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Structure feature and antitumor activity of a novel polysaccharide isolated from *Lactarius deliciosus* Gray

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ABSTRACT

A novel heteropolysaccharide was isolated from the fruiting bodies of *Lactarius deliciosus* Gray through DEAE-cellulose column and Sephadex G-100 column. The *L. deliciosus* Gray polysaccharide (LDG-A) had a molecular weight of 1.1×10^4 Da and was mainly composed of L-mannose and D-xylose (L-Man and D-Xyl) which ratio was 3:1. As a precondition to understand the bioactivity, structural feature of *L. deliciosus* Gray polysaccharide (LDG-A) were investigated by a combination of total hydrolysis, methylation analysis, gas chromatography–mass spectrometry (GC–MS), scanning electron microscope (SEM), infrared (IR) spectra, nuclear magnetic resonance (NMR) spectroscopy and dynamical analysis of the atomic force microscope (AFM) studies. The results indicated that *L. deliciosus* Gray polysaccharide (LDG-A) had a backbone of 1,6-disubstituted-α-L-mannopyranose which branched at O-2 and the branches were mainly composed of a →3)-α-D-xylopyranose residue. LDG-A exhibited significant anti-tumor activates in vivo. *L. deliciosus* Gray may be one ideal sources of antitumor development.

1. Introduction

Polysaccharides are long carbohydrate molecules, of repeating units joined together by glycosidic bonds. They are often linear, but may also be even highly branch. Polysaccharides are often quite heterogeneous, containing slight modifications of the repeating unit. Depending on the structure, these macromolecules can have distinct properties from their monosaccharide building blocks. They may be amorphous or even insoluble in water (Bertozzi & Kiessling, 2001). Polysaccharides known as "biological response modulators", reflect its significant immunomodulatory activity, such as promoting the proliferation and differentiation of immune cells, activating T cells and B cells, secreting a variety of lymphokines, regulating the balance of nerve–endocrine–immune regulatory network and promoting the DNA, RNA, protein synthesis, etc. (Pauline, Time, Peter, Ian, & Raymond, 2001). In recent years, as more and more polysaccharides have been reported to exhibit a variety of biological activities, including anti-tumor (Wasser, 2002), immunostimulation (Yamada, 1994), anti-oxidation, etc. (Li et al., 2003; Liu, Ooi, & Chang, 1997) the polysaccharides have emerged as one important class of bioactive natural products. In many oriental countries, several immunoceuticals composed of polysaccharides have been accepted such as lentinan, schizophyllan and krestin (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Liu, Ooi, & Fung, 1999).*Lactarius deliciosus* Gray is a kind of fungi belonging to Lactarius which grows in Xiaojin country of Sichuan province in China at an elevation of 3750 m. In this work, one novel water-soluble polysaccharide was extracted and purified from the fruiting bodies of *L. deliciosus* Gray using a DEAE-cellulose column chromatography and a Sephadex G-200 column chromatography. Its chemical structures were characterized and the antitumor activity was evaluated for the first time. The result of this study introduced *L. deliciosus* Gray as a possible valuable source which helped to exhibit unique antitumor properties.

2. Materials and methods

2.1. Chemicals

The fruiting bodies of *L. deliciosus* Gray was collected in Xiaojing country of Sichuan province, China, and was authenticated by Prof. Zhirong Yang (College of Life Sciences, Sichuan University, Chengdu, China). At the same time, a voucher specimen had been preserved in Key Laboratory of Southwest China Wildlife Resources Conservation, College of Life Sciences, China West Normal University. DEAE-Cellulose 52 and Sephadex G-100 were purchased from Sigma–Aldrich (mainland, China). Monosaccharide standards, Dextran T-500, T-110, T-70, T-40, and T-10KDa, were purchased from Beijing Biodee Biotechnology Co., Ltd (Beijing, China). All other reagents used were of analytical grade.

