



Structural characterization and hepatoprotective activity of an acidic polysaccharide from *Ganoderma lucidum*

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ABSTRACT

In this study, *Ganoderma lucidum* crude polysaccharide (GLP) was found to have protective effect on liver damage in mice caused by restraint stress through improving oxidative status. Two polysaccharides, including a neutral β-glucan (GLPB2) and an acidic β-glucan (GLPC2) were purified from GLP through anion-exchange chromatography (AEC) combined with gel permeation. GLPC2, with an average molecular weight of 20.56 kDa, exhibited stronger hepatoprotective effect against H₂O₂-induced liver injury in HepG2 cells compared to GLPB2. Glycosidic residues and NMR analysis comprehensively revealed that GLPC2 contained D-Glcp-(1→, →3)-D-Glcp-(1→, →4)-D-Glcp-(1→, →6)-D-Glcp-(1→, →3, 6)-D-Glcp-(1→ and →4)-D-GlcpA-(1→. AEC can be an effective technique for separating β-glucans into neutral and acidic fractions by different ionic strength buffer. The findings provided a theoretical basis for the potential application of *G. lucidum* polysaccharides as a hepatoprotective in food and pharmaceutical industry.

1. Introduction

Mushroom polysaccharides act generally as an obligatory part of cell walls in fruiting bodies, mycelia and other parts. More than that, they display effective bioactivities including immunomodulatory, anti-tumor, and anti-inflammatory etc. (He et al., 2020; Kothari, Patel, & Kim, 2018; Ruthes, Smiderle, & Iacomini, 2016; Synytsya & Novák, 2013; Y. Wang et al., 2017). Besides their benefit to human health, it was worthy to mentioned that most mushroom polysaccharides were found to be comparatively nontoxic against liver and kidneys and then even no significant side effects (Zhao et al., 2014), which make them a research hot spot in natural medicine and functional food industries, especially for liver protection (Y. Wang et al., 2017).

As a vital organ, the liver acts an important role in metabolism, taking biological transformation and detoxification (J. Xiao et al., 2019). Stress, excessive diet, alcohol and drugs abuse can cause liver injure, which may further develop into chronic fatigue, coagulopathy, liver failure and even cancers (Luo et al., 2021). Liver diseases which develop from liver injury have brought enormous burdens for society and economics. Thus, it is highly demanded to search for effective and safe

hepatoprotective agents, for which mushrooms are a vital source.

Ganoderma lucidum (Polyporaceae) is a well-known edible and medicinal mushroom, which has been used widely in medicinal and functional food industry (Lu, He, Sun, Zhang, Linhardt, & Zhang, 2020). The polysaccharides in *G. lucidum* are believed as one of the most important contributors to its multiple medicinal and health benefits, such as anti-cancer, anti-diabetes, and immune-modulation (Lu, He, Sun, Zhang, Linhardt, & Zhang, 2020). Over 200 polysaccharides with different molecular weights and different glycosidic linkages had been published from *Ganoderma* spp. (Y. Wang et al., 2017), and the number continually increased recently. However, most of them were neutral. Furthermore, hetero-polysaccharide occupied *Ganoderma* polysaccharides, their detailed characterization especially in terms of specific glycosidic linkages is still unclear, which made the structure–activity relationship research very difficult.

In this study, we reported the protective effects of *G. lucidum* crude polysaccharides (GLP) against acute liver injury induced by restraint stress *in vivo*. Two main polysaccharides, including a neutral β-glucan (GLPB2) and an acidic β-glucan (GLPC2) were purified from GLP. Moreover, the chemical properties and hepatoprotective effect *in vitro* of

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GLPB2 and GLPC2 were investigated. Knowing the relationship between structural features and activities of *G. lucidum* polysaccharides gave novel insights into the application of *G. lucidum* polysaccharides as a hepatoprotective and antioxidant agent or adjuvant in medicine or food industry.

2. Experimental

2.1. Materials and reagents

The fruiting bodies of *G. lucidum* were obtained from Yuewei Edible Fungi Technology Co. Ltd., Guangzhou, China (S. Chen, Yong, Zhang, Su, Jiao, & Xie, 2017). DEAE Sepharose™ FF and Sephacryl S-200 HR were purchased from Cytiva (GE healthcare). Monosaccharide standards, series dextrans kit, trifluoroacetic acid (TFA), and 1-phenyl-3-methyl-5-pyrazolone (PMP) were purchased from Sigma-Aldrich LLC. Dialysis bags (MWCO = 3500 Da), dimethyl sulfoxide (DMSO), sodium borohydride (NaBH₄), iodomethane (CH₃I), and other chemical reagents were obtained from Guangzhou Mingwang Bio-Technology Co., Ltd.. Deuterium oxide (99.8% D) was obtained from CIL Technology. Vitamin C (V_C) powder was purchased from Shanghai Yuanye Biotech Co.. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), catalase (CAT) and superoxide dismutase (SOD) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute.

2.2. Extraction and isolation

Before extraction, the fruiting bodies of *G. lucidum* (2.5 kg) was dried overnight at 60 °C, and then powdered and defatted by soaking with 95% ethanol for two times. Then the defatted residue was air-dried and extracted with deionized water for two times (80 °C, 30 L and 3 h for each). The extracting solution was filtrated, vacuum concentrated and then dialyzed with deionized water for 36 h. The retentate was precipitated by adding four times the initial volume of 95% ethanol. After storage at 4 °C for 48 h, the precipitate was obtained after siphon treatment, and the precipitate was re-dissolved in deionized water. *G. lucidum* crude polysaccharides (GLP) were acquired after deproteination by Savage method and freeze-drying. The total carbohydrates content was measured by phenol-sulfuric method.

