Development of Ganoderma lucidum spore powder based proteoglycan and its application in hyperglycemic, antitumor and antioxidant function

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and antioxidant function

Li-Fang Zhu,^{1,2} Yuanfa Yao,^{1,2} Zeeshan Ahmad,³ Ming-Wei Chang^{1,2,4*}

¹ Key Laboratory for Biomedical Engineering of Education Ministry of China, Zhejiang University, Hangzhou, 310027, PR China.

² Zhejiang Provincial Key Laboratory of Cardio-Cerebral Vascular Detection Technology and Medicinal Effectiveness Appraisal, Zhejiang University, Hangzhou, 310027, PR China.

³ Leicester School of Pharmacy, De Montfort University, The Gateway, Leicester, LE1 9BH, UK.

⁴ Nanotechnology and Integrated Bioengineering Centre, University of Ulster, Jordanstown Campus, Newtownabbey, BT37 0QB, Northern Ireland, UK.

*corresponding author: Ming-Wei Chang, Ph.D., Assoc. Professor

Tel: +86(0)571-87951517, Email: mwchang@zju.edu.cn

obtained via solvent extraction and optimized using response surface methodology (RSM). The differences among the characteristics of the new proteoglycan from cracked (proteoglycan-C) and uncracked GLSP (proteoglycan-UC) were explored. The SDS-PAGE results showed the molecular weight of protein contained in proteoglycan-UC (55, 72, 95 kDa) was different to that proteoglycan-C (43, 95 kDa). The differences of these amino acids (Glu, Arg, Leu and Lys) content and monosaccharides (Fuc, Gal, Glc, Man and Gal-AC) content between proteoglycan-C and proteoglycan-UC were determined by HPLC/ ion chromatography. The 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities of proteoglycan-C were higher than that proteoglycan-UC. The proteoglycan-UC exhibited stronger hypoglycemic and antibacterial effects against *E. coli* and *S. aureus*, and the proteoglycan-C and proteoglycan-UC showed antitumor effects with potential for therapeutic applications.

Keywords: proteoglycan; *Ganoderma lucidum* spore powder; hypoglycemic; antibacterial; antitumor.

39 effects and minor side effects of the products in prevention and alleviation of cancer,
40 hypertension and diabetes [1]. For example, Lentinan, a polysaccharide obtained from
41 the stipe portion of *Lentinus edodes* mushroom, revealed anticancer activities against
42 human colon cancer [2]. In addition, the proteoglycan obtained from *Ganoderma*
43 *lucidum* (*G. lucidum*) fruiting bodies has demonstrated protective effects on the kidneys
44 and amelioration of diabetic nephropathy [3].

45 *G. lucidum* is a well-known traditional Chinese medicine herb that has been used to
46 promote health and longevity for several thousands of years [4]. During the past few
47 decades, applications of *G. lucidum* have been explored and include alleviating
48 symptoms of asthma [5], promoting wound healing [6], as antitumor agents [7] and also
49 as an oral multidrug [8, 9]. Among the bioactive ingredients, the proteoglycans and
50 polysaccharides have shown important roles in medicinal potencies [10]. The
51 proteoglycan is a kind of glycoprotein consisting of a core protein linked to one or more
52 glycosaminoglycan chains via covalent bonding [11], and shows efficient
53 hypoglycemic activity in vitro [12] as well as excellent anti-herpetic activities against
54 type 1 and type 2 herpes simplex virus has been revealed [13]. This suggests the
55 polysaccharides presence during the extraction of protein is advantageous with regards
56 to the bioactive effects of the proteoglycan.

61 firstly confirmed. The differences between physicochemical properties, amino acid, and
62 monosaccharide in cracked GLSP and uncracked GLSP were then explored through
63 Fourier transform infrared spectroscopy (FTIR), High Performance Liquid
64 Chromatography (HPLC) and ion chromatography. Subsequently, the antioxidant,
65 antitumor and antibacterial effects of proteoglycan from cracked and uncracked GLSP
66 were compared. In consideration of the widespread applications of proteoglycans and
67 *G. lucidum*, the results in this study may broaden the source of proteoglycans and
68 application of GLSP.

