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Sulfonation of (1→6)-β-D-Glucan (Lasiodiplodan) and Its Antioxidant and Antimicrobial Potential

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Abstract: The objective of this study was to investigate the sulfonation of (1→6)-β-D-glucan (lasiodiplodan) as a potentiating mechanism for biological functionalities. Lasiodiplodan was sulfonated by the chlorosulfonic acid-pyridine method. The modified exopolysaccharide was characterized by FT-IR and ^{13}C NMR spectroscopy, X-ray diffraction and SEM. Antioxidant activity was assessed by the methods of H_2O_2 and OH radical removal and reducing power. Antimicrobial potential was evaluated by the broth-microdilution method. Sulfonation resulted in a derivative with DS of 0.24. FT-IR analysis indicated the introduction of sulfonyl groups in the macromolecule structure through specific bands in the regions of 1,240 cm^{-1} and 810 cm^{-1}. ^{13}C NMR analysis suggested that sulfonation occurred at carbon 2 of the glucose residue. Sulfonation led to morphological changes in the structure of the biopolymer resulting in a heterogeneous structure with the presence of fibrils. Derivatization promoted an increase in the antioxidant ability of the macromolecule, with a high OH removal potential (74.32%). Bacteriostatic activity against *E. coli* (*Escherichia coli*) and *S. enterica* (*Salmonella enterica*) typhimurium and fungicidal activity against *C. albicans* (*Candida albicans*) and *C. tropicalis* (*Candida tropicalis*) were found in the sulfonated sample. Sulfonation potentiated the antioxidant and antimicrobial activities of the biomacromolecule, suggesting that it is a potentiating mechanism of biological functions.

Key words: Chemical derivatization, microbial polysaccharides, bioactivity, sulfonate groups.

1. Introduction

β-Glucans are polysaccharides composed of D-glucose units linked by β-glucosidic bonds and may have linear or branched structures [1, 2]. These biomacromolecules present peculiar physical, chemical and biological characteristics that are associated with their molecular structure and conformation, type of branching and glycosidic bonds, which makes them interesting for different applications in the medical, pharmaceutical, cosmetic and food sectors [1, 3, 4].

Different biological functionalities such as immunomodulatory activity [5], antitumor [6], anti-aging [7], anti-inflammatory [8], hypocholesterolemic [9], antioxidant [10] and antimicrobial potential [11] have been described in the scientific literature on β-glucans. These biomacromolecules can be found in the cell wall of yeasts and filamentous fungi, algae and also in cereal grains such as oats and barley. In addition, some microorganisms can produce and excrete β-glucans into the extracellular medium as exopolysaccharides [12-14].

Lasiodiplodan is an exocellular (1→6)-β-D-glucan produced by the filamentous fungus *Lasiodiplodia theobromae* MMPI when grown in sugar rich medium, especially glucose and sucrose [12, 15]. This carbohydrate biopolymer has demonstrated different biological functions, among them being antioxidant

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activity [13, 16], protective activity against doxorubicin-induced DNA damage [17], hypoglycemic and transaminase activities [18], and anticancer activity in breast cancer cells [19].

Chemical derivatization of these biopolymers has been shown to be an important tool to enhance their biological functionalities. Among the chemical modifications described in the scientific literature, is sulfonation (Fig. 1), which involves the substitution of hydroxyl groups by sulfonate groups (S^+O_2OH) [20, 21].

The sulfonation reaction is usually performed using chlorosulfonic acid, anhydrous sulfuric acid and complexes of sulfur trioxide-pyridine as derivatizing agents [21]. The DS (degree of substitution) of the molecule can be influenced by structural or intrinsic factors, which include the chemical properties of the biomolecule, molecular weight, type and configuration of the glycosidic bonds, and reaction or extrinsic factors, such as reaction temperature and time, type of derivatizing agent employed and the ratio of the organic solvent to the sulfonation reagent used [21].

Studies have shown that sulfonation increases the biological potential of β-glucans, especially the relation to anticoagulant activity [22, 23], antiviral [24], and anti-proliferation against cancer cells [11], antioxidant activity [25], and antibacterial activity [23]. Such biological potential functions make the sulfonated polysaccharides important biomaterials that can be widely used in the medical field, especially in the pharmaceutical and cosmetic sectors [21, 22].

In this context, the objectives of the work reported herein was to chemically modify the exocellular (1→6)-β-D-glucan (lasiodiplodan) from *Lasiodiplodia theobromae* by sulfonation, and to characterize and compare the native and sulfonated polysaccharides by FT-IR and ^{13}C NMR spectroscopy, scanning electron microscopy and X-ray diffraction. Antioxidant ability and antimicrobial potential were also evaluated on the native and sulfonated polysaccharides.

2. Method and Materials

2.1 Materials

Pyridine, chlorosulfonic acid and dialysis membranes (12,000 Da, 1.3 in. Width, MWCO 11,331) were obtained from Sigma-Aldrich (St Louis, MO, USA). Glucose, dimethylsulfoxide, sodium bicarbonate, hydrochloric acid and trichloroacetic acid were obtained from Vetec Química Fina Ltda (Duque de Caxias-RJ, Brazil). All reagents used in this study were of analytical grade.