GLP was fully dissolved in distilled water (20 mg/mL) and centrifugated (8000 rpm × 10 min). The supernatant was then subjected on a DEAE Sepharose™ FF column (50 cm × 3.6 cm) and fractioned with 0.05, 0.1, 0.3 and 0.5 M NaCl solution stepwise, and the collected eluants were assayed by phenol-sulfuric method. Two major fractions (GLPB and GLPC) collected from 0.3 M and 0.5 M NaCl solution eluants were further subjected on a Sephacryl S-200 HR column (60 cm × 2.6 cm) to afford two main polysaccharides GLPB2 and GLPC2, respectively. The procedures were repeated to obtain adequate GLPB2 and GLPC2.

2.3. Molecular weights (Mw) of GLPB2 and GLPC2

The Mw of GLPB2 and GLPC2 were evaluated by high performance gel permeation chromatography (HPGPC) equipped with two connected columns of ACQUITY APC AQ450 and AQ200 (Waters, Germany) at 35 °C. The mobile phase was 25 mmol/L NaH₂PO₄-Na₂HPO₄ (pH = 6.7) with a flow rate of 0.6 mL/min. Dextrans (Mw from 5 kDa to 670 kDa) was used to draw the standard curve. Mw was calculated by Waters Empower™ GPC software.

2.4. Monosaccharide composition

Monosaccharide composition analysis of GLP, GLPB2 and GLPC2 was performed using PMP pre-column derivation and HPLC analysis (Dai et al., 2010; S. Zhang, He, Chen, & Ding, 2019). Samples (each 3 mg) were fully hydrolyzed with TFA respectively. After removing TFA, the

hydrolysate were reacted with NaOH and PMP, and stopped by adding HCl. The PMP derivatives were analyzed by HPLC with a YMC-Pack ODS-AM column (250 mm × 4.6 mm, 5 μm) eluted with 15% acetonitrile containing KH₂PO₄-K₂HPO₄ (0.1 M, pH = 6.7) at a flow rate of 1.0 mL/min. The monosaccharide standards were processed and analyzed as above.

2.5. Linkage patterns

GLPC2R was firstly obtained from carboxyl reduction reaction of GLPC2 (Kim & Carpita, 1992). GLPC2 and GLPC2R were methylated using Haworth's method (Haworth, 1915) and Ciucanu's method (Ciucanu & Kerek, 1984). The methylated polysaccharide was fully hydrolyzed with TFA, the hydrolysate was prepared for partially methylated alditol acetates (PMAA), and then detected on a Thermo Fisher GC-MS apparatus equipped with a TR-5 column (30 m × 0.25 mm × 0.25 μm). The temperature programming was 120–250 °C at 3 °C/min, followed by 250 °C held for 5 min.

2.6. FT-IR and NMR spectra

Dried GLPC2 (1 mg) and KBr powder were mixed and fully ground to make a sheet. FT-IR spectrum was acquired on a Jasco FT-IR 480 plus spectrophotometer with frequency range of 4000–500 cm⁻¹.

GLPC2 (35 mg) was exchanged with D₂O and centrifuged. The supernatant was lyophilized and then re-dissolved with 0.5 mL of D₂O. NMR spectroscopic data were measured on a Bruker AVANCE III 600 spectrometer at 25 °C.

2.7. Scanning electron microscopy (SEM) and atomic force microscope (AFM) analysis

In SEM analysis, GLPC2 was mounted on aluminum stubs and coated with a gold layer. The surface morphologies and section-cross microstructure were observed by employing a Hitachi S-3000 N scanning electron microscope (JEOL Ltd., Tokyo, Japan).

In AFM analysis, GLPC2 solution (10 μg/mL) was prepared and then the solution (5 μL) was dropped onto the surface of a clean mica sheet and dried. The surface topology was scanned using a Bruker MultiMode8 atomic force microscope.

2.8. In vivo hepatoprotective effect of *G. lucidum* crude polysaccharides GLP

The animal experimental protocols were approved by Institute of Microbiology, Guangdong Academy of Sciences (IMGAS) Ethics Committee (GT-IACUC201807032). Male BALB/c mice (7 weeks) were purchased from Guangdong Medical Laboratory Animal Center, Guangzhou. The mice were housed in cages under controlled humidity (50 ± 5%), temperature (23 ± 2 °C) and 12 h day/night cycle. The mice were free to get food and water during the experiments. After one week of adjustable feeding, the mice were randomly divided into six groups (*n* = 8): normal control (N), model control (M), positive control (Vc), GLP-100, GLP-200 and GLP-400 groups. Mice in Vc group were administered with Vc at a dose of 200 mg/kg/day. Mice in GLP-100, GLP-200 and GLP-400 groups were given GLP at a dose of 100, 200 and 400 mg/kg/day, respectively. Mice in N and M groups were given equal volume of water, respectively. After 7 days, each mouse except those in N group was confined to a 50 mL centrifuge tube drilled at the bottom for 18 h after the last administration. Then, the mice were sacrificed. Blood was collected from eyeballs and liver was harvested. Serum was obtained after centrifugation for 10 min at 4000 r/min. The content of ALT, AST, GSH-Px, MDA, CAT and SOD were determined in accordance with the kits' instructions.

