Structure elucidation and anti-tumor activities of water-soluble oligosaccharides from *Lactarius deliciosus* (L. ex Fr.) Gray

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ABSTRACT

Background: Oligosaccharides are composed of a variable number of monosaccharide units and very important in the biologically diverse of biological systems. **Materials and Methods:** Crude water-soluble oligosaccharide was extracted from the fruiting bodies with water and then successively purified by DEAE–cellulose 52 and Sephadex G-100 column chromatography, yielding one major oligosaccharides fractions: LES-A. Structural features of *Lactarius deliciosus* (L. ex Fr.) Gray oligosaccharide (LDGO-A) were investigated by a combination of monosaccharide component analysis by thin layer chromatography, infrared spectra, nuclear magnetic resonance spectroscopy, scanning electron microscopy, and high-performance gel permeation chromatography analysis. **Result:** The results indicated that LDGO-A was composed of D-glucose and D-xylose, and the average molecular sizes was approximately 945 Da. The anti-tumor activity of LDGO-A was evaluated *in vivo*. The inhibitory rate in mice treated with 40 mg/kg LDGO-A can reach 40.02%, being the highest in the three doses, which may be comparable to mannatide. Histology of immune organs shows that the tissues arranged more regular and firmer, but the tumor tissue arranged looser in LDGO-A group than those in the control group. Meanwhile, there is no obvious damage to other organs, such as heart. The anti-tumor activity of the LDGO-A was usually believed to be a consequence of the stimulation of the cell-mediated immune response because it can significantly promote the lymphocyte and macrophage cells in the dose range of 100–400 μg/mL *in vitro*. LDGO-A also effected the expression of some housekeeping genes mRNA in S180 tumor. **Conclusion:** Accordingly, the LDGO-A might serve as an effective healthcare food and source of natural anti-tumor compounds.

Key words: Anti-tumor assay, *Lactarius deliciosus* (L. ex Fr.) Gray, oligosaccharide, structure elucidation, water-soluble

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INTRODUCTION

An oligosaccharide is a saccharide polymer containing a small number (typically 3–10) of component sugars, also known as simple sugars (monosaccharides). Oligosaccharides can have many functions; for example, they are commonly found on the plasma membrane of animal cells where they can play a role in cell-cell recognition. An example is ABO blood type specificity. A and B blood types have two different oligosaccharide glycolipids embedded in the cell membranes of the red blood cells, AB-type blood has both, while O blood type has neither.

Mannan oligosaccharides (MOS) are widely used animal feed to improve gastrointestinal health, energy levels, and performance. They are normally obtained from the yeast cell walls of *Saccharomyces cerevisiae*. Research at the University of Illinois has demonstrated that MOS differ from other oligosaccharides in that they are not fermentable, and their primary mode of actions include agglutination of type-1 fimbriae pathogens and immunomodulation.[1] When oligosaccharides are consumed, the undigested portion serves as food for the intestinal microflora. Depending on the type of oligosaccharide, different bacterial groups are stimulated or suppressed.[2] Clinical studies

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have shown that administering fructo-oligosaccharides, galacto-oligosaccharides, or inulin can increase the number of these friendly bacteria in the colon while simultaneously reducing the population of harmful bacteria.[3]

Lactarius deliciosus (L. ex Fr.) Gray is a kind of fungi belonging to *Lentinus*, which grows in Xiaojin Country of Sichuan Province in China at an elevation of 3700 m.[4] In this work, one water-soluble oligosaccharide was extracted and purified from the fruiting bodies of *L. deliciosus* (L. ex Fr.) Gray using a DEAE-cellulose 52 column chromatography and a Sephadex G-200 column chromatography. Its chemical structures were characterized for the first time. The anti-tumor activity of *L. deliciosus* (L. ex Fr.) Gray oligosaccharide (LDGO-A) was evaluated *in vivo*.

MATERIALS AND METHODS

Chemicals

The fruiting bodies of *L. deliciosus* (L. ex Fr.) Gray were collected in Xiaojing Country of Sichuan Province, China, and were authenticated by Professor 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, School of Life Sciences, China West Normal University. DEAE-cellulose 52 and Sephadex G-200 were purchased from Sigma-Aldrich (Mainland, China). Monosaccharide standards, dextran T-500, T-110, T-70, T-40, and T-10 were purchased from Beijing Biodee Biotechnology Co., Ltd., (Beijing, China). All other reagents used were of analytical grade.

