



JMB Papers in Press. First Published online Feb 24, 2016

DOI: 10.4014/jmb.1510.10018

**Manuscript Number:** JMB15-10018

**Title:** Antimicrobial properties and cytotoxicity of sulfated (1,3)- $\beta$ -D-glucan from the mycelium of the mushroom *Ganoderma lucidum*

**Article Type:** Research article

**Keywords:** *Ganoderma lucidum*, (1,3)- $\beta$ -D-glucan sulfate, Antimicrobial activity, Cytotoxicity

ACCEPTED

1 **Antimicrobial properties and cytotoxicity of sulfated (1,3)- $\beta$ -D-glucan from the mycelium of**  
2 **the mushroom *Ganoderma lucidum***

3 Wan Abd Al Qadr Imad Wan-Mohtar<sup>1\*</sup>, Louise Young<sup>2</sup>, Gráinne M. Abbott<sup>2</sup>, Carol Clements<sup>2</sup>  
4 Linda M. Harvey<sup>1</sup>, Brian McNeil<sup>1</sup>

5 <sup>1</sup> *Fermentation Centre, SIPBS, 161 Cathedral Street, Strathclyde University, Glasgow, U.K.*

6 <sup>2</sup> *SIDR, SIPBS, Strathclyde University, Glasgow, U.K.*

7 **Abstract**

8 *Ganoderma lucidum* BCCM 31549 has a long established role for its therapeutic activities. In this  
9 context, much interest has focused on the possible functions of the (1,3)- $\beta$ -D-glucan (G)  
10 produced by these cultures in a stirred-tank bioreactor and extracted from their underutilized  
11 mycelium. In the existing study, we report on the systematic production of G, and its sulfated  
12 derivative (GS). The aim of this study was to investigate the G and its GS from *G. lucidum* in  
13 terms of antibacterial properties, and cytotoxicity spectrum against Human-Prostate-Cell (PN2TA)  
14 and Human-Caucasian-Histiocytic-Lymphoma (U937). <sup>1</sup>H NMR for both G and GS compounds  
15 showed  $\beta$ -glycosidic linkages and structural similarities when compared with two standards  
16 (Laminarin and Fucoidan). The existence of characteristic absorptions at 1,170 and 867  $\text{cm}^{-1}$  in  
17 the FTIR for GS demonstrated the successful sulfation of G. Only GS exhibited antimicrobial  
18 activity against a varied range of test bacteria of relevance to foodstuffs and human health.  
19 Moreover, both G and GS did not show any cytotoxic effects on PN2TA cells, thus helping  
20 demonstrate the safety on these polymers. Also, GS shows 40% antiproliferation against  
21 cancerous U937 cells at low concentration (60  $\mu\text{g}/\text{mL}$ ) applied in this study compared to G (10%).

---

\*Corresponding author: [Tel:+447990716141](tel:+447990716141), Fax:+4401415482125, Email:wan.bin-wan-mohtar@strath.ac.uk

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

## 25 **Keywords**

26 *Ganoderma lucidum*, (1,3)- $\beta$ -D-glucan sulfate, Antimicrobial activity, Cytotoxicity

27 **Running title:** Antimicrobial activity of *G. lucidum* glucan sulfate

## 28 **1. INTRODUCTION**

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

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

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

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

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

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

## 74 **2. MATERIALS AND METHODS**

### 75 **2.1 Reagents**

76 Gentamicin susceptibility test discs (30 mcg/mL) were supplied by Thermo Scientific  
77 Oxoid (Fisher Scientific, Loughborough, UK). In this experiment, (U937) Human-Caucasian-  
78 Histiocytic-Lymphoma and Human-Prostate-Normal cell (PNT2A) were obtained from ECAAC,  
79 European Collection of Cell Cultures, supplied by (Sigma-Aldrich, Dorset, UK). DMEM and  
80 TrypLE™ Express were provided by Gibco (Life Technologies, Paisley, UK). RPMI - Bio  
81 Whittaker® without L-glutamine was supplied by Lonza, Vergiers, Belgium. HBBS –Hank's  
82 balanced salt solution was provided by Sigma-Aldrich, St. Louis, USA. The 96-wells plate, TPP  
83 92096 was provided by TPP, Trasadingen, Switzerland. Cell culture spectroscopy analysis was  
84 done using Wallac, Victor2™<sub>H20</sub> Multilabel Counter with IR, high-density TR-Fluorometry,  
85 robot loading and stacker (PerkinElmer, Waltham, MA, USA). Entire solvents and chemicals  
86 were analytical grades.

## 87 **2.2 Fungal material**

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

## 95 **2.3 Extraction, isolation, and sulfation**

96 Distilled water (D<sub>2</sub>O) was functioned to rinse the mycelium (biomass) off the sieves from  
97 the fermented culture broth. Through Whatman filter paper; they were filtered and vaporized to  
98 50 mL at 60-80 °C. This volume was added to 150 mL of ethanol, for macromolecules  
99 precipitation, containing the desired polysaccharide-derived β-glucan. A glass rod was used to  
100 obtained the product by twirling. Based on the macromolecules precipitation, the precipitate was  
101 attached or adsorbed onto the glass rod and harvested from the solution. The glucose, however,  
102 may be confined within the extracted precipitate, which was then splashed using 96% (v/v)  
103 ethanol. Subsequently, the solution was dialyzed against distilled water for three days (MW cut-  
104 off = 10,000 Da) using a dialysis tube (Fisher Scientific, Loughborough, UK). The residual  
105 glucan was aerated and pre-chill in -20 °C freezer. After a couple of hours (h), the samples were  
106 transferred to -80 °C freezer for 24 h and then freeze-dried for 48 h. Later on, the build-up  
107 moisture surrounding the precipitated glucan were completely evaporated. It was then re-  
108 suspended in distilled water, freeze-dried in -80 °C freezer and evaluated to yield a 1,3-β-D-  
109 glucan (G).

110 The G produced from the bioreactor fermentation processes was water-insoluble;  
111 therefore an inevitable process needs to be implemented to increase its solubility in water. Suzuki  
112 et al., [32] and Williams et al., [37] did sulfation of active 1,3-β-D-glucans to increase their  
113 solubility or increase their bioavailability. Hence, sulfation of the current water-insoluble G was  
114 executed in this experiment. The improvised method of G sulfation of Williams et al., [37] was  
115 followed. Soluble 1,3-β-D-glucan sulphate (GS) was produced as outlined in Fig. 1. Firstly, 1 g  
116 of microparticulate G was liquefied in 50 mL of dimethyl sulfoxide (DMSO) containing 6 M urea.  
117 8 mL of concentrated sulphuric acid was added drop-wise directly erstwhile to heating. In a water  
118 bath, the solution was heated at 100 °C, and the reaction process continued for 3 to 6 h. By 90  
119 minutes, a crystalline precipitate (ammonium sulfate) was formed. The mixture solution was then  
120 vented at room temperature, and 1 L of ultrapure, pyrogen-free, D<sub>2</sub>O (Millipore, Bedford, MA)  
121 was added. The GS solution was then pre-filtered to remove unreacted polymer in G. The GS  
122 solution was dialyzed using a Vivaflow 200, using a 10,000 MW cut-out filter (Sartorius Stedim  
123 Lab Ltd, Binbrook, UK). The final volume was reduced to 500 mL and lyophilized to dryness.