2.2 Production and Isolation of Native Lasiodiplodan

Lasiodiplodan (LAS-N) was produced by submerged fermentation (250 mL Erlenmeyer flasks) by the ascomyceteous fungus, *Lasiodiplodia theobromae* MMPI in minimum salts medium (100 mL) [26], containing glucose (20 g·L^{-1}), and standard inoculum (10 mL). The flasks were incubated at 28 °C for 72 hours under 150 rpm agitation as described by Cunha et al. [12]. At the end of the fermentation, the culture fluid was separated from the fungal biomass by centrifugation (1,500 × *g*, 15 minutes, 4 °C), and lasiodiplodan was precipitated with 3 volumes of absolute ethanol at 4 ºC (overnight). The precipitate (LAS-N) was resolubilized in distilled water by heating

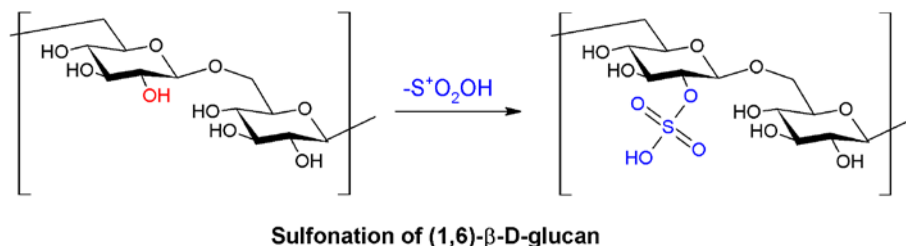

Fig. 1 Derivatization of lasiodiplodan by sulfonation.

at 60 ºC for 2 hours, and then subjected to intensive dialysis against distilled water for five days using dialysis membranes, and the lasiodiplodan solution lyophilized and stored at 4 °C.

2.3 Sulfonation

Sulfonation was performed according to protocols described by Vasconcelos et al. [22] and Wang and Zhang [27], with subtle modification: LAS-N (50 mg) was solubilized in dimethylsulfoxide (DMSO, 10 mL) under vigorous stirring for 24 hours at environmental temperature. Subsequently, pyridine (10 mL) was added and the solution stirred for two hours at environmental temperature. Then, chlorosulfonic acid (4 mL) was added dropwise, and the solution was kept in an ice bath for 2 hours, and the resulting mixture left at 4 ºC for 15 hours. Sulfonation was terminated by the addition of ice-cold water (5 mL) and the mixture neutralized by the addition of sodium bicarbonate (10%, v/v) until complete elimination of CO_2. The resulting solution was extensively dialyzed against distilled water for six days, and then lyophilized producing a product denoted as LAS-S.

The DS was determined according to the protocol described by Mendes et al. [28] with minor modifications. Initially, samples of LAS-S (1 mg) were hydrolyzed with concentrated hydrochloric acid (12.23 mol·L^{-1}) in a thermostatic bath at 100 ºC for 10 minutes. Next, 0.2 mL of the LAS-S hydrolyzates were added to test tubes containing 3.8 mL of trichloroacetic acid (TCA, 3% w/v), and then 1.0 mL of protective solution (6 g NaCl, 0.5 mL concentrated. HCl, 0.1% gelatin (w/v) and 47 mL of distilled water) and 0.03 g of barium chloride were added. The tubes were shaken vigorously for 1 minute and left standing for 15 minutes. The resulting barium sulfate was quantified spectrophotometrically at 420 nm. The DS, which designates the average number of sulfonate groups on each glucose residue of lasiodiplodan, was established from the sulfur content described in Eqs. (1) and (2), where S = % sulfur, 162 = average molecular weight of glycosyl residue (180 - 18) in polysaccharide, 3200 = molecular weight of sulfur (32 x 100), 102 ($Mw_{NaHSO4} - MwH_20$) = molecular weight of sulfonate group after sufonation (120 - 18):

$$S(\%) = \frac{BaSO_4(\mu g) \text{ X } 0.1314 \text{ X } 100}{\text{Sample } (\mu g)} \quad (1)$$

$$DS = \frac{162 \text{ X } S}{3200 - 102 \text{ X } S} \quad (2)$$

2.4 Characterization of Native and Sulfonated Lasiodiplodan

2.4.1 FT-IR (Fourier Transformation-Infrared Spectroscopy)

FT-IR spectra were obtained on a Frontier spectrometer (Perkin Elmer®, USA), in the spectral range of 400-4,000 cm^{-1}, with a resolution of 2 cm^{-1} and the number of accumulations of 16 scans for each spectrum using the potassium bromide (KBr) disc technique.

2.4.2 ^{13}C Nuclear Magnetic Resonance

The ^{13}C solid-state NMR spectra were obtained on a nuclear magnetic resonance imaging spectrometer (Bruker Avance III 300 Spectrometer, 7.1 T), combining the cross-polarization technique with magic-angle spinning (CP/MAS) at 10 kHz and proton decoupling in a 5 mm MAS broadband probe. Pulses of 90º were used with a repetition time of 3 seconds, and CP time of 3 minutes.

2.4.3 SEM (Scanning Electron Microscopy)

SEM was used to examine the surface morphology of the native and derivatized samples of lasiodiplodan. Micrographs were acquired in a scanning electron microscope (Hitachi TM3000, USA) using lyophilized samples. Images were taken at magnifications of 200× and 1,500×.

2.4.4 XRD (X-Rays Diffraction)

X-ray diffractogram patterns were obtained on a Rigaku MiniFlex600 diffractometer, using copper radiation font (CuKα = 1.5418Å), 15 mA current, 40 kV voltage, scanning range of 10° to 60° (2θ), a scanning speed of 5° min^{-1} and a step width of

0.02° (2*θ*).