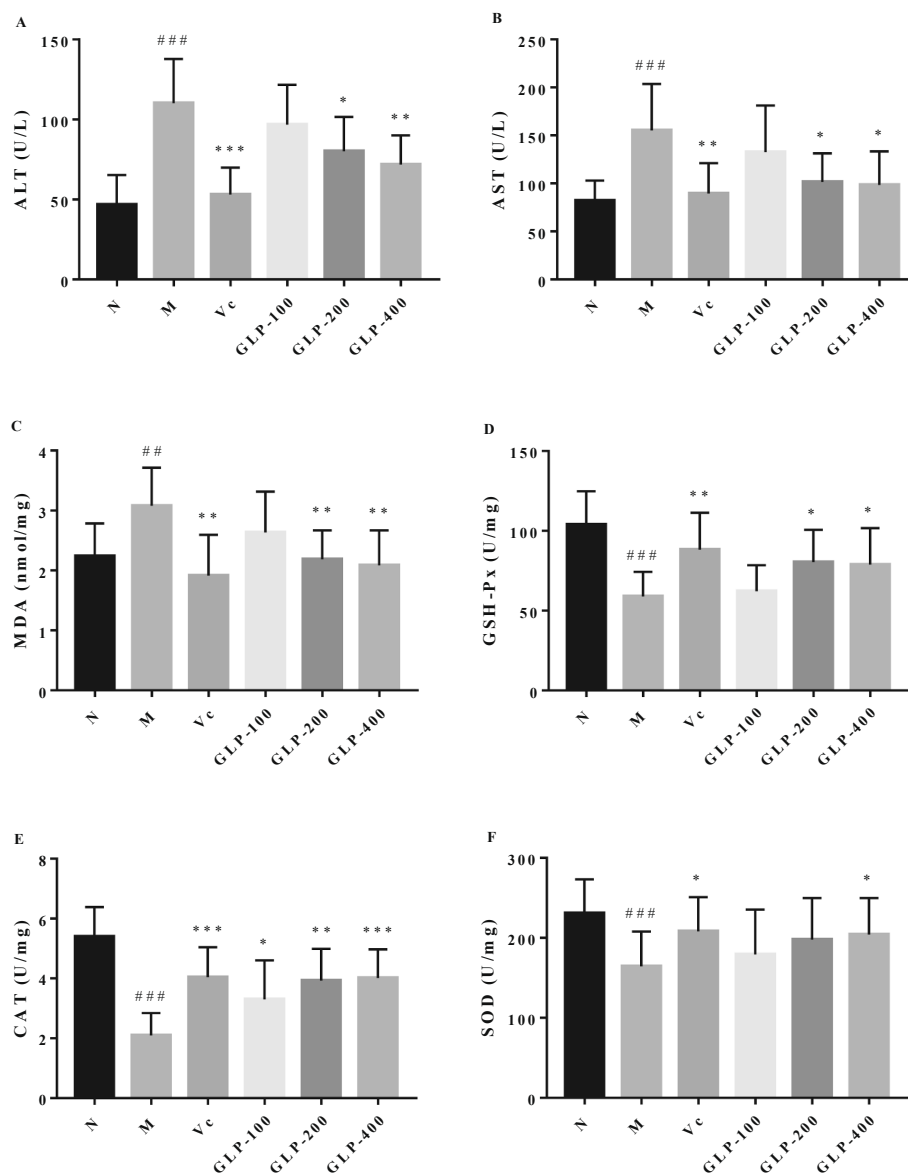


Fig. 1. Effect of GLP on ALT (A), AST (B), MDA (C), GSH-Px (D), CAT (E) and SOD (F) levels in mice with restraint stress. Data were presented as mean \pm SD. ^{##} $P < 0.01$, ^{###} $P < 0.001$, compared to N group; ^{*} $P < 0.05$, ^{**} $P < 0.01$, ^{***} $P < 0.001$, compared to M group.

2.9. In vitro hepatoprotective activities

2.9.1. Cell viability

HepG2 cells (1×10^4 per well) were incubated in 96-well plates for 24 h, and then treated with different concentrations of GLPB2 or GLPC2 (0.1, 0.2, and 0.4 mg/mL). After 24 h treatment, the medium was replaced with 200 μ L complete DMEM containing 10 μ L of CCK-8. The plate was incubated at 37 $^{\circ}$ C for 4 h and its OD was measured at 450 nm on a microplate reader.

2.9.2. Determination of ALT and AST levels

As described above, HepG2 cells were cultured in 96-well plates for 24 h and then added with H_2O_2 (100 μ g/mL) and further incubated for 2 h. Then the cells were treated with different concentrations of GLPB2 and GLPC2 (0.1, 0.2, and 0.4 mg/mL) and incubated for 24 h. ALT and AST were measured using commercially kits according to the manufacturer's instructions.