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2.2. Extraction, purity and fractionation of polysaccharides from L. deliciosus Gray

After the fruiting bodies of *L. deliciosus* Gray (200 g) were soaked with 95% EtOH, the residues were dried and then extracted with boiling water for three times (5 h for each). After the filtrate was concentrated, dialyzed (MWCO 8000, Sigma), and centrifuged, the supernatant was added with 3 volumes of 95% EtOH to precipitate crude polysaccharides (named LDG, 25.7 g, recovery 12.85%). After Sevag method (Staub, 1965) was used for the deproteination, LDG (2 g) was subjected to a DEAE-cellulose column (Tris–Hcl, pH 7.0, Cl^-) and eluted stepwise with 0, 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0 M NaCl. The eluate was monitored by the phenol–sulfuric acid method (Dubois, Gillis, Hamilton, Rebers, & Smith, 1956). The 0 M NaCl elution was concentrated, lyophilized and purified on a Sephadex G-100 column (2.6 cm × 60 cm). The resulting *L. deliciosus* Gray polysaccharide, named LDG-A, was obtained by the above processes and the yield rate of LDG-A was 0.215% (0.430 g) for the starting material.

2.3. Measurement of molecular weight and monosaccharide composition analysis of LDG-A

High performance gel permeation chromatography (HPGPC) was carried out to measure molecular weight (Yamamoto, Nunome, Yamauchi, Kato, & Sone, 1995). The column was calibrated with standard T-series Dextran (T-500, T-110, T-70, T-40 and T-10 KDa). The data were processed with Waters GPC (Millennium32 software). The polysaccharide LDG-A (6.0 mg) was hydrolyzed with 2 M TFA at 110 °C for 6 h on the mechanism of acid-catalyzed hydrolysis (Yu et al., 2009). Excess acid was removed by co-distillation with Methyl Alcohol (MeOH) after the hydrolysis was completed. One part of the hydrolysate (1.0 mg) was used for thin layer chromatography (TLC) analysis as described previously developing solvent: acetoacetate–pyridine–ethanol–water solution (8:5:1.5:1). The developer system: diphenylamine–aniline system (85% phosphoric acid solution 140 mL containing 8 mL diphenylamine, 8 g aniline) (Partridge, 1949), and the other (1.0 mg) was dissolved in pyridine (0.2 mL). The derivatization reaction was initiated by the addition of hexamethyl-disilazane (0.2 mL) and trimethyl chloro-silicane (0.2 mL) according to the method described by Dong and Guentas (Dong, Zhang, Lin, & Fang, 1995; Guentas et al., 2001). The resulting supernatant was examined by GC–MS at a temperature program of 50–230 °C with a rate of 2 °C/min (Chen, Xie, Nie, Li, & Wang, 2008).

2.4. Methylation analysis

The polysaccharide, LDG-A (10 mg), was methylated using methyl iodide (MeI) according to the Hakomori method (Hakomori, 1964). After completed methylation, the permethylated polysaccharide was depolymerized with 90% aqueous formic acid (3 mL) for 10 h at 100 °C in a sealed tube. The methylated sugars were derivatized using the method described method and analyzed by GC–MS.

2.5. UV and infrared (IR) spectra analysis

LDG-A was tested in UV from 200 nm to 600 nm. And infrared analysis of the samples was obtained by grinding a mixture of polysaccharide with dry KBr and then pressing in a mold. Spectra were run in the 4000–400 cm^{-1} region.

2.6. Nuclear magnetic resonance (NMR) experiment

^1H NMR spectra and ^{13}C NMR spectra were recorded on a Varian Unity INOVA 400/45 in D_2O with Tetramethylsilane as internal standard.