2.5 Antioxidant Activity

The antioxidant potential of samples of LAS-N and LAS-S was evaluated by the methods of hydrogen peroxide removal, hydroxyl radical removal and reducing power, according to the methodology described by Liu et al. [29]. DMSO (20%, v/v) was used as the solubilizing agent of the native and sulfonated polysaccharide samples (0.88 g·L^{-1}).

2.6 Antimicrobial Activity

Antimicrobial activity was evaluated by the broth-microdilution method according to the procedure described by Krichen et al. [30], with modifications. LAS-N and LAS-S samples were evaluated against two Gram-positive bacterial strains (*Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 19659), and two Gram-negative strains (*Salmonella enterica* Typhimurium ATCC 0028 and *Escherichia coli* ATCC 25922). The antimicrobial performance of the native and sulfonated samples was also assessed against the yeasts, *Candida tropicalis* ATCC 13803 and *Candida albicans* ATCC 118804.

Aliquots (200 μL) of LAS-N and LAS-S diluted in 20% DMSO at a concentration of 3.84 mg·mL^{-1} were added in tubes together with 200 μL of Mueller-Hinton broth and 10 μL of a previously standardized microbial suspension at the McFarland scale of 0.5 (1.5×10^8 CFU mL^{-1}). The tubes were incubated for 24 hours at 37 °C (bacteria) or 28 °C (yeast). Subsequently, 20 μL (100 mg L^{-1}) of the resazurin dye-reagent (Sigma-Aldrich, USA) was added to the tubes to confirm the presence of viable cells. Samples with positive results (inhibition of the microorganism) were transferred to Petri dishes containing nutrient-agar (bacteria) or Sabouraud-agar with chloramphenicol (yeast-grown cultures), which were, in turn, incubated for 24 hours in a bacteriological oven at 37 °C (bacteria) or 28 °C (yeast) to verify microbial activity. Tetracycline and fluconazole were used as positive controls for bacteria and yeast, respectively. Saline solution (0.9%) and DMSO (20%) were used as negative controls.

3. Results

3.1 Chemical Characterization

The sulfonation reaction conditions employed led to the production of a sulfonated derivative with a degree of substitution of 0.24, which corresponds to 0.24 sites occupied per D-glucose residue along the biomacromolecule backbone chain. Considering the degree of purity (97%) of lasiodiplodan, and that there are three potential substitution sites (hydroxyl groups) per monomeric (glucose) unit in the macromolecule, the yield of sulfonation derivatization was approximately 8%.

The effectiveness of the sulfonation reaction was confirmed by FT-IR analysis and spectra of LAS-N and LAS-S are shown in Fig. 2. Both LAS-N and LAS-S samples displayed typical polysaccharide signals in the region between 4,000 and 400 cm^{-1}. Bands in the regions at 3,337 cm^{-1} and 3,418 cm^{-1} (stretching OH), 2,938 cm^{-1} and 2,927 cm^{-1} (stretching CH methylene), 1,640 cm^{-1} and 1,646 cm^{-1} (stretching vibration of the glucose ring), 1,068 cm^{-1} and 1,074 cm^{-1} (CO elongation of the pyranose ring) were confirmed for the respective polysaccharides, LAS-N and LAS-S. The band at 886 cm^{-1} in the LAS-N spectrum is characteristic of β-type configuration [31-34].

FT-IR spectra of LAS-S show a band of medium intensity in the 1,240 cm^{-1} region, which corresponds to asymmetric vibrational stretching (strong) S = O associated with the distribution of sulfonate groups on the molecule [22, 28, 31, 33, 34]. Bands in the 810 cm^{-1} region correspond to the C-O-S symmetrical vibration, associated with the C-O-SO_3 group, indicating that the native polysaccharide was sulfonylated [20, 22, 28, 34, 35].

The ^{13}C NMR spectra from LAS-N and LAS-S are presented in Fig. 3.

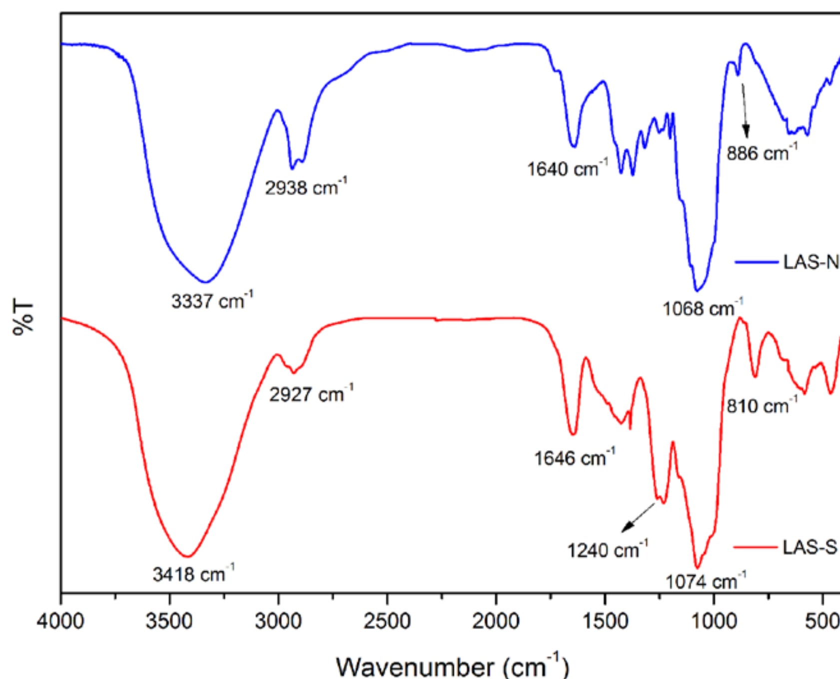

Fig. 2 FT-IR spectra of native (LAS-N) and sulfonated (LAS-S) lasiodiplodan.