3. Results and discussion

3.1. Extraction of *G. ludicum* crude polysaccharides (GLP) and purification of polysaccharides GLPB2 and GLPC2

G. ludicum crude polysaccharides (GLP) were prepared through water extraction and different impurity removal methods (Fig. S1). The total carbohydrate content of GLP was $52.4 \pm 4.1\%$, and it was composed of mannose (10.1%), glucose (74.7%), glucuronic acid (6.5%), galactose (3.9%), xylose (3.4%) and fucose (1.4%) by PMP pre-column derivation and HPLC analysis (Fig. S3). Then, GLP was further purified by different chromatographic columns. As shown in Fig. S2, two polysaccharides GLPB2 and GLPC2 were obtained from GLP on a DEAE SepharoseTM FF column by different concentrations of sodium chloride aqueous solutions, followed by a Sephacryl S-200 column. According to the HPGPC analysis, GLPB2 and GLPC2 showed single symmetric peaks, respectively. The M_w of GLPB2 was calculated as 22.51 k_D and GLPC2 was 20.56 k_D.

3.2. Monosaccharide composition

Monosaccharide composition of GLPB2 and GLPC2 were analyzed by PMP-HPLC method. As shown in Fig. S3, GLPB2 was mainly composed of mannose (7.5%) and glucose (85.3%), with trace glucuronic acid (0.9%), galactose (1.6%), xylose (1.9%) and fucose (1.8%). GLPC2 consisted of mannose (5.9%), glucuronic acid (9.0%) and glucose (80.4%), with small amount of galactose (1.8%), xylose (1.8%) and fucose (0.9%). The results of monosaccharide composition analysis implied that both GLPB2 and GLPC2 may be glucans.

3.3. Protective effect of GLP on liver damage in restraint stress-induced mice

Dysfunctional mitochondria or reduced antioxidant defense may increase the production of free radicals, which further lead to the development of oxidative stress (Sies, 2015). Long-term exposure to stress may trigger problems in mental and physical health and cause lifestyle diseases (Moore & Cunningham, 2012). It has been reported that stress can cause glucose and lipid metabolism disorder, immunocompromise and acute liver damage (M. Pan et al., 2020; Yan et al., 2020). Nowadays, diseases caused by stress-related have received widespread attention. Relieving stress was very important in keeping health (López-Otín & Kroemer, 2021). Previous studies have reported that *G. lucidum* triterpenoids and *G. lucidum* crude polysaccharides presented hepatoprotective effect against liver injury caused by chemicals (Hu et al., 2020; Wu, Kan, Wu, Yi, Chen, & Wu, 2016). However, the protective effect of *G. lucidum* polysaccharides on oxidative stress induced liver damage was rarely reported. Hence, we focused on the effect of GLP on liver damage caused by oxidative stress.

ALT and AST are considered as the major markers of liver damage. In this *in vivo* study, plasma ALT and AST in M group apparently increased to 110.18 ± 9.19 and 155.29 ± 16.11 U/L respectively, compared to those in N group (46.79 ± 6.19 and 82.19 ± 6.95 U/L), which indicated the liver was damaged after being stressed for 18 h. It was worthy to note that GLP decreased plasma ALT and AST levels in a dose-dependent manner (Fig. 1), depicting that GLP could attenuate acute liver damage induced by restraint stress.

Free radical reactions induced by stress can further cause harmful change for cell membranes, proteins and DNA, increase lipid peroxidation, and reduce antioxidant substances endogenously (Zhai et al., 2012). The related mechanism of GLP for attenuating stress was further investigated by observing the improvement effect of GLP on oxidative damage. The MDA, GSH-Px, CAT and SOD level in liver were determined, and the results were also shown in Fig. 1. Compared to N group (2.24 ± 0.18 nmol/mg), the hepatic MDA content of M group (3.08 ± 0.21 nmol/mg) was significantly higher ($P < 0.01$). However, the MDA content was significantly decreased by administration of GLP (200 and 400 mg/kg) as well as V_C (200 mg/kg). The hepatic GSH-Px level (103.92 ± 6.95 U/mg) significantly was decreased to 58.93 ± 5.13 U/mg by being stressed ($P < 0.001$). Obviously, GLP (200 and 400 mg/kg) increased the hepatic GSH-Px level to 80.46 ± 6.75 and 78.76 ± 7.62 U/mg, respectively. As the positive control, V_C also significantly increase the hepatic GSH-Px level (88.31 ± 7.66 U/mg) compared with the stressed mice ($P < 0.01$). Similarly, stress decreased the hepatic CAT level, and GLP (100, 200 and 400 mg/kg) and V_C increased CAT activity. Hepatic SOD activity significantly was decreased after being stressed (164.5 ± 12.6 vs. 230.9 ± 12.2 U/mg), and 400 mg/kg of GLP significantly increased the hepatic SOD level to 204.1 ± 13.2 U/mg ($P < 0.05$). Above all, GLP may relieve acute liver damage by enhancing the activity of antioxidant enzymes and inhibiting the production of lipid peroxide.