#### 124 **2.4 Elemental analysis**

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

$$129 \quad DS = \frac{72s}{32c} \quad (A)$$

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

## 132 **2.5 Infrared Spectroscopy**

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

## 136 **2.6 <sup>1</sup>H NMR Spectroscopy**

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

## 144 **2.7 Bioassay of antimicrobial activity**

145 The test bacteria used for antimicrobial sensitivity testing comprise the bacteria  
146 *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Staphylococcus*  
147 *epidermis*, *Escherichia coli* that were obtained from the General Microbiology Lab Collections  
148 SIPBS, Glasgow, UK. In addition, *Escherichia coli* EPIC S17, *Salmonella* BA54 SL1344 pSsaG,  
149 *Listeria monocytogenes*, *Shigella sonnei* 20071599, and Methicillin-Susceptible-*Staphylococcus*  
150 *aureus* (MSSA) ATCC 292123 were kindly supplied by Dr. Jun Yu, SIPBS, Glasgow, UK. At 20  
151 °C, the strains were kept in the suitable freshly-prepared medium and rejuvenated two times  
152 before being applied in the proposed assays. Bacteria were cultured with the oxygen supplied  
153 environment at 37 °C (Incubator- Bruker 200, Thermo, UK) in nutrient agar (NA) medium for  
154 bacteria.



### 155 **2.7.1 Kirby-Bauer disk diffusion assay, MIC, and MBC**

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

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

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

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

## 190 **2.8 Bioassay of cytotoxicity**

### 191 **2.8.1 On normal cells**

192 Cell lines were grown in the appropriate freshly-prepared complete medium in a cell  
193 culture incubator (gaseous composition 95% air, 5% CO<sub>2</sub>) at 37 °C. The PN2TA normal human  
194 prostate cell line was sustained in a complete medium comprising RPMI, penicillin-streptomycin  
195 (5 mL), 50 mL fetal bovine serum (FBS), L-glutamine (5 mL), and pH at 7.4. AlamarBlue®  
196 assay determined the cytotoxic effect of both G and GS. Initially, 96-well microtitre plates were  
197 seeded with the PN2TA cells at  $2 \times 10^4$  cells/mL for each well. Cells were permitted to cultivate  
198 one day afore being introduced to GS: 500, 300, 50, 30, 5, and 3  $\mu$ g/mL. For the negative control

199 group, 4% (v/v) of Triton-X was added to the medium. After the incubation for the indicated  
200 hours, 10% (working volume per well) of alamarBlue® reagent was decanted to each well and  
201 incubated for an extra 6 h in a humidified incubator. Once 6 h of incubation completed, the  
202 resazurin in the alamarBlue® undergoes oxidation-reduction change in response to cellular  
203 metabolic change. The reduced form resazurin is pink and extremely fluorescent, and the strength  
204 of fluorescence produced is proportional to some living cells undergone respiration. The  
205 wavelength of 570 nm was used for absorbance reading. For analysis, cytotoxic activity was  
206 calculated based on cell survival ratio (%).

### 207 **2.8.2 On cancer cells**

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

218

### 219 **2.9 Statistical analysis**

220

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

## 224 **3. RESULTS AND DISCUSSION**

### 225 **3.1 Glucan solubility**

226

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

236 The introduction of sulfate group has several purposes. Based on the present study's  
237 findings, the aqueous solubility of the extracted G from the fermenter was as poor as that of G  
238 prepared from other procedures. Astonishingly, this was mentioned in the literature that G was  
239 less suitable for medicinal applications [13]. In terms of the commercial importance of bioactive  
240 glucan, the water insoluble G show slight bioactivity, although G by-products such as pullulan  
241 sulfate, lentinan sulfate, and dextran sulfate have been suggested to display high anti-HIV  
242 activities and small anticoagulant activities [36]. Wang and Zhang [6] also revealed that the  
243 sulfation process on the fruiting bodies of *G. lucidum* producing G have led to enhanced

244 antitumor and antiviral activities [23, 27]. However, comparable studies on the antimicrobial and  
245 cytotoxicity of mycelial-sourced GS are limited.

## 246 3.2 Compositional analysis

### 247 3.2.1 Elemental analysis

248 Elemental analysis was accomplished to attain the composition of the GS and, therefore,  
249 its degree of sulfation (DS). Basic examination of lyophilized GS gives a composition of (w/w):  
250 24.5% C, 5.72% H, 49.92% O, 9.85% S, and 10.01% N (Table 2). When compared with standard  
251 fucoidan, GS had the same C and H values but slight different in H, O, and S. This was due to  
252 different sulfation technique applied on each GS and fucoidan, respectively, and this might  
253 generate different molecular weight.

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

### 258 3.2.2 IR spectroscopy

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

265 In the functional group region of the G-spectra, there were significant absorptions at  
266 3,400, 1,077, 2,925, 1,374, 1,647, 1,246, 1,540, 1,077, and 892  $\text{cm}^{-1}$ , which resembles the

267 elongating absorption bands of poly -OH, C=O=C, -CH<sub>2</sub>, -CH<sub>3</sub>, C=O, amide, pyranose ring and β-  
268 configuration of D-glucose units. As compared with the previous work by Wang et al., [36] and  
269 Kiho et al., [22], the specific absorption of G at 892.9 cm<sup>-1</sup> demonstrates that the compound is a  
270 β-glucan. The characteristic peak of the β-configuration at 892.9 cm<sup>-1</sup> was also noted in the  
271 spectra of GS with two new absorption peaks at 1,170 and 867 cm<sup>-1</sup> also present (Fig. 2), which  
272 match the to S=O asymmetrical stretching and C-S-C symmetrical vibration [35]. These  
273 confirmed that the GS had been efficiently synthesized from G.

### 274 3.2.3 NMR spectroscopy

275 As can be seen in Fig. 3 and Fig. 4, <sup>1</sup>H NMR spectroscopic analysis of the G and GS from  
276 *G. lucidum* was conducted at 80 °C using D<sub>2</sub>O-*d*<sub>6</sub> as a solvent. Using ppm as the standardized unit  
277 for NMR studies, <sup>1</sup>H NMR spectra of the G were compared with the standard laminarin (β-1,3-D-  
278 glucan) from *L. digitata* while the GS spectra were compared with the standard fucoidan  
279 (sulfated-β-1,3-D-glucan) from *F. vesiculosus*. The spectrum chemical shifts of δ 3.9 to 5.4 ppm  
280 and δ 2.6 to 5.5 ppm exhibited indicate that both compounds were glucans, as can be observed in  
281 both Fig. 3 and Fig. 4, respectively. The current work is comparable with previous research by Ji  
282 et al., [16], which analyzed laminarin and sulfated laminarin in the area of <sup>1</sup>H-NMR spectrum of  
283 δ 4.49-5.5 ppm. Thus, these spectra indicate that the glycosidic bonds in both G (Fig. 3) and GS  
284 (Fig. 4) were β-type.

285 Evaluation of the ‘anomeric region’ of <sup>1</sup>H NMR spectra in this study with those  
286 described previously specifies that they are of similar pattern [22, 33, 36]. For G (Fig. 3) <sup>1</sup>H  
287 NMR spectra, the signals at δ 5.08, 4.50 and 4.40 were assigned to OH-2, OH-6, and OH-4 when  
288 compared with the reported work by Wagner et al., [33]. The GS (Fig. 4) <sup>1</sup>H NMR also exhibits  
289 similarity to the G with the signals at δ 5.21, 4.52 and 4.40. When compared, the anomeric

290 signals for both compounds in the present study (G and GS) were at  $\delta$  4.5 ppm and  $\delta$  4.2 ppm,  
291 respectively indicating  $\beta$ -configuration for glucopyranosyl units as reported by Liu et al., [22].