Extraction, purity, and fractionation of oligosaccharides from *Lactarius deliciosus* (L. ex Fr.) Gray

After the fruiting bodies (200 g) of *L. deliciosus* (L. ex Fr.) Gray were soaked with 95% EtOH, the residue was dried and then extracted with boiling water for three times (3 h for each). After the filtrate was concentrated, dialyzed, and centrifuged, the supernatant was added with three volumes of 95% EtOH to precipitate crude oligosaccharides LDGO (oligosaccharides from *L. deliciosus* (L. ex Fr.) Gray, 17.4 g, recovery 8.7%). After Sevag method[5] was used for the deproteination, LDGO (5 g) was subjected to a DEAE–cellulose 52 column (Tris-Hcl, pH 7.0 nmm, 4.5 cm × 50 cm, Cl^-) and eluted stepwise with distilled water, 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0M NaCl. The eluate was monitored by the phenol–sulfuric acid method.[6] The distilled water eluation was concentrated, lyophilized, and purified on a Sephadex G-200 column (2.6 cm × 60 cm). The resulting *L. deliciosus* (L. ex Fr.) Gray oligosaccharide, named LDGO-A, was obtained by the above processes, and the yield rate of LDGO-A was 0.09% (0.180 g) for the starting material.

Measurement of molecular weight of *Lactarius deliciosus* (L. ex Fr.) gray oligosaccharide

High-performance gel permeation chromatography was carried out to measure molecular weight.[7] The column was calibrated with standard T-series dextran (T-500, T-110, T-70, T-40, and T-10). The data were processed with waters gel permeation chromatography Millennium 32 software, Millennium Software Developers, Inc., NY, USA.

Monosaccharide composition analysis of *Lactarius deliciosus* (L. ex Fr.) gray oligosaccharide

The oligosaccharide LDGO-A (5.0 mg) was hydrolyzed with 2M trifluoroacetic acid at 110°C for 6 h on the mechanism of acid-catalyzed hydrolysis.[8] Excess acid was removed by co-distillation with methyl alcohol after the hydrolysis was completed. The hydrolysate 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).[9]

Ultraviolet and infrared spectra analysis

LDGO-A was tested in ultraviolet (UV) from 200 nm to 600 nm. And infrared (IR) analysis of the sample LDGO-A was obtained by grinding a mixture of oligosaccharide with dry KBr and then pressing in a mold. Spectra were run in the 4000–400 cm^{-1} region.[10]

Nuclear magnetic resonance experiment

^{1}H-Nuclear magnetic resonance (^{1}H-NMR) spectra and ^{13}C-NMR spectra were recorded on a varian unity Inova 400/45 (Varian Associates, NY, USA) in D_2O with tetramethylsilane as an internal standard.[11]

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). The animal experiments were conducted according to the "Guidelines for Animal Experimentation" of the NSMC.

Assay of anti-tumor activity *in vivo*

S180 tumor cells (3×10^6) were implanted subcutaneously into the right hind groin of the Kunming strain male mice. Mice were randomly divided into five groups ($n = 6$). One day after inoculation, LDGO-A was dissolved in distilled water and administered intra-peritoneally (i.p.) to the mice at the doses of 10, 20, and 40 mg/kg, respectively. The positive control was given with 0.2 mL mannatide (20 mg/kg) and a 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) × 100, where A and B were the average tumor weights of the negative control and treated groups, respectively.

Histopathology and morphological observations

After treating the mice with LDGO-A as described above, a portion of the tissues were cut into small pieces, fixed in Heidenhain's Suea Fluid ($HgCl_2$ 4.5 g; NaCl: 0.5 g; distilled water: 80.0 mL; formalin: 20.0 mL; acetic acid: 4.0 mL; trichloroacetic acid: 2.0 mL), and then stained with hematoxylin and eosin, examined and photographed under an OLYMPUS microscope (Olympus, Tokyo, Japan).

Preparation of lymphocyte cells

The extirpated spleens were treated in germ-free condition. Single spleen cells suspension were prepared in 0.15 mol^{-1} NaCl/0.02 mol^{-1} sodium phosphate, pH 7.0 phosphate buffered saline containing 0.1% (w/v) bovine serum albumin by forcing spleen fragments through a fine wire mesh. Washed samp LDGO twice in phosphate buffered saline/0.1% (w/v) bovine serum albumin and in low-speed centrifugation, 200 rpm, 5 min. The cells were resuspended to a concentration of 5 × 10^6 cells/mL in Roswell Park Memorial Institute (RPMI1640) completely cultivated liquid (HyClone, USA).

Assay of lymphocyte proliferation

One hundred microliter (5 × 10^6 cells/mL) single spleen cells sample was placed on a 96-well microplate and cultured for 24 h. 20 μL LDGO-A of different concentrations (25, 50, 100, 200 μg/mL) was added to each test well as the experimental group, 20 μL ConA (50 μg/mL) was added as a positive control group, and 20 μL RPMI1640 cultivated liquid as a blank control group. And then 80 μL RPMI1640 cultivated liquid was added to each hole for the total volume of 200 μL for each test. Eight repeated wells were used for each concentration. The cells samp LDGO were incubated in a 5% CO_2-air mixture at 37°C for 68 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cellular viability assay was used for lymphocyte proliferation analysis. Calculation of lymphocyte proliferation was done: Rate of lymphocyte proliferation (%) = ([T − C]/C) ×100, where *T* is optical density value of test well; *C* is that of control well.

