Antimicrobial properties and cytotoxicity of sulfated (1,3)-β-D-glucan from the mycelium of the mushroom Ganoderma lucidum

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Abstract

Ganoderma lucidum BCCM 31549 has a long established role for its therapeutic activities. In this context, much interest has focused on the possible functions of the (1,3)-β-D-glucan (G) produced by these cultures in a stirred-tank bioreactor and extracted from their underutilized mycelium. In the existing study, we report on the systematic production of G, and its sulfated derivative (GS). The aim of this study was to investigate the G and its GS from G. lucidum in terms of antibacterial properties, and cytotoxicity spectrum against Human-Prostate-Cell (PN2TA) and Human-Caucasian-Histiocytic-Lymphoma (U937). ^{1}H NMR for both G and GS compounds showed β-glycosidic linkages and structural similarities when compared with two standards (Laminarin and Fucoidan). The existence of characteristic absorptions at 1,170 and 867 cm^{-1} in the FTIR for GS demonstrated the successful sulfation of G. Only GS exhibited antimicrobial activity against a varied range of test bacteria of relevance to foodstuffs and human health. Moreover, both G and GS did not show any cytotoxic effects on PN2TA cells, thus helping demonstrate the safety on these polymers. Also, GS shows 40% antiproliferation against cancerous U937 cells at low concentration (60 µg/mL) applied in this study compared to G

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(10%). Together, this demonstrates that sulfation clearly improved the solubility and therapeutic activities of G. The water-soluble GS demonstrates the potential multi-functional effects of these materials in foodstuffs.

Keywords

Ganoderma lucidum, (1,3)-β-D-glucan sulfate, Antimicrobial activity, Cytotoxicity

Running title: Antimicrobial activity of G. lucidum glucan sulfate

1. INTRODUCTION

Bacterial infection is one of the most significant causes of food degradation, and there is little attention on the role of food producers to prevent this phenomenon. Foodstuffs represent a rich source of nutrients often stored under conditions of permissible temperature and humidity. In addition to food degradation by microorganisms, high levels of multiplying microorganisms present in the food may initiate food poisoning which can contribute to public health problems [1] and disrupting supply chain issues worldwide. Ideally, improving the safety and spoilage characteristics of foodstuffs by including other naturally occurring products which may possess both antimicrobial, and other desirable biological activities (e.g. cytotoxicity on cancer cells, health-giving), potentially offers a route to safer foods with enhanced health-imparting characteristics. This approach makes use of the potential for ‘’bifunctional’’ effects [24] of glucan materials derived from traditional food sources, including some species of mushrooms [21]. These natural foods have been shown to be a relatively unexplored source for improvements in food safety, preservation while providing extra health benefits [40].

Mushrooms of the genus Ganoderma, have been eaten for many centuries in Asia to encourage well-being, durability and endurance [11, 22]. To date, more than 120 species of

Ganoderma have been identified across the world. In the last 30 years, there has been significant scientific interest in the species Ganoderma lucidum. This fungus has been lately shown to possesses varied health benefits, such as anti-bacterial effects [14] and antiproliferative effects on cancer cells [19]. In this study, β-glucan produced by the cultures with potential bioactivities are extracted from the mycelia.

The extracted mycelial G. lucidum β-glucan (G) is known to act as biological response modifier. Therefore, much research has focused on this fungal polysaccharide as a functional foodstuff and source for the development of biomedical drugs [14]. The clinical utilization of β-glucans has one main difficulty in addition to the limited availability referred to above, that is, their comparative absence of solubility in aqueous solution, which leads to difficulties in product analysis, formulation and delivery. This is usually ascribed to the high number of –OH groups in the β-glucan leading to the native polymer adopting a compact triple stranded helix conformation, which determines their poor solubility in aqueous condition [36]. These demonstrate the failure of existing glucan products and the proposed glucan sulfate would not.

Upon preliminary isolation from G. lucidum, the β-glucan mainly, (1-3)-β-D-glucan exists as an insoluble microparticulate. Thus, a technique such as sulfation is needed to alter the molecule's hydrophobicity thus making it water-soluble and potentially more bioactive in aqueous systems. The proposed sulfation technique has been used as an effective approach to improve the antibacterial, antiproliferative, anti-inflammatory, antitumor, and immunomodulatory activity of a range other polysaccharides [5, 17, 38, 40]. A previous effort by Williams et al.,[37] demonstrated that insoluble (1-3)-β-glucan was able to dissolve in water by a sulfation process, while increasing the positive biological functions [5].

To date, the cytotoxicity and antimicrobial activity of extracts from G. lucidum mycelia particularly the glucan sulfate (GS) have not been completely characterized. In the current study, glucan from G. lucidum mycelia was sulfated. Both glucan (G) and sulfated glucan (GS) structures were matched to known standards and screened antimicrobial and cytotoxic effects. The results showed that GS exhibited significant antimicrobial activities as well as antiproliferative responses while showing no toxic effects and hence could be utilized as a potential additive in food systems. With that, it's presence would inhibit both spoilage and pathogenic bacteria, and impart significant health benefits noted in this study.

2. MATERIALS AND METHODS

2.1 Reagents

Gentamicin susceptibility test discs (30 µg of concentration) were supplied by Thermo Scientific Oxoid (Fisher Scientific, Loughborough, UK). In this experiment, (U937) Human-Caucasian-Histiocytic-Lymphoma and Human-Prostate-Normal cell (PNT2A) were obtained from ECAAC, European Collection of Cell Cultures, supplied by (Sigma-Aldrich, Dorset, UK). DMEM and TrypLE™ Express were provided by Gibco (Life Technologies, Paisley, UK). RPMI - Bio Whittaker® without L-glutamine was supplied by Lonza, Vergiers, Belgium. HBBS – Hank's balanced salt solution was provided by Sigma-Aldrich, St. Louis, USA. The 96-wells plate, TPP 92096 was provided by TPP, Trasadingen, Switzerland. Cell culture spectroscopy analysis was done using Wallac, Victor2 ™ $_{H20}$ Multilabel Counter with IR, high-density TR-Fluorometry, robot loading and stacker (PerkinElmer, Waltham, MA, USA). Entire solvents and chemicals were analytical grades.

2.2 Fungal material

G. lucidum BCCM 31549 was obtained from the Belgian Coordinated Collections of Microorganisms (BCCM/MUCL), [Agro] Industrial Fungi and Yeast Collection (Leuven, Belgium). The fungus was subcultured onto potato dextrose agar (PDA, Oxoid Limited, Hampshire, UK) upon receipt from the supplier to avoid any contamination and ensure viability as suggested from previous research [10]. Plates were inoculated and incubated at 30 ^{o}C for seven days and stored at 4 ^{o}C. The strain was preserved on PDA slants. The fermentation strategy was implemented in a stirred-tank bioreactor, and the mycelial pellets were extracted.

2.3 Extraction, isolation, and sulfation

Distilled water (D_2O) was functioned to rinse the mycelium (biomass) off the sieves from the fermented culture broth. Through Whatman filter paper; they were filtered and vaporized to 50 mL at 60-80 ^{o}C. This volume was added to 150 mL of ethanol, for macromolecules precipitation, containing the desired polysaccharide-derived β-glucan. A glass rod was used to obtained the product by twirling. Based on the macromolecules precipitation, the precipitate was attached or adsorbed onto the glass rod and harvested from the solution. The glucose, however, may be confined within the extracted precipitate, which was then splashed using 96% (v/v) ethanol. Subsequently, the solution was dialyzed against distilled water for three days (MW cut-off = 10,000 Da) using a dialysis tube (Fisher Scientific, Loughborough, UK). The residual glucan was aerated and pre-chill in -20 ^{o}C freezer. After a couple of hours (h), the samples were transferred to -80 ^{o}C freezer for 24 h and then freeze-dried for 48 h. Later on, the build-up moisture surrounding the precipitated glucan were completely evaporated. It was then re-suspended in distilled water, freeze-dried in -80 ^{o}C freezer and evaluated to yield a 1,3-β-D-glucan (G).