3.4. Protective effect of GLPB2 and GLPC2 against H_2O_2 -induced HepG2 cells *in vitro*

Cellular oxidative damage is a well-established general mechanism

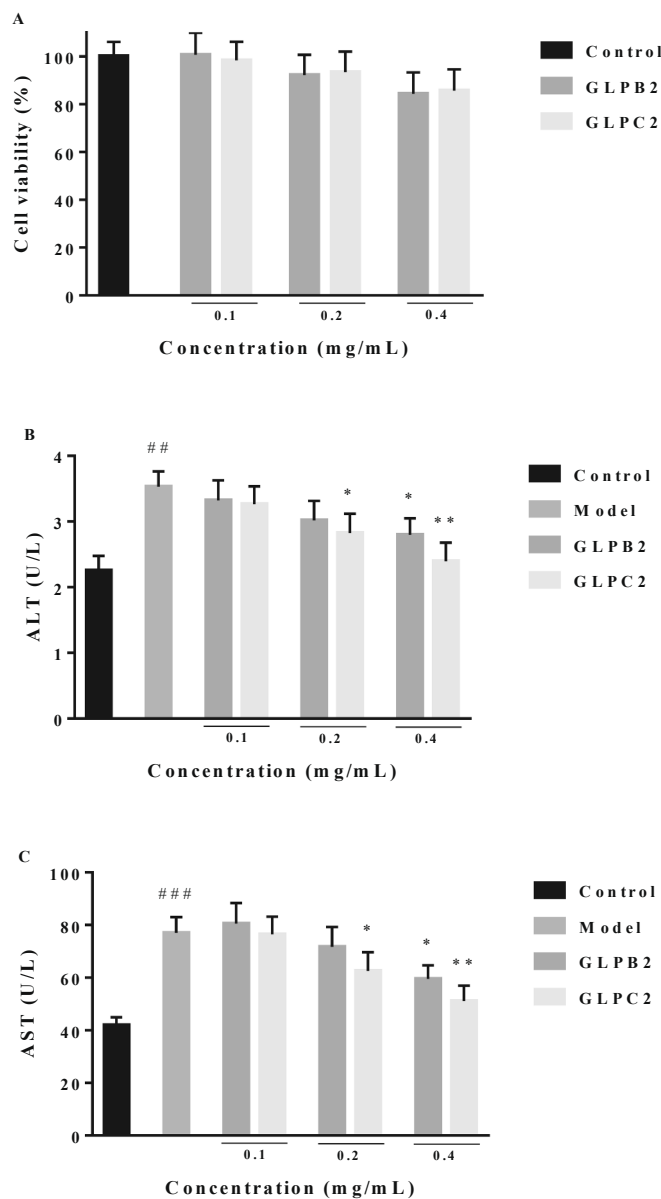


Fig. 2. Hepatoprotective activities of GLPB2 and GLPC2. Cell viability (A) and ALT (B) and AST (C) activities in H_2O_2 -induced HepG2 cells treated with various concentrations of GLPB2 and GLPC2. ## $P < 0.01$, ### $P < 0.001$, compared to N group; * $P < 0.05$, ** $P < 0.01$, compared to M group.

for cell and tissue injury and primarily caused by ROS. GLP had protective effects on restraint stress-induced acute liver damage by improving the oxidative status. Thus, the protective effects of GLPB2 and GLPC2 against H_2O_2 -induced HepG2 cells were tested. Hydrogen peroxide is a toxic substance for the liver cells and can be converted into hydroxyl radicals and oxygen radicals. Previous studies had confirmed the hepatoprotective effect of polysaccharides based on their effect on the viability, ALT and AST activity of H_2O_2 -induced HepG2 cells (Qu et al., 2020). ALT and AST activities were considered as indicators to measure the degree of hepatocyte injury. Preliminarily, the cytotoxicity of GLPB2 and GLPC2 were determined based on the viability of HepG2 cells. As shown in Fig. 2A, both GLPB2 and GLPC2 exerted no obvious toxicity against HepG2 cells at the concentration from 0.1 to 0.4 mg/mL. Then, Both GLPB2 and GLPC2 were further examined for their protective effects against H_2O_2 -induced HepG2 cells damage. As shown in Fig. 2B and C, ALT and AST activities in HepG2 cells were increased after being induced by H_2O_2 . Pretreatment with GLPB2 and GLPC2