2.7. Atomic force micrograph (AFM)

The normal sample preparation procedure consisted of spreading of a dilute (25 μg/mL) polymer solution onto a freshly cleaved mica surface and successive air-drying under ambient pressure, temperature, and humidity (Kirby, Gunning, & Morris, 1995; Michela, Meredith, Paola, Neil, & Rizzo, 2009). The atomic force microscopy was operated in the tapping-mode (Gunning et al., 2003; Morri, Gunning, Kirby, Round, & Waldron, 1997).

2.8. Animals

S180 tumor cells were maintained in peritoneal cavities of Kunming strain male mice obtained from Institute of Biochemistry and Molecular Immunology of North Sichuan Medical College (NSMC) (Nanchong, China). Male Kunming strain mice, weighed 25.0 ± 1.0 g, purchased from NSMC, were housed six per plastic cages with wood chip bedding in an animal room with a 12 h light and 12 h dark cycle at room temperature (25 ± 2 °C) and allowed free access to standard laboratory diet (purchased from the Institute of Biochemistry and Molecular Immunology of the North Sichuan Medical College). The animal experiments were conducted according to the 'Guidelines for Animal Experimentation' of the North Sichuan Medical College.

2.9. Assay of anti-tumor activity in vivo

S180 tumor cells (3×10^6) were implanted subcutaneously into right hind groin of the Kunming strain male mice. Mice were randomly divided into five groups ($n = 6$). One day after inoculation, LDG-A was dissolved in distilled water and administered intraperitoneally (i.p.) to the mice at the doses of 20, 40, and 80 mg/kg. Positive and negative controls were set for comparison. The positive control was given with 0.2 mL mannatide (20 mg/kg) and negative one with physiological saline instead of the test solution. Animals were sacrificed after 2 weeks. The body weights were measured. Tumors, spleens and livers were excised and the tumor inhibitory ratio were calculated by following formula: Inhibition ratio (%) = $[(A - B)/A] \times 100$, where A and B were the average tumor weights of the negative control and treated groups, respectively.

2.10. Statistical analysis

All data were presented as means ± standard deviation (SD) of three replications. Statistical analyses were performed using Student's t-test and one-way analysis of variance. Values of $P < 0.05$ or less were considered to be a statistically significant finding.

3. Results and discussion

3.1. Extraction, purity and composition of polysaccharides

The crude polysaccharide, named LDG, was obtained from the fruiting bodies of *L. deliciosus* Gray with a yield of 12.85%. The yield was calculated by the following formula: yield (%) = $[A/A_0] \times 100\%$, where A is the weight of LDG and A_0 is the weight of the fruiting bodies of *L. deliciosus* Gray. After fractionation on DEAE-Cellulose 52 and Sephadex G-200 column chromatography, 430 mg of LDG-A was obtained from the 0 M NaCl eluate and detected by the

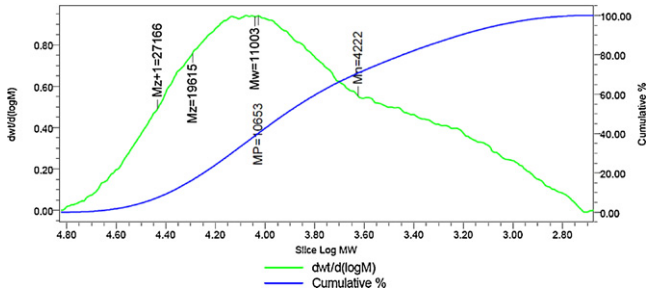

Fig. 1. Molecular weight determination spectrum of LDG-A by HPGPC.

phenol–sulfuric acid assay. The homogeneity of the polysaccharide was elucidated by the following tests. LDG-A was eluted from gel-filtration chromatography on Sephadex G-100 column and was detected by the phenol–sulfuric acid assay as a single peak. No absorption at 280 and 260 nm in UV absorption spectra of LDG-A demonstrated the absence of protein and nucleic acid in this polysaccharide and it had the same optical rotation: $[\alpha]^{20}_{D}$ 10.7° (c0.5, water) in different low concentrations of ethanol using HK7-SGW-1 automatic optical polarimeter at room temperature. Weight-average molecular weight was around 1.1×10^4 Da (Fig. 1). The two monosaccharides, L-mannose (L-Man) and D-xylose (D-Xyl) were also identified using the hydrolysate of LDG-A by GC–MS and the ratio was 3:1. LDG-A was supposed to contain the L-mannose and D-xylose configuration monosaccharide according to GC–MS analysis.