69 70 **2. Materials and methods**

71 **2.1 Materials**

72 GLSP (cracked and uncracked wall) was offered by TianHe Agricultural Group
73 (ZheJiang LongQuan, China). Sodium hydroxide (troche), ammonium sulfate (powder),
74 methanol, absolute ethanol and hydrochloric acid were purchased from Sinopharm
75 Chemical Reagent Co., Ltd (Shanghai, China). Fetal bovine serum (FBS) was
76 purchased from Sijiqin (Sijiqin, Hangzhou, zhejiang, China), Dulbecco's modified
77 eagle's medium (DMEM) was purchased from Gibico (Gibco, USA) and deionized
78 water (DI) were offered by the Millipore Milli-Q Reference ultrapure water purifier

83 electrophoresis (SDS-PAGE) Gel Quick Preparation Kit were offered by Shanghai
84 Beyotime biotechnology Co., Ltd (Shanghai, China). Violet red bile agar, baird-parker
85 agar base, adding egg-yolk tellurite emulsion, nutrient broth, 7.5% sodium chloride
86 broth were from Qingdao Hope Bio-Technology Co., Ltd (Shandong, China). Universal
87 indicator paper (pH 1-14, Shanghai SSS reagent Co., Ltd). All chemicals were
88 analytical grade with no further purification before experimentation.

89

90 **2.2 Extraction of proteoglycan**

91 The proteoglycan was obtained using conventional water extraction via a water bath
92 (DZKW-D-4, Suzhou Jiangdong precise instrument Co., Ltd, Suzhou, China).
93 Approximately 1g of dried GLSP (uncracked) was dispersed with DI water according
94 to a predetermined solid-to-liquid ratio, and then kept in the water bath at the
95 predetermined temperature for predetermined time. The mixture was then centrifuged
96 at 8000 rpm, at 4 °C for 30 min using Centrifuge 5810 R (Eppendorf, Germany),
97 according the method in previous work [14]. The supernatant was collected and
98 concentrated using Rotavapor (RE-52A, Shanghai, China) at -0.09 MPa and 55 °C to
99 one-tenth of the volume. Ammonium sulfate was introduced to the concentrated
100 mixture until saturation. NaOH was introduced to achieve a neutral pH and left to stand

protein assay reagent (Bio-Rad, USA). Since proteoglycan is similar to glycoprotein and there is no single standard for proteoglycan, BSA was used as a standard protein. Absorbance of BSA was examined at 595 nm using UV spectrophotometer (UV-2600, Shimadzu, Japan). The standard curve of concentration-absorbance was plotted (in Fig. S1), and fitting equation is shown in Eq. (1):

$$y = 9.1755x + 0.011 \quad (1)$$

where, y was the absorbance, x was the concentration (mg/mL).

Box-behnken design was used to optimize the extraction parameters according to the results from response surface methodology (RSM) (Design-Expert 8.0.6 Trial, Stat-Ease, Inc. USA). Four three-level independent variables (Table S1) were selected based on the single factorial assays.

2.3 Molecular weight determination of protein

The protein molecular weight was determined using Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis (SDS-PAGE) [16]. Each sample (20 μ L, 10 mg/mL) was determined in SDS-PAGE with a 6% resolving gel and a 5% stacking gel (SDS-PAGE Gel Quick Preparation Kit). The operation was performed at 100 V through stacking gel followed by 120 V to the end of resolving gel using Power Pac™ Basic

127 The proteoglycans were determined using Fourier transform infrared (FT-IR)
128 spectroscopy (IR Affinity 1, Shimadzu, Japan). The samples were grounded with KBr
129 powder and then pressed into pellet using powder compressing machine (FW-4A,
130 Tianjin TUOPU instrument Co., Ltd, Tianjin, China) under the pressure ~14 MPa for 2
131 min, and getting the spectrum with 20 scans at a resolution of 4 cm⁻¹ (4000–400 cm⁻¹).
132