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

### 297 **3.3 Assessment of antimicrobial activity**

298  
299 The antimicrobial effect of the GS from *G. lucidum* was tested against ten species of  
300 bacteria as G was not evident. Their strength was measured quantitatively and qualitatively by the  
301 absence or presence of inhibition zones, zone diameters, MBC and MIC values. The findings of  
302 these tests are summarized in Table 3 (inhibition zone diameters). Among the bacterial strains  
303 tested in Table 3, when the GS reached 500 mg/mL, the diameters (mm) of the inhibition zone  
304 were  $34 \pm 3.2$ ,  $24 \pm 2.6$ ,  $32 \pm 1.0$ ,  $25 \pm 2.6$ ,  $23 \pm 2.8$ ,  $27 \pm 1.5$ ,  $28 \pm 0.5$ ,  $26 \pm 1.0$ ,  $30 \pm 1.0$ , and  $30$   
305  $\pm 3.1$ , for *E. coli* EPIC S17, *E. coli*, *L. monocytogenes*, *Shigella sonnei* 20071599, *P. aeruginosa*,  
306 *S. enteritidis*, *Salmonella* BA54 SL 1344 (pSsaG), *Staph. aureus*, *Staph. epidermis*, and  
307 Methicillin-Susceptible *Staph. aureus* (MSSA) ATCC 292123, respectively. The inhibition zone  
308 diameters increased with increasing GS prepared concentrations (Table 3). These reactions  
309 displayed that the antimicrobial effect of GS was dose-dependent and that the gentamicin positive  
310 control was clearly effective against all the test bacteria.

311 Furthermore, the MIC concentrations for bacterial strains were in the range of 1- 5 mg/mL  
312 and the MBC concentrations range was 5-10 mg/mL except the resilient *Shigella sonnei*

20071599 (Table 3, no.3). Among four species of Gram-positive bacteria verified, the greatest active antimicrobial activity of GS was shown against *Staph. aureus* (Table 3, no.8), and its MIC was 2 mg/mL. Meanwhile, the antimicrobial activity of GS was verified against six species of Gram-negative microbial strains. GS exhibited fairly strongest antimicrobial activity against *E. coli* (Table 3, no.2) (MIC = 1 mg/mL), and seven species of microbial strains were shown to have MIC concentrations at respective 3 mg/mL while the most resistant bacterium was *Staph. epidermis* (Table 3, no.9) (MIC = 5 mg/mL).

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

The antimicrobial activity of some bacteria including *K. pneumoniae* ATCC 13883 and *M. marinum* ATCC BAA 535 were tested via 96-well microtitre plates to assess the antimicrobial effects of GS. Overall, the results showed some clear inhibition of growth of both these test species (Table 4). The Gram-negative *K. pneumoniae* ATCC 13883 exhibited a survival of  $52.8 \pm$



336 5.66 % (at 500  $\mu\text{g/mL}$  and 24 h incubation) while the acid-fast bacteria *M. marinum* ATCC BAA  
337 535 gave a survival value of  $65 \pm 3.39$  % (at 100  $\mu\text{g/mL}$  and 24 h incubation) compared to  
338 positive growth controls. Due to the significant and increasing occurrence of nosocomial  
339 infections and destructive changes to human lungs as mentioned by Daligault et al.,[6] by  
340 antibiotic-resistant *K. pneumoniae* ATCC 13883 the possibility of using novel antimicrobials  
341 from processed natural sources such as GS extracted from *G. lucidum* merits further  
342 investigation and refinement. Meanwhile, GS might have some potential in controlling the  
343 occurrence of common granulomatous diseases arising from *M. marinum* ATCC BAA 535 that  
344 affect individuals who work with fish or keep aquaria as described by Slany [31].

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

356 Slany et al., [31] discuss the impact of sulfation on the structure and biological activity. In  
357 general, the sugar chain conformation becomes modified by the process of sulfation such that  
358 non-covalent bonds form more readily when the  $-\text{OH}$  groups in a  $\beta$ -glucan element are replaced

359 with sulfate groups. Similarly, repulsions between the anionic groups lead to elongation of the  
360 sugar chain. They propose that these events result in the polymer developing an active  
361 conformation, thus initiating the bioactivity surge.

362 In the last 20 years, there have been insufficient reports on antimicrobial activities of  
363 biopolymers from *Ganoderma* species [6, 14, 20, 39]. This genus has been commonly considered  
364 for its therapeutic properties, but less widely explored as a source of novel antibacterial agents  
365 [12, 14]. However, certain polysaccharides from *Ganoderma* species employ antibacterial  
366 activity by hindering the growth of bacteria and, in some events, by eliminating pathogenic  
367 bacteria [30]. Nearly all antibacterial investigations on *Ganoderma* species have been  
368 accomplished on the fruiting body and not on extracts from the liquid cultivated mycelium, a  
369 point which is made strongly in the recent review by Ferreira et al., [11]. Meanwhile, most of the  
370 positive antibacterial compounds were from alcoholic extracts, hot-water extracts and  
371 triterpenoids of fruiting bodies. The current work is the first to show positive results using GS  
372 extracted from *G. lucidum* mycelium produced in the bioreactor.

### 373 **3.4 Assessment of cytotoxicity activity**

374

375 The current extracted and processed GS from *G. lucidum* was reactive against pathogenic  
376 bacteria. Yet, to ensure whether these compounds might have clinical impact on healthy patient  
377 cells and before their introduction as new antimicrobial drugs, some preliminary assessment of  
378 the impact of such biomolecules upon normal host cells is of interest. Likewise, assessment of the  
379 effects of such derivatized polymers on tumors is of value given the widely reported impact of  
380 other fungal macromolecules on such cell types. Accordingly, in the present study cytotoxicity  
381 assays using alamarBlue® reagent were carried out on healthy human prostate cells (PN2TA).

382 The *in vitro* effects of both GS and its G from *G. lucidum* on PN2TA were studied in the current  
383 work (Fig. 5).

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

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

400 The current concentration of GS (60  $\mu\text{g/mL}$ ) applied is considerably lower than that used  
401 in the earlier study on of sulfated glucan (sourced from *Hypsizigus marmoreus*) which showed  
402 only 39% of antiproliferative activity at 1000  $\mu\text{g/mL}$  [2], thus further concentration increment for  
403 the current work would highly beneficial. As reported, the molecular weight, chemical

404 configuration, degree of branching, and structure of the polymeric backbone were crucial for  
405 antiproliferative activities stimulation for both G and GS [25]. Therefore, the biochemical aspects  
406 and mechanism of the antiproliferative reactions stimulated by GS from mycelium of *G. lucidum*  
407 is still not fully unspoken and requests further study.

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

## 420 **Acknowledgements**

421 This work was financially supported by the Majlis Amanah Rakyat (MARA) London under  
422 Malaysian Government Sponsorship. Greatest appreciation to Dr Jun Yu, microbiology  
423 department, SIPBS, University of Strathclyde, Glasgow for supplying some tested bacteria.

424

425

426 **Figures captions**

427 **Fig. 1.** Homogeneous reaction for sulfated (1-3)- $\beta$ -D-glucan (GS) preparation: process scheme.

428 Improved from Wang et al [36].

429 **Fig. 2.** Comparison of  $\beta$ -glucan IR spectra. A: glucan (G); B: glucan sulfate (GS) derived from  
430 extended batch cultures of *G. lucidum* BCCM 31549 mycelium.

431  
432 **Fig. 3.**  $^1\text{H}$  NMR spectra of (1-3)- $\beta$ -D-glucan (G) derived from extended batch cultures of *G.*  
433 *lucidum* BCCM 31549 mycelium and laminarin (*Laminaria digitata*) standard in  $\text{D}_2\text{O}$ - $d_6$  at 80 °C.

434 **Fig. 4.**  $^1\text{H}$  NMR spectra of sulfated (1-3)- $\beta$ -D-glucan (GS) derived from extended batch cultures  
435 of *G. lucidum* BCCM 31549 mycelium and fucoidan (*Fucus vesiculosus*) standard in  $\text{D}_2\text{O}$ - $d_6$  at 80  
436 °C.

437

438 **Fig. 5.** Cytotoxicity effects of both glucan (G) and glucan sulfate (GS) derived from extended  
439 batch cultures of *G. lucidum* BCCM 31549 mycelium in normal human Prostate-cell-line  
440 (PN2TA). After the cells were incubated with G and GS treatments [Control, 500, 300, 50, 30, 5,  
441 3, Triton X  $\mu\text{g/mL}$ ], the viability was measured by alamarBlue® assay. Both G and GS had the  
442 same morphological observation under the microscope at 10x magnification. Each data was  
443 presented S.D  $\pm$  mean, and the P value was  $> 0.05$  when compared to control. If the error bars do  
444 not appear then, they are less than the size of the icon or symbol.

