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Characterization and cytotoxic activity of sulfated derivatives of polysaccharides from *Agaricus brasiliensis*

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Abstract

Agaricus brasiliensis cell-wall polysaccharides isolated from fruiting body (FR) and mycelium (MI) and their respective sulfated derivatives (FR-S and MI-S) were chemically characterized using elemental analysis, TLC, FT-IR, NMR, HPLC, and thermal analysis. Cytotoxic activity was evaluated against A549 tumor cells by MTT and sulforhodamine assays. The average molecular weight (Mw) of FR and MI was estimated to be 609 and 310 kDa, respectively. FR-S (127 kDa) and MI-S (86 kDa) had lower Mw, probably due to hydrolysis occurred during the sulfation reaction. FR-S and MI-S presented ~14 % sulfur content in elemental analysis. Sulfation of samples was characterized by the appearance of two new absorption bands at 1253 and 810 cm^{-1} in the infrared spectra, related to S=O and C-S-O sulfate groups, respectively. Through ^{1}H and ^{13}C NMR analysis FR-S was characterized as a (1→6)-(1→3)-β-D-glucan fully sulfated at C-4 and C-6 terminal and partially sulfated at C-6 of (1→3)-β-D-glucan moiety. MI-S was shown to be a (1→3)-β-D-gluco-(1→2)-β-D-mannan, partially sulfated at C-2, C-3, C-4, and C-6, and fully sulfated at C-6 of the terminal residues. The combination of high degree of sulfation and low

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Conflict of interest
Authors disclose any actual or potential conflict of interest in this work.

molecular weight was correlated with the increased cytotoxic activity (48 h of treatment) of both FR-S (EC_{50}=605.6 μg/mL) and MI-S (EC_{50}=342.1 μg/mL) compared to the non-sulfated polysaccharides FR and MI (EC_{50}>1500 μg/mL).

Keywords

Agaricus brasiliensis; sulfated polysaccharide; chemical characterization; cytotoxic activity

1. Introduction

Agaricus brasiliensis is an edible Basidiomycete fungus belonging to the Brazilian biota and has traditionally been used to treat cancer and other diseases. In the last few decades, numerous studies have reported the cytotoxic and antitumor properties of *A. brasiliensis* polysaccharides, which mainly act through immunomodulatory mechanisms, but also by direct cytotoxic effects on tumor cells [1].

The species found in Brazil was originally named *Agaricus blazei* Murrill *sensu* Heinemann. Since 2005, this binominal nomenclature has been considered incorrect and replaced by two botanical names: *Agaricus subrufescens* Peck or *Agaricus brasiliensis* Wasser, with the latter being adopted for the fungus cultivated in Brazil [2–5].

Polysaccharides are a structurally diverse class of macromolecules for which physicochemical and biological properties are dependent on a combination of factors such as sugar composition, molecular weight, and chain conformation. For sulfated polysaccharides, the degree of substitution and position of sulfated groups are also important [6]. There are many reports demonstrating that sulfation improves the biological activity of polysaccharides, including anti-coagulant [7], antiviral [8], immunostimulant [9], hypoglycemic [10], anti-oxidant [11], cytotoxic [12, 13], and antitumor [14] properties.

In our previous work [15], we evaluated the anti-herpetic activity of an *A. brasiliensis* mycelial polysaccharide (MI) and its sulfated derivative (MI-S) and found that sulfation of MI significantly improved its antiviral activity. Since *A. brasiliensis* polysaccharides and sulfated derivatives have potential therapeutic applications, the goal of this work was to chemically characterize MI and MI-S as well as *A. brasiliensis* fruiting body polysaccharide (FR) and its sulfated derivative (FR-S). Analytical methods included scanning electron microscopy, elemental and thermogravimetric analysis, high performance gel permeation and liquid chromatography (HPGPC and HPLC), FT-IR, and NMR. The cytotoxicity of MI, MI-S, FR, and FR-S also was evaluated against tumor (A549) and non-tumor cell lines (Vero). To the best of our knowledge, this is the first report on the cytotoxic activity of sulfated derivatives of polysaccharides from this species.

2. Materials and methods

2.1 Chemical reagents

The chemical reagents were purchased from Sigma-Aldrich (USA). All the reagents used in High Performance Chromatography analysis were HPLC grade. All the others reagents were analytical grade.

2.2 Fungal materials

The fruiting bodies of *Agaricus brasiliensis* Wasser (syn *A. subrufescens* Peck) were collected in Biguaçu, Santa Catarina state, Brazil, and designed as strain UFSC 51. A voucher specimen is deposited in the FLOR Herbarium at UFSC (FLOR 11797) and at *Coleção Brasileira de Micro-organismos de Ambiente e Indústria* -CBMAI/UNICAMP (available at http://webdrm.cpqba.unicamp.br/catalogo/pycat/index.py, code number: 1449). The *A. brasiliensis* mycelium was isolated and cultivated as previously described [15].