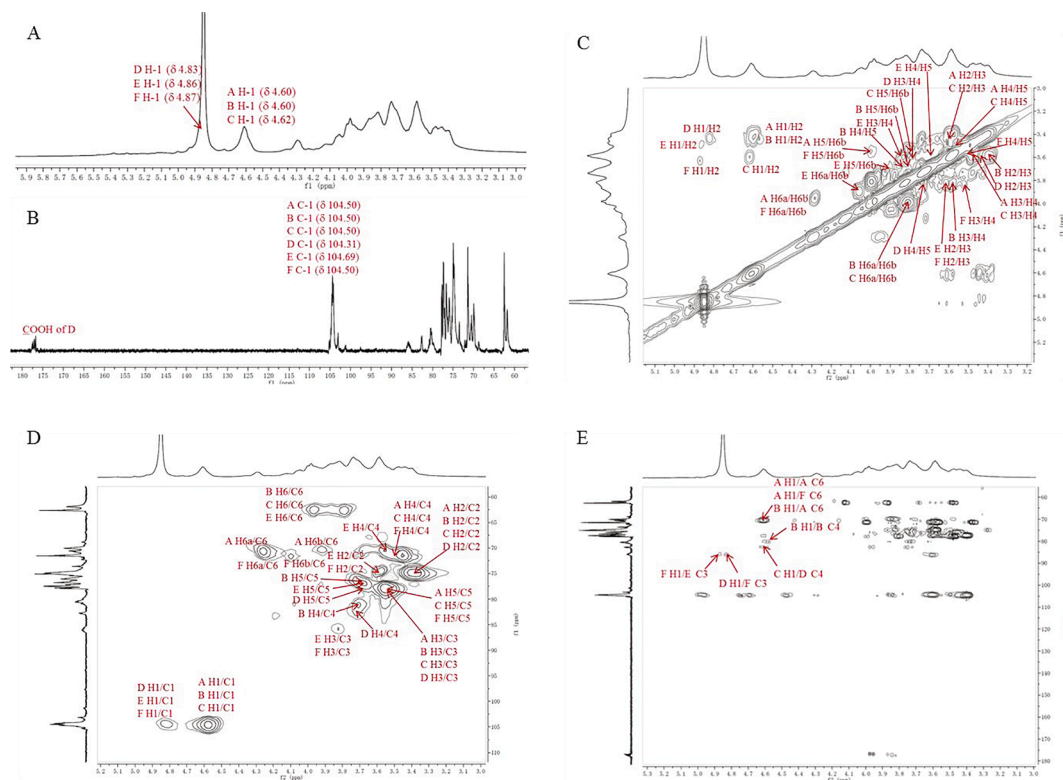


Fig. 3. ^1H (A), ^{13}C (B), ^1H - ^1H COSY (C), HSQC (D) and HMBC (E) spectra of GLPC2.

prominently inhibited ALT and AST activities in a concentration-dependent manner. Moreover, GLPC2 showed stronger inhibitory activity than GLPB2. According to the results of the monosaccharide analysis and the hepatoprotective assay, it is concluded that the stronger hepatoprotective activity of GLPC2 may be partly attributed to the glucuronic acid content.

Continuous research has reported that polysaccharides exerted hepatoprotective effect through various mechanisms including the pathological process of inflammation, apoptosis and oxidative stress. Polysaccharides could regulate NF- κB , JAK/STAT, TGF- β , PI3K/AKT, MAPK, caspase cascade, p53 and Nrf2-Keap1 pathways, lipid metabolism as well as cytochrome P450 enzymes (Qu et al., 2020). Therefore, further research is needed to verify the hepatoprotective effect of GLPC2 *in vivo* and to study the underlying mechanism.

3.5. Linkage patterns analysis

Carboxyl reduction and methylation analysis were performed to determine the linkage types of GLPC2 and the GC-MS data were listed in Table S1. GLPC2 mainly contained T-Glcp, 1, 3-Glcp, 1, 4-Glcp, 1, 6-Glcp, and 1, 3, 6-Glcp, with a molar ratio of about 1:2:3:2:1. Besides, GC-MS analysis also revealed small trace of T-Manp and 1, 6-Galp. GLPC2R, the reduction products of GLPC2, included T-Glcp, 1, 3-Glcp, 1, 4-Glcp, 1, 6-Glcp, and 1, 3, 6-Glcp, with a molar ratio of 1:2:4:2:1. The results implied that GlcA in GLPC2 existed as 1, 4-linkages.

3.6. FT-IR and NMR spectra

The typical absorption signals of GLPC2 can be observed from its FT-IR spectrum (Fig. S4). The peak at 3415.0 cm^{-1} was attributed to the characteristic O-H stretching absorption. Bands at 2923.5 , 1375.3 and 1262.8 cm^{-1} were assigned to the C-H stretching absorption, C-H bending vibration and H-C-H twisting vibration, respectively (Venkatesan, Qian, Ryu, Kumar, & Kim, 2011; S. Zhang, He, Chen, & Ding, 2019). The band at 895 cm^{-1} indicated the β -glucopyranosyl linkages of

Table 1
 ^1H and ^{13}C NMR assignment of GLPC2 (in D_2O).

	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6
\rightarrow 6)- β -D-Glcp-(1 \rightarrow (A)	4.60 104.53	3.45 74.97	3.58 77.52	3.49 71.55	3.55 77.86	4.28, 3.93 70.59
\rightarrow 4)- β -D-Glcp-(1 \rightarrow (B)	4.59 104.53	3.39 75.24	3.58 77.52	3.84 80.49	3.68 75.70	3.99, 3.81 62.69
β -D-Glcp-(1 \rightarrow (C)	4.60 104.53	3.45 74.97	3.58 77.52	3.49 71.55	3.55 77.86	3.99, 3.81 62.53
\rightarrow 4)- β -D-GlcpA-(1 \rightarrow (D)	4.82 104.31	3.42 74.92	3.59 76.85	3.79 82.89	3.71 77.95	176.94
\rightarrow 3)- β -D-Glcp-(1 \rightarrow (E)	4.86 104.69	3.63 75.15	3.85 86.02	3.58 69.94	3.69 76.70	4.05, 3.89 61.97
\rightarrow 3,6)- β -D-Glcp-(1 \rightarrow (F)	4.87 104.63	3.61 74.93	3.84 85.72	3.47 71.38	3.55 77.44	4.28, 3.93 70.62

GLPC2 (Fu, Shi, & Ding, 2019; Shi, Zhong, Zhang, & Yan, 2020).