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

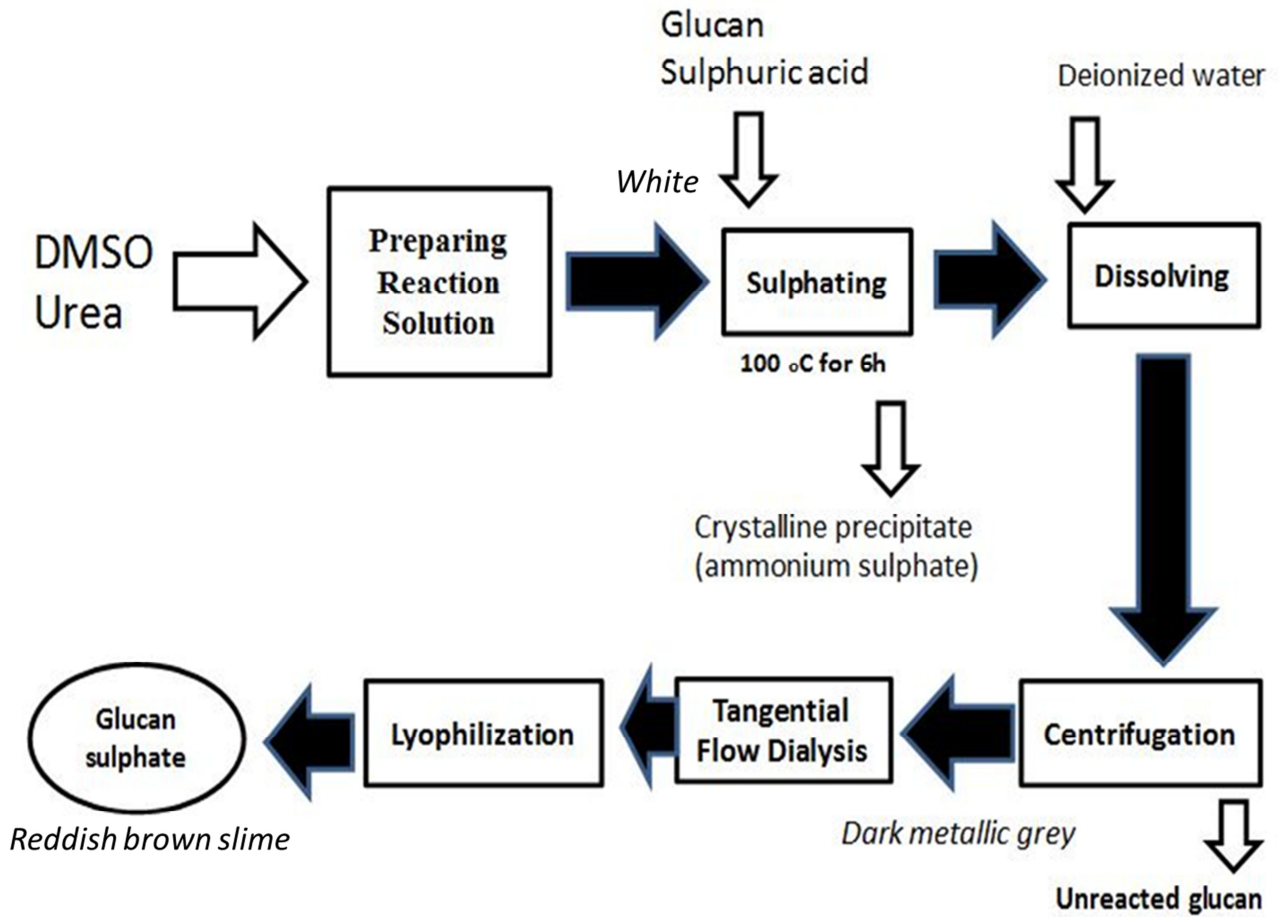
452 **Fig. 7.** Cytotoxicity effects of glucan sulfate (GS) derived from glucan (G) against cancerous  
453 human Caucasian-Histiocytic-lymphoma cell line (U937) from a 37-year-old male patient. After  
454 the cells were incubated with GS treatments [Control, 60, 50, 30, 10, Triton X  $\mu\text{g/mL}$ ], the  
455 viability was measured by alamarBlue® assay. GS had morphological observation under the  
456 microscope at 10x magnification. Each data was presented S.D  $\pm$  mean, and the P value was  $<$   
457  $0.05$  when compared to control. If the error bars do not appear then, they are less than the size of  
458 the icon or symbol.