Preparation of peritoneal macrophage cells

BALB/c albino mice of 6–8-week-old were injected i.p. with 1 mL of 3% thioglycollate (Sigma Chemical Co., St. Louis, MO, USA). Four days after injection, mice were euthanized, and peritoneal exudate cells were collected by lavage with 5 mL of sterile cold D-Hank's. The exudate cells were collected and cultured in 60 mm dishes with RPMI-1640 (Gibco, USA) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 μg/mL) (RPMI-FBS). After 1 h incubation at 37°C, the cultures were washed twice with RPMI-1640 to remove nonadherent cells and the adherent cells were collected by gently scraped. The viability of macrophages was assessed by trypan blue exclusion.

Assay of macrophage stimulation

Macrophages (1 × 10^6 cells/mL) were plated in a 96-well plate (Corning, NY, USA). The cells were cultured in phenol red-free RPMIFBS medium containing increasing concentrations of LDGO-A from 50 to 400 μg/mL (20 μL per well) as the experimental group, and 20 μL LPS (50 μg/mL) was added as positive control group, respectively. And then 80 μL phenol red-free RPMIFBS cultivated liquid was added to each hole for the total volume of 200 μL for each test. Eight repeated wells were used for each concentration. The cells samp LDGO were incubated in a 5% CO_2-air mixture at 37°C for 4 h. Then added neutral red solution 50 μL to a final concentration of 0.72 mg/L, continue cultured for 30 min. Aspirate the neutral red solution, washed three times and added 200 μL lysis buffer (glacial acetic acid: Ethanol = 1:1), standing overnight until the cell lysis completely, evaluated the absorbance value (OD).

Quantitative real time-polymerase chain reaction detection of related gene expression

The total RNA was extracted using Trizol Reagent (Invitrogen, USA) and reverse-transcribed into cDNA using Oligo (dT) 18 primers (Invitrogen,

Table 1: The result of primer design

Gene name	Gen bank accession numbers	Primer name	Sequences (5′-3′)	Position	Temperature (°C)	Amplicon size
Apc	NM_007462	Apc-F	CGTTGTCCATGAGGACATTC	10425	58.93	108
		Apc-R	AATTATGGAGCGTGTGTGGA	10513	59.00	
Cdkn2b	NM_007670	Cdkn2b-F	CTCCAGCTCGACAAGGAAAT	1147	59.43	123
		Cdkn2b-R	CAATTCATCACTGGGCTTTG	1250	59.12	
Run×3	NM_019732	Run×3-F	GTTTCACGCTCACAATCACC	952	59.14	84
		Run×3-R	GTCCATCCACAGTGACCTTG	1016	58.94	
Mdm2	NM_010786	Mdm2-F	CTAGCTTCTCCCTGAATGCC	1529	59.03	114
		Mdm2-R	TTGCACACGTGAAACATGAC	1623	59.14	

Cdkn2b: Cyclin-dependent kinase inhibitor 2B; APC: Adenomatous polyposis coli; Run×3: Runt-related transcription factor 3; Mdm2: Mouse double minute 2 homolog

USA). The result of primer design is shown in Table 1. Amplification of each target cDNA was performed in the Icycler System (Bio-rad, USA). Polymerase chain reaction (PCR) products were quantified using SYBR Green I. b-actin was used as an endogenous control to normalize levels among samples. The relative expression abundance was calculated by the following formula:

Relative expression abundance = mols of detected mRNA/mols of b-actin mRNA

Statistical analysis

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

RESULTS AND DISCUSSION

Extraction, purity, and composition of oligosaccharides

The crude oligosaccharide, named LDGO, was obtained from the fruiting bodies of *L. deliciosus* (L. ex Fr.) Gray with a yield of 8.7% [Figures 1 and 2]. After fractionation on DEAE-cellulose 52 and Sephadex G-200 column chromatography, 180 mg of LDGO-A was obtained from the distilled water eluate and detected by the phenol–sulfuric acid assay. The homogeneity of the oligosaccharide was elucidated by the following tests. LDGO-A was eluted from gel-filtration chromatography on Sephadex G-200 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 LDGO-A demonstrated the absence of protein and nucleic acid in this oligosaccharide and it had the same optical rotation: $[\alpha]^{20}D - 8.1°$ (c 0.5, water) in different low concentration of ethanol using HK7-SGW-1 automatic optical polarimeter (Shanghai INESA Physico Optical Instrument Co., Ltd., Shanghai, China) at room temperature. Weight-average molecular weight of LDGO-A was around 945 Da [Figure 3]. The two monosaccharides, D-glucose, D-xylose (D-Glu and D-Xyl) were identified using the hydrolysate of LDGO-A by the TLC [Figure 4].