133 **2.5 Amino acid and monosaccharide composition of proteoglycan**

134 The amino acid content of the protein was determined using a High Performance
135 Liquid Chromatography system (HPLC, UitMate 3000, Thermo-Fisher Scientific,
136 USA), which was equipped with a DAD-3000 (RS) fluorescence detector. The analyte
137 was separated using an analytical column ZORBAX Eclipse-AAA (4.6 mm×150 mm,
138 Agilent ZORBAX Eclipse, Agilent Technology, USA). The mobile phase consisted of
139 Na₂HPO₄ (pH 7.8) with a concentration of 40 mM and acetonitrile-methanol-DI (45:
140 45: 10, v/v/v).

141 The monosaccharide was detected using an ion chromatograph system (Dionex ICS-
142 5000, Thermo-Fisher Scientific Dionex, USA) combining a chromatographic guard
143 column (3 mm×50mm, Dionex Carbo PACTM PA20) and anion exchange column (3
144 mm×250 mm, Dionex Carbo PACTM PA20). The ICS-5000 electrochemical detector

149 The ABTS radical scavenging activity was determined using a total antioxidant
150 capacity assay kit. In brief, the ABTS radical solution and trolox standard solution with
151 various concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mM) were prepared according to
152 manufacturer instructions. Peroxidase stock solution was added to a 96-well plate (20
153 $\mu\text{L}/\text{well}$). DI water was used as blank control, trolox standard solution was added for
154 the positive control and sample solution was added for the sample group (10 $\mu\text{L}/\text{well}$).
155 Following that, ABTS radical solution was added (170 $\mu\text{L}/\text{well}$) and the plate was
156 cultured for 6 minutes at 25°C. The absorbance was measured at 414 nm using Spectra
157 Max 190 microplate reader (NanoDrop, USA). The antioxidant activity of sample was
158 shown as Trolox equivalent antioxidant capacity (TEAC) and calculated as Eq. (2),
159 according to previous works [14, 17] :

$$160 \text{ TEAC (mM Trolox/ mg protein of sample)} = \frac{A_s - A_b}{A_b} \times \frac{1}{mX} \quad (2)$$

161 Where, the A_s is the absorbance of sample; the A_b is the absorbance of blank
162 control; the m is slope of a plot between antioxidant capacity and mM Trolox; the X
163 is mg protein of sample.

164 The DPPH radical scavenging activity was measured using the previous method of
165 Yang et al. [18]. Using DI water and ethanol to dissolve proteoglycan and DPPH, to the
166 final concentration of 100 $\mu\text{g}/\text{mL}$ and 0.2 mM, respectively. Mixing 1 mL sample

171 DPPH radical scavenging activity (%) = $[1 - (A_s - A_c)/A_b] \times 100\%$ (3)

172 Where, the A_s is the absorbance of sample solution; the A_c is the absorbance of
173 control, which the DPPH was replaced by ethanol in equal volume; the A_b is the
174 absorbance of blank, which the sample solution was replaced by DI water in equal
175 volume.

176

177 **2.7 Hypoglycemic activity assay in vitro**

178 **2.7.1 Hypoglycemic effect on normal HepG2 cells**

179 HepG2 (human hepatocellular liver carcinoma) cells (ca. 1.4×10^5 cells/mL) were
180 cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in cell
181 culture dishes (diameter, $\Phi = 6$ cm) under standard conditions (37 °C, 5% CO₂) for 48
182 h. Then, pipetted cell suspensions into a 96-well plate (100 µL/well) and incubated in
183 serum-free DMEM at standard conditions for 12 h. Following this, cells for blank
184 control were incubated in serum-free DMEM, and cells for positive control were
185 incubated in serum-free DMEM supplemented with metformin (1×10^{-3} mol/L). The
186 treatment groups were incubated in serum-free DMEM supplemented with various
187 concentrations of proteoglycan (0.1, 1, 10 mg/mL). After being incubated 24 h,
188 measuring the glucose content of supernatant of media using Micro Blood Sugar