^{13}C NMR analysis showed that lasiodiplodan has a β-configuration as judged by a typical chemical shift at δ103.7 (C-1) and previously described by Kagimura et al. [13]. The chemical shifts observed in the LAS-S sample at δ86.5 and δ62.4 were attributed to carbons 3 and 5, respectively. The C-2 signal was not observed on both the LAS-N or LAS-S spectra, suggesting an overlap with the C-4 signal, which presents itself as a broadband. The regio-selective esterification at the C-2 position in the LAS-S spectrum can be observed from the chemical shift at 170.8 ppm attributed to the carbon bound to the sulfonate group.

Fig. 4 shows the micrographs obtained by scanning electron microscopy. The LAS-N sample presented a microstructure containing thin films with a relatively homogeneous surface, but with regions with folds and twist-like formations (Figs. 3A and 3B). After sulfonylation, morphological changes were observed on the surface of microstructure of the biopolymer, and LAS-S was found to have a heterogeneous surface containing fibre structures along its surface (Figs. 4C and 4D).

In order to evaluate the crystallinity, the LAS-N and LAS-S samples were subjected to X-ray diffraction analysis and the diffraction profiles of native and sulfonated samples are shown in Fig. 5. The X-ray diffractogram profile of the native and sulfonated samples indicated that lasiodiplodan has an amorphous matrix with crystalline regions.

Three diffraction peaks at 2θ with values near to 21.17°, 23.58° and 39.96° were identified in both diffractograms (LAS-N and LAS-S) indicating coherent crystalline planes. Actually, organic macromolecular compounds such as β-glucans present diffractographic profiles typical of amorphous materials. After the sulfonation reaction, peaks in the regions near 11.64°, 29.15° and 31.15° at 2θ were observed, suggesting that the introduction of sulfonate groups in the lasiodiplodan structure contributed to the formation of regions with certain molecular organization as shown in Fig. 4.

3.2 Biological Activities of Native and Sulfonated Lasiodiplodan

Although the introduction of sulfonate groups onto polysaccharide macromolecules is increasingly

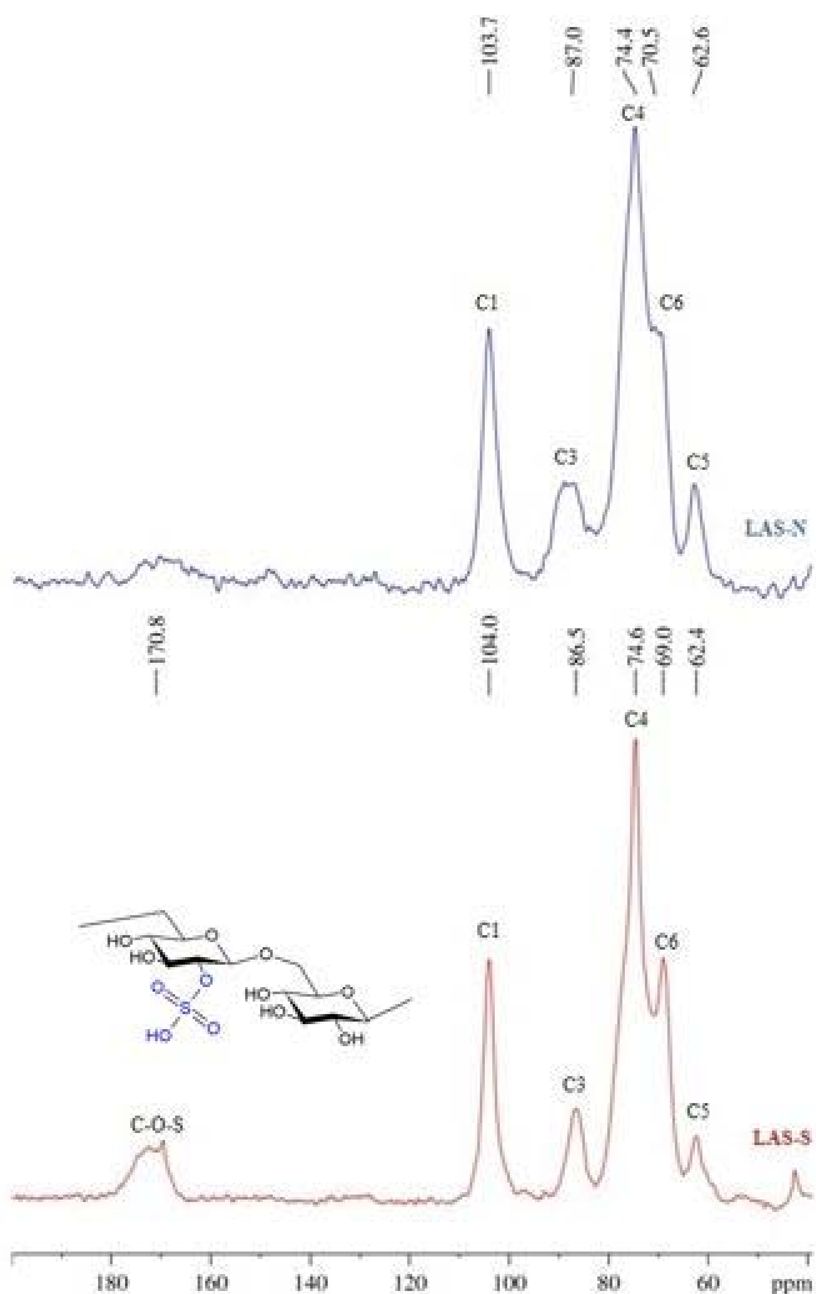

Fig. 3 Solid-state ^{13}C NMR spectra of native (LAS-N) and sulfonated (LAS-S) lasiodiplodan.