The G produced from the bioreactor fermentation processes was water-insoluble; therefore an inevitable process needs to be implemented to increase its solubility in water. Suzuki et al., [32] and Williams et al., [37] did sulfation of active 1,3-β-D-glucans to increase their solubility or increase their bioavailability. Hence, sulfation of the current water-insoluble G was executed in this experiment. The improvised method of G sulfation of Williams et al., [37] was followed. Soluble 1,3-β-D-glucan sulphate (GS) was produced as outlined in Fig. 1. Firstly, 1 g of microparticulate G was liquefied in 50 mL of dimethyl sulfoxide (DMSO) containing 6 M urea. 8 mL of concentrated sulphuric acid was added drop-wise directly erstwhile to heating. In a water bath, the solution was heated at 100 ^{o}C, and the reaction process continued for 3 to 6 h. By 90 minutes, a crystalline precipitate (ammonium sulfate) was formed. The mixture solution was then vented at room temperature, and 1 L of ultrapure, pyrogen-free, D_2O (Millipore, Bedford, MA) was added. The GS solution was then pre-filtered to remove unreacted polymer in G. The GS solution was dialyzed using a Vivaflow 200, using a 10,000 MW cut-out filter (Sartorius Stedim Lab Ltd, Binbrook, UK). The final volume was reduced to 500 mL and lyophilized to dryness.

2.4 Elemental analysis

The content of C, H, O, N, and S were estimated using a Perkin Palmer 2400 Series II CHNS/O Elemental Analysis (Waltham, MA, USA) device. According to the recorded results of the elemental analysis, the degree of sulfation (DS) is defined by the following equation (A) according to Wang et al., [36].

$$\mathrm{DS} = \frac{72s}{32c} \qquad \text{(A)}$$

Where s is the mass ratio of S element in the product glucan sulfate (GS). From now on, DS signifies the number of sulfate groups per glucose residue.

2.5 Infrared Spectroscopy

FTIR spectra of the G and GS samples were taken using a FTIR 3000 spectrophotometer, (Jusco, Japan) following the method of Shi [29]. For jelly-like specimens (GS), FTIR Attenuated Total Reflectance (ATR) [Perkin Elmer, USA] was used to acquire the spectrum.

2.6 ^{1}H NMR Spectroscopy

The NMR spectra of both G and GS were taken using a DXM 500 FT-NMR spectrometer (Bruker, Switzerland). Both compounds were liquefied in deuterium oxide $-d_6$ at the concentration of about 10 mg/mL to 30 mg/mL. All spectra were carried out at 80 ^{o}C, respectively. Scan number was 16, and the chemical shifts (δ) indicated in parts per million (ppm). Laminarin from Laminaria digitata (Sigma-Aldrich, Dorset, UK) was used as the comparison standard for G while Fucoidan originated from Fucus vesiculosus (Sigma-Aldrich, Dorset, UK) was used as the comparison standard for GS.

2.7 Bioassay of antimicrobial activity

The test bacteria used for antimicrobial sensitivity testing comprise the bacteria Pseudomonas aeruginosa, Salmonella enteritidis, Staphylococcus aureus, Staphylococcus epidermis, Escherichia coli that were obtained from the General Microbiology Lab Collections SIPBS, Glasgow, UK. In addition, Escherichia coli EPIC S17, Salmonella BA54 SL1344 pSsaG, Listeria monocytogenes, Shigella sonnei 20071599, and Methicillin-Susceptible-Staphylococcus aureus (MSSA) ATCC 292123 were kindly supplied by Dr. Jun Yu, SIPBS, Glasgow, UK. At 20 ^{o}C, the strains were kept in the suitable freshly-prepared medium and rejuvenated two times

153 before being applied in the proposed assays. Bacteria were cultured with the oxygen supplied
154 environment at 37 ^{o}C (Incubator- Bruker 200, Thermo, UK) in nutrient agar (NA) medium for
155 bacteria.

156 **2.7.1 Kirby-Bauer disk diffusion assay, MIC, and MBC**

157 Determination of antimicrobial activity was carried out using the Kirby-Bauer disk
158 diffusion assay method. First, 20 mL of NA medium were decanted into each Petri dish. All test
159 microorganisms were adjusted to 0.5 McFarland standards using sterile broth medium. Once
160 hardened, about roughly 200 µL of suspension of the test bacteria was smeared on the prepared
161 agar. The standardized 11 mm sterile discs (blank) (Sigma-Aldrich, Dorset, UK) with an identical
162 absorbed GS volume were soaked with a known amount of extract. It was positioned moderately
163 onto the agar overlay. The plates were carefully incubated overnight at 37 ^{o}C or 48 h or 30 ^{o}C for
164 two days depending on the growth requirement of the bacterium. Gentamicin was applied as the
165 positive control while ethanol was the negative control. After the incubation, the diameters (mm)
166 of the inhibition zone were measured. Inhibition zones that were higher than 11 mm were
167 considered positive for antimicrobial reactions.

168 The minimal inhibitory concentration (MIC) was evaluated by microdilution using 96-
169 well microtitre plates according to Li et al., [20] with slight modifications. Sterile broth medium
170 in conjunction with 0.5 McFarland standards was used as bacterial suspensions adjustment. GS
171 compounds were dissolved in sterile ultrapure water and serially diluted into (mg/mL) 200, 100,
172 20, 10, 8, 5, 3, 2 and 1. The final mixture was 25 µL of compounds with 75 µL of a suspension of
173 each bacterium (working volume of 100 µL). Each test culture was pipetted onto the plates and
174 incubated for 24 h at 30 ^{o}C. Once the incubation time ended, the turbidity or cloudiness was
175 taken as the signal or indication for bacterial growth. The lowest diluted concentration at which

the incubated mixture persisted clear after microscopic assessment (at the binocular microscope) was thus selected as the MICs.

Based on the MIC observation, the level at which the incubated mixture stayed clear after the microscopic estimation was selected as the MIC. The microscopic growth range were then pipetted (100 µL) to the NA. Sterile L-spreaders were used to make the spreading even. Following that, the concentration indicating the MIC and at least two of the more concentrated dilutions were plated and enumerated to determine viable colonies specifically for minimum bactericidal concentration (MBC) determination. The media were cultured at 30 ^{o}C for 24 h to observe for any microorganism growth. For the MBC, the minimum or lowest concentration in the medium that had less than five colonies was used.

The method by SIDR (Strathclyde Institute of Drug Research) was used for the antimicrobial test on the bacteria included Klebsiella pneumoniae ATCC 13883 and Mycobacterium marinum ATCC BAA 535 using the 96-well microtitre plates [4, 18]. These tests were in triplicate, and the GS was supplied at 10 mg/mL. Gentamicin was used as a positive control for the bacteria, and DMSO as the negative one.

2.8 Bioassay of cytotoxicity

2.8.1 On normal cells

Cell lines were grown in the appropriate freshly-prepared complete medium in a cell culture incubator (gaseous composition 95% air, 5% CO_2) at 37 ^{o}C. The PN2TA normal human prostate cell line was sustained in a complete medium comprising RPMI, penicillin-streptomycin (5 mL), 50 mL fetal bovine serum (FBS), L-glutamine (5 mL), and pH at 7.4. AlamarBlue® assay determined the cytotoxic effect of both G and GS. Initially, 96-well microtitre plates were

198 seeded with the PN2TA cells at 2 x 10^4 cells/mL for each well. Cells were permitted to cultivate
199 one day afore being introduced to GS: 500, 300, 50, 30, 5, and 3 µg/mL. For the negative control
200 group, 4% (v/v) of Triton-X was added to the medium. After the incubation for the indicated
201 hours, 10% (working volume per well) of alamarBlue® reagent was decanted to each well and
202 incubated for an extra 6 h in a humidified incubator. Once 6 h of incubation completed, the
203 resazurin in the alamarBlue® undergoes oxidation-reduction change in response to cellular
204 metabolic change. The reduced form resazurin is pink and extremely fluorescent, and the strength
205 of fluorescence produced is proportional to some living cells undergone respiration. The
206 wavelength of 570 nm was used for absorbance reading. For analysis, cytotoxic activity was
207 calculated based on cell survival ratio (%).

208 **2.8.2 On cancer cells**

209 The cytotoxicity of both G and GS were also tested on the cancerous cell U937 by 96-
210 well microtitre plate using alamarBlue® assay. The U937 cells at a density of 3 x 10^5 cells/well
211 were being exposed to 60, 50, 30, and 10 µg/mL of both G and GS at day one prior incubation.
212 As for the control group, an identical volume of complete sterile medium was applied (positive
213 control) while Triton X (4%) as the negative control. After incubation for the designated period,
214 10% of alamarBlue® reagent was pipetted to each well and incubated for an extra 6 h in a
215 humidified incubator. Following 6 h incubation, the alamarBlue® reagent initiated resazurin to
216 undergoes oxidation-reduction change in response to the cellular metabolic modification. The
217 wavelength of 570 nm was used for absorbance reading. For analysis, cytotoxic activity was
218 calculated based on cell survival ratio (%).