2.3 Isolation of mycelial and fruiting body polysaccharides and sulfation method

A. brasiliensis polysaccharides were isolated as previously described [15, 16], with minor modifications. Briefly, 50 g of dried fruiting bodies or mycelial biomass from the submerge-cultivated state were blended twice with 0.5 L of distilled water, refluxed at 100°C for 3 h and filtered through a Whatman filter paper no. 42. The extracts were precipitated with three volumes of 95 % ethanol and recovered by centrifugation (2000 x g, 15 min) to obtain the crude mycelial (cMI) and fruiting body (cFR) polysaccharide fractions. Finally, the higher molecular weight polysaccharides from the mycelium and fruiting body were obtained through dialysis (5 kDa cutoff membrane - Spectrum Laboratories, New Brunswick, USA) and, after lyophilization, were designated as MI and FR, respectively. Both polysaccharides were chemically sulfated using the chlorosulfonic acid/pyridine method as described by Zhang et al. [17], generating their respective sulfated derivatives MI-S and FR-S.

2.4 Sample characterization

2.4.1 Scanning electron microscopy (SEM)—The surface morphology of gold coated samples was analyzed by scanning electron microscopy (JSM-6390 LV, Jeol, Japan).

2.4.2 Thermogravimetry combined with differential thermogravimetric analysis (TGA-DTGA)—Thermograms of samples (1 mg) were obtained in a thermogravimetric analyzer (TGA-50, Shimadzu) from room temperature up to 900°C, at a scan rate of 10°C per min.

2.4.3 High-performance gel permeation chromatography (HPGPC)—The molecular weight (Mw) determination was carried out by HPGPC using a Perkin-Elmer series 200 instrument (USA) equipped with a refractive index detector and a gel filtration column (TSK-Gel 5000 PW 7.8 × 300 mm connected to a TSK PWH 5 × 75 mm guard column; Tosoh, Japan). Samples were eluted with 0.2 M NaCl mobile phase at a flow rate of 1 mL/min. The Mw was estimated by reference to the calibration curves of standard dextrans (5, 12, 50, 150, 410, and 670 kDa).

2.4.4 Analytical methods—Total sugar content was determined using the phenol-sulfuric acid method [18] adapted to a 96 well microassay plate [19] using glucose as standard. The Bradford method [20] was used to determine the protein content using calibration curves built with bovine serum albumin. The sulfate content was determined by the $BaCl_2$ method [21]. Results, determined from calibration curves obtained on three different days, are expressed as mean ± standard deviation (% w/w ± s.d.).

2.4.5 Elemental analysis—Elemental analysis (carbon, hydrogen, nitrogen, and sulfur) was performed with a Perkin Elmer 2400 series II elemental analyzer. The percentage of sulfur (% S) and carbon (% C) were used to calculate the degree of substitution (DS) according to the formula:

$$\mathrm{DS}=2.25\times \%\,\mathrm{S}/\%\,\mathrm{C}.$$

[22]

2.4.6 Monosaccharide composition analysis—The qualitative monosaccharide composition of MI and FR was evaluated by thin layer chromatography (TLC) using xylose, arabinose, mannose, glucose, and galactose as reference compounds. Briefly, the samples were hydrolyzed with 3M trifluoroacetic acid (TFA) for 4 h and analyzed on PEI-cellulose sheets (Merck, Germany), developed with *n*-butanol/ethyl acetate/pyridine/water (6:1:5:4). The chromatograms were visualized after spraying aniline-*o*-phthalic acid reagent followed by heating [23].

Quantification of monosaccharides was performed using High Performance Liquid Chromatography (HPLC) in a Perkin-Elmer series 200 instrument equipped with an UV detector, at 250 nm, according to Lv et al. [24]. Briefly, hydrolyzed samples (4 M TFA, 2 h) were derivatized with 1-phenil-3-methyl-5-pyrazolone (PMP). Calibration curves, constructed with PMP-labeled standard monomers (mannose, glucose, galactose, and glucuronic acid), were used for determining the sugar concentrations of the samples. Arabinose was employed as an internal standard. Analyses were undertaken at room temperature (25 °C) on a C18 column (4.6 mm × 250 mm, 5 μm, Perkin-Elmer, USA) using a gradient elution of 0.045 % KH_2PO_4 - 0.05 % triethylamine buffer (A) / acetonitrile (B) as follows: 10 % to 14 % B over 40 min, at a flow rate of 1.0 mL/min.

2.4.7 Fourier transformation infrared (FT-IR) spectroscopy—The FT-IR spectrum was recorded on a Perkin-Elmer Spectrum One spectrometer in the region between 650 and 4.000 cm^{-1}.

2.4.8 Nuclear Magnetic Resonance (NMR) analysis—The spectra were recorded at room temperature for samples (60 mg/mL) dissolved in D_2O in a Bruker Avance III 500 NMR instrument, operating at 500 MHz for ^1H and 125 MHz for ^{13}C. Chemical shifts were expressed in ppm relative to internal acetone (= 32 ppm).