193 HepG2 cells (ca. 1.4×10^5 cells/mL) were seeded into a 12-well plate for 24 h in serum-
194 free DMEM. The cells were then treated with serum-free DMEM with insulin (1×10^{-7}
195 mol/L) for 1 h. Subsequently, the cells were incubated in serum-free DMEM with
196 metformin (1×10^{-3} mol/L) or various concentrations of proteoglycan (1, 5, 10 mg/mL)
197 for 24 h. Finally, the glucose content of supernatant of media was measured by using
198 UV-Vis.

199

200 **2.8 Antitumor activities assay *in vitro***

201 **2.8.1 Cell viability assay**

202 The antitumor effects of proteoglycan on tumor cell lines were determined using
203 CCK-8 (cell counting Kit-8 reagent, Dojindo Laboratories, Kumamoto, Japan) assay
204 [20]. In brief, HeLa cell suspension (ca. 1.4×10^5 cells/mL) was obtained after being
205 cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in cell
206 culture dishes ($\Phi = 6$ cm) under standard conditions for 48 h. 100 μ L cell suspensions
207 were pipetted into a 96-well plate and incubated at standard conditions for 24 h. For
208 treatment group, 100 μ L each well of proteoglycan solution was added after being
209 disinfected using UV radiation for 4 h; and 100 μ L medium was added to the control.
210 After being cultured for 24 h, the cells were added 10 μ L CCK-8 solution and the

215 blank group, respectively.

216

217 **2.8.2 AO/EB staining**

218 HeLa cells were stained using AO/EB dye mixture. In brief, cell suspension (1.4×10^5
219 cells/mL) were seeded into a 6-well plate. After being incubated 24 h for adherence,
220 the cells were treated with proteoglycan solution with various concentrations (1 or 5
221 mg/mL) for another 48 h. 20 μ L of AO/EB dye mixture (100 μ g/mL EB and 100 μ g/mL
222 AO into the 6-well plate and left to stand for 30 min at 37°C. The plate was analyzed
223 using fluorescence microscopy (Olympus, BX61W1-FV1000, Tokyo, Japan).

224

225 **2.9 Antibacterial activity assay**

226 The antibacterial effects of proteoglycan on *E. coli* (NW1014 (8099), Nanjing
227 Maojie Microbiology Technology Co., Ltd, Jiangsu, China) and *S. aureus*
228 (CMCC(B)26003, Shanghai Luwei Microbial SCI. & TECH. Co., Ltd, China) was
229 studied using the method [22]. Pressing proteoglycan powder (ca. 100 mg) to a disk by
230 the powder compressing machine. Coating 0.2 mL (1.2×10^6 CFU/mL) inoculums of *E.*
231 *coli* and *S. aureus* on violet red bile agar and baird-parker agar base plates adding egg-
232 yolk tellurite emulsion, respectively. Then, put the agar plate ($\Phi \approx 3$ cm) in biochemical

Meanwhile, 0.01 mL (ca. 1.2×10^6 CFU/mL) inoculum of *E. coli* and *S. aureus* was added into 100 mL nutrient broth and 7.5% sodium chloride broth respectively, and then incubated in the biochemical incubator at 37°C for 24h. After that, using 25 µg/mL FDA and PI to stain the bacteria for 20 min at 25°C and was kept away from light. The fluorescence value was measured using a fluorescence microplate reader (FlexStation II, NanoDrop, USA), and the fluorescence value was calculated using previous method [22].

2.10 Statistical analysis

All experiments were performed in triplicate and data is given as mean $\pm$ standard deviation (n=3). Statistical analysis was performed using SPSS software (SPSS Statistics v18, IBM, UK). The one-way analysis of variance (ANOVA) were revealed using RSM (Design- Expert 8.0.6 Trial, Stat-Ease, Inc., USA). All the statistical plots were plotted using Origin software (OriginLab, USA).