The ^1H and ^{13}C NMR spectra of GLPC2 were presented in Fig. 3A and B. In ^1H NMR spectrum, the main peaks of δ_{H} 4.60–4.62 and δ_{H} 4.82–4.87 ppm indicated that all the glycosidic residues were β -configured. Correspondingly, the signals of the anomeric carbons were observed at about 104.50 ppm in ^{13}C NMR spectrum. The downfield signals at about δ_{C} 86 and 80 were assigned to the substituted C-3 and C-4 of glycosidic residues, respectively. The downfield peak at about δ_{C} 70 ppm was attributed to the substituted C-6 of glycosidic residues. The strong peak at δ_{C} 62.69 was from the unsubstituted C-6 of glycosidic residues. Based on NMR assignments reported in literatures (Y. Chen et al., 2017; Dong et al., 2012; Fu, Shi, & Ding, 2019; D. Huang, Hou, Zhang, Zhang, & Yan, 2020; Y. Wang et al., 2017; H. Zhang, Nie, Cui, Xu, Ding, & Xie, 2017), the HSQC correlation observed at 4.60/104.53 and 4.59/104.53 (Fig. 3C) were assigned as H-1/C-1 of 1, 6- β -D-Glcp (marked as residue A) and 1, 4- β -D-Glcp (B), respectively. Another correlation of 4.60/104.53 was ascribed to H-1/C-1 of T- β -D-Glcp (C). 4.82/104.31 was from H-1/C-1 of 1, 4- β -D-GlcpA (D). Signals at 4.86/104.69

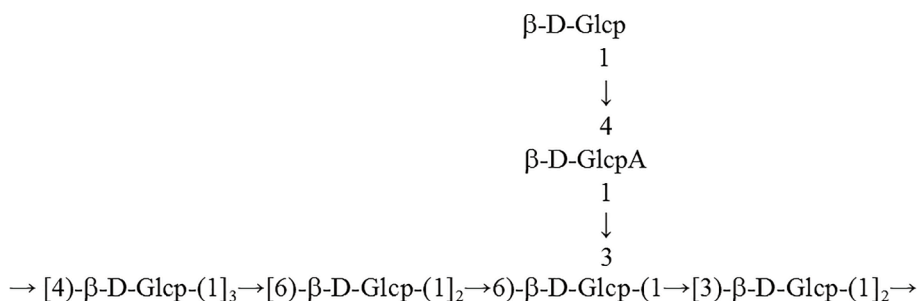


Fig. 4. Possible repeated unit of GLPC2.

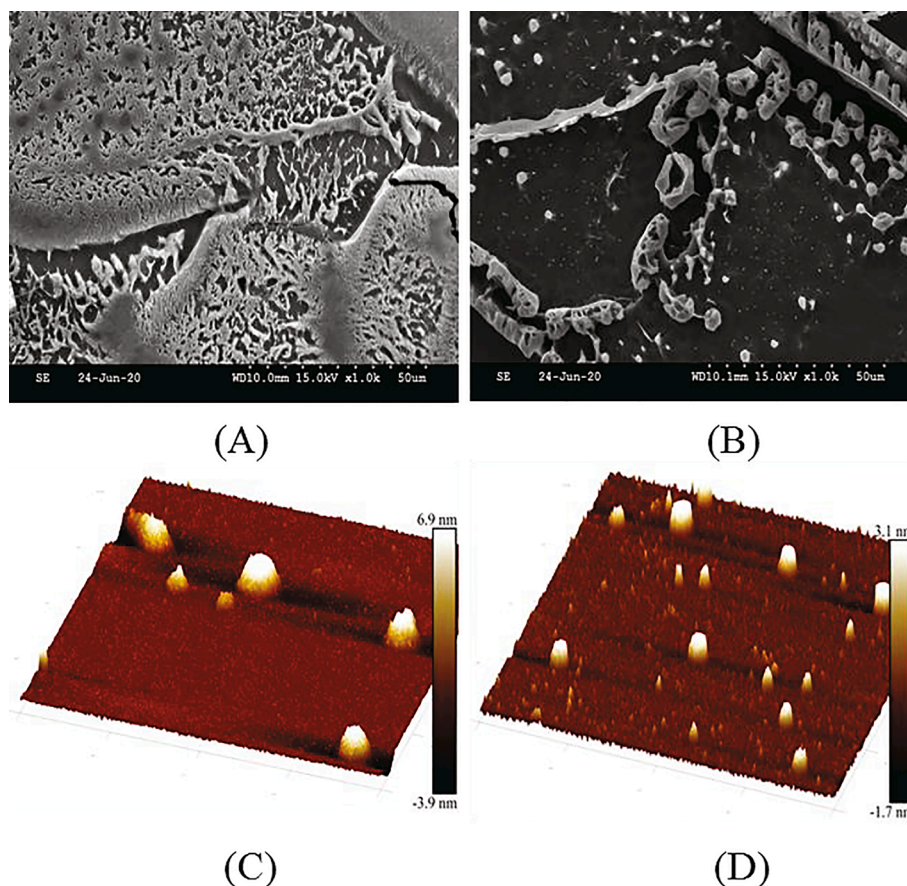


Fig. 5. SEM (A, B) and AFM (C, D) analysis of GLPC2.

was assigned as H-1/C-1 of 1, 3- β -D-Glcp (E) and 4.87/104.63 was assigned as H-1/C-1 of 1, 3, 6- β -D-Glcp (F). The cross peaks of 3.84/85.87 and 3.85/86.02 were from H-3/C-3 of 1, 3- β -D-Glcp (E) and 1, 3, 6- β -D-Glcp (F), respectively. 4.27, 4.02/70.59, and 4.28, 3.93/70.62 were assigned as H₂-6/C-6 of 1, 6- β -D-Glcp (A) and 1, 3, 6- β -D-Glcp (F) respectively. In the same way, the other proton and carbon signals for these residues were assigned by analysis of HSQC combined with ¹H-¹H COSY spectra (Fig. 3D). The NMR assignments of glycosidic residues A – E were listed in Table 1.