Structure elucidation of *Lactarius deliciosus* (L. ex Fr.) gray oligosaccharide

The intensity of bands around 3416.39 cm^{-1} in the IR spectrum [Figure 5] was due to the hydroxyl stretching vibration of the oligosaccharide, and as expected, they were broad. The bands in the region of 2938.79 cm^{-1} were due to C–H stretching vibration, and the bands in the region of 1648.55 cm^{-1} were due to associated water.[12] The strong absorption bands at 1155.75 cm^{-1}, 1077.61 cm^{-1} in the range of 1200–1000 cm^{-1} in the IR spectrum suggested that the monosaccharide in LC-1 had a pyranose-ring.[13] The strong absorption bands at 1402.73 cm^{-1} were due to C–H bending vibration and the bands in the region of 917.88–707.78 cm^{-1} were due to C–H rocking vibration. Moreover, the characteristic absorptions at 707.78 cm^{-1} indicated α-configurations existing in the oligosaccharide, which was in good agreement with the anomeric proton signals at δ 5.05, δ 5.06 in the ^{1}H-NMR (400 MHz) spectrum [Figure 6]. The signals at

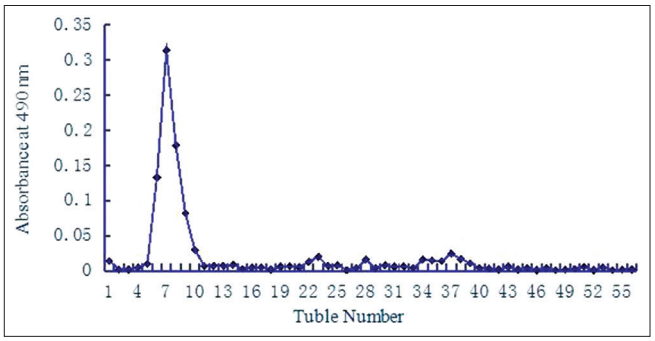

Figure 1: Chromatogram of oligosaccharide from *Lactarius deliciosus* Gray on a DEAE–cellulose 52 column eluted with distilled water and the stepwise gradient of NaCl (0, 0.1, 0.2, 0.3, 0.4, and 0.5 M)

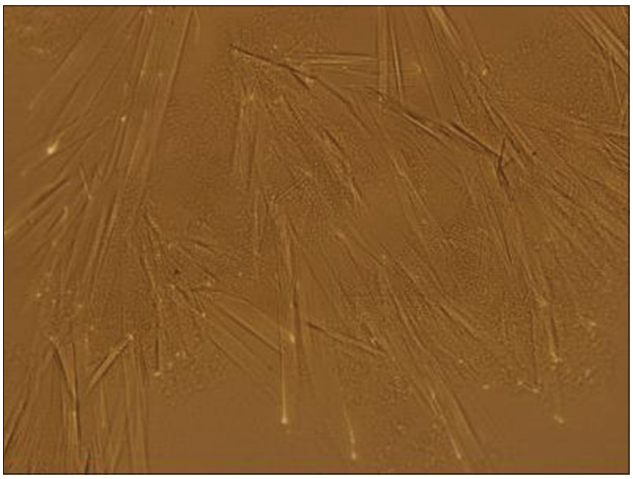

Figure 2: The oligosaccharide from *Lactarius deliciosus* Gray was imaged by scanning electron microscopy (×200)

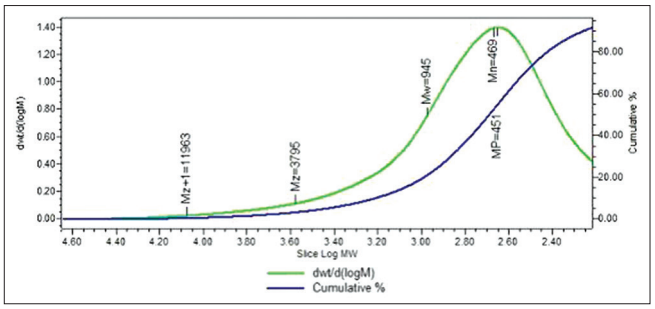

Figure 3: The molecular weight of oligosaccharide from *Lactarius deliciosus* Gray Mw: Weight-average molecular weight; Mn: Number-average molecular weight; Mz: Z-average molecular weight; Mp: Peak molecular weight

δ 3.0–3.8 are the signal peak of remaining proton which mostly formed by a number of signal peaks overlapped. Among them, signals at δ 3.78, 3.73, 3.71 are the signal peak of α-H of methene in xylose and signals at δ 3.59 are the signal peak of β-H of methene in xylose. Signals at δ 3.52, 3.50, 3.44 are the signal peak of other hydrogen in xylose. The signals at δ 3.67, 3.65 are the signal peak of hydroxy-methyl in glucose and signals at δ 3.77 indicate the hydrogen signal peak in C-3 of glucose. δ 4.676 was the hydrogen signal of water. The hydrogen spectrum data were in good agreement with monosaccharide analysis data. According to the literature, the resonances in the region of 95–100 ppm in the ^{13}CNMR (400 MHz) spectrum of LC-1 were attributed to the anomeric carbon atoms of D-Glu and D-Xyl[13] [Figure 7].