3. Results and discussion

3.1 Optimization of the proteoglycan extraction

This study of controlling parameters (e.g. time, temperature, extraction times, solid-

effects on yields. The experimental data and results are shown in Table S2. Then, the regression equation was obtained according to the results of ANOVA:

$$Y=3.93+0.027\times A-0.044\times B+0.044\times C+0.12\times D-0.20\times A\times B+0.099\times A\times C+0.079\times A\times D-0.12\times B\times C+4.75\times B\times D\times 10^{-3}-0.24\times C\times D-0.63\times A^2-0.78\times B^2-0.94\times C^2-0.88\times D^2$$

(5)

where, Y is the proteoglycan yield (%) in the resultant product probably; A, B, C and D was the solid-liquid ratio, time, temperature and pH value, respectively.

The RSM results (Fig. 1 & Fig. S3) revealed the interactions among the experimental parameters. As the results shown (Table S3), the fitting model F-value was 3.23 and p-value was 0.024 (< 0.05), which revealed the quadratic model was significant. The linear coefficients (A, B, C, D), cross product coefficients (AB, AC, AD, BC, BD, CD) were found insignificant ($p > 0.05$), whereas the quadratic coefficients (A^2 , B^2 , C^2 , D^2) exhibited significant effect ($p < 0.05$). The significant order priority of the four factors on the result was pH value > time = temperature>solid-liquid ratio, which confirmed the fact that the little longer extraction time [24] with appropriate temperature [25], and neutral pH [26] may be improved protein extraction.

After calculations, the predictive maximum protein yield (4.525%) was obtained on the optimizing parameters: 1:40 (g: mL), 2.93 h, 35.19 °C and 10.07 (pH value). For

optimizing extraction parameters of proteoglycan.

Then, the difference in physicochemical properties, amino acid, and monosaccharide content of the proteoglycan from cracked well GLSP (proteoglycan-C) and uncracked well GLSP (proteoglycan-UC) were explored through FTIR, HPLC and Ion chromatography. Following that, the antioxidant, antitumor and antibacterial effects of proteoglycan-C and proteoglycan-UC were compared.

3.2 FT-IR analysis

FT-IR is frequently used to analyze functional groups of a compound [27-29]. The FT-IR spectra of proteoglycan-C and proteoglycan-UC are presented in Fig. 2a. The peaks at 1527 and 1664 cm^{-1} were due to the presence of amide II and amide I respectively, which revealed the isolated compound contained protein fractions [30]. The signals at 2920, 2850, 1478, 1160, 1078 cm^{-1} were attributed to the presence of pyranose ring [31]. Moreover, the absorption peaks around 2920, 1620 and 1400 cm^{-1} were the characteristic absorption peaks of polysaccharide [32] due to C-H and -COOH groups, respectively [24]. The FT-IR results confirmed that the isolated proteoglycan was a polysaccharide and protein complex. Also, there were no obvious differences in the functional group between the proteoglycan from cracked and uncracked GLSP; just

303 stained using Coomassie brilliant blue, which revealed the protein was a non-
304 monomeric protein. Comparing with standard protein molecular weight marker (lane
305 1), the molecular weight of protein in proteoglycan-C (lane 2-4) was 43 and 95 kDa;
306 and the protein contained in proteoglycan-UC (lane 5-7) possessed molecular weight
307 as follows: 55, 72 and 95 kDa. The band width of protein with molecular weight 95
308 kDa of proteoglycan-UC (lanes 5-7) is wider than that of proteoglycan-C (lanes 2-4),
309 which indicates proteoglycan-UC contains a higher protein content than proteoglycan-
310 C. The protein obtained in this work was consistent with the protein with molecular
311 range 12 to 150 kDa from *Ganoderma boninense* [33].