The observed HMBC correlation (Fig. 3E) of δ 4.60/70.59 (AH1/AC6), 4.60/70.62 (AH1/FC6), and 4.60/70.59 (BH1/AC6) suggested that C-6 of residue A was linked to O-1 of residue A, O-1 of residue B, as well as O-1 of residue F. Cross signal at 4.60/80.49 (BH1/BC4) indicated that C-4 of residue B was connected to O-1 of residue B. The correlation of 4.87/86.02 (FH1/EC3) indicated C-3 of residue E was linked to O-1 of residue F. These results suggested that GLPC2 had the backbone of \rightarrow 3)- β -D-Glcp-(1 \rightarrow , \rightarrow 4)- β -D-Glcp-(1 \rightarrow , \rightarrow 6)- β -D-Glcp-(1 \rightarrow , and \rightarrow 3, 6)- β -D-

Glcp-(1 \rightarrow , with a branching point at C-3 of \rightarrow 3, 6)- β -D-Glcp-(1 \rightarrow . The branch was consisted of \rightarrow 4)- β -D-Glcp-(1 \rightarrow (D) and T- β -D-Glcp (C) from the observed correlations of 4.82/85.57 (DH1/FC3) and 4.62/82.78 (CH1/DC4), respectively. Finally, Combined with the results of mono-saccharide composition, and GC-MS analysis, a putative structure for GLPC2 was proposed as shown in Fig. 4.

Previous studies have revealed that both glucans and heteroglycans were found in *G. lucidum* (Y. Liu et al., 2014; Lu, He, Sun, Zhang, Linhardt, & Zhang, 2020; J. Wang, Ma, Zhang, Fang, Jiang, & Phillips, 2011). Glucans were composed of 1, 3-, 1, 4-, 1, 6-, 1, 3, 6-, and 1, 4, 6- β -D-Glcp residues with different Mw (Bao, Wang, Dong, Fang, & Li, 2002; Kao et al., 2012; Li et al., 2020; W. Liu, Wang, Pang, Yao, & Gao, 2010). However, *G. lucidum* heteroglycans possessed different monosaccharide combination including mannose, rhamnose, glucose, galactose, xylose, fucose and arabinose with different linkage patterns (Bao, Wang, Dong, Fang, & Li, 2002; S. Huang, Li, Li, & Wang, 2011; Li et al., 2020; D. Pan, Wang, Chen, Hu, & Zhou, 2015; C. Xiao, Wu, Zhang, Xie, Cai, & Tan,

2017). Different strains, cultivated patterns, growing periods may cause the complexity and diversity of *Ganoderma* polysaccharides. Besides, most of *G. lucidum* polysaccharides are neutral polysaccharides and the acidic polysaccharides had yet received little attention. Our results revealed that acid glucans were also contained in *G. lucidum*, which further enriched the understanding of chemical diversity of *G. lucidum* polysaccharides.

3.7. Morphological analysis of GLPC2

SEM and AFM are considered as powerful tools to observe the microstructure and morphological characteristics of macromolecular. As shown in Fig. 5A and B, GLPC2 had spherical or columnar crannied conglomerations on the smooth surface. It can be seen from Fig. 5C and D visually that GLPC2 comprised rigid short-rod conformation. The AFM images were in accordance with the results of SEM. It is demonstrated that β -D-glucans polysaccharide possessed a high Mw ($>2.5 \times 10^4$) and presented a triple helix tertiary structure (Synytsya & Novák, 2013). The low Mw would destruct the hydrogen bond, which lead to the failure of the triple helix structure formation (Li et al., 2020).

4. Conclusion

G. lucidum crude polysaccharide GLP was found to relieve oxidative stress of acute liver injury in restraint mice. Two glucans, named GLPB2 and GLPC2, were purified from GLP. GLPB2 and GLPC2 possessed similar Mw but different monosaccharide compositions. In addition, GLPC2 showed stronger hepatoprotective activity compared to GLPB2 *in vitro*, indicating that the content of uronic acid may influence the hepatoprotective bioactivities of polysaccharides. Glycosidic residues and NMR analysis comprehensively revealed that GLPC2 contained D-Glcp-(1 \rightarrow , \rightarrow 3)-D-Glcp-(1 \rightarrow , \rightarrow 4)-D-Glcp-(1 \rightarrow , \rightarrow 6)-D-Glcp-(1 \rightarrow , \rightarrow 3, 6)-D-Glcp-(1 \rightarrow and \rightarrow 4)-D-GlcpA-(1 \rightarrow . Besides, AEC could be an effective technique for separating glucans into neutral and ionic fractions by utilizing different ionic strength buffer. Our current study may contribute to the improved utilization of *G. lucidum* polysaccharides for liver protection. Future work on structure–activity relationship of GLPs and the hepatoprotective mechanism is in progress.

CRedit authorship contribution statement

Shaodan Chen: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Funding acquisition. **Xiaoying Guan:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Tianqiao Yong:** Writing – original draft. **Xiong Gao:** Writing – review & editing. **Chun Xiao:** Writing – review & editing. **Yizhen Xie:** Writing – review & editing. **Diling Chen:** Writing – review & editing. **Huiping Hu:** Writing – review & editing. **Qingping Wu:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2022.100204>.

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