219

2.9 Statistical analysis

All analysis were carried out in triplicate, and the respective mean ± S.D determined using the software, GraphPad Prism 5 (Version 5.01) and shown as error bars. If the error bars do not appear then, they are less than the size of the icon or symbol.

3. RESULTS AND DISCUSSION

3.1 Glucan solubility

In this study, the method for solubilization of G employs DMSO to dissolve initially the water-insoluble G preceding sulfation [11]. The DMSO and other reaction products were removed from G by extensive dialysis to ensure the purity of the GS produced. The solubility of the GS in water was measured post-sulfation to assess the effects of sulfation on G. In ultrapure distilled water, the final solubility of G was below 5% (w/v), but that of its GS was above 95% (w/v). Table 1 recaps the solubility and yields of the insoluble G and soluble GS. Furthermore, the GS was readily dissolved without heating while the G needs 0.1 M of NaOH at 80 ^{o}C to assist dilution in water. The improved tractability of GS about G represents a significant aid in developing and implementing assays.

The introduction of sulfate group has several purposes. Based on the present study's findings, the aqueous solubility of the extracted G from the fermenter was as poor as that of G prepared from other procedures. Astonishingly, this was mentioned in the literature that G was less suitable for medicinal applications [13]. In terms of the commercial importance of bioactive glucan, the water insoluble G show slight bioactivity, although G by-products such as pullulan sulfate, lentinan sulfate, and dextran sulfate have been suggested to display high anti-HIV

activities and small anticoagulant activities [36]. Wang and Zhang [6] also revealed that the sulfation process on the fruiting bodies of G. lucidum producing G have led to enhanced antitumor and antiviral activities [23, 27]. However, comparable studies on the antimicrobial and cytotoxicity of mycelial-sourced GS are limited.

3.2 Compositional analysis

3.2.1 Elemental analysis

Elemental analysis was accomplished to attain the composition of the GS and, therefore, its degree of sulfation (DS). Basic examination of lyophilized GS gives a composition of (w/w): 24.5% C, 5.72% H, 49.92% O, 9.85% S, and 10.01% N (Table 2). When compared with standard fucoidan, GS had the same C and H values but slight different in H, O, and S. This was due to different sulfation technique applied on each GS and fucoidan, respectively, and this might generate different molecular weight.

Based on the composition of GS, the DS of GS is thus 0.90 indicating 90 sulfate groups are present on every 100 glucose subunits within the polysaccharide on average. When compared with the previous DS value (0.94) of sulfated polysaccharide (S-GL) of G. lucidum reported by Wang and Zhang [34], the current DS value of GS (0.90) was broadly similar to each other.

3.2.2 IR spectroscopy

Table 3 and Fig. 2, summarize the results of using FTIR spectroscopy to assess the structural characteristics of the G and GS. Both molecules showed the typical IR absorptions of polysaccharides at 1,250 and 1,650 cm^{-1}: 1,170 and 1,651 cm^{-1}, respectively. These IR absorptions as well as those in the 'anomeric region' at 950 – 700 cm^{-1} allow us to differentiate β from α glucans spectroscopically [35]. Overall, the D-glucosidic linkage arrangement is β-type both prior to and the following sulfation.

In the functional group region of the G-spectra, there were significant absorptions at 3,400, 1,077, 2,925, 1,374, 1,647 , 1,246, 1,540, 1,077, and 892 cm^{-1}, which resembles the elongating absorption bands of poly -OH, C=O=C, -CH_2, -CH_3, C=0, amide, pyranose ring and β-configuration of D-glucose units. As compared with the previous work by Wang et al., [36] and Liu et al., [22], the specific absorption of G at 892.9 cm^{-1} demonstrates that the compound is a β-glucan. The characteristic peak of the β-configuration at 892.9 cm^{-1} was also noted in the spectra of GS with two new absorption peaks at 1,170 and 867 cm^{-1} also present (Fig. 2), which match the to S=0 asymmetrical stretching and C-S-C symmetrical vibration [35]. These confirmed that the GS had been efficiently synthesized from G.

3.2.3 NMR spectroscopy

As can be seen in Fig. 3 and Fig. 4, ^{1}H NMR spectroscopic analysis of the G and GS from G. lucidum was conducted at 80 ^oC using D_20-d_6 as a solvent. Using ppm as the standardized unit for NMR studies, ^{1}H NMR spectra of the G were compared with the standard laminarin (β-1,3-D-glucan) from L. digitata while the GS spectra were compared with the standard fucoidan (sulfated-β-1,3-D-glucan) from F. vesiculosus. The spectrum chemical shifts of δ 3.9 to 5.4 ppm and δ 2.6 to 5.5 ppm exhibited indicate that both compounds were glucans, as can be observed in both Fig. 3 and Fig. 4, respectively. The current work is comparable with previous research by Ji et al., [16], which analyzed laminarin and sulfated laminarin in the area of ^{1}H-NMR spectrum of δ 4.49-5.5 ppm. Thus, these spectra indicate that the glycosidic bonds in both G (Fig. 3) and GS (Fig. 4) were β-type.

Evaluation of the 'anomeric region' of ^{1}H NMR spectra in this study with those described previously specifies that they are of similar pattern [22, 33, 36]. For G (Fig. 3) ^{1}H NMR spectra, the signals at δ 5.08, 4.50 and 4.40 were assigned to OH-2, OH-6, and OH-4 when

compared with the reported work by Wagner et al., [33]. The GS (Fig. 4) ^{1}H NMR also exhibits similarity to the G with the signals at δ 5.21, 4.52 and 4.40. When compared, the anomeric signals for both compounds in the present study (G and GS) were at δ 4.5 ppm and δ 4.2 ppm, respectively indicating β-configuration for glucopyranosyl units as reported by Liu et al., [22].

Moreover, the ^{1}H-NMR spectrum of the GS displayed that the chemical shift of hydrogen usually stimulated downfield relative to G, which showed that most of the hydroxyl groups in the G had been sulfated and similarly specified that GS had β-glycosidic bonds. From the IR and ^{1}H NMR analyzed, it is possible to conclude that the G compound is composed of (1-3)-β-D-linkages which gave the polymer structure apparently as a1,3-β-D-glucan.

3.3 Assessment of antimicrobial activity

The antimicrobial effect of the GS from G. lucidum was tested against ten species of bacteria as G was not evident. Their strength was measured quantitatively and qualitatively by the absence or presence of inhibition zones, zone diameters, MBC and MIC values. The findings of these tests are summarized in Table 3 (inhibition zone diameters). Among the bacterial strains tested in Table 3, when the GS reached 500 mg/mL, the diameters (mm) of the inhibition zone were 34 ± 3.2, 24 ± 2.6, 32 ± 1.0, 25 ± 2.6, 23 ± 2.8, 27 ± 1.5, 28 ± 0.5, 26 ± 1.0, 30 ± 1.0, and 30 ± 3.1, for E. coli EPIC S17, E. coli, L. monocytogenes, Shigella sonnei 20071599, P. aeruginosa, S. enteritidis, Salmonella BA54 SL 1344 (pSsaG), Staph. aureus, Staph. epidermis, and Methicillin-Susceptible Staph. aureus (MSSA) ATCC 292123, respectively. The inhibition zone diameters increased with increasing GS prepared concentrations (Table 3). These reactions displayed that the antimicrobial effect of GS was dose-dependent and that the gentamicin positive control was clearly effective against all the test bacteria.

312 Furthermore, the MIC concentrations for bacterial strains were in the range of 1- 5 mg/mL
313 and the MBC concentrations range was 5-10 mg/mL except the resilient Shigella sonnei
314 20071599 (Table 3, no.3). Among four species of Gram-positive bacteria verified, the greatest
315 active antimicrobial activity of GS was shown against Staph. aureus (Table 3, no.8), and its MIC
316 was 2 mg/mL. Meanwhile, the antimicrobial activity of GS was verified against six species of
317 Gram-negative microbial strains. GS exhibited fairly strongest antimicrobial activity against E.
318 coli (Table 3, no.2) (MIC = 1 mg/mL), and seven species of microbial strains were shown to have
319 MIC concentrations at respective 3 mg/mL while the most resistant bacterium was Staph.
320 epidermis (Table 3, no.9) (MIC = 5 mg/mL).