313 **3.4 Amino acid content and constitution of polysaccharide**

314 The amino acid content of proteoglycan was determined using HPLC (Fig. 3a & b).
315 The content of amino acids (e.g. Asp, Glu, Lys) were calculated (Table S4). The Arg
316 content of proteoglycan-UC (10.74%) was did not appeared in proteoglycan-C; the
317 content of Lys in proteoglycan-C (6.32%) was not detected in proteoglycan-UC. The
318 Asn and Gln were not detected in proteoglycan-C and proteoglycan-UC.

319 The monosaccharide contained in proteoglycan was determined using Ion
320 chromatograph system. The results (Fig. 3c & d) revealed the presence of several kinds

proteoglycan-C. Besides, the Glc and Man contents of proteoglycan-UC (524.281, 6.386 $\mu\text{g}/\text{mg}$, respectively) were more than twice of that proteoglycan-C (247.959, 2.667 $\mu\text{g}/\text{mg}$, respectively). While the Fuc and Gal content of proteoglycan-UC was 1.186 $\mu\text{g}/\text{mg}$ and 7.421 $\mu\text{g}/\text{mg}$ respectively, higher content was found in proteoglycan-C (3.308 $\mu\text{g}/\text{mg}$ and 13.320 $\mu\text{g}/\text{mg}$, respectively). The Xyl, Fru, Rib and Glc-AC were not detected in proteoglycan-C and proteoglycan-UC. The results confirmed that the effect of GLSP (cracked or uncracked) on the monosaccharide content was significant. Differences in amino acid and monosaccharide content of proteoglycan between cracked and uncracked wall GLSP may be attributed to two main causes. The first is oxidation reactions which occur in cracked wall GLSP. Secondly, Maillard reaction between monosaccharides and amino acids influence ion chromatography detection. The latter can also occur (and impact) during ion chromatography experimental conditions [34].

3.5 Free radical scavenging activity

The scavenging effect of ABTS was expressed as Trolox equivalent antioxidant capacity (TEAC), as shown in Fig. 4. The ABTS scavenging activity of proteoglycan-C and proteoglycan-UC (1 mg/mL) was $73.3 \pm 6.7\%$ and $47.2 \pm 5.9\%$, respectively while

C was higher than that of proteoglycan-UC, which was the same for both ABTS and DPPH test. The differences among these amino acid contents might be the key causes responsible for the antioxidant activities differences of the proteoglycans as the peptides contained in the amino acids (His, Phe and Pro) (Tables S4 & S5) have shown antioxidant activity [35].

3.6 Hypoglycemic activity *in vitro*

Metformin is used for the treatment of type 2 diabetes, and is a commercially available hypoglycemic drug [36]. Numerous studies have used this drug to demonstrate hypoglycemic activity, typically as a positive control when examining hypoglycemic effects between various treatment groups. Therefore, metformin was selected to compare hypoglycemic activity of proteoglycan in the current work. The glucose concentration of the supernatant of media after being cultured for 24 h indicated the capacity of glucose consumption of HepG2 cell. The hypoglycemic potency of proteoglycan on normal HepG2 cells is shown in Fig. 5a. The glucose concentration in the proteoglycan treatment (10 mg/mL) was comparable with the metformin treatment group (10^{-3} mol/L). Differences in hypoglycemic effect on normal HepG2 cells between treatment and control groups was not significant. This may be due to normal HepG2

369 proteoglycan-C was stronger than proteoglycan-UC, and indicates proteoglycan
370 (concentration of 10 mg/mL) exhibits a similar hypoglycemic effect as metformin at a
371 concentration of 10^{-3} mol/L on normal HeG2 cells.