3.2. Structure elucidation of LDG-A

The intensity of bands around 3412.67 cm^{-1} in the IR spectrum (Fig. 2) were due to the hydroxyl stretching vibration of the polysaccharide and as expected they were broad. The bands in the region of 2925.85 cm^{-1} were due to C—H stretching vibration, and the bands in the region of 1638.75 cm^{-1} were due to associated water (Cao et al., 2006). The strong absorption bands at 1148.96 cm^{-1}, 1075.57 cm^{-1} in the range of 1200–1000 cm^{-1} in the IR spectrum was due to the C—O—H stretching vibration of —COOH and the C—O—C stretching vibration of ether in pyranose-ring which suggested that the monosaccharide in LDG-A had a pyranose-ring (Barker, Bourne, Stacey, & Whiffen, 1954). Moreover, the characteristic absorptions at 800.72 cm^{-1} indicated α-configurations existing in the polysaccharide (Wu & Tu, 2005), which was in good

Table 1
^{13}C NMR chemical shift data (δ, ppm) for polysaccharide LDG-A.

Sugar residues	Chemical shifts, δ (ppm)					
	C_1	C_2	C_3	C_4	C_5	C_6
→6)-α-L-Man*p*-(1→	96.78	68.68	71.83	72.78	70.75	68.63
→2,6)-α-L-Man*p*-(1→	95.92	66.38	71.46	72.48	70.30	68.49
α-D-Xyl*p*-(3→	102.60	63.23	70.88	71.88	69.31	61.13

agreement with the anomeric proton signals at δ 5.049, δ 5.141 in the ^1H NMR (400 MHz) spectrum (Fig. 3) (Kim et al., 2000). According to the literature (Wang, Luo, & Liang, 2004), the resonances in the region of 98–106 ppm in the ^{13}C NMR (400 MHz) spectrum of LDG-A were attributed to the anomeric carbon atoms of α-L-mannopyranose (α-L-Man*p*) and α-D-xylopyranose (α-D-Xyl*p*). In the anomeric carbon region, signals at δ 96.78 could be attributed to C-1 of →6)-α-L-Man*p*-(1→; δ 95.92 to C-1 of →2,6)-α-L-Man*p*-(1→; δ 102.60 to C-1 of α-D-Xyl*p*-(3→, respectively. All the assignment of the carbon atoms signals was shown in Table 1.

After methylation according to the Hakomori method for four times, the hydroxyl group absorption at 3600–3200 cm^{-1} in IR disappeared (date not shown), indicating the completeness of methylation. The methylated polysaccharide was depolymerized and converted into partially methylated ramifications. The analysis of the methylated monosaccharide was conducted by GC–MS. The information in MS showed that the mannose and xylose residues (L-Man, SI: 96 and D-Xyl, SI: 92) were L and D-configuration, respectively. Methylation analysis for LDG-A proved that the α-L-mannopyranose residues were 3,4-bis-substituted and 2,3,4-trisubstituted and the α-D-xylopyranose residue was 1,2,4,6-tetra-subsituted (Table 2). Results of methylated linkage analysis of LDG-A indicated that (1 → 6)-linked-α-L-mannopyranose was one of the largest amounts residue of the polysaccharide structure, the branched residue was (1 → 2,6)-linked-α-L-mannopyranose revealing that (1 → 6)-linked-α-L-mannopyranose should be possible to form the backbone structure. The relative amounts of (1 → 2,6)-linked-α-L-mannopyranose indicating that approximate branch ratios could theoretically be 33.3%, namely on average one branching point for each three residues of backbone. Residues of branch structure were (2 → 3)-linked-α-D-xylopyranose. It was concluded that a repeating unit of LDG-A had a backbone of (1 → 6)-α-L-mannopyranose residues which branched at O-2 based on the experimental results. The branch was supposed to be the composition