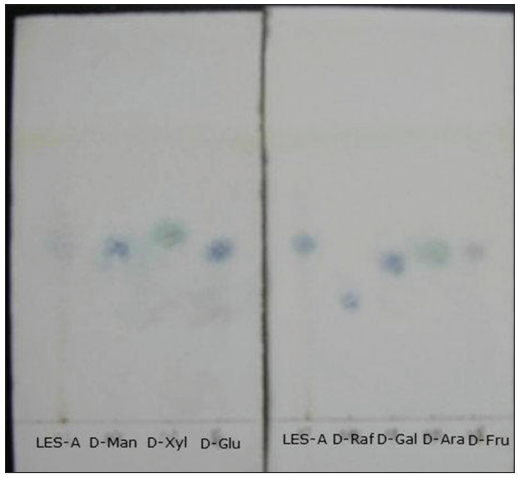

Figure 4: Thin layer chromatography of oligosaccharide from *Lactarius deliciosus* Gray hydrolyzed by trifluoroacetic acid (sample) and standard monosaccharide (LES: Samples, D-Man: D-Mannose, D-Xyl: D-Xylose, D-Glu: D-Glucose, D-Raf: D-Raffinose Pentahydrate, D-Gal: D-Galactose, D-Ara: D-Arabinose, D-Fru: D-Fructose)

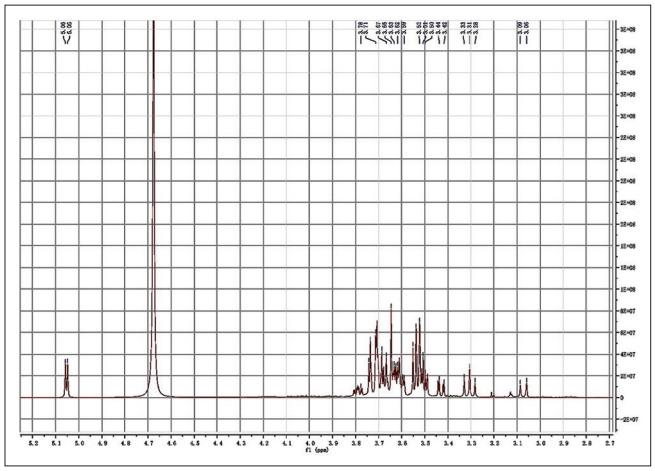

Figure 6: ^{1}H-nuclear magnetic resonance spectra of oligosaccharide from *Lactarius deliciosus* Gray

Anti-tumor activity of *Lactarius deliciosus* (L. ex Fr.) gray oligosaccharide *in vivo*

To detect the anti-tumor activity of LDGO-A *in vivo*, we used the mice transplanted S180 to evaluate the effects, and the results are summarized in Table 2. The weight and the histological preparations of the vital organ in each female rat in the control group were compared to that in the treated group to measure the effect of the drug. LDGO-A could inhibit the growth of the tumors ($P < 0.01$) in a dose-dependent manner. The inhibitory rate in mice treated with 40 mg/kg LDGO-A was 40.02%, being the highest in the three doses. Furthermore, during the experiments, the appetite, activity, and coat luster of each animal in LDGO-A groups were better than the mice treated with mannatide. Histology of immune organs of liver, spleen, and thymus shows that tissues arranged more regular and firmer, and the tumor tissue is more loosely in LDGO-A group than those in the control group. But there is no obvious damage to other organs, such as heart [Figure 8]. The results also showed little change in average liver weight in test groups which indicating that LDGO-A did not cause serious liver damage. On the 14th day, the average tumor weight of negative control mice was 3.47 g, whereas the average tumor weight of mice in LDGO-A group at dose of 40 mg/kg

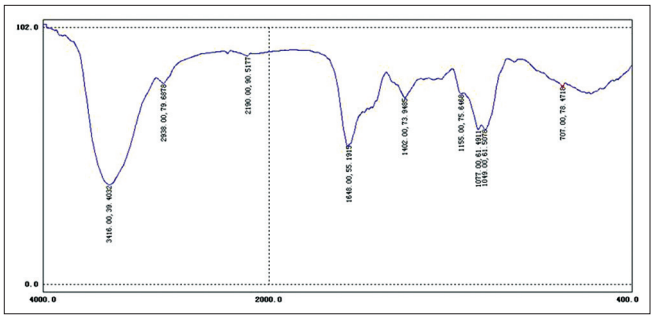

Figure 5: Fourier transform infrared spectra of oligosaccharide from *Lactarius deliciosus* Gray