459

460

461 **Figures**

462 Fig. 1



463

464

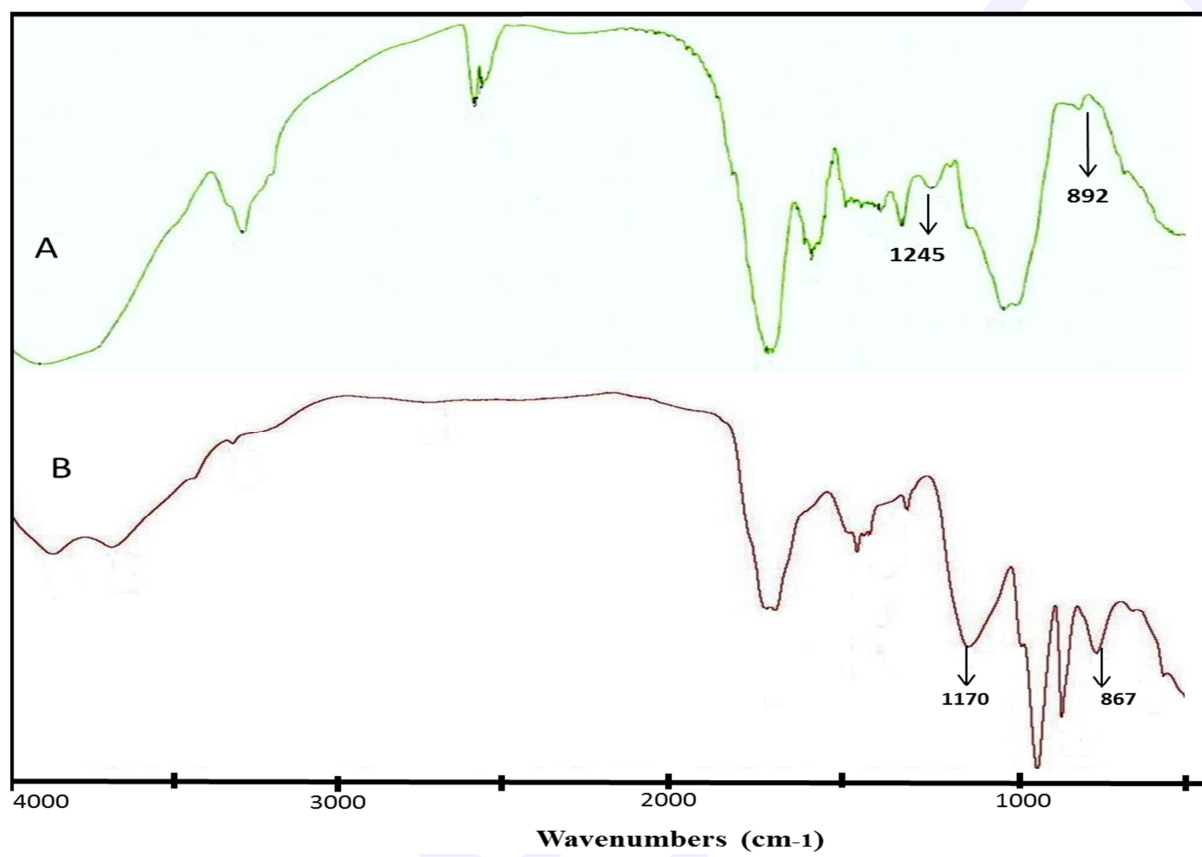
465

466

467

468

469 Fig. 2



470

471



Fig. 3

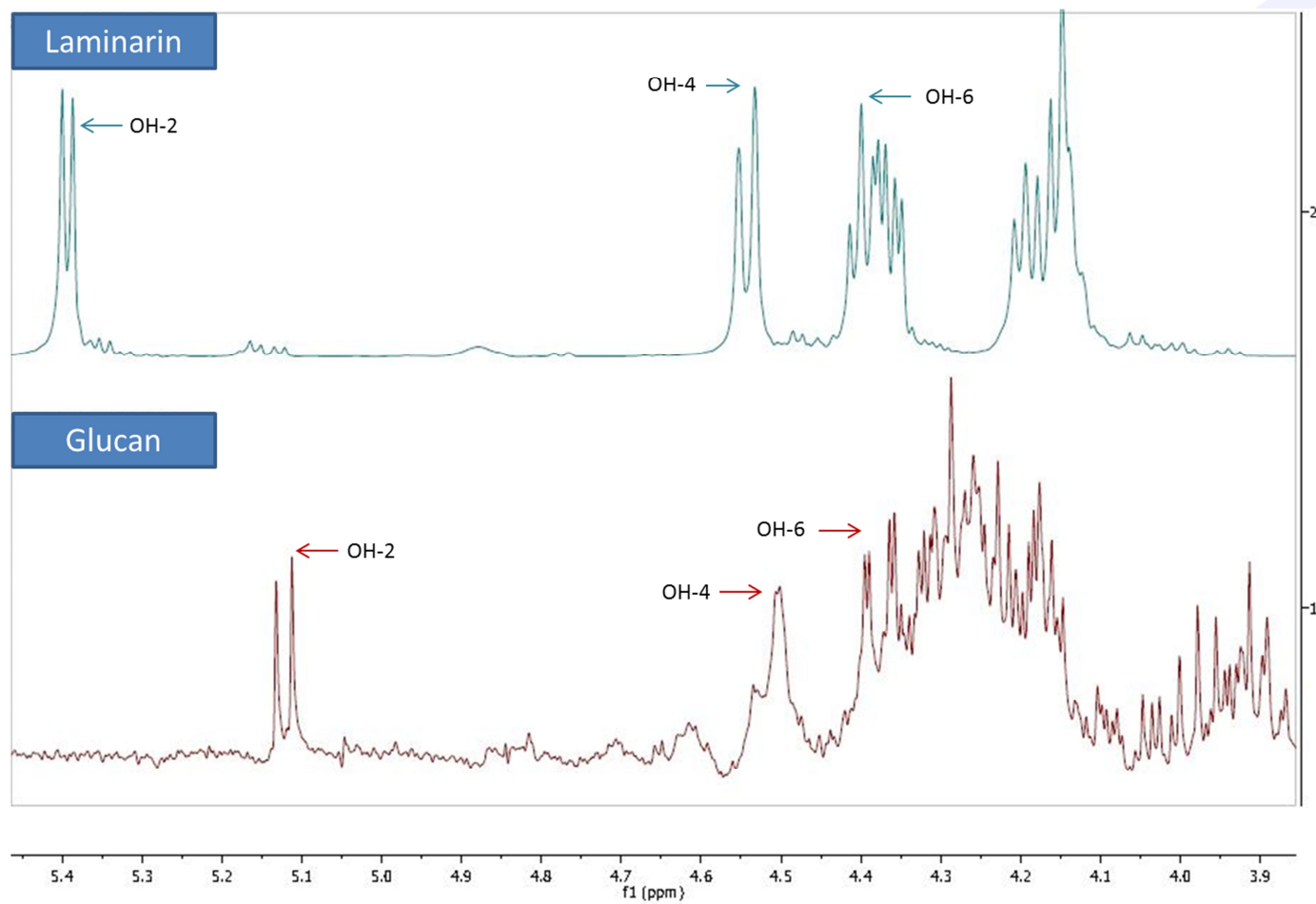


Fig. 4

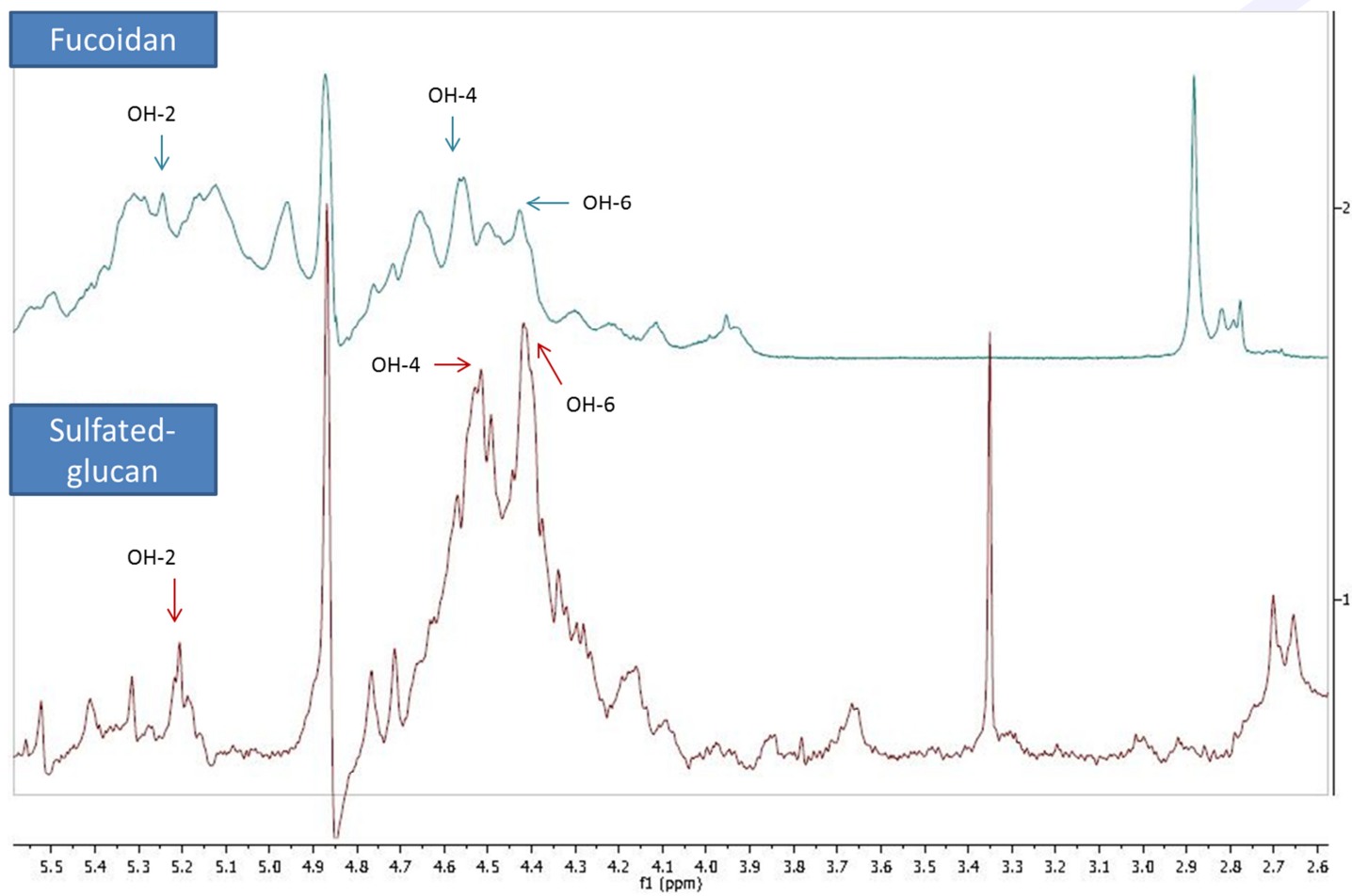


Fig. 5.