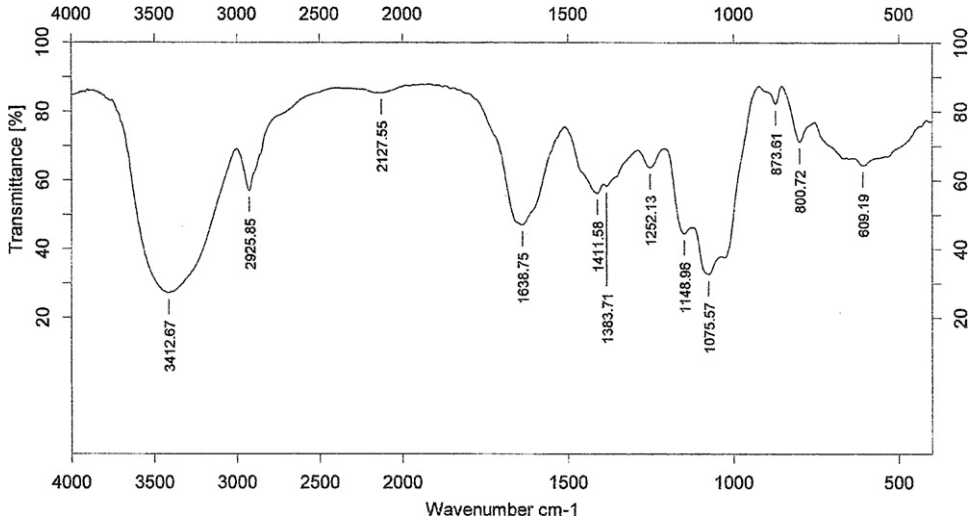

Fig. 2. FTIR spectra of polysaccharide LDG-A.