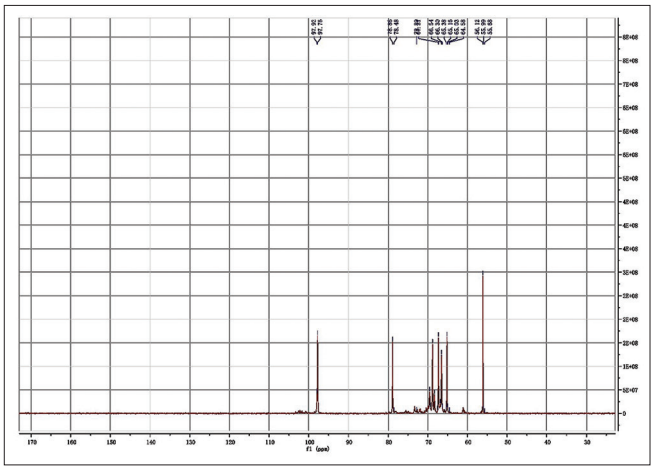

Figure 7: ^{13}C-nuclear magnetic resonance spectra of oligosaccharide from *Lactarius deliciosus* Gray

was 2.082 g, and also significantly reduced in doses of 10 mg/kg and 20 mg/kg, which were 3.180 g and 2.873 g, respectively. It is noteworthy that the average weights of the spleens and thymus in test groups were significantly greater in doses of 20 mg/kg than that in the mannatide mice, and even that of the negative control mice, indicating that LDGO-A could increase the weights of immune organs in moderate doses [Table 2]. These results suggested that activating immune responses in the host might be one of the mechanisms of anti-tumor activity of LDGO-A, as many anti-tumor oligosaccharide found in the world.

Immune activity of *Lactarius deliciosus* (L. ex Fr.) gray oligosaccharide *in vitro*

The anti-tumor activity of the oligosaccharide was usually believed to be a consequence of the stimulation of the cell-mediated immune response. The present study showed the immune activity of the LDGO-A by assay of lymphocyte proliferation and macrophage stimulation. The proliferation of splenocytes is an indicator of immunoactivation. The purified oligosaccharide LDGO-A was able to proliferate splenocytes as shown in Figure 9. LDGO-A can significantly promote the proliferation of spleen cells at the concentration of 50 μg/mL and 100 μg/mL dose ($P < 0.05$), while it can highly significantly promote the proliferation of spleen cells in the concentration of 200–400 μg/mL dose range compared with the control group ($P < 0.01$).

Oligosaccharide is good stimulators of macrophage owing to the presence of various receptors on the macrophage membrane. In this study, LDGO-A also can significantly promote the phagocytosis of mouse peritoneal macrophages at the concentration 50 μg/mL