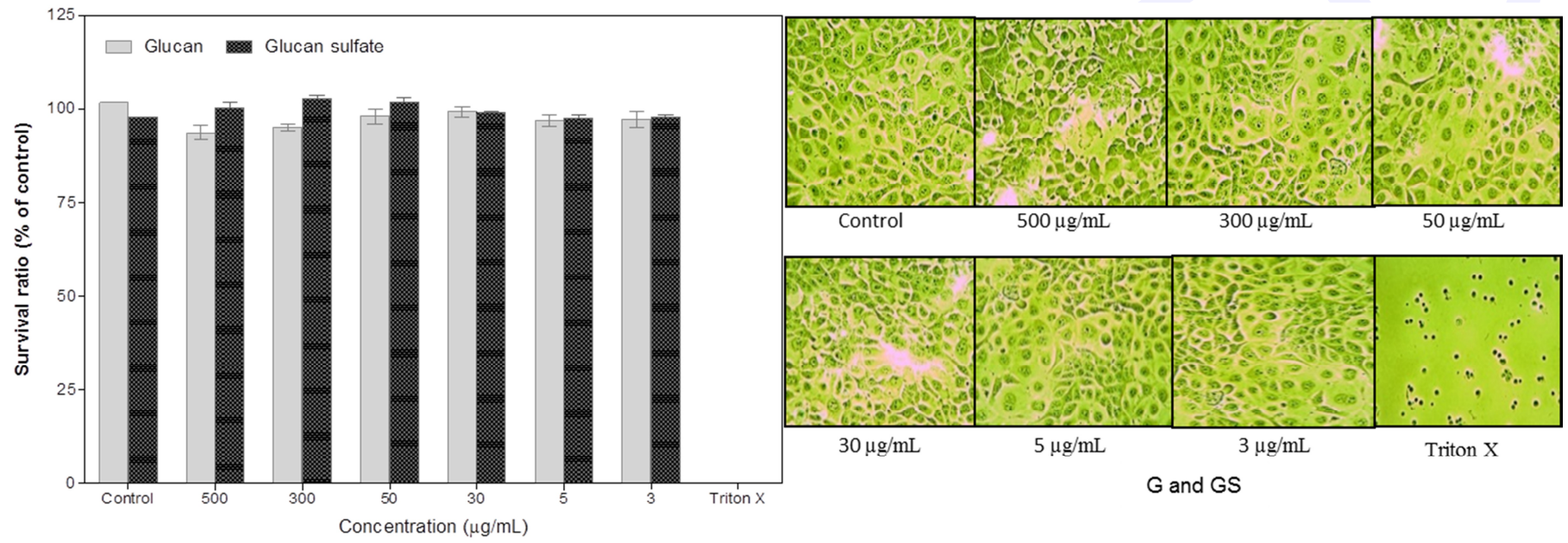


Fig. 6.

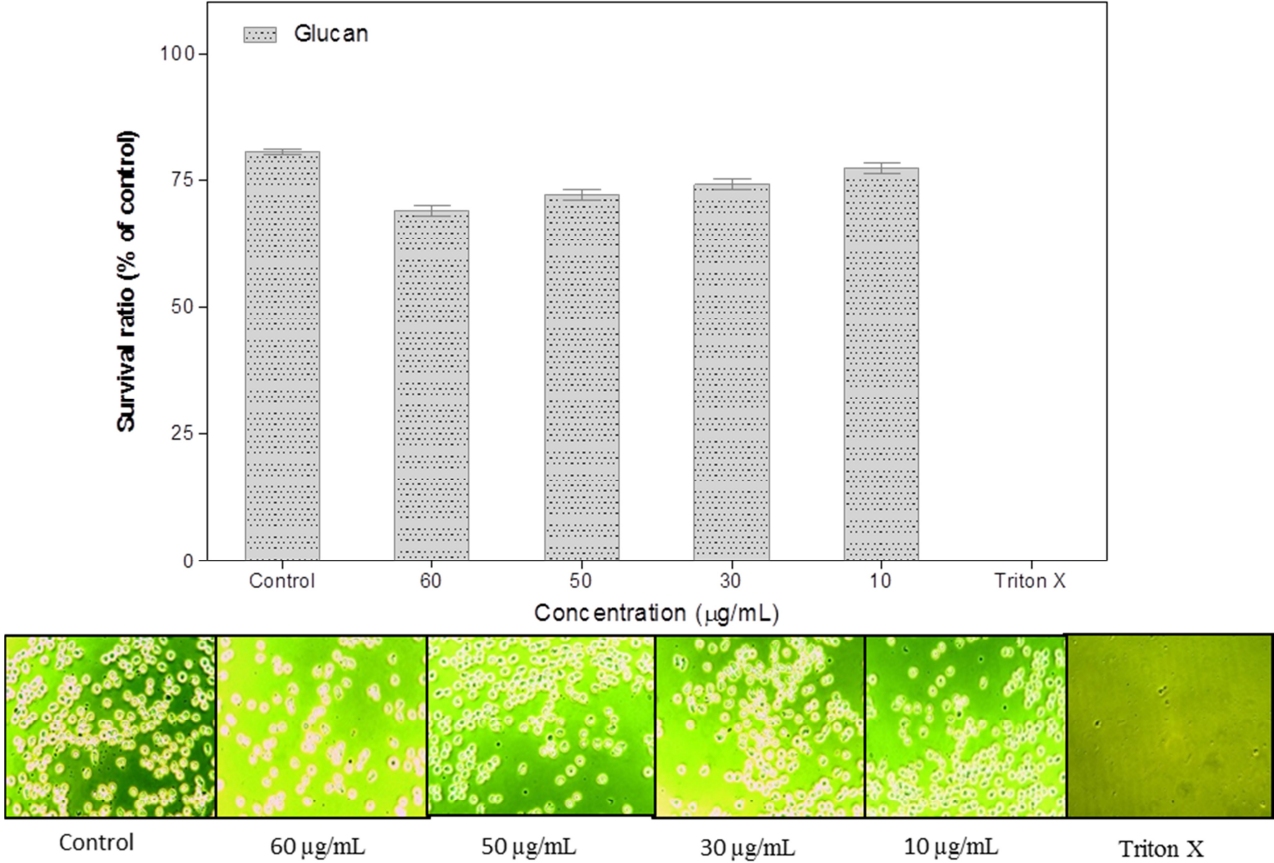
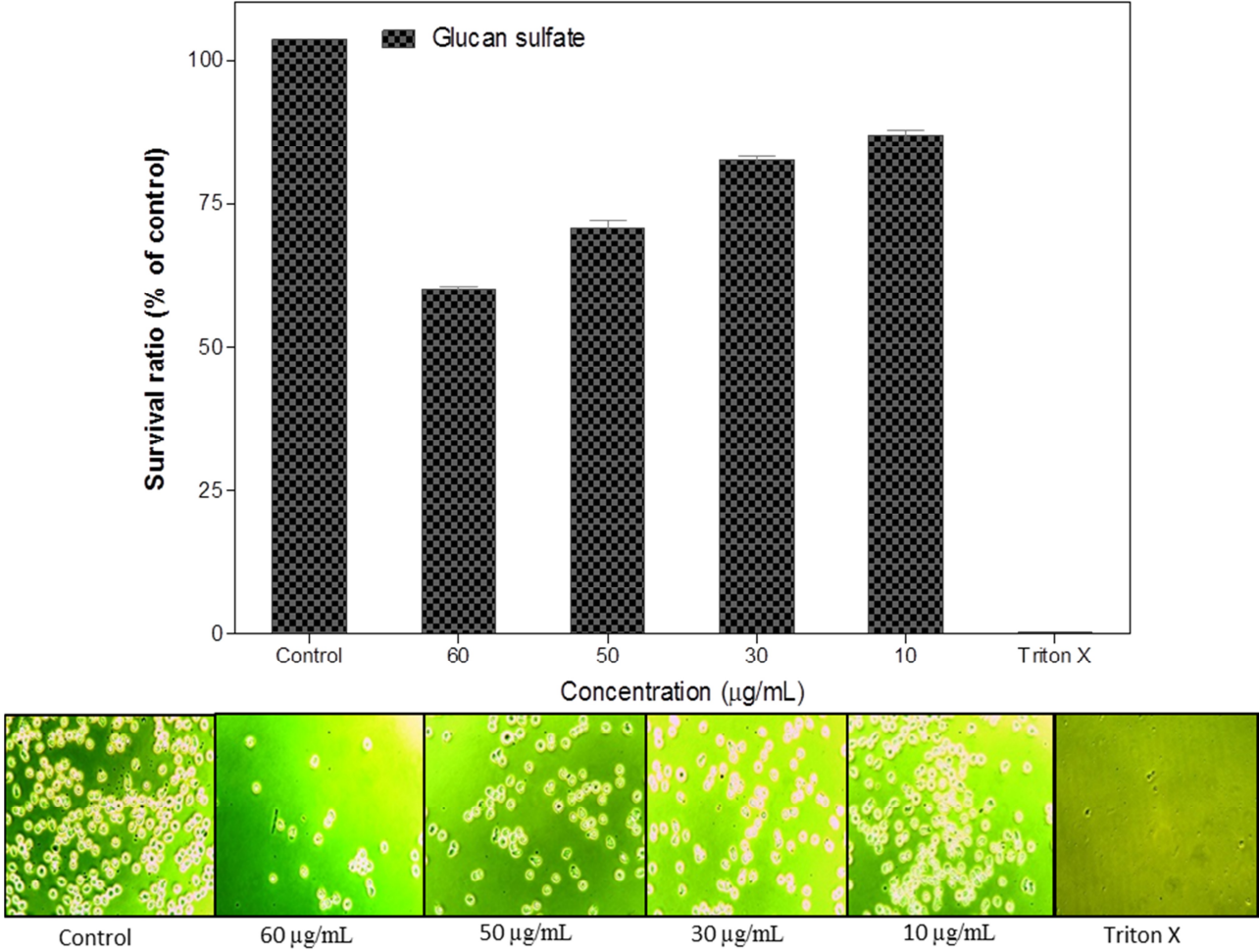


Fig. 7



## 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

Sample	(w/w) %					
	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 <sup>a</sup>

No./ G	Bacteria	GS						GENT <sup>c</sup> 30 mcg	Ethanol <sup>c</sup> 100%
		Diameter of inhibition zone <sup>b</sup> (mm)				MIC <sup>d</sup> mg/mL	MBC <sup>e</sup> mg/mL		
		200 mg/mL	300 mg/mL	400 mg/mL	500 mg/mL				
1 (G-)	<i>Escherichia coli</i> 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-)	<i>Escherichia coli</i>	20 ± 1.5	22 ± 1.5	20 ± 1.1	24 ± 2.6	1	2	23 ± 3.1	11.3 ± 0.1
3 (G-)	<i>Shigella sonnei</i> 20071599	16 ± 1.0	21 ± 0.5	23 ± 2.1	25 ± 2.6	3	20	22 ± 1.5	11.4 ± 0.1
4 (G-)	<i>Pseudomonas aeruginosa</i>	16 ± 1.0	20 ± 1.0	20 ± 0.5	23 ± 2.8	3	5	23 ± 1.1	11.2 ± 0.1
5 (G-)	<i>Salmonella enteritidis</i>	17 ± 1.5	20 ± 1.0	23 ± 1.0	27 ± 1.5	3	10	24 ± 2.5	11.1 ± 0.1
6 (G-)	<i>Salmonella</i> 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+)	<i>Listeria monocytogenes</i>	26 ± 2.1	28 ± 1.0	30 ± 1.5	32 ± 1.0	3	8	34 ± 1.0	11.1 ± 0.1
8 (G+)	<i>Staphylococcus aureus</i>	18 ± 1.0	20 ± 0.5	21 ± 2.3	26 ± 1.0	2	5	21 ± 1.0	11.1 ± 0.1
9 (G+)	<i>Staphylococcus epidermis</i>	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- <i>Staphylococcus aureus</i> 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

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

Bacteria	<i>Klebsiella pneumoniae</i> ATCC 13883	<i>Mycobacterium marinum</i> ATCC. BAA. 535	Gentamicin	DMSO
Gram	(-)	Acid-fast bacteria		
GS ( $\mu\text{g/mL}$ )	500	100	100	100
Survival ratio <sup>b</sup> (% of control)	52.8 $\pm$ 5.66	65 $\pm$ 3.39	1 $\pm$ 1.20	99 $\pm$ 1.05
Inhibition status	Positive	Positive	(+) control	(-) control

<sup>a</sup> Values represent averages  $\pm$  S.D (P < 0.05) for triplicate experiments

<sup>b</sup> Lower percentage of control value means the greater antibacterial effect



## References

1. Alvarez-Suarez JM, Tulipani S, Díaz D, Estevez Y, Romandini S, Giampieri F, *et al.* 2010. Antioxidant and antimicrobial capacity of several monofloral Cuban honeys and their correlation with color, polyphenol content and other chemical compounds. *Food Chem Toxicol.* **48**: 2490-2499.
2. Bao H, Choi W-S, and You S. 2010. Effect of sulfated modification on the molecular characteristics and biological activities of polysaccharides from *Hypsizigus marmoreus*. *Biosci Biotech Biochem.* **74**: 1408-1414.
3. Bao XF, Wang XS, Dong Q, Fang JN, and Li XY. 2002. Structural features of immunologically active polysaccharides from *Ganoderma lucidum*. *Phytochem.* **59**: 175-181.
4. Cechinel-Filho V. 2012. *Plant bioactives and drug discovery: principles, practice, and perspectives*. John Wiley & Sons, Inc., Hoboken, New Jersey.
5. Chen S, Wang J, Xue C, Li H, Sun B, Xue Y, and Chai W. 2010. Sulfation of a squid ink polysaccharide and its inhibitory effect on tumor cell metastasis. *Carbohydr Polym.* **81**: 560-566.
6. Daligault H, Davenport K, Minogue T, Bishop-Lilly K, Bruce D, Chain P, *et al.* 2014. Draft genome assembly of *Klebsiella pneumoniae* type strain ATCC 13883. *Genome Announc.* **2**: e00939-00914.
7. Devlieghere F, Vermeulen A, and Debevere J. 2004. Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. *Food Microbiol.* **21**: 703-714.
8. Dutta P, Tripathi S, Mehrotra G, and Dutta J. 2009. Perspectives for chitosan based antimicrobial films in food applications. *Food Chem.* **114**: 1173-1182.
9. Ellington MJ, Hope R, Livermore DM, Kearns AM, Henderson K, Cookson BD, *et al.* 2010. Decline of EMRSA-16 amongst methicillin-resistant *Staphylococcus aureus* causing bacteraemias in the UK between 2001 and 2007. *J Antimicrob Chemoth.* **65**: 446-448.
10. Fazenda ML, Harvey LM, and McNeil B. 2010. Effects of dissolved oxygen on fungal morphology and process rheology during fed-batch processing of *Ganoderma lucidum*. *J Microbiol Biotechnol.* **20**: 844-851.
11. Ferreira IC, Heleno SA, Reis FS, Stojkovic D, Queiroz MJR, Vasconcelos MH, and Sokovic M. 2015. Chemical features of *Ganoderma* polysaccharides with antioxidant, antitumor and antimicrobial activities. *Phytochem.* **114**: 38-35.