2.5 Cytotoxic activity evaluation

2.5.1. Cell lines—Human lung adenocarcinoma (A549, ATCC, CCL-185) and Vero cells (ATCC, CCL-81) were grown in Minimal Essential Medium supplemented with 10 % fetal bovine serum, 100 U/mL penicillin G, 100 μg/mL streptomycin and 25 μg/mL amphotericin B in a humidified 5 % CO_2 atmosphere at 37°C.

2.5.2 MTT assay—The effect on cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cellular viability assay [25]. In brief, 1×10^4 cells/well were seeded in 96-well microplates and incubated for 24 h. Cells were then treated with different concentrations of the samples for 48 or 72 h. Negative controls were treated with medium only. After the exposure period, the culture medium was replaced by MTT solution (1 mg/mL) and plates were further incubated for 4 h. Formazan crystals were dissolved by addition of DMSO (Merk, Germany) and the optical densities were read at 540 nm (Infinite 1200 TECAN, Austria). The 50 % effective concentration (EC_{50}) was defined as the concentration that reduced cell proliferation by 50 % when compared to untreated controls. Paclitaxel (Glenmark, Brazil) was used as the positive control.

2.5.3 Sulforhodamine assay—The sulforhodamine assay [26] was also used to evaluate the cytotoxicity of selected samples. Briefly, A549 or Vero (ATCC: CCL 81) cells, cultured in 96-well plates, at a density of 1×10^4 cells/well, were exposed to eight concentrations of samples for 48 h. After subtracting the absorbance values of the initial cell cultures (time zero cell control), the GI_{50} (50 % growth inhibitory activity), TGI (total growth inhibition, cytostatic activity), and LC_{50} (50 % lethal concentration, cytotoxic activity) values were calculated.

2.6 Statistical analysis

GraphPad Prism 5 Software was used to calculate EC_{50} values and their 95 % confidence intervals through a nonlinear fit- curve (log of compound concentration *versus* normalized response - variable slope). GI_{50}, TGI, and LC_{50} values were calculated using linear fit-curves in the same program.

3 Results and Discussion

Since the introduction of sulfate groups with an appropriate degree of substitution can improve the bioactivity of polysaccharides, the goal of this study was to modify polysaccharides isolated from *A. brasiliensis* fruiting body and mycelium and evaluate their physicochemical properties and cytotoxic activity. Analysis of surface morphology by scanning electron microscopy (SEM) is a qualitative tool to characterize fungal polysaccharides, comparing with standards and assessing morphological differences of modified derivatives. Microstructural irregularities observed in SEMs presented in Fig. 1 show that *A. brasiliensis* polysaccharides are an amorphous solid. The FR polysaccharide (Fig. 1 A,) showed a rough appearance with no porosity that may be due to high molecular packing as a result of inter and intramolecular hydrogen bonds. Following sulfation, FR-S (Fig. 1 C, D) displayed a smoother surface with an internal porous structure, suggesting that

the sulfate groups expanded intermolecular spaces. The MI polysaccharide (Fig. 1 E, F) consisted of flakes that were smaller in size than those of FR and FR-S. MI-S (Fig. 1 G, H) presented particles of variable size with smoother surfaces and rounded shapes. Porous structures were also observed in the MI-S preparation. These particle structures are consistent with results obtained by others. For example, an *A. brasiliensis* mycelial exopolysaccharide was previously reported to be an amorphous solid by Lima et al. [27]. In contrast, Hong and Choi [28] described a spherical shape for an *A. blazei* protein-polysaccharide complex, prepared by spray-drying process. This variation on morphology may be related to the different methods used for fungus cultivation, sample extraction and drying.

Data from thermal analysis performed by TGA-DTGA are compiled in Table 1 and thermograms are available in Supplementary Fig. 1. According to the thermograms, polysaccharide decomposition occurred in three steps. The small initial drop in mass represents the loss of water. The second stage of decomposition began above 266°C for the non-modified compounds (FR and MI) and above 218°C for the sulfated derivatives (FR-S and MI-S) suggesting that sulfation reduced the thermal stability. The temperature peak (Tmax) recorded in the DTGA curve is characteristic of an endothermic reaction and could be attributed to thermal decomposition of the polysaccharides [29]. The Tmax obtained for FR and MI was around 300°C. Hong and Choi [28] found a similar profile for a polysaccharide-protein complex isolated from *A. blazei* fruiting body. A similar behavior has been described for other polysaccharides such as chitosan [30] and galactomannan [31]. Jayakumar et al. [32] also reported a slight decrease in the thermostability of chitin after sulfation.

The data on the apparent molecular weights (Mw) of *A. brasiliensis* polysaccharides are presented in Table 2. The polysaccharide isolated from the *A. brasiliensis* fruiting body (FR) was found to have a Mw of 609 kDa, which falls within the range of 390–2000 kDa previously described for similar preparations [33–36]. The Mw of 310 kDa determined for the mycelial polysaccharide (MI) showed good correspondence with the values reported by Fujimiya et al. (380 kDa) [37] and Lin and Yang (274 kDa) [38]. The sulfation process carried out in acid medium at high temperature probably induced hydrolysis of the polymers, thus accounting for the lower Mw of the sulfated derivatives (127 and 86 kDa for FR-S and MI-S, respectively). This effect has been previously described by Lanteri [39].

The monosaccharide composition of cFR and cMI was initially accessed by TLC analysis (Supplementary Fig. 2). The cFR was found to contain primarily glucose, while cMI had predominantly glucose, with minor amounts of other sugars.

The quantitative carbohydrate composition was assayed by HPLC-UV analysis of PMP derivatized samples. As shown in Table 2, the major sugar found in FR was glucose (63.67 %), while MI contained predominantly mannose (58.65 %), with significant amounts of glucose (19.35 %), which is characteristic of a heteropolymeric glucomannan. No detectable uronic acids were found in the samples since there was no peak at retention time of glucuronic acid standard. The total carbohydrate content varied from 66 % to 79 %, similar to the concentrations (80–95 %) previously reported for *A. blazei* polysaccharides purified

by anion exchange chromatography [33, 35]. Similarly, the low percentage of protein (1–1.8 %) was comparable to values obtained previously [16, 27]. The low nitrogen content (Table 3) is in agreement with the results of protein determination by the Bradford assay (Table 2). According to Fernandes et al. [40] the temperature, solvent, and pH used for the extraction and drying procedures can break peptide bonds thereby reducing the protein content. Therefore, different extraction and drying procedures may explain the differences in protein levels reported by some groups for polysaccharides obtained from *A. brasiliensis* and *A. blazei* fruiting body [28, 35, 41, 42] and mycelium [43]. Sulfate analysis (Table 2) confirmed that the parental polysaccharides have no detectable sulfate content, whereas the derivatized samples contain over 35 % sulfate. These results were also confirmed by data from elemental analysis (Table 3).

The IR spectra recorded for the samples confirmed their polysaccharide constitution, disclosed by the intense stretching bands between 3200–3470 cm^{-1} (ν O-H) and 1075 cm^{-1} (ν C-O) [44]. A reduction in the intensity of the bands, observed for the derivatives, was related to the sulfation of the hydroxyl groups [45]. The absence of uronic acids was confirmed by the lack of carbonyl bands around 1700 cm^{-1} [16]. Polysaccharides can easily be hydrated due to their affinity for water and this is consistent with the presence of the water absorption bands at 1623–1636 cm^{-1}. The small peaks at 1370–1540 cm^{-1} confirmed that the preparations contained low amounts of protein. The weak absorption bands at 876–898 cm^{-1} present in all the *A. brasiliensis* polysaccharides spectra are indicative of β stereochemistry [6]. The sulfation was confirmed by the appearance of two new absorption bands around 1200 and 800 cm^{-1} in FR-S and MI-S spectra, characteristic of asymmetric (S=O) and symmetric (C-O-S) vibrations, respectively [17].

The attribution of the chemical shifts from the ^{13}C and ^{1}H NMR spectra obtained for the native polysaccharides and the sulfated derivatives are listed in Table 4. FR was characterized as a glucan, with a majority of β-(1→6) linkages, and a minor amount of (1→3)-β-linkages. The β-(1→3)-linked H-1 of the side chain appeared as one resolved doublet centered at 4.95 ppm, whose coupling constant value (J= 8.28 Hz) designates the β-configuration [46, 47]. Although the signal around 86 ppm ascribed to C-3 of (1→3)-β-linkage was not detected in the FR spectrum, as reported by Ohno et al. [48], the remaining signals indicate the presence of a (1→3)-β-glucan component. These results show that FR consists of a backbone of (1→6)-β-D-glucan with (1→3)-β-D-glucan side chains attached to C-3, as previously described for the polysaccharides isolated from fruiting bodies of *A. brasiliensis* [16] as well as of *A. blazei* cultivated in Brazil [35] and China [48].

The sulfation of hydroxyl groups results in downfield shift of the carbons bearing sulfates and the protons linked to them by about 7–10 ppm and 0.5–2 ppm, respectively [49]. Hence, the ^{13}C and ^{1}H NMR spectra of FR-S showed downfield shifts of signals at 70.92/3.68 ppm and 63.34/4.30–4.46 ppm respectively to 76.08/4.65 and 71.59/5.32 ppm, indicating that the (1→6)-β-D-glucan portion was fully sulfated at C-4 and C-6 of the terminal residues. Similarly, the hydroxyl groups of (1→3)-β-D-glucan moiety in FR-S appear to have been fully sulfated at positions 4 and 6 of the terminal residues. Although the signal at 60.69 assigned to C-6 in the 1,3-β-chain was displayed in both FR and FR-S ^{13}C NMR spectra, the corresponding H-6 signal (3.90 ppm) was barely detected in ^{1}H NMR spectrum of FR-S and

the appearance of a signal at 5.07 ppm most likely indicates the partial sulfation of C-6. The low reactivity of other carbon positions could be attributed to steric hindrance [50].