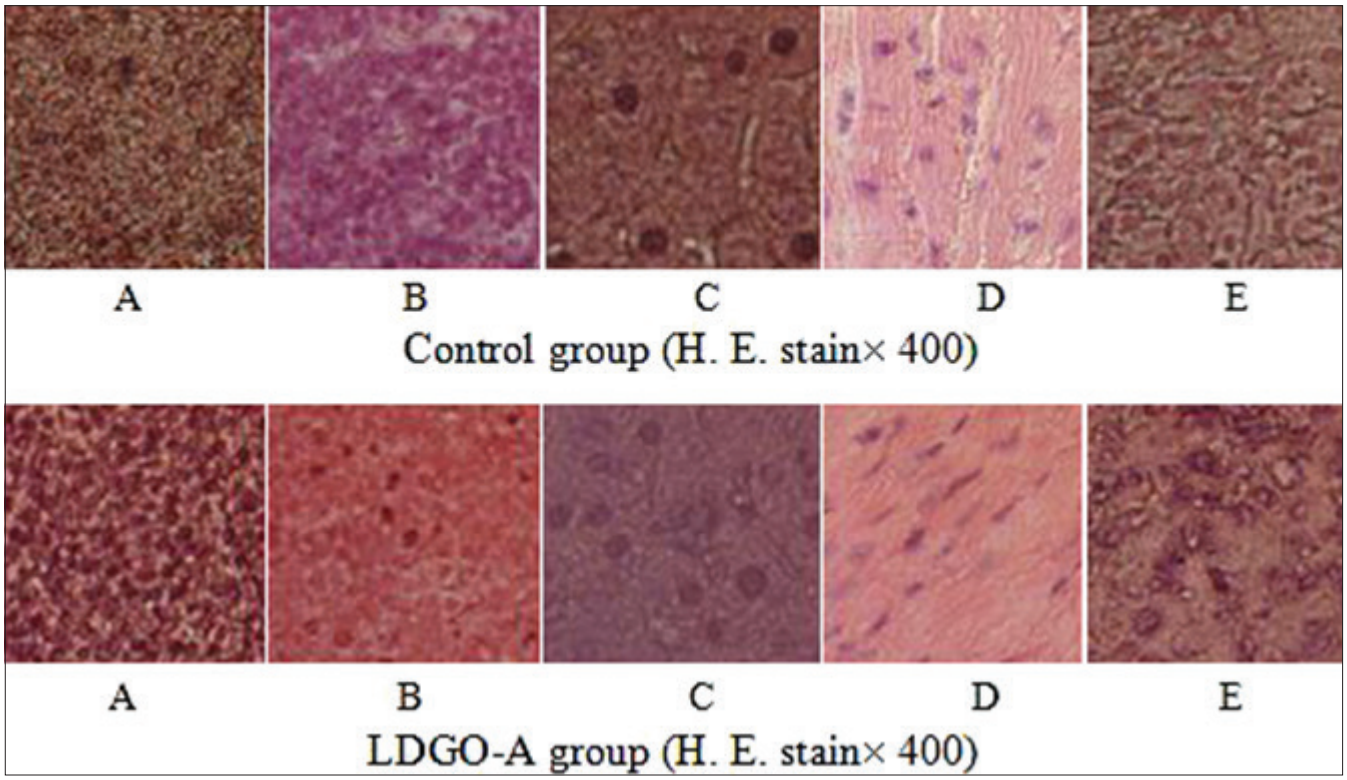

Figure 8: Histological preparations of the spleen (a) thymus (b) liver (c) heart (d) tumor (e) in oligosaccharide from *Lactarius deliciosus* Gray group (concentration of 40 mg/kg) compared with control group

Table 2: Anti-tumor activities of LDGO-A on S180 tumor (mean ± SD, *n*=6)

Group	Spleen index (mg/g)	Liver index (mg/g)	Thymus index (mg/g)	Average tumor weight (g)	Inhibitory rate of tumor (%)
N	4.604±2.108	57.302±2.334	1.249±0.607	3.470±0.311	-
S1	6.619±2.414	55.812±2.910	2.174±0.293	3.180±0.610	8.36*
S2	7.709±3.241	62.156±3.399	4.078±3.431	2.873±0.406	17.20**
S3	5.043±0.786	58.727±4.676	1.319±0.522	2.082±0.702	40.02**
Man	6.304±0.501	58.471±0.219	2.268±0.720	1.672±0.811	51.82**

N: Negative control group; S1, S2, S3: LDGO-A groups of 10 mg/kg, 20 mg/kg, 40 mg/kg, respectively; Man: positive control group of Mannatide. Significant differences from negative control group and positive control group were evaluated using Student's *t* test: *P<0.05, **P<0.01. SD: Standard deviation, LDGO-A: *Lactarius deliciosus* (*L. ex Fr.*) Gray oligosaccharide

dose ($P < 0.05$), and can very significantly promote the phagocytosis of mouse peritoneal macrophages within the dose range of 100–400 μg/mL compared with the control group ($P < 0.01$) [Figure 10].

The promote capacity of both lymphocyte and macrophage cells and the concentration of the LDGO-A was positively correlated.

Lactarius deliciosus (L. ex Fr.) Gray oligosaccharide effected the expression of some housekeeping genes mRNA in S180 tumor

Housekeeping genes are typically constitutive genes that are required for the maintenance of basic cellular functions and are expressed in all cells under normal or pathophysiological conditions. The proteins produced by a housekeeping gene vary, but are involved in some way in processes necessary to the survival of a cell. Some may be involved in sustaining cell function, while others may be involved in cell maintenance. These genes tend to produce proteins at steady rates, and errors in their expression will lead to cell death. Like a literal housekeeper, they keep the vital systems of a cell running smoothly so that it can continue to function, and they also contribute to the overall function of the larger organism.

Adenomatous polyposis coli (*APC*) known as deleted in polyposis 2.5 (DP2.5) is a protein that in humans is encoded by the *APC* gene. *APC* is classified as a tumor suppressor gene. Tumor suppressor genes prevent the uncontrolled growth of cells that may result in cancerous tumors. The protein made by the *APC* gene plays a critical role in several cellular processes that determine whether a cell may develop into a tumor. The *APC* protein helps control how often a cell divides, how it attaches to other cells within a tissue, or whether a cell moves within or away from a tissue. This protein also helps ensure that the chromosome number in cells produced through cell division is correct.