321 When compared to other studies where derivatised fungal polymers have been examined
322 as food preservatives and their antimicrobial activity has been assessed [8], it showed that SC2
323 sulfated-polysaccharide (chitosan) has MIC values higher than 2 mg/mL [28] for Staph. aureus,
324 L. monocytogenes, Vibrio parahaemolyticus, P. aeruginosa, Shigella dysenteriae, V. cholera,
325 Aeromonas hydrophila and S. typhimurium. SC2 shows a much higher MIC's against Gram-
326 positive than Gram-negative bacteria. Devlieghere [7], Muzzarelli [26] and Hernandez-Lauzardo
327 [15] also tested the antimicrobial activity of chitosan as food preservatives and gave results for
328 MIC's at or above 2.5 mg/mL. The closest comparison to the present study involved an
329 assessment of MIC values of an ethanolic extract of G. atrum sourced from powdered fruiting
330 bodies varying from 1.6 to 6.25 mg/mL for the common bacterial food contaminants [20] which
331 also reported by Ferreira et al., [11].Thus, the MIC's recorded for the GS in the present study are
332 broadly similar to those reported in other studies for fungal-derived polymers.

333 The antimicrobial activity of some bacteria including K. pneumoniae ATCC 13883 and
334 M. marinum ATCC BAA 535 were tested via 96-well microtitre plates to assess the antimicrobial

335 effects of GS. Overall, the results showed some clear inhibition of growth of both these test
336 species (Table 4). The Gram-negative K. pneumoniae ATCC 13883 exhibited a survival of 52.8 ±
337 5.66 % (at 500 µg/mL and 24 h incubation) while the acid-fast bacteria M. marinum ATCC BAA
338 535 gave a survival value of 65 ± 3.39 % (at 100 µg/mL and 24 h incubation) compared to
339 positive growth controls. Due to the significant and increasing occurrence of nosocomial
340 infections and destructive changes to human lungs as mentioned by Daligault et al.,[6] by
341 antibiotic-resistant K. pneumoniae ATCC 13883 the possibility of using novel antimicrobials
342 from processed natural sources such as GS extracted from G. lucidum merits further
343 investigation and refinement. Meanwhile, GS might have some potential in controlling the
344 occurrence of common granulomatous diseases arising from M. marinum ATCC BAA 535 that
345 affect individuals who work with fish or keep aquaria as described by Slany [31].

346 Overall, at present it is not entirely clear what the mechanism(s) of the antimicrobial
347 activity of a sulfated polysaccharide such as GS is likely to be, as there are few studies in this
348 area, meanwhile the G was negative in terms of antibacterial impact (results not shown). The
349 steric and repulsive electrostatic properties of sulfate groups and how these might alter the spatial
350 construction of the glucan were proposed by Ji et al., [31] as a possible contributor to the
351 observed behavior of GS. Others suggested that changes in the flexibility of the polysaccharide
352 backbone, and the altered water solubility could lead to variations in biotic response [3, 9, 30],
353 which may also include the antimicrobial effects. The mechanisms of sulfation on the structure G
354 were proposed for these positive reactions by GS. Consequently, it is essential to further studies
355 in order investigate glucan structure-activity relationships, which might deliver a detailed
356 foundation for their development and improvement.

Slany et al., [31] discuss the impact of sulfation on the structure and biological activity. In general, the sugar chain conformation becomes modified by the process of sulfation such that non-covalent bonds form more readily when the –OH groups in a β-glucan element are replaced with sulfate groups. Similarly, repulsions between the anionic groups lead to elongation of the sugar chain. They propose that these events result in the polymer developing an active conformation, thus initiating the bioactivity surge.

In the last 20 years, there have been insufficient reports on antimicrobial activities of biopolymers from Ganoderma species [6, 14, 20, 39]. This genus has been commonly considered for its therapeutic properties, but less widely explored as a source of novel antibacterial agents [12, 14]. However, certain polysaccharides from Ganoderma species employ antibacterial activity by hindering the growth of bacteria and, in some events, by eliminating pathogenic bacteria [30]. Nearly all antibacterial investigations on Ganoderma species have been accomplished on the fruiting body and not on extracts from the liquid cultivated mycelium, a point which is made strongly in the recent review by Ferreira et al., [11]. Meanwhile, most of the positive antibacterial compounds were from alcoholic extracts, hot-water extracts and triterpenoids of fruiting bodies. The current work is the first to show positive results using GS extracted from G. lucidum mycelium produced in the bioreactor.

3.4 Assessment of cytotoxicity activity

The current extracted and processed GS from G. lucidum was reactive against pathogenic bacteria. Yet, to ensure whether these compounds might have clinical impact on healthy patient cells and before their introduction as new antimicrobial drugs, some preliminary assessment of the impact of such biomolecules upon normal host cells is of interest. Likewise, assessment of the

effects of such derivatized polymers on tumors is of value given the widely reported impact of other fungal macromolecules on such cell types. Accordingly, in the present study cytotoxicity assays using alamarBlue® reagent were carried out on healthy human prostate cells (PN2TA). The in vitro effects of both GS and its G from G. lucidum on PN2TA were studied in the current work (Fig. 5).

In this study, a series of dose-response assays were implemented to define the cytotoxic reactions in PN2TA. Once the cells exposed to different concentrations (3, 5, 30, 50, 300 and 500 µg/mL) of GS and G for 24 h, and the alamarBlue® reagent assay displayed no loss of cell viability. Morphological observations of the treated cells were the same as the control cells, therefore; these data indicated that GS and G did not exhibit cytotoxicity in PN2TA normal human cell. When compared with the previous work by Li et al., [20], the β-glucan from G. atrum did not react on the viability of healthy cells, thus confirming the clinical safety of G. lucidum β-glucan extracts from the current strain.

The cytotoxicity of G and GS against the development of cancer cells (U937) were examined using the alamarBlue® reagent in this study. As revealed in Fig. 6, GS displayed a dose-dependent antiproliferative reactions within the value range of 10 - 60 µg/mL and exhibited stronger antiproliferation than G. GS showed the most potent antiproliferative effect at 60 µg/mL with approximately 40% antiproliferation compared to 10% for G, as the Figure shows the fewest cell growth with ascending growth towards lower concentrations. As reported, it demonstrates that the antiproliferative activity of cancer cell growth was enriched by the sulfation process (GS) as matched to the unprocessed glucan (G) [2, 35].

401 The current concentration of GS (60 µg/mL) applied is considerably lower than that used
402 in the earlier study on of sulfated glucan (sourced from Hypsizigus marmoreus) which showed
403 only 39% of antiproliferative activity at 1000 µg/mL [2], thus further concentration increment for
404 the current work would highly beneficial. As reported, the molecular weight, chemical
405 configuration, degree of branching, and structure of the polymeric backbone were crucial for
406 antiproliferative activities stimulation for both G and GS [25]. Therefore, the biochemical aspects
407 and mechanism of the antiproliferative reactions stimulated by GS from mycelium of G. lucidum
408 is still not fully unspoken and requests further study.

409 In summary, it has been shown that the compounds extracted from these mycelial cultures
410 were polysaccharide with a proposed structure of β-1,3-D-glucan when compared with both
411 standards, laminarin, and fucoidan. The antimicrobial activity of the GS from G. lucidum was
412 effective against tested microbes in the used assays. Also, cytotoxicity of GS was evaluated with
413 normal human prostate cells and no such effects were noted at the levels tested in this study. The
414 GS may also have potential in antiproliferative work based on its cytotoxicity of Human-
415 Caucasian-Histiocytic-Lymphoma cancer cells (U937). These GS activities indicate that sulfate
416 substitution on the G not only improved solubility, they also had an impact on therapeutic
417 activities, suggesting that sulfation was an effective way to enhance these activities. In relation,
418 the GS might have a role as a natural additive in many foods with multi-functional benefits
419 (preservative, antiproliferative, immune-stimulation). Further examination of these functions for
420 such polymers and their derivatives will be required.

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Figures captions

Fig. 1. Homogeneous reaction for sulfated (1-3)-β-D-glucan (GS) preparation: process scheme. Improvised from Wang et al [36].

Fig. 2. Comparison of β-glucan IR spectra. A: glucan (G); B: glucan sulfate (GS) derived from extended batch cultures of G. lucidum BCCM 31549 mycelium.

Fig. 3. ^{1}H NMR spectra of (1-3)-β-D-glucan (G) derived from extended batch cultures of G. lucidum BCCM 31549 mycelium and laminarin (Laminaria digitata) standard in D_20-d_6 at 80 ^{o}C.