12. Gao Y, Zhou S, Huang M, and Xu A. 2003. Antibacterial and antiviral value of the genus *Ganoderma* P. Karst. species (aphyllophoromycet-ideae): a review. *Int J Med Mushrooms*. **5**: 235–246.
13. Han MD, Han YS, Hyun SH, and Shin HW. 2008. Solubilization of water-insoluble  $\beta$ -glucan isolated from *Ganoderma lucidum*. *J Environ Biology*. **29**: 237-242.
14. Heleno SA, Ferreira IC, Esteves AP, Ćirić A, Glamočlija J, Martins A, *et al.* 2013. Antimicrobial and demelanizing activity of *Ganoderma lucidum* extract, p-hydroxybenzoic and cinnamic acids and their synthetic acetylated glucuronide methyl esters. *Food Chem Toxicol*. **58**: 95-100.
15. Hernández-Lauzardo A, Bautista-Baños S, Velázquez-del Valle M, Méndez-Montealvo M, Sánchez-Rivera M, and Bello-Pérez L. 2008. Antifungal effects of chitosan with different molecular weights on in vitro development of *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. *Carbohydr Polym*. **73**: 541-547.
16. Ji CF, Ji YB, and Meng DY. 2013. Sulfated modification and anti-tumor activity of laminarin. *Exp Ther Med*. **6**: 1259-1264.
17. Karnjanapratum S, Tabarsa M, Cho M, and You S. 2012. Characterization and immunomodulatory activities of sulfated polysaccharides from *Capsosiphon fulvescens*. *Int J Biol Macromol*. **51**: 720-729.
18. Khalaf AI, Bourdin C, Breen D, Donoghue G, Scott FJ, Suckling CJ, *et al.* 2012. Design, synthesis and antibacterial activity of minor groove binders: the role of non-cationic tail groups. *Eur J Med Chem*. **56**: 39-47.
19. Kimura Y, Taniguchi M, and Baba K. 2002. Antitumor and antimetastatic effects on liver of triterpenoid fractions of *Ganoderma lucidum*: mechanism of action and isolation of an active substance. *Anticancer research*. **22**: 3309–3318.
20. Li WJ, Nie SP, Liu XZ, Zhang H, Yang Y, Yu Q, and Xie MY. 2012. Antimicrobial properties, antioxidant activity and cytotoxicity of ethanol-soluble acidic components from *Ganoderma atrum*. *Food Chem Toxicol*. **50**: 689-694.
21. Liao SF, Liang CH, Ho MY, Hsu TL, Tsai TI, Hsieh YS, *et al.* 2013. Immunization of fucose-containing polysaccharides from Reishi mushroom induces antibodies to tumor-associated Globo H-series epitopes. *Proc Natl Acad Sci USA*. **110**: 13809-13814.
22. Liu Y, Zhang J, Tang Q, Yang Y, Guo Q, Wang Q, *et al.* 2014. Physicochemical characterization of a high molecular weight bioactive beta-D-glucan from the fruiting bodies of *Ganoderma lucidum*. *Carbohydr Polym*. **101**: 968-974.
23. Liu YJ, Shen J, Xia YM, Zhang J, and Park HS. 2012. The polysaccharides from *Ganoderma lucidum*: Are they always inhibitors on human hepatocarcinoma cells? *Carbohydr Polym*. **90**: 1210-1215.

24. Llauro G, Morris HJ, Ferrera L, Camacho M, Castan L, Lebeque Y, *et al.* 2015. In-vitro antimicrobial activity and complement/macrophage stimulating effects of a hot-water extract from mycelium of the oyster mushroom *Pleurotus* sp. *Innov Food Sci Emerg.* **30**: 177-183.
25. Ma CW, Feng MY, Zhai XF, Hu MH, You LJ, Luo W, and Zhao MM. 2013. Optimization for the extraction of polysaccharides from *Ganoderma lucidum* and their antioxidant and antiproliferative activities. *J Taiwan Inst Chem E.* **44**: 886-894.
26. Muzzarelli R, Tarsi R, Filippini O, Giovanetti E, Biagini G, and Varaldo P. 1990. Antimicrobial properties of N-carboxybutyl chitosan. *Antimicrob Agents Ch.* **34**: 2019-2023.
27. Peng Y, Zhang L, Zhang Y, Xu X, and Kennedy JF. 2005. Solution properties of water-insoluble polysaccharides from the mycelium of *Ganoderma tsugae*. *Carbohydr Polym.* **59**: 351-356.
28. Shahidi F, Arachchi JKV, and Jeon Y-J. 1999. Food applications of chitin and chitosans. *Trends Food Sci Tech.* **10**: 37-51.
29. Shi M, Zhang Z, and Yang Y. 2013. Antioxidant and immunoregulatory activity of *Ganoderma lucidum* polysaccharide (GLP). *Carbohydr Polym.* **95**: 200-206.
30. Skalicka-Wozniak K, Szypowski J, Los R, Siwulski M, Sobieralski K, Glowinski K, and Malm A. 2012. Evaluation of polysaccharides content in fruit bodies and their antimicrobial activity of four *Ganoderma lucidum* (W Curt. Fr.) P. Karst. strains cultivated on different wood type substrates. *Acta Soc Bot Pol.* **81**: 17-21.
31. Slany M, Jezek P, Fiserova V, Bodnarova M, Stork J, Havelkova M, *et al.* 2011. *Mycobacterium marinum* infections in humans and tracing of its possible environmental sources. *Canadian J Microbiol.* **58**: 39-44.
32. Suzuki T, Ohno N, Adachi Y, Cirelli AF, Covian JA, and Yadomae T. 1991. Preparation and biological activities of sulfated derivatives of (1-3)-beta-D-glucans. *J Pharmacobiodyn.* **14**: 256-266.
33. Wagner R, Mitchell DA, Sasaki GL, Amazonas MALD, and Berovic M. 2003. Current techniques for the cultivation of *Ganoderma lucidum* for the production of biomass, ganoderic acid and polysaccharides. *Food Technol Biotech.* **41**: 371-382.
34. Wang J and Zhang L. 2009. Structure and chain conformation of five water-soluble derivatives of a  $\beta$ -D-glucan isolated from *Ganoderma lucidum*. *Carbohydr Res.* **344**: 105-112.
35. Wang J, Zhang L, Yu Y, and Cheung PC. 2009. Enhancement of antitumor activities in sulfated and carboxymethylated polysaccharides of *Ganoderma lucidum*. *J Agr Food Chem.* **57**: 10565-10572.

36. Wang Y-J, Yao S-J, Guan Y-X, Wu T-X, and Kennedy J. 2005. A novel process for preparation of (1→3)-β-D-glucan sulphate by a heterogeneous reaction and its structural elucidation. *Carbohydr Polym.* **59**: 93-99.
37. Williams DL, Pretus HA, McNamee RB, Jones EL, Ensley HE, and Browder IW. 1992. Development of a water-soluble, sulfated (1→3)-beta-D-glucan biological response modifier derived from *Saccharomyces cerevisiae*. *Carbohydr Res.* **235**: 247-257.
38. Xu Z, Chen X, Zhong Z, Chen L, and Wang Y. 2011. *Ganoderma lucidum* polysaccharides: immunomodulation and potential anti-tumor activities. *Am J Chin Med.* **39**: 15-27.
39. Yoon SY, Eo SK, Kim YS, Lee CK, and Han SS. 1994. Antimicrobial activity of *Ganoderma lucidum* extract alone and in combination with some antibiotics. *Arch Pharm Res.* **17**: 438-442.
40. Zhang J, Liu Y-j, Park H-s, Xia Y-m, and Kim G-s. 2012. Antitumor activity of sulfated extracellular polysaccharides of *Ganoderma lucidum* from the submerged fermentation broth. *Carbohydr Polym.* **87**: 1539-1544.