Cyclin-dependent kinase inhibitor 2B (*Cdkn2b*) gene lies adjacent to the tumor suppressor gene *CDKN2A* in a region that is frequently mutated and deleted in a wide variety of tumors. This gene encodes a *Cdkn2b*, which forms a complex with CDK4 or CDK6, and prevents the activation of the CDK kinases, thus the encoded protein functions as a cell growth regulator that controls cell cycle G1 progression. The expression of this gene was found to be dramatically induced by transforming growth factor beta (TGF beta), which suggested its role in the TGF beta induced growth inhibition.

Runt-related transcription factor 3 (*Runx3*) gene encodes a member of the runt domain-containing family of transcription factors. It can interact with other transcription factors. It functions as a tumor suppressor, and the gene is frequently deleted or transcriptionally silenced in cancer.

Mouse double minute 2 homolog (*Mdm2*) also known as E3 ubiquitin-protein ligase *Mdm2* is a protein that in humans is encoded by the *Mdm2* gene. *Mdm2* is an important negative regulator of the p53 tumor suppressor. *Mdm2* protein functions both as an E3 ubiquitin ligase and an inhibitor of p53 transcriptional activation. There are several known mechanisms for regulation of *Mdm2*. One of these mechanisms is phosphorylation of the *Mdm2* protein. *Mdm2* is phosphorylated at multiple sites in cells. Following DNA damage, phosphorylation of *Mdm2* leads to changes in protein function and stabilization of p53. In addition, phosphorylation at certain residues within the central acidic domain of *Mdm2* may stimulate its ability to target p53 for degradation. Quantitative RT-PCR results showed a significant reduction in the level of *Apc*, *Cdkn2b*, and *Runx3* mRNA in LDGO-A-treated and mannatide treated S180 tumor compared to those untreated. Especially, the expression level of *Runx3* mRNA in the mannatide group increased 38.85, but in LDGO-A-treated group downed-263.20 [Table 3]. The quantitative RT-PCR results also showed there were no changes in *Mdm2* gene. The

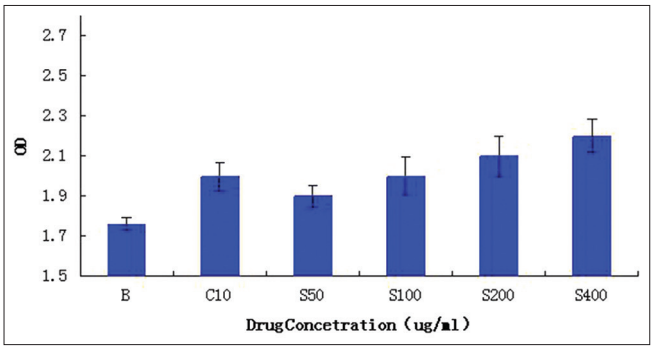

Figure 9: Effect of oligosaccharide from *Lactarius deliciosus* Gray on the proliferation of mouse spleen cell. B: Blank control group; C: ConA group; S: Oligosaccharide from *Lactarius deliciosus* Gray group (mean ± standard deviation, $n = 6$)

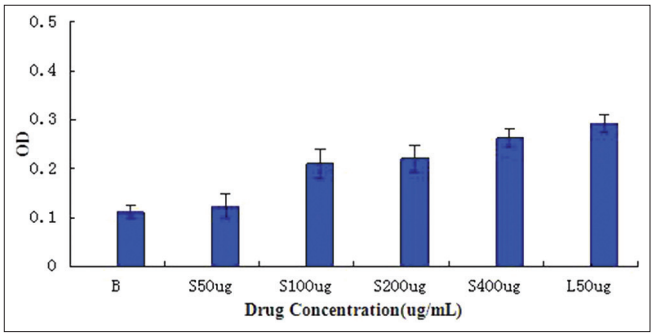

Figure 10: Effect of oligosaccharide from *Lactarius deliciosus* Gray on the proliferation of mouse macrophage cell *in vitro*. B: Blank control group; L: LPS group; S: Oligosaccharide from *Lactarius deliciosus* Gray group (mean ± standard deviation, $n = 6$)

Table 3: Expression of housekeeping genes mRNA in S180 tumor effected by LDGO-A

Gene	Model group	Mannatide group	LDGO-A group
Apc	−10.70	−11.08	−14.03
Cdkn2b	−15.78	−20.25	−42.22
Run×3	-	38.85	−263.20
Mdm2	4.89	−2.89	-

Cdkn2b: Cyclin-dependent kinase inhibitor 2B; APC: Adenomatous polyposis coli; Run×3: Runt-related transcription factor 3; Mdm2: Mouse double minute 2 homolog; LDGO-A: *Lactarius deliciosus* (L. ex Fr.) Gray oligosaccharide

results indicated that the anticancer activity of LDGO-A may not involve the inhibition of p53 transcriptional activation. Further research is ongoing to determine the bioactive principle (s) of LDGO-A responsible for its anticancer activity. Accordingly, