527 cm^{-1} refer to the proteins. This was also observed by other authors when studying another basidiomycete genus with recognized medical potential [22].

Due to complexity of the spectra and monosaccharide composition in glucose, galactose, and mannose, it is possible that the fractions FI and FII are mixtures of polysaccharides, including the possibility of a heteropolymer.

Conclusion

The FI and FII fractions extracted from *P. sajor-caju* CCB 019 fruiting bodies presented lower number of neoplasic cells (14.2×10^6 cells/mL for FI and 15.4×10^6 cells/mL for FII), after 6 days of treatment, representing about 85% reduction of the number of these cells in relation to the positive control group. FI and FII fractions were characterized in terms of their monosaccharide composition (63.9% and 71.4% glucose, 23.9% and 15.5% galactose, and 12.0% and 4.2% mannose for FI and FII, respectively) and protein content (11.3% and 4.2% for FI and FII, respectively). The ^{13}C NMR and the infrared spectra confirmed the presence of mainly polysaccharides and protein. These results are very promising and show the antineoplasic potential of *P. sajor-caju* CCB 019 fruiting body extracts.

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