Fig. 4. ^{1}H NMR spectra of sulfated (1-3)-β-D-glucan (GS) derived from extended batch cultures of G. lucidum BCCM 31549 mycelium and fucoidan (Fucus vesiculosus) standard in D_20-d_6 at 80 ^{o}C.

Fig. 5. Cytotoxicity effects of both glucan (G) and glucan sulfate (GS) derived from extended batch cultures of G. lucidum BCCM 31549 mycelium in normal human Prostate-cell-line (PN2TA). After the cells were incubated with G and GS treatments [Control, 500, 300, 50, 30, 5, 3, Triton X µg/mL], the viability was measured by alamarBlue® assay. Both G and GS had the same morphological observation under the microscope at 10x magnification. Each data was presented S.D ± mean, and the P value was > 0.05 when compared to control. If the error bars do not appear then, they are less than the size of the icon or symbol.

Fig. 6. Cytotoxicity effects of glucan (G) derived from extended batch cultures of G. lucidum BCCM 31549 mycelium against cancerous human Caucasian-Histiocytic-lymphoma cell line (U937) from a 37-year-old male patient. After the cells were incubated with G treatments [Control, 60, 50, 30, 10, Triton X µg/mL], the viability was measured by alamarBlue® assay. G had morphological observation under the microscope at 10x magnification. Each data was presented S.D ± mean, and the P value was < 0.05 when compared to control. If the error bars do not appear then, they are less than the size of the icon or symbol.

Fig. 7. Cytotoxicity effects of glucan sulfate (GS) derived from glucan (G) against cancerous human Caucasian-Histiocytic-lymphoma cell line (U937) from a 37-year-old male patient. After the cells were incubated with GS treatments [Control, 60, 50, 30, 10, Triton X µg/mL], the viability was measured by alamarBlue® assay. GS had morphological observation under the microscope at 10x magnification. Each data was presented S.D ± mean, and the P value was < 0.05 when compared to control. If the error bars do not appear then, they are less than the size of the icon or symbol.

462 **Figures**

463 Fig. 1

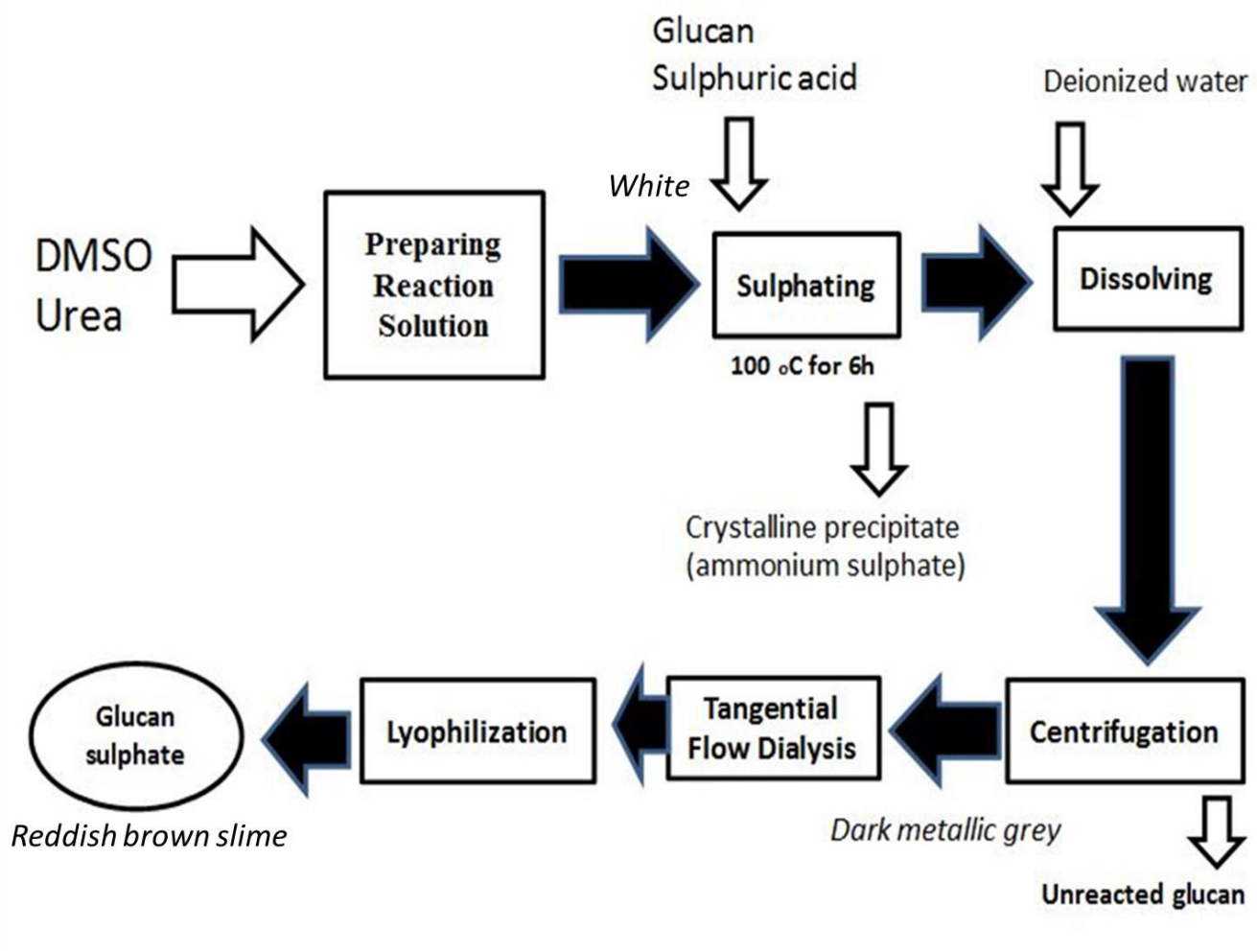

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Fig. 2

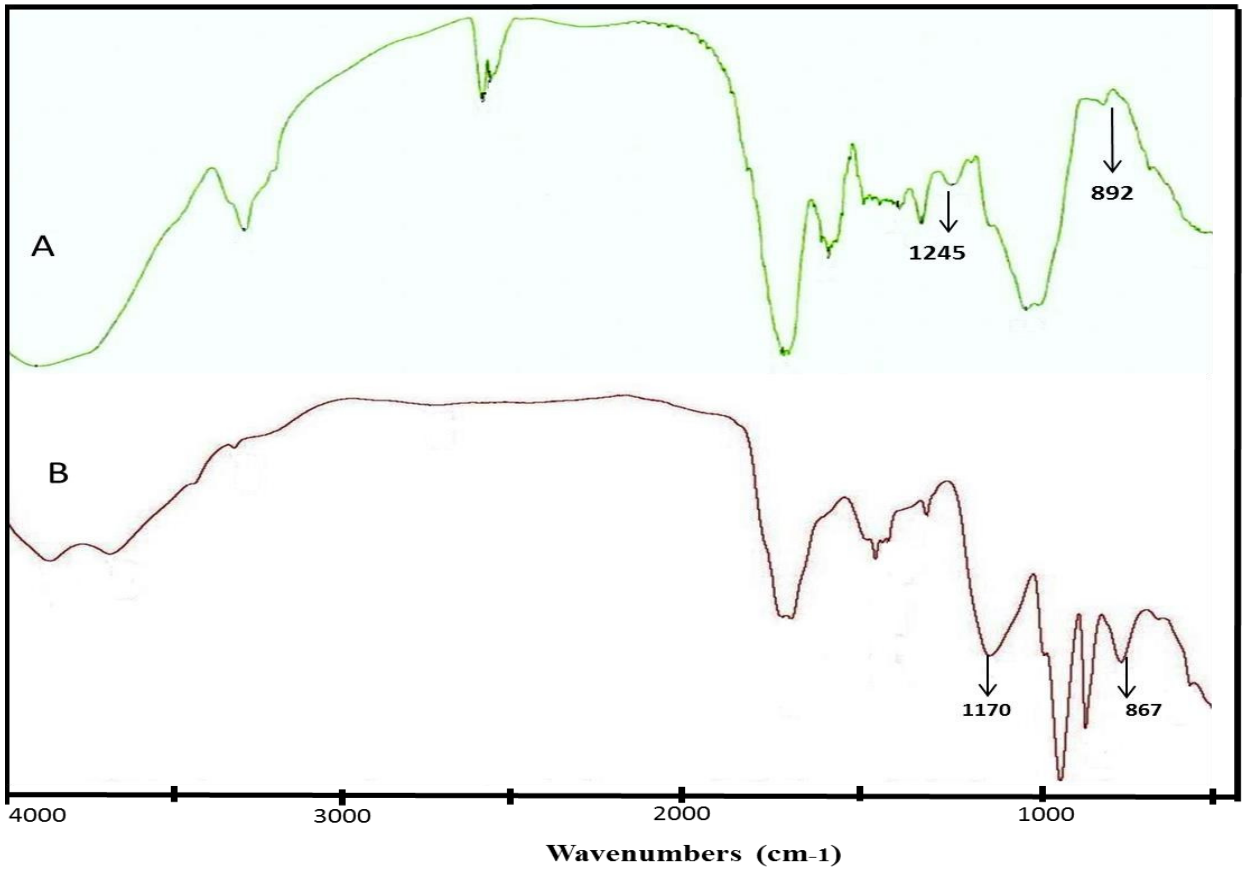


Fig. 3

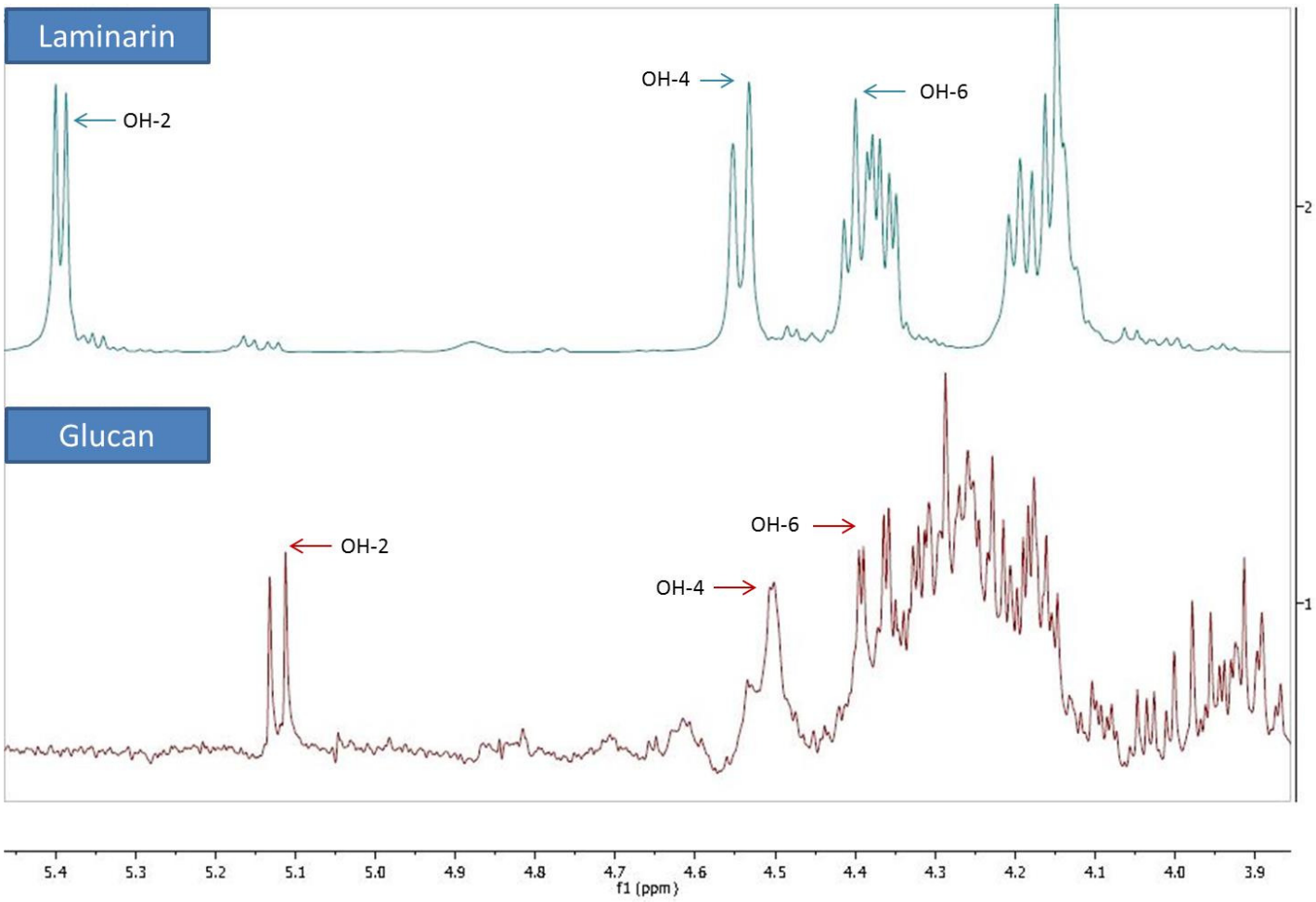

Laminarin
OH-2
OH-4
OH-6
Glucan
OH-2
OH-4
OH-6
2
1
5.4
5.3
5.2
5.1
5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4.0
3.9
f1 (ppm)

Fig. 4

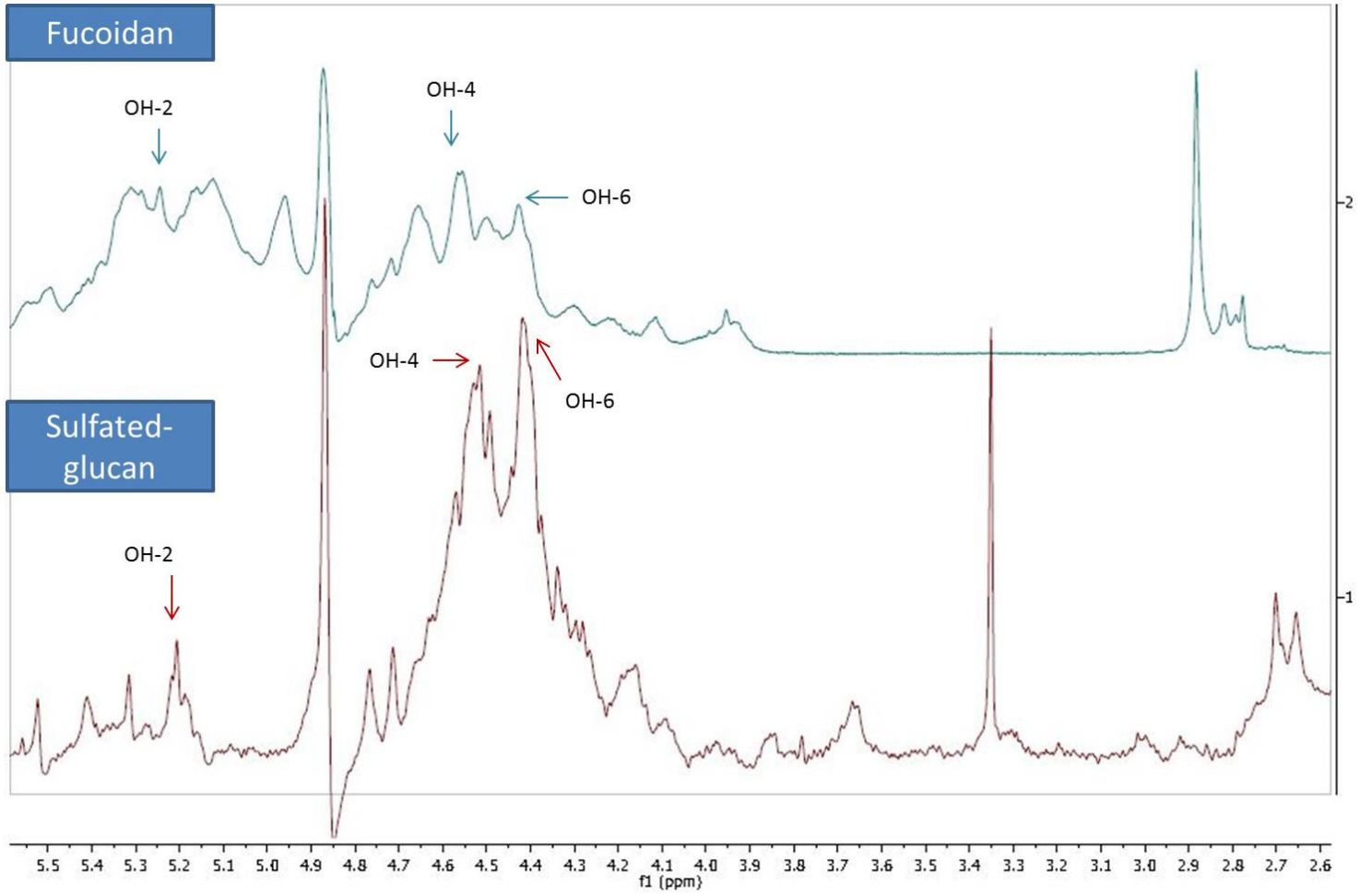


Fig. 5.

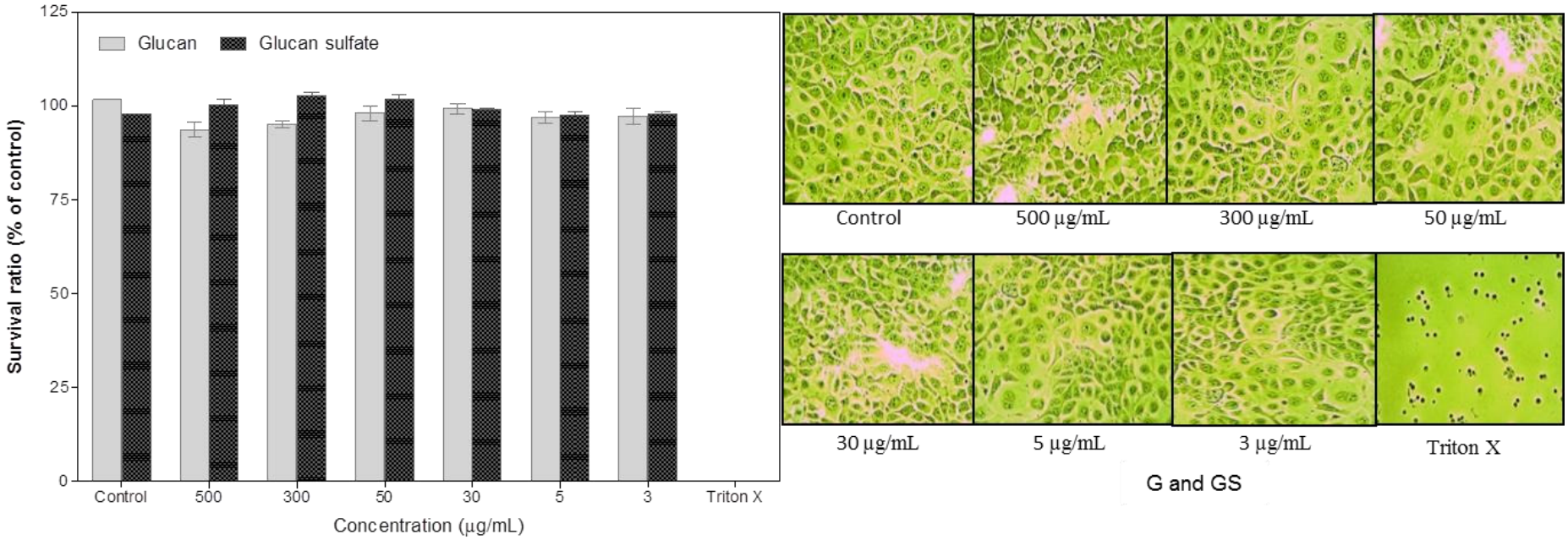

Glucan
Glucan sulfate
Survival ratio (% of control)
125
100
75
50
25
0
Control
500
300
50
30
5
3
Triton X
Concentration (μg/mL)
Control
500 μg/mL
300 μg/mL
50 μg/mL
30 μg/mL
5 μg/mL
3 μg/mL
Triton X
G and GS

Fig. 6.

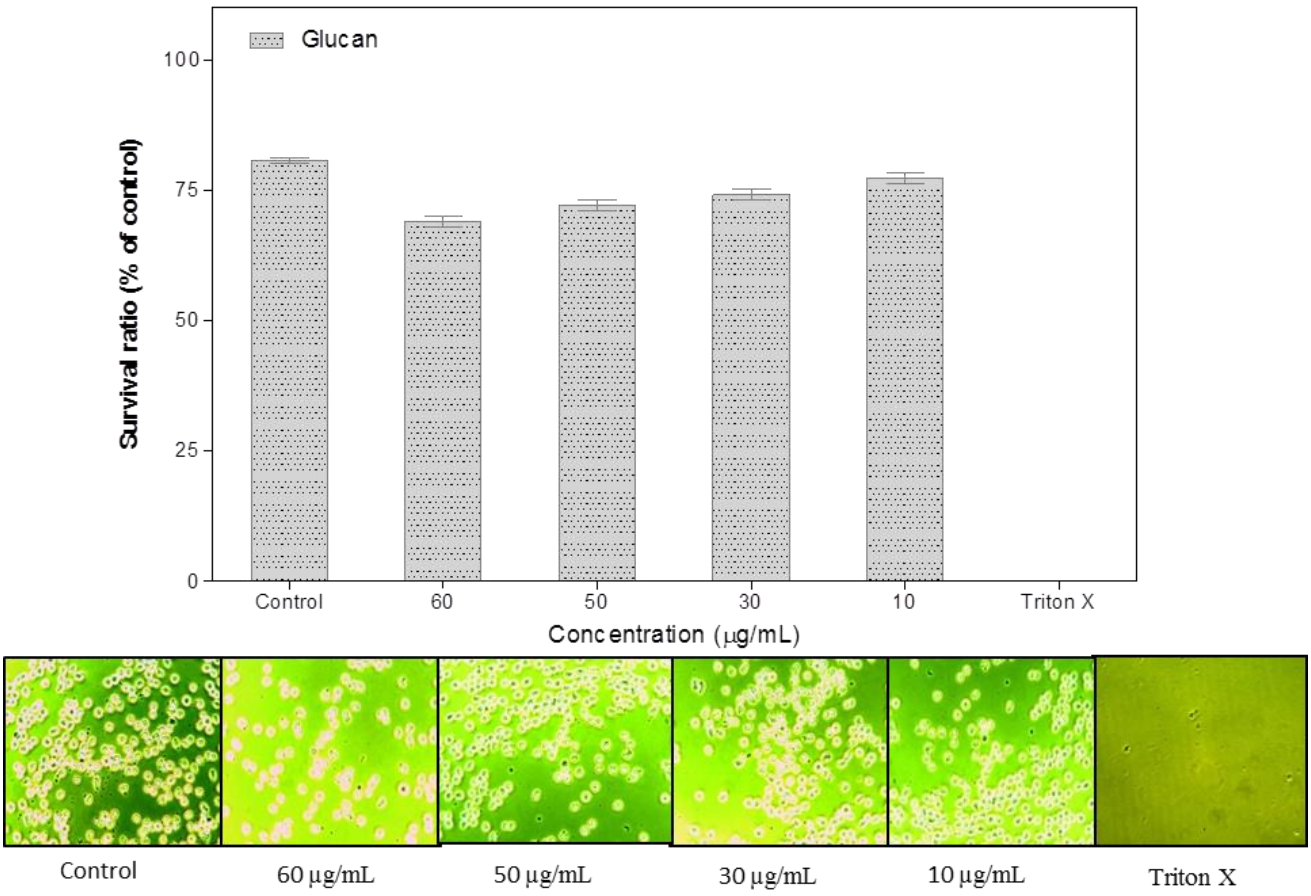
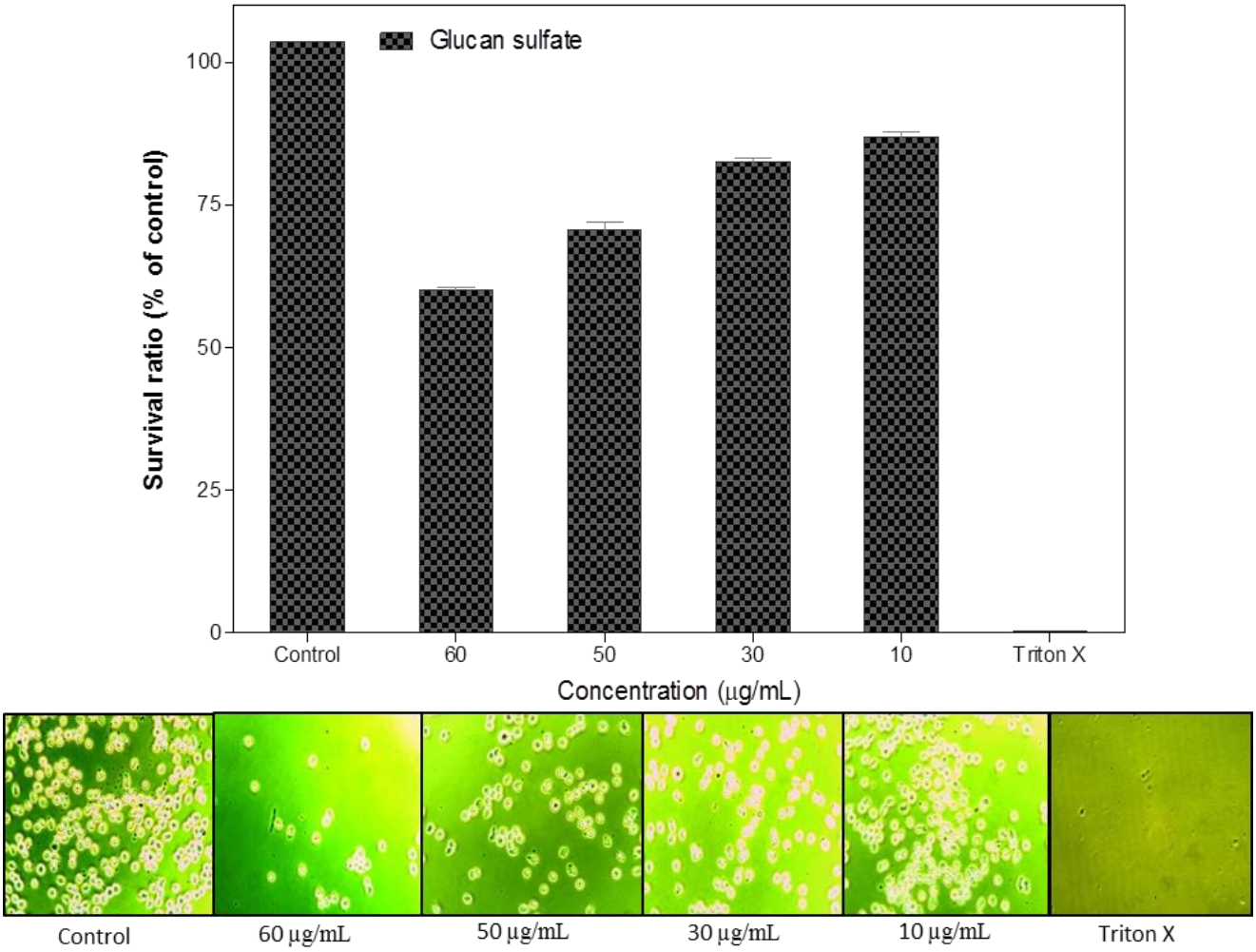