As expected, the signals of the hydrogens linked to carbons bearing sulfate groups experienced downfield shifts from 0.60 to 2.03 ppm with respect to the unsubstituted polysaccharides in the ^{1}H NMR spectra of sulfated derivatives. Minor changes in chemical shifts were also observed on protons located in the vicinity of sulfation sites [51].

According to ^{13}C and ^{1}H NMR data, MI was characterized as a (1→3)-β-D-gluco-(1→2)-β-D-mannan, as previously described [15, 52]. The signal at 65.86 ppm probably results from the branching in C6 of the main chain. With regard to MI-S, sulfation at C-6 position of the terminal residue was nearly complete, both in the main and side chains, disclosed by the downfield shift of C-6 to a broad peak at 69.10 ppm. Partial sulfation was observed at C-3, C-4, and C-6 positions of the (1→2)-β-D-mannan moiety, as well as at C-2, C-4, and C-6 sites of the (1→3)-β-D-glucan side chain. A splitting pattern of C-3 signal (74.00 ppm) from (1→2)-β-D-mannan and C-2 (75.59 ppm) from (1→3)-β-D-glucan moiety can be attributed to the sulfation of adjacent carbons.

It is well known that sulfation of polysaccharides is responsible for changes in the original chain conformation usually resulting in alterations in their biological effects, including antiviral, cytotoxic, and antitumor activities [13, 53–59]. The inhibitory effects on A549 cell proliferation were concentration- and time-dependent and the results, expressed as EC_{50} values for the 48 and 72 h treatment period, are presented in Table 5. FR and MI had no cytotoxic effect after 48 h of treatment at 1,500 μg/ml. Sulfation of both preparations increased the cytotoxic activity with EC_{50} values of 605.6 and 342.1 μg/ml, respectively for FR-S and MI-S. Similarly DEX-S was more cytotoxic than DEX. Besides the introduction of sulfate groups, the Mw reduction of the sulfated derivatives (FR-S and MI-S) in comparison to the native polysaccharides (FR and MI) might have contributed to their higher cytotoxic effect. Similarly, Yang et al., [60] found a significantly higher cytotoxic activity for partially hydrolyzed fucoidans (Mw= 490 kDa) compared to the native polymers (Mw= 5,100 kDa).

In order to differentiate the cytotoxic (LC_{50}) and cytostatic (GI_{50} and TGI) effects, FR-S and MI-S were evaluated by the sulforhodamine assay using A549 and Vero cells. Table 6 shows that FR-S and MI-S were cytotoxic at the higher concentrations tested, with MI-S appearing to be slightly more active. Vero cells were found to be more resistant to the cytotoxic effects of FR-S and MI-S. These results raise the possibility that these sulfated polysaccharides might display selective cytotoxicity for human cancer cells, but further studies are needed to confirm this hypothesis. As observed by other authors, the antiproliferative effect of sulfated polysaccharides depends on cell type [53, 61].

Previous studies have shown that there is an optimum degree of sulfation (DS) to reach the maximal biological response, which varies according to the polysaccharide type. For instance, Liu et al. [54], comparing polysaccharides with similar Mw (~20 kDa), observed a stronger inhibition of Hep 2 cell growth by polysaccharides with a DS of 1.8 in comparison to those with lower DS values (1.52), while the activity was reduced when the DS was

increased to 2.02. Similarly, Bao et al. [57] demonstrated that sulfated polysaccharides with low DS (0.11–0.14) were less cytotoxic than those with higher values (0.28–066), whereas further increases in DS (1.06) reduced the activity. No direct relationship between the cytotoxic activity and DS values were observed in the present work. However, it is important to note that the sulfated polysaccharides evaluated herein have different sugar compositions, chain conformations, and sulfation positions, and these features probably also contribute to the differences in their cytotoxicity.

4. Conclusions

The current study showed that the sulfation increased the cytotoxic activity of *A. brasiliensis* fruiting body polysaccharide, and was essential for the activity of the mycelial polysaccharide at the tested concentrations. Despite using identical conditions for chemical derivatization, distinct patterns of sulfation were obtained for MI-S and FR-S, most likely due to the differences in their native carbohydrate composition and structure. FR was determined to be a glucan with predominantly β-(1→6) linkages with some β-(1→3) linkages. FR-S was fully sulfated at C-4 and the terminal C-6. MI was found to be a (1→3)-β-D-gluco-(1→2)-β-D-mannan. Sulfation in MI-S was nearly complete at C-6 with partial sulfations at C-3, C-4, and C-6 of the mannan moiety and C-2, C-4, and C-6 of the glucan side chains.