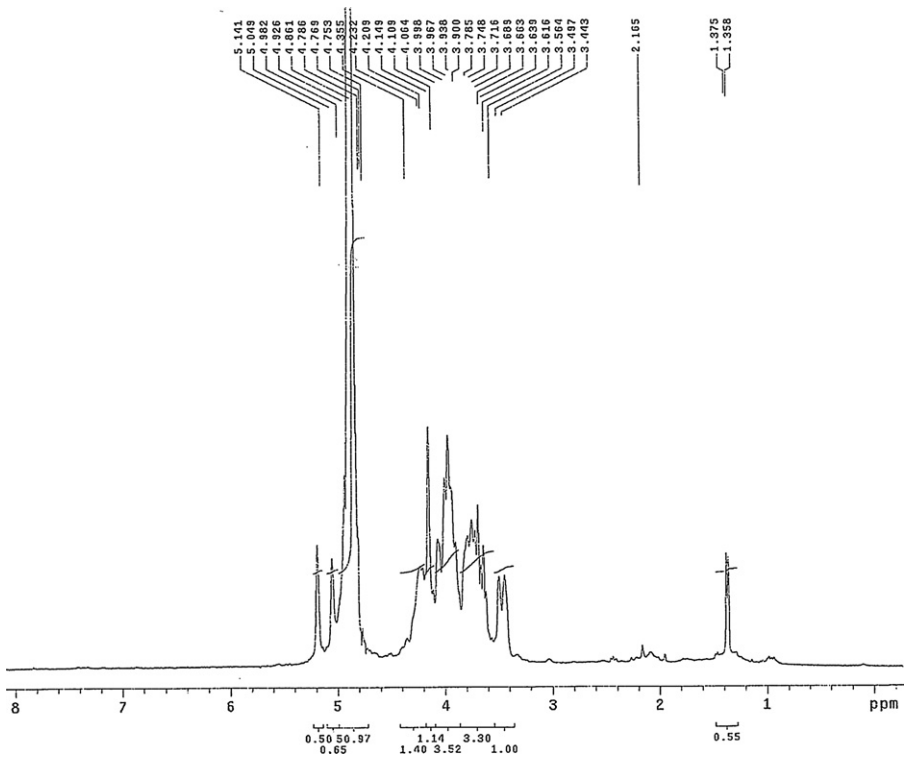

Fig. 3. The ^{1}H NMR spectra of polysaccharide LDG-A.

Table 2
GC–MS results of methylation analysis of LDG-A.

Methylated sugar	Linkage	*m*/*z*
2,3,4-Me_3-Man	1,6-	61, 73, 85, 103, 130, 147, 155, 191, 204, 217, 231
3,4-Me_2-Man	1,2,6-	45, 59, 73, 103, 130, 147, 155, 191, 204, 217
1,2,4,6-Me_4-Xyl	T-	45, 59, 73, 89, 114, 131, 146, 159, 189, 217, 276

of an (2 → 3)-α-D-xylopyranose residue. The predicted structure of the novel polysaccharide LDG-A was shown in Fig. 4.

3.3. Atomic force micrograph (AFM)

The topographical AFM planar image of LDG-A deposited from a 25 μg/mL water solution was shown in Fig. 5. Only spherical lumps could be seen and the diameter and height of the lumps ranged from 20 to 80 nm. The heights of the spherical structures are much higher than that of a single polysaccharide chain (about 0.1–1 nm), suggesting that molecular aggregation was involved. We can see that irregularly shaped large structures were formed together like worm from AFM cubic image of LDG-A, which suggested that LDG-A could take random coil compact conformation. The conformation might be related to its side chains.

3.4. Anti-tumor activity of LDG-A

The anti-tumor activity of the polysaccharide was usually believed to be a consequence of the stimulation of the cell-mediated immune response (Ooi & Liu, 2000). To detect the anti-tumor activity of LDG-A in vivo, we used the mice transplanted S180 to evaluate the effects and the results were summarized in Table 3. LDG-A could inhibit the growth of the tumors ($P < 0.01$) in a dose-dependent manner. The inhibitory rate in mice treated with 80 mg/kg LDG-A was 68.422%, being the highest in the three doses. Furthermore, during the experiments, the appetite, activity and coat luster of

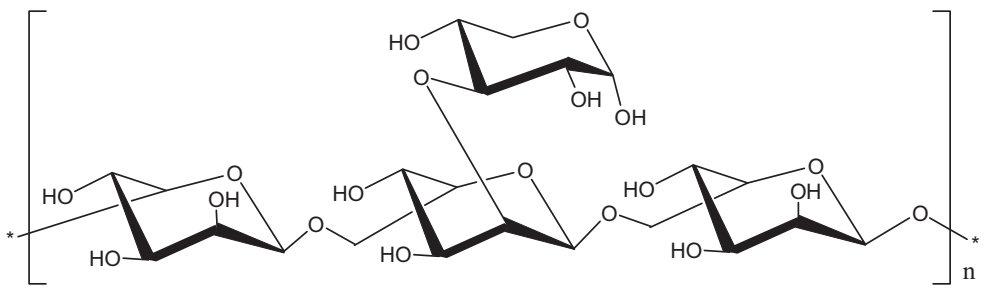

Fig. 4. Predicted chemical structure of polysaccharide LDG-A.

Table 3
Anti-tumor activities of LDG-A on S180 tumor.