Glucan
Survival ratio (% of control)
100
75
50
25
0
Control
60
50
30
10
Triton X
Concentration (μg/mL)
Control
60 μg/mL
50 μg/mL
30 μg/mL
10 μg/mL
Triton X

Fig. 7


Glucan sulfate
Survival ratio (% of control)
100
75
50
25
0
Control
60
50
30
10
Triton X
Concentration (μg/mL)
Control
60 μg/mL
50 μg/mL
30 μg/mL
10 μg/mL
Triton X

Tables

Table 1

Solubility properties of glucan sulfate (GS) prepared from glucan (G) derived from extended batch cultures of G. lucidum BCCM 31549 mycelium

Characteristics	G	GS
Appearance	White powder	Reddish brown slime
Dry weight (g)	1.6	1.4
Yield (%)	-	87.5
Water solubility (%) (w/v)	Below 5	Above 95

*Values are means of four batches

Table 2

Elemental analysis of glucan (G) and glucan sulfate (GS) derived from extended batch cultures of G. lucidum BCCM 31549 mycelium

	(w/w) %					
Sample	C	H	N	O	S	(DS)
G	28.60 ±1.2	3.28 ±1.2	1.79 ±0.5	65.08 ±1.3	1.25 ±0.5	-
Laminarin	39.36 ±2.2	6.47 ±1.5	0.32 ±0.01	52.84 ±1.8	1.01 ±0.2	-
GS	24.50 ±2.1	5.72 ±1.6	10.01 ±0.9	49.92 ±1.3	9.85 ±1.1	0.90
Fucoidan	24.10 ±1.9	4.24 ±1.4	0.3 ±0.01	64.49 ±2.1	6.87 ±1.3	0.64

* Laminarin is a standard for (1,3)-β-D-glucan from Laminaria digitata

* Fucoidan is a standard for sulfated-(1,3)-β-D-glucan from Fucos vesiculosus

* Values were in triplicate and presented in mean ± S.D. P value is <0.05.

Table 3

Antimicrobial activities of glucan sulfate (GS) derived from extended batch cultures of G. lucidum BCCM 31549 mycelium [a]

No./ G	Bacteria	GS						GENT [c] 30 µg	Ethanol [c] 100%
		Diameter of inhibition zone [b] (mm)				MIC [d] mg/mL	MBC [e] mg/mL		
		200 mg/mL	300 mg/mL	400 mg/mL	500 mg/mL				
1 (G-)	Escherichia coli EPIC S17	22 ± 2.6	26 ± 1.2	29 ± 1.0	34 ± 3.2	3	10	19 ± 2.5	11.4 ± 0.1
2 (G-)	Escherichia coli	20 ± 1.5	22 ± 1.5	20 ± 1.1	24 ± 2.6	1	2	23 ± 3.1	11.3 ± 0.1
3 (G-)	Shigella sonnei 20071599	16 ± 1.0	21 ± 0.5	23 ± 2.1	25 ± 2.6	3	20	22 ± 1.5	11.4 ± 0.1
4 (G-)	Pseudomonas aeruginosa	16 ± 1.0	20 ± 1.0	20 ± 0.5	23 ± 2.8	3	5	23 ± 1.1	11.2 ± 0.1
5 (G-)	Salmonella enteritidis	17 ± 1.5	20 ± 1.0	23 ± 1.0	27 ± 1.5	3	10	24 ± 2.5	11.1 ± 0.1
6 (G-)	Salmonella BA54SL1344 (pSsaG)	20 ± 2.1	24 ± 1.5	26 ± 1.0	28 ± 0.5	3	5	28 ± 2.0	11.2 ± 0.1
7 (G+)	Listeria monocytogenes	26 ± 2.1	28 ± 1.0	30 ± 1.5	32 ± 1.0	3	8	34 ± 1.0	11.1 ± 0.1
8 (G+)	Staphylococcus aureus	18 ± 1.0	20 ± 0.5	21 ± 2.3	26 ± 1.0	2	5	21 ± 1.0	11.1 ± 0.1
9 (G+)	Staphylococcus epidermis	22 ± 1.5	23 ± 4.0	28 ± 1.5	30 ± 1.0	5	10	27 ± 1.5	11.2 ± 0.1
10 (G+)	Methicillin-Susceptible-Staphylococcus aureus ATCC 292123 ATCC 292123	22 ± 2.1	24 ± 2.0	27 ± 1.2	30 ± 3.1	3	10	25 ± 2.5	11.2 ± 0.1

[a] Values represent mean ± S.D ($P < 0.05$) for triplicate experiments. G indicates Gram positive (G+) or Gram negative (G-) bacteria
[b] Sterile disc size was 11 mm indicating negative reactions and positive reactions were more than 11 mm.
[c] Ethanol was used as the negative control while Gentamicin (GENT) was used as the positive control.
[d] The minimum inhibiting concentration (MIC) (as mg/mL). [e] The minimum bactericidal concentration (MBC) (as mg/mL)

Table 4

Activity of glucan sulfate (GS) derived from extended batch cultures of G. lucidum BCCM 31549 mycelium using 96-well microtitre plates [a]

Bacteria	Klebsiella pneumoniae ATCC 13883	Mycobacterium marinum ATCC. BAA. 535	Gentamicin	DMSO
Gram	(-)	Acid-fast bacteria		
GS (µg/mL)	500	100	100	100
Survival ratio [b] (% of control)	52.8 ± 5.66	65 ± 3.39	1 ± 1.20	99 ± 1.05
Inhibition status	Positive	Positive	(+) control	(-) control

a Values represent averages ± S.D ($P < 0.05$) for triplicate experiments

b Lower percentage of control value means the greater antibacterial effect

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