After chemical modification, structural analysis revealed that the chain structure of the compounds was preserved. The main modifications obtained by sample derivatization besides sulfation were: 1) reduction in Mw, 2) slight decrease in thermal stability, 3) minor reduction on protein content, and 4) improvement of cytotoxic activity. The present findings increase the understanding of FR-S and MI-S structure-activity relationships and raise the possibility that these sulfated polysaccharides might display selective toxicity against tumor cells. Additional studies of these polysaccharides are clearly warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Polysaccharides were isolated from *Agaricus brasiliensis* fruiting body (FR) and mycelia (MI)
- The sulfated derivatives FR-S and MI-S were prepared
- FR, FR-S, MI, and MI-S were chemically characterized
- FR-S and MI-S presented promising cytotoxic activity against A549 lung tumor cells
- The cytotoxic effect was related to the presence of sulfate groups and the lower molecular weight of the sulfated polysaccharides.

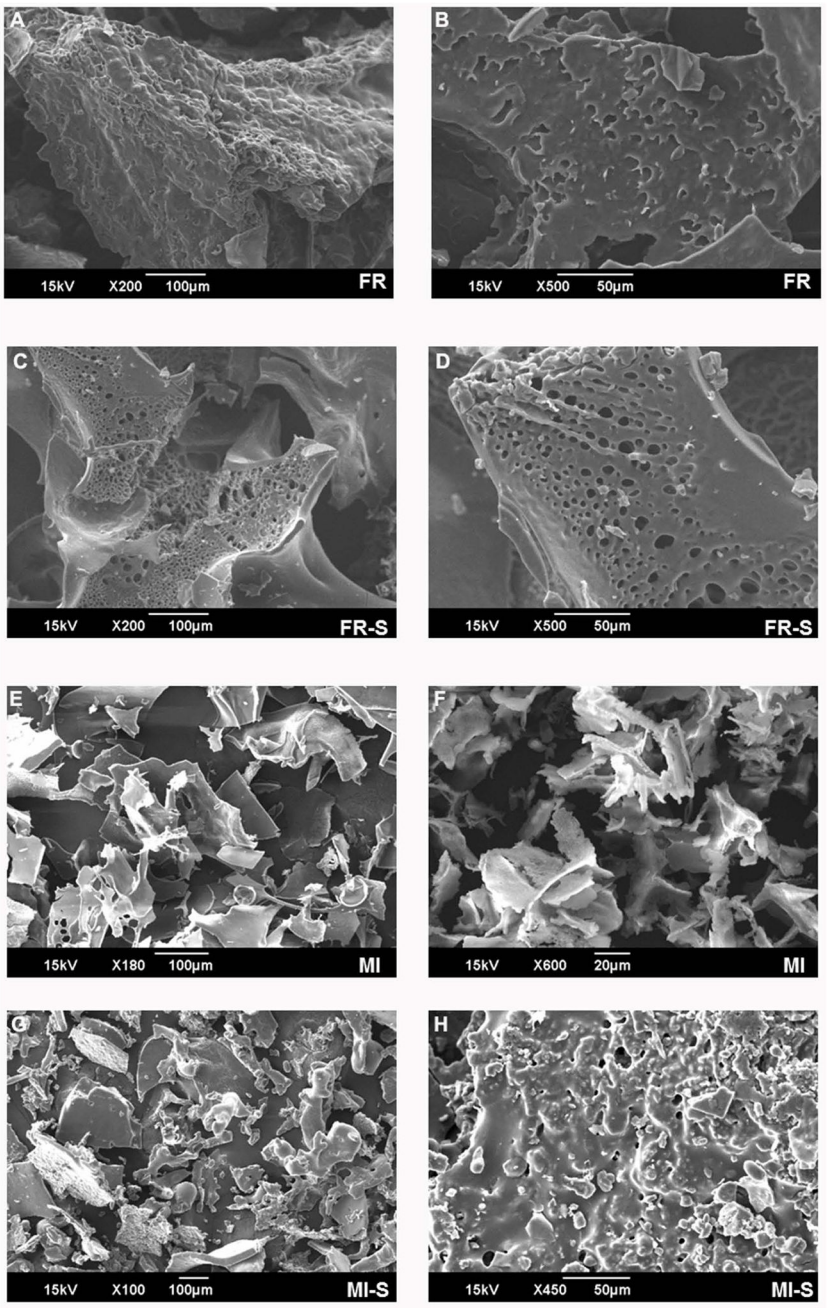

Fig. 1.

Agaricus brasiliensis polysaccharides observed under scan electron microscopy. Lyophilized samples were gold coated and analyzed by a JSM-6390 LV microscope. A and B: FR, *A. brasiliensis* fruiting body polysaccharide; C and D: FR-S, sulfated derivative of *A. brasiliensis* fruiting body polysaccharide; E and F: MI, *A. brasiliensis* mycelial polysaccharide; G and H: MI-S, sulfated derivative of *A. brasiliensis* mycelial polysaccharide.