recognized as a function of fundamental importance, the sulfonation of endogenous molecules is still a biological phenomenon not always easily understood [36]. Many studies have demonstrated that the sulfonation of natural polysaccharides is an important way of obtaining new antioxidants agents and the sulfonate groups take an important role in antioxidant activities [37]. The sulfonation of polysaccharides could not only enhance their water solubility, but also changes the chain conformation, thus resulting in the enhanced bioactivities [38]. According to Wang et al. [39], the introduction of chemical groups onto polysaccharide molecules could lead to weaker dissociation energy of hydrogen bonding, increasing therefore, the hydrogen donating ability of the macromolecule. Furthermore, chemical derivatization

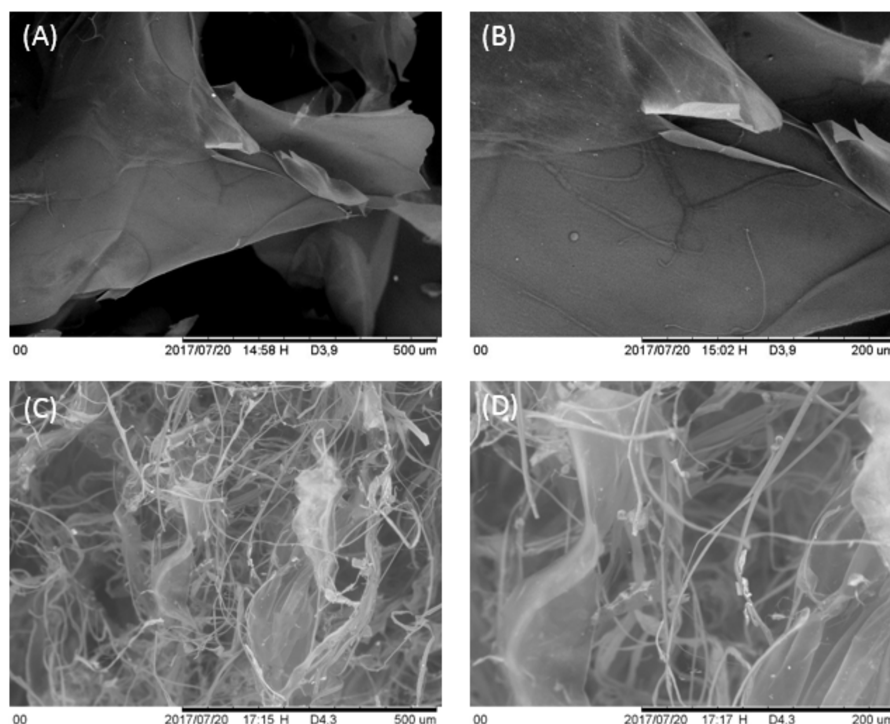

Fig. 4 Micrographs (SEM) of LAS-N (A, B) and LAS-S (C, D) at 200× and 500×.

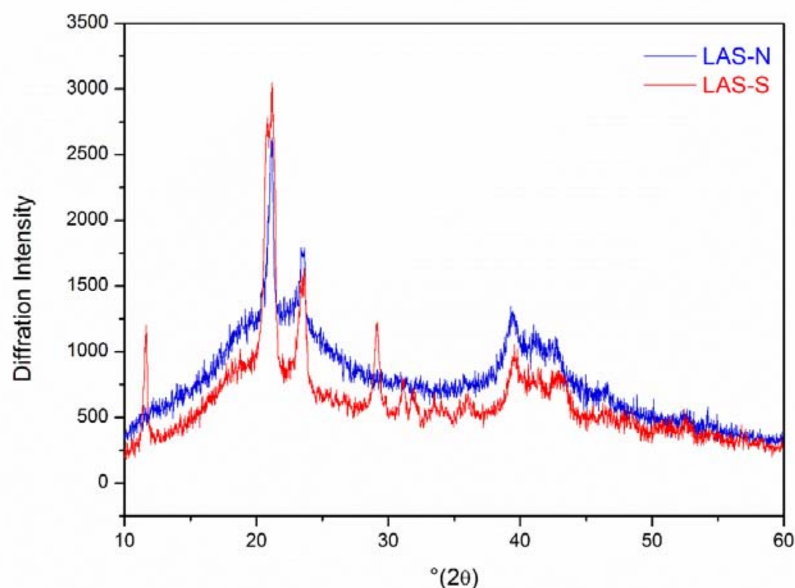

Fig. 5 X-ray diffractograms of LAS-N and LAS-S.

can sometimes promote a decrease in molecular weight, hence improving the antioxidant potential of polysaccharides. The sulfonated polysaccharides usually trap free radicals in an electrostatic manner since the sulfonate groups usually generate a highly acidic environment and the sulfur substitution may also weaken hydrogen bond interactions between polysaccharides [39].

The mechanism of antimicrobial action of polysaccharides also is not yet clear. HE et al. [40] proposed that these biomolecules act in the rupture of the cell wall and cytoplasmic membrane, leading to

the extravasation of essential molecules, in addition to causing degradation of DNA of the microorganism.

3.2.1 Antioxidant Potential

The ability to remove hydrogen peroxide by the LAS-N and LAS-S samples, glucose (Glc) and ascorbic acid (AAc) is shown in Fig. 6. Ascorbic acid was evaluated as an antioxidant standard, and the antioxidant activity of glucose was assessed as it is the monomeric repeat residue of the macromolecule lasiodiplodan.

Glucose monomer demonstrated low hydrogen peroxide removal capacity (1.58%), whereas LAS-N did not display hydrogen peroxide removal activity at the concentration evaluated. On the other hand, after sulfonation the macromolecule (LAS-S) was able to remove 4.8% of the hydrogen peroxide, although this potential was lower to that demonstrated by the antioxidant standard ascorbic acid (22%) (Fig. 6).

In relation to the reducing power, both LAS-N and LAS-S samples demonstrated ferric ion reduction ability, as can be observed in Fig. 7. In addition, an increase in the antioxidant capacity of the sample was

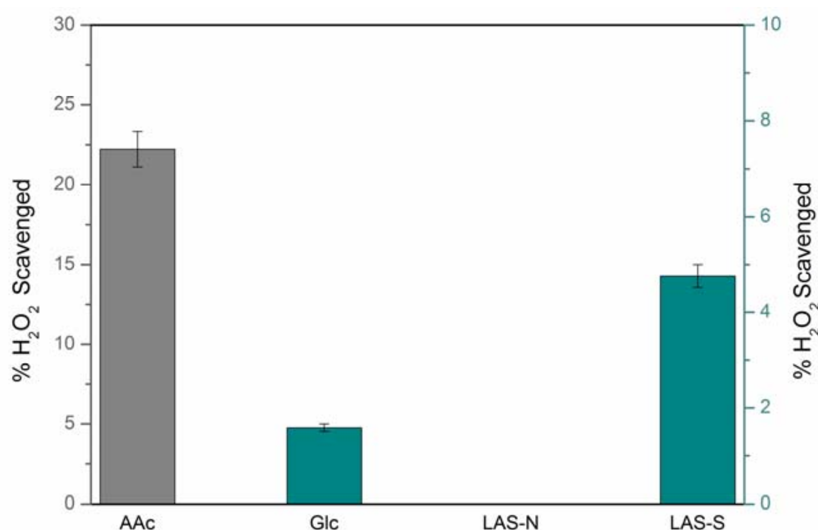

Fig. 6 Antioxidant potential of ascorbic acid (AAc; positive control), glucose (Glc) and native (LAS-N) and sulfonated (LAS-S) lasiodiplodan as measured by H_2O_2 removal capacity.

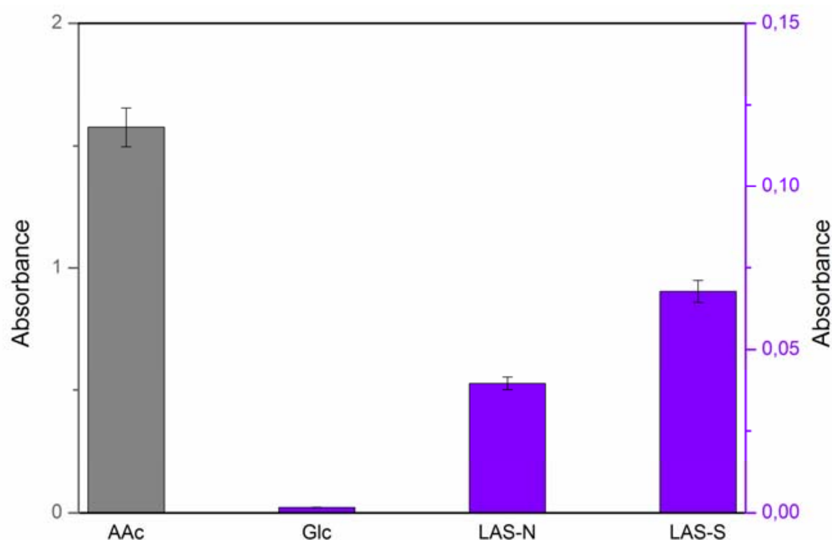

Fig. 7 Antioxidant potential of ascorbic acid (AAc; positive control), glucose (Glc) and native (LAS-N) and sulfonated (LAS-S) lasiodiplodan as measured by ferric ion reducing power.

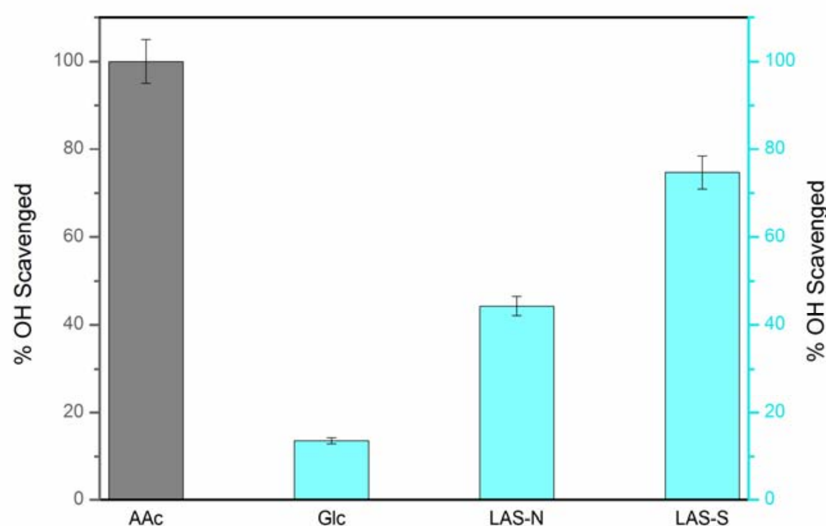

Fig. 8 Antioxidant potential of ascorbic acid (AAc; positive control), glucose (Glc) and native (LAS-N) and sulfonated (LAS-S) lasiodiplodan as measured by OH radical removal capacity.

observed after sulfonation. Similarly to that observed in the H_2O_2 removal assay, the ascorbic acid standard showed a reduction potential higher than the lasiodiplodan samples. Glucose monomer did not display antioxidant potential, which differed to that observed in the H_2O_2 removal assay.

Fig. 8 shows the results of the assessment of hydroxyl radical removal capacity for the analyzed samples and ascorbic acid as the standard. LAS-N presented a 44.32% hydroxyl radical removal activity, while the sulfonated sample (LAS-S) exhibited an increase in the antioxidant potential, reaching a maximum removal capacity of 74.32%. In this assay procedure, glucose also presented free hydroxyls and showed removal activity of 13.68%. Ascorbic acid, the reference antioxidant standard presented 100% removal capacity, and was used for normalization of the data.

3.2.2 Antimicrobial Potential

Table 1 shows the results regarding the antimicrobial potential of the LAS-N and LAS-S samples as evaluated against different bacteria and yeasts. LAS-N did not show antimicrobial activity against any of the microbial strains evaluated. However, after sulfonylation bacteriostatic effects were displayed against the Gram-negative bacteria: *S. enterica* Typhimurium and *E. coli*. In addition, a fungistatic and fungicidal effect against the yeasts *C. tropicalis* and *C. albicans* was observed. On the other hand, no antimicrobial effects were observed against the Gram-positive bacterial strains evaluated.

4. Discussions

The chlorosulfonic acid-pyridine method is the most used protocol for the derivatization of polysaccharides by sulfonation. However, it is necessary to carefully control the reaction conditions to avoid the degradation of the macromolecule. Some studies have shown the re-sulfonation is a tool to obtain derivatives with higher degrees of substitution, and is a better step than using more drastic reaction conditions to improve the DS. In our work, we opted to use the standard condition for sulfonation without re-sulfonation, seeking to preserve the integrity of the polysaccharide structure. This procedure resulted in producing a sulfonated derivative of a relatively low degree of substitution (DS: 0.24). It's to be noted that even a low degree of substitution may promote appreciable enhancements in the chemical properties and biological functions of the polysaccharide. Similar results were observed by Zhang et al. [34] after derivatization of an exocellular heteropolysaccharide

composed of glucose and galactose (molar ratio 1.80:1.03) from *Streptococcus thermophilus* GST-6 (DS: 0.26) that showed improved antioxidant activity following derivatization. They also found that the derivatized exopolysaccharide showed stronger inhibitory activity against several bacteria, *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus*.

The spectra obtained in the infrared region (Fig. 1) presented characteristic bands of carbohydrates, constituted by monomeric units linked by β-glycosidic bonds [27, 32]. After sulfonation, bands characteristic of the insertion of sulfonate groups in the derivatized sample appeared in the regions of 1,240 and 810 cm^{-1} [20]. Similar observations have been reported by Vasconcelos et al. [22] and Zhang et al. [34] for sulfonated exopolysaccharides.

Broader signals in the ^{13}C NMR spectrum were observed after the sulfonation of the hydroxyl groups of β-glucans, which make its interpretation more complicated [41]. Furthermore, the carbons directly attached to the electronegative sulfonated ester groups shift signals to downfield. In contrast, the carbons indirectly attached (i.e., C-3) to the sulfonyl groups shift to an upfield position [22]. In the present study, we assumed that the sulfonation of lasiodiplodan occurred at C-2, since a chemical shift to an upfield position in the C-3 neighboring carbon (Δ = 1.3 ppm) of the sulfonate group in LAS-S was observed, which is absent in the Las-N sample. There is no indication of sulfonation at the C-4 and C-5 positions, since there was no significant variation (Δ < 0.3 ppm) of chemical signals shift in C-4 (Δ < 0.2 ppm) and C-6 (Δ < 0.1 ppm) in the LAS-N and LAS-S samples.

Significant changes of the surface structure of lasiodiplodan occurred after sulfonation and the arising of fibrils along the microstructure of the derived polysaccharide (Figs. 3C and 3D) can possibly be associated with a decrease in the hydrogen interactions along the macromolecule structure after substitutions of hydroxyls by the sulfonate groups. Wang et al. [20] also observed structural morphological changes in a polysaccharide from *Cyclocarya paliurus* after sulfonation by the chlorosulfonic acid-pyridine method, compared to the unmodified native polysaccharide.

The scientific literature emphasizes that sulfonation derivatization may confer an increase in the biological potential of the polysaccharides [4, 21]. Such behavior was observed in the present study in relation to antioxidant (Figs. 4-6) and antimicrobial activities (Table 1).