372 The hypoglycemic effect of proteoglycan on insulin-induced insulin resistance type
373 HepG2 cells, which used to imitate T2DM patients [12] is shown in Fig. 5b. The
374 glucose concentration in insulin treatment group (10.91 ± 0.56 mM) was comparable
375 with that of control (10.92 ± 0.18 mM), indicating that the insulin-induced insulin
376 resistance type HepG2 cells were constructed successfully. Comparing with control, the
377 hypoglycemic effect of resulting proteoglycan-C and proteoglycan-UC on insulin
378 resistance type HepG2 cells were obvious, for the $P < 0.05$ (p-value was 0.03 and 0.02,
379 respectively). Furthermore, the glucose concentration treated by proteoglycan-C was
380 little lower than that of proteoglycan-UC.

381 The mechanism under the hypoglycemic effect of the proteoglycan is most likely an
382 interaction between proteoglycan and protein tyrosine phosphatase 1B (PTP1B), which
383 was inhibited via interactions between the carboxyl groups of Asp and Glu and Tyr20,
384 Arg24 and Arg254 active sites of PTP1B [37]. Thus, the differences of Asp and Glu
385 content (Table S4) in proteoglycan-C and proteoglycan-UC were the major causes for
386 the difference in hypoglycemic activity.

391 antitumor activity *in vitro* [16, 38]. As shown in Fig. 6a, the HeLa cell viability
392 decreased as treatment dose and time increases. When HeLa cells were treated with 0.5
393 or 1.0 mg/mL of proteoglycan-C for 12 h, the cell viability was $95.2 \pm 1.5\%$ and 72.5
394 $\pm 1.9\%$, respectively, and decreased further (to $69.9 \pm 2.6\%$ and $62.3 \pm 0.9\%$) at the
395 treatment of 48 h. The result of proteoglycan-UC on the cell viability was $90.3 \pm 0.9\%$
396 and $78.2 \pm 5.3\%$ for 0.5 or 1.0 mg/mL for 12 h, respectively, and then decreased to
397 $69.1 \pm 1.0\%$ and $58.2 \pm 0.9\%$ when treated for 48 h, respectively.

398 The antitumor effect can be visualized via AO/EB dual staining results (Fig. 6). The
399 blank control (Fig. 6b) presented bright green with slight orange fluorescence,
400 indicating most vital cells. At the treatment of proteoglycan-C or proteoglycan-UC (1.0
401 mg/mL) (Fig. 6b1 & c1), the green fluorescence was weaker and orange fluorescence
402 was brighter, while little less orange fluorescence showed with lower concentration (0.5
403 mg/mL) treatment (Fig. 6b2 & c2). This indicates the number of viable cells was less
404 than that of the control and the number of necrotic cells had increased; consistent with
405 previously reported studies [39]. The results confirmed the antitumor activity of
406 proteoglycan, which probably due to the polysaccharide fraction in proteoglycan can
407 stimulate the immune system [40] or suppress the cell growth by inhibiting the activity
408 of Akt and transcription factors AP-1 and nuclear factor-kappa B (NF- κ B) [41] .

413 Compared to control, the proportion of viable cells for *E. coli* decreased to $89.6 \pm 9.9\%$
414 and the necrotic cells proportion increased to $112.5 \pm 9.1\%$ (Fig. 7a) at the treatment of
415 proteoglycan-C (1mg/mL). The proportion of viable and necrotic cells of was $93.6 \pm$
416 9.2% and $107.5 \pm 3.9\%$ respectively when *E. coli* was treated with proteoglycan-UC
417 (1mg/mL). For *S. aureus* (Fig. 7b), at the treatment of proteoglycan-C (1 mg/mL), the
418 proportion of viable and necrotic cells was $90.8 \pm 8.7\%$ and $124.9 \pm 6.5\%$ respectively.
419 The same treatment of proteoglycan-UC induced the proportion of viable and necrotic
420 cells was $97.5 \pm 5.4\%$ and $108.4 \pm 7.1\%$ respectively. Thus, these results indicate the
421 antibacterial activities against *E. coli* and *S. aureus* of proteoglycan-UC were stronger
422 than proteoglycan-C.