Table 1

Thermal analysis of *Agaricus brasiliensis* polysaccharides

	TGA[a]			DTGA[b]
Sample	**Range of temperature (°C)**	**Tf-Ti**	**Mass loss (%)**	**Tmax (°C)**
FR	272.68 – 343.72	71.04	51.59	313.92
FR-S	224.96 – 277.36	52.40	39.09	248.89
MI	266.12 – 343.98	77.86	44.83	301.33
MI-S	217.21 – 260.30	43.09	27.23	236.32

FR and FR-S, *A. brasiliensis* fruiting body polysaccharide and its sulfated derivative;

MI and MI-S, *A. brasiliensis* mycelial polysaccharide and its sulfated derivative.

[a] Thermogravimetric analysis; (Tf - Ti)= range of reaction (final temperature - initial temperature);

[b] Differential thermogravimetric analysis; Tmax= maximal temperature of degradation.

Table 2

Molecular weights and chemical composition of *Agaricus brasiliensis* polysaccharides

Sample	Molecular Weight (kDa)	Monosaccharides (%)			Content mean ± SD [% (w/w)]		
		Glucose	Mannose	Galactose	Total sugar	Protein	Sulfate
cFR	617.93 ± 12.01 and 17.88 ± 0.98	60.19 ± 2.12	8.86 ± 3.46	0.94 ± 0.03	n.t.	n.t.	n.t.
FR	608.73 ± 4.11	63.67 ± 4.08	1.76 ± 0.13	4.55 ± 1.44	78.97 ± 7.52	1.76 ± 0.22	n.d.
FR-S	127.20 ± 6.95	68.98 ± 1.26	1.16 ± 0.34	2.86 ± 1.31	65.91 ± 9.39	n.d. (< 0.16)	40.25 ± 1.78
cMI	309.89 ± 2.22 and 30.20 ± 9.22	65.05 ± 5.12	9.95 ± 0.21	n.d.	n.t.	n.t.	n.t.
MI	310.11 ± 1.08	19.35 ± 3.12	58.65 ± 2.74	n.d.	77.33 ± 10.41	1.06 ± 0.06	n.d.
MI-S	85.52 ± 5.33	24.72 ± 4.12	55.28 ± 3.12	n.d.	76.60 ± 12.66	0.97 ± 0.09	36.07 ± 2.74
DEX	410	n.t.	n.t.	n.t.	75.11 ± 12.07	n.d. (< 0.16)	n.d.
DEX-S	>500	n.t.	n.t.	n.t.	70.70 ± 10.90	n.d. (< 0.16)	48.67 ± 1.40

cFR and cMI, *A. brasiliensis* fruiting body and mycelial crude polysaccharide fractions, respectively.

FR and FR-S, *A. brasiliensis* fruiting body polysaccharide and its sulfated derivative.

MI and MI-S, *A. brasiliensis* mycelial polysaccharide and its sulfated derivative.

DEX and DEX-S, dextran and dextran sulfate, purchased from Sigma (USA).

n.d., not detected or the concentration is below the detection limit between parentheses.

n.t. not tested.

Table 3

Elemental analysis of polysaccharides from *Agaricus brasiliensis*

	Elements (% w/w)				
Sample	**Carbon**	**Hydrogen**	**Nitrogen**	**Sulfur**	**DS[a]**
FR	34.42	5.56	2.50	0	0
FR-S	17.15	3.52	1.62	14.37	1.88
MI	32.07	4.36	1.63	0	0
MI-S	21.10	3.60	1.80	14.77	1.58
DEX-S	14.90	3.10	0	10.72	1.62

FR and FR-S, *A. brasiliensis* fruiting body polysaccharide and its sulfated derivative;

MI and MI-S, *A. brasiliensis* mycelial polysaccharide and its sulfated derivative.

DEX-S, dextran sulfate, purchased from Sigma.

[a]Degree of substitution DS= 2.25 x (S % /C %)

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Table 4

^{13}C and ^{1}H NMR spectroscopic data of *Agaricus brasiliensis* polysaccharides and its sulfated derivatives