In relation to the ability to remove hydrogen peroxide, glucose was more effective (1.58%) than LAS-N (Fig. 4). This behavior may possibly be explained by the fact that glucose in its cyclic form presents five hydroxyls available for reaction with hydrogen peroxide, whereas LAS-N presents a smaller number of free hydroxyls, which can participate in intramolecular and intermolecular hydrogen interactions, thus reducing their potential for hydrogen peroxide removal.

Table 1 Antimicrobial potential of the LAS-N and LAS-S samples against different microorganisms: (-) resistance; (+) inhibition.

Microorganism	Inhibition observed		Bacteriostatic/fungistatic activity		Bactericidal/fungicidal activity	
	LAS-N	LAS-S	LAS-N	LAS-S	LAS-N	LAS-S
Staphylococcus aureus ATCC 25923	-	-	-	-	-	-
Bacillus subtilis ATCC 19659	-	-	-	-	-	-
Salmonella enterica Typhimurium ATCC 0028	-	+	-	+	-	-
Escherichia coli ATCC 25922	-	+	-	+	-	-
Candida tropicalis ATCC 13803	-	+	-	+	-	+
Candida albicans ATCC 118804	-	+	-	+	-	+

The reduction power is based on the electron donation ability of the polysaccharide to potassium ferricyanide [$K_3Fe(CN)_6$], which is reduced to potassium ferrocyanide [$K_4Fe(CN)_6$] [42]. As shown in Fig. 5, LAS-N and LAS-S presented similar results, demonstrating that sulfonation did not promote a significant increase in the macromolecule reducing potential.

For the increase of the reducing power, an adjustment in the degree of sulfonation may be necessary, since this can promote increased solubility and consequently in the potentialities of the biomolecule [21]. Such behavior was observed by Zhang et al. [25] who found a dose-dependent relationship between the concentration of the sulfonated polysaccharide and antioxidant capacity.

The ability to remove the hydroxyl radical is related to the chelation of transition metal ions, such as Fe^{2+} and Cu^{2+}, in order to make them inactive for the elimination of these radicals [33]. This ability was found for LAS-N (44.32%) and LAS-S (74.32%), since they presented high percentages of OH removal, especially after derivatization. This event increased the antioxidant capacity of the macromolecule to 30% relative to native lasiodiplodan (Fig. 6). Results similar to those obtained in the present study were reported by Zhang et al. [25], which verified a 76.2% hydroxyl radical removal capacity in the polysaccharide from *Diospyros kaki* L. after sulfonation (DS 0.88).

As demonstrated in the present study, a notable ability of hydroxyl radical removal by a derivatized polysaccharide with low degree of substitution was also reported by Xie et al. [37]. These authors evaluated the sulfonation of polysaccharides extracted from *Cyclocarya paliurusi* and found a 63.14% OH radical removal capacity in the sulfonated polysaccharide with DS of 0.25.

Scientific reports have indicated that polysaccharides following chemical derivatization by sulfonylation present an increase in antimicrobial capacity [11, 23]. This effect was also observed in the present study with sulfonated lasiodiplodan with DS of 0.24 (Table 1), which presented antimicrobial potential against Gram-negative bacteria (*E. coli* and *S. enterica* Typhimurium) and the yeasts (*C. albicans* and *C. tropicalis*). Similarly, Zhang et al. [34] evaluated the antimicrobial potential of a sulfonated polysaccharide with DS close to 0.24, and also found inhibitory activity against the Gram-negative bacteria (*E. coli* and *S. enterica* Typhimurium), suggesting that sulfonation is an effective method to improve the bioactivity of polysaccharides. Similarly, Wan-Mohtar et al. [11] observed antimicrobial activity of sulfonated polysaccharides against strains of *E. coli* and *S. enterica* Typhimurium evaluating sulfonated polysaccharide concentrations close to those employed in this study.

The mechanism of antimicrobial action of these biomolecules is not well understood, but it is believed that these can act on multiple targets including rupture of the cell wall and cytoplasmic membrane, causing extravasation of the intracellular contents and degradation of essential molecules to cell life, besides promoting damage to the DNA of the microorganism [43].

5. Conclusions

The exocellular (1→6)-β-D-glucan (lasiodiplodan) from *L. theobromae* MMPI was sulfonated by the chlorosulfonic acid-pyridine method, which produced a derivative with degree of substitution of 0.24. The effectiveness of sulfonation derivatization was evaluated by FT-IR spectroscopy and ^{13}C nuclear magnetic resonance. FT-IR spectral analysis indicated that lasiodiplodan was sulfonated as revealed by specific bands associated with sulfonate groups, and ^{13}C NMR analysis suggested that sulfonation occurred at the C-2 position. Sulfonation promoted significant morphological changes in the microstructure of lasiodiplodan, which acquired a heterogeneous appearance with fibril structures distributed along the

surface. XRD analysis revealed that sulfonation contributed to the appearance of semi-crystalline regions in the biopolymer structure. The antioxidant ability of lasiodiplodan was improved after sulfonation, especially in relation to the hydroxyl radical removal capacity. Sulfonation promoted the antimicrobial potential of lasiodiplodan against *E. coli*, *S. enterica* Typhimurium, *C. albicans* and *C. tropicalis*. Derivatization of lasiodiplodan can be an important tool for the enhancement of its biological activities or contributing to producing new bioactivities, which can make the macromolecule attractive for applications in the pharmacological and chemical sectors. The increase in the degree of sulfonation may possibly increase the bioactive potential of lasiodiplodan.

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