423 The antibacterial properties can be visualized using disk diffusion method [42]. Figs.
424 7 c1-d2 presents a clear inhibition zone with agar plates coated with *E. coli* and *S.*
425 *aureus*. The diameter of inhibition zone for *S. aureus* is larger than that for *E. coli* (Table
426 1). The difference in activity is attributed to the bacterial cell type. *S. aureus* is Gram-
427 positive bacteria and restricting entry of nutrients into cells leads to death. *E. coli* is
428 Gram-negative bacteria and an increase in cell permeability results in death [43]. This
429 is the same case for both proteoglycan-C and proteoglycan-UC, which was consistent
430 with previous works [44]. The probable mechanism was that binding of polysaccharides

434

435 4. Conclusion

436 In summary, the proteoglycan from GLSP was obtained using conventional water
437 extraction. The extraction parameters were optimized through RSM results. The results
438 revealed that the proteoglycan obtained from cracked and uncracked GLSP differed its
439 characteristics with respect to molecular weight, amino acid content, monosaccharide
440 content and antibacterial activity. The proteoglycan-C (from cracked GLSP) possessed
441 smaller molecular weight, higher content amino acid of Lys and monosaccharide of Gal
442 comparing with that proteoglycan-UC (from uncracked GLSP). Moreover, the
443 proteoglycan-UC contained higher content amino acids of Arg, Glc, monosaccharide
444 of Man, and revealed stronger antibacterial effects against *E. coli* and *S. aureus* and
445 stronger hypoglycemic effect in vitro than that of proteoglycan-C. Meanwhile, the
446 proteoglycan-C exhibited stronger scavenging effects against ABTS and DPPH radicals.
447 The antitumor effects against HeLa cells of proteoglycan-C were similar to that of
448 proteoglycan-UC. Overall, the obtained results regarding antioxidant, hypoglycemic,
449 antitumor and antibacterial activity revealed that the resulting proteoglycan from GLSP
450 is useful as a functional additive as well as a potential candidate for a therapeutic agent.

451

452

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459

460 **Competing interests**

461 There are no conflicts of interests to declare.

462

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599 Fig. 1. Contour plots (2D) for response surface plots results: a-f corresponding to temperature and
600 time, pH and temperature, pH and time, time and solid-to-liquid ratio, pH and solid-to-liquid-ratio,
601 temperature and solid-to-liquid ratio, respectively.

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603 Fig. 2. FT-IR spectroscopy of proteoglycan (a) and SDS-PAGE of crude protein contained in
604 proteoglycan (b): lane 1, molecular weight markers; lane 2-4, proteoglycan-C in triplicate; lane 5-
605 7, proteoglycan-UC in triplicate.

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607 Fig. 3. Amino acids content of proteoglycan-UC (a) and proteoglycan-C (b); monosaccharides
608 content of proteoglycan-UC (c) and proteoglycan-C (d).

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610 Fig. 4. The antioxidant effects of proteoglycan-C and proteoglycan-UC.

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612 Fig. 5. The hypoglycemic capacity against normal HepG2 cells (a) and insulin resistance type
613 HepG2 cells (b) of proteoglycan.

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615 Fig. 6. Effects of proteoglycan on HeLa cell viability using CCK-8 assay (a); Fluorescent images of
616 HeLa cells morphology of blank control group (b) and treated with proteoglycan-C of 1.0 mg/mL
617 (b1), 0.5 mg/mL (b2) and proteoglycan-UC of 1.0 mg/mL (c1), 0.5 mg/mL (c2).

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619 Fig. 7. Effects of proteoglycan on HeLa cell viability using CCK-8 assay (a); and on proliferation
620 of *E. coli* (a) and *S. aureus* (b); inhibition zone results of proteoglycan-UC (c1, d1) and
621 proteoglycan-C (c2, d2) against *E. coli* (c1, c2) and *S. aureus* (d1, d2).

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Inhibition zone diameter (Φ , mm)		
	Proteoglycan-UC	Proteoglycan-C
<i>E. coli</i>	20.1	20.8
<i>S. aureus</i>	25.2	27.2

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