Sample	Residue	Chemical shift (ppm)						
		C/H-1	C/H-2	C/H-3	C/H-4	C/H-5	C/H-6	C/H-6 term[c]
FR	(1→6)-β-D- glucose	103.14 (4.60–4.73)	73.18 3.47	75.71 3.75	70.92, 68.97[b] 3.68	75.03 3.88	69.60 (4.06–4.13)	63.34 (4.30–4.46)
FR-S	(1→6)-β-D- glucose	103.14 (4.72–4.78)	73.18 3.50	75.71 (3.70–3.90)	n.d. **(76.07)**[a], 68.95[b] n.d. **(4.65)**[a]	75.03 (4.00–4.22)	69.60 4.39	n.d. **(71.59)**[a] n.d. **(5.32)**[a]
FR	(1→3)-β-D- glucose	103.14 (4.96, 4.94)	73.18 3.86	n.d. (3.94, 3.96)	69.35 3.79	75.71 3.77	60.69 (3.90, 3.91)	63.34 (4.20–4.29)
FR-S	(1→3)-β-D- glucose	103.14 (4.85, 4.90)	73.18 (3.70–3.90)	n.d. (4.00–4.22)	n.d. (**74.25**)[a] n.d. **(4.59)**[a]	75.71 (3.70–3.90)	60.69 3.90 **(5.07)**[a]	n.d. **(71.59)**[a] n.d. **(5.27)**[a]
MI	(1→2)-β-D- mannose	104.88, 98.55 5.30	78.59 (3.20–4.00)	74.11 (3.20–4.00)	72.26 (3.20–4.00)	78.40 (3.20–4.00)	63.25, 3.40 (3.20–4.00)	60.08 (3.20–4.00)
MI-S	(1→2)-β-D- mannose	105.71, 98.48 5.35	78.63 (3.80–4.10)	74.00 **(78.91–79.69)**[a] 3.70 **(4.30)**[a]	72.05 **(77.64)**[a] 3.60 **(4.20)**[a]	78.33 3.37	63.21 **(70.43)**[a] (3.80–4.10) **(5.46)**[a]	n.d. **(69.10)**[a] (3.80–4.10) **(5.92)**[a]
MI	(1→3)-β-D- glucose	102.56, 94.74 (4.60–4.90)	75.40 (3.20–4.00)	n.d. (3.20–4.00)	72.00 (3.20–4.00)	76.79 (3.20–4.00)	63.40 (3.20–4.00)	60.08 (3.20–4.00)
MI-S	(1→3)-β-D- glucose	102.27, 94.60 (4.60–4.90)	75.59 **(80.87–80.93)**[a] 3.60 **(4.20)**[a]	86.71 (3.80–4.10)	71.25 **(77.28)**[a] 3.48 **(4.13)**[a]	76.72 3.37	63.41 **(70.55)**[a] (3.80–4.10) **(5.85)**[a]	n.d. **(69.10)**[a] (3.80–4.10) **(5.92)**[a]

FR and FR-S, *A. brasiliensis* fruiting body polysaccharide and its sulfated derivative.

MI and MI-S, *A. brasiliensis* mycelial polysaccharide and its sulfated derivative.

[a] Assignments corresponding to the respective sulfation sites are shown in bold font between parentheses.

[b] Assignment for the C4 of (1→6)-β-D-glucose linked to the side chain in C3.

[c] term.: corresponding to the terminal sugar residues.

n.d.: not detected.

Chemical shifts were assigned using the CASPER program [60] and according to previously published data [16, 35, 42, 52].

Table 5

Inhibitory effect of *Agaricus brasiliensis* polysaccharides in A549 cell proliferation (MTT assay)

	48 h		72 h		Increase in Cytotoxicity
Sample	**EC_{50}[a]**	**95 %Confidence Interval**	**EC_{50}[a]**	**95 %Confidence Interval**	**EC_{50} 48 h/ EC_{50} 72 h**
FR	>1500	-	1147	1006 to 1307	>1.3
FR-S	605.6	440.6 to 832.5	222.5	153 to 323.6	2.7
MI	>1500	-	>1500	-	-
MI-S	342.1	275.4 to 425.1	60.66	50.41 to 73	5.6
DEX	>1500	-	>1500	-	-
DEX-S	991.6	600.5 to 1638	783.1	560.6 to 1094	1.3
Paclitaxel	0.31	0.22 to 0.45	0.056	0.03 to 0.11	5.5

FR and FR-S, *A. brasiliensis* fruiting body polysaccharide and its sulfated derivative;

MI and MI-S, *A. brasiliensis* mycelial polysaccharide and its sulfated derivative;

DEX and DEX-S, dextran (410 kDa) and dextran sulfate (> 500 kDa), Sigma.

[a] 50 % effective concentration (μg/mL)

Table 6

Inhibitory effect of sulfated *Agaricus brasiliensis* polysaccharides in A549 and Vero cells growth by the sulforhodamine method

	FR-S	MI-S	Paclitaxel
Parameter	**A549**		
GI_{50}[a]	155.40 ± 24.32	160.90 ± 11.95	0.23 ± 0.03
TGI[b]	598.80 ± 63.89	488.30 ± 63.43	0.19 ± 0.01
LC_{50}[c]	1042.00 ± 97.43	815.70 ± 80.59	0.62 ± 0.04
	Vero		
GI_{50}[a]	>1500	>1500	0.12 ± 0.01
TGI[b]	>1500	>1500	0.46 ± 0.05
LC_{50}[c]	>1500	>1500	1.05 ± 0.12
SI[d]	>1.4	>1.8	1.7

FR-S, sulfated derivative of *A. brasiliensis* fruiting body polysaccharide; MI-S, sulfated derivative of *A. brasiliensis* mycelial polysaccharide.

[a] Median growth inhibition;

[b] Total growth inhibition;

[c] Median lethal inhibition. Values represent the mean ± S.D. of two independent experiments and are expressed in μg/mL.

[d] Selectivity index: calculated as LC_{50} Vero/LC_{50} A549.