Group	Spleen index (mg/g)	Liver index (mg/g)	Thymus index (mg/g)	Average tumor weight (g)	Inhibitory rate of tumor (%)
N	5.784 ± 2.108	60.307 ± 9.395	1.429 ± 0.706	3.740 ± 0.423	–
L1	7.619 ± 2.414	56.468 ± 4.509	4.741 ± 0.301	2.081 ± 0.740	44.358[a]
L2	7.807 ± 3.427	66.351 ± 8.802	7.790 ± 3.208	1.783 ± 0.430	52.326[a]
L3	5.202 ± 0.989	59.977 ± 4.786	0.903 ± 0.401	1.181 ± 0.241	68.422[a]
Man	7.0439 ± 3.105	58.263 ± 5.399	2.862 ± 1.727	1.581 ± 0.706	57.727[a]

N: negative control group; L1, L2, L3 indicating LDG-A groups of 20 mg/kg, 40 mg/kg, 80 mg/kg, respectively; Man: positive control group of mannatide.
[a] Significant differences from negative control group and positive control group were evaluated using student's t test: $P < 0.01$

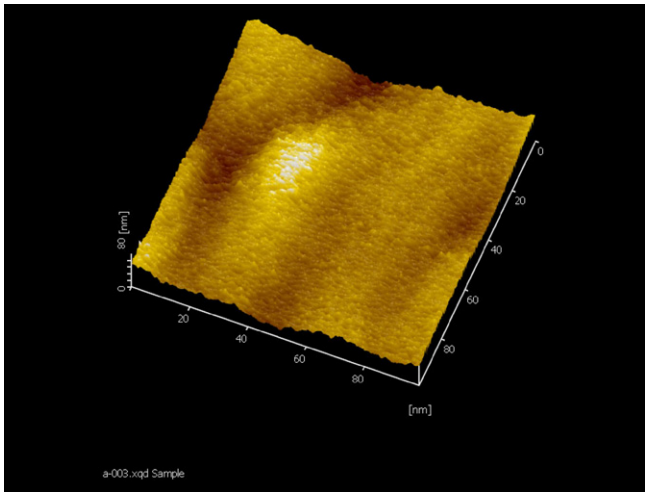

Fig. 5. Atomic force microscopy (AFM) cubic images of molecular structure of polysaccharide LDG-A.

each animal in LDG-A groups were better than the mice treated with mannatide. The results showed little change in average liver weight in test groups which indicating that LDG-A did not cause serious sliver damage. On the 14th day, the average tumor weight of negative control mice was 3.74 g, whereas the average tumor weight of mice in LDG-A group at dose of 80 mg/kg was 1.181 g, and also significantly reduced in doses of 20 mg/kg and 40 mg/kg, which were 2.081 g and 1.783 g, respectively. It is noteworthy that the average weights of the spleens and thymus in test groups were significantly greater in doses of 40 mg/kg than that in the mannatide mice, and even that of the negative control mice, indicating that LDG-A could increase the weights of immune organs in moderate doses. These results suggested that activating immune responses in the host might be one of the mechanisms of anti-tumor activity of LDG-A, as many anti-tumor polysaccharides found in the world.

4. Conclusions

According to the results above, it was concluded that the novel polysaccharide obtained from *L. deliciosus* Gray was a heteropolysaccharide, namely LDG-A. And the purified polysaccharide (LDG-A) was confirmed of high purity. The present study also showed that LDG-A consisted of two monosaccharides, namely L-Man and D-Xyl and their ratio was 3:1 by GC–MS. Structure study demonstrated that LDG-A had a backbone of (1 → 6)-α-L-mannopyranose residues which branched at O-2 based on the experimental results. The branch was mainly composed of an (2 → 3)-α-D-xylopyranose residue. Purification polysaccharides prepared in our work were confirmed of high purity. LDG-A exhibited significant anti-tumor activates in vivo. The structural and pharmacological results obtained might help enlarge the knowledge of structural correlation to anti-tumor effects of polysaccharides. Overall, *L. deliciosus* Gray may be one ideal sources of antitumor development.

Conflict of interest statement

The authors have declared that no conflict of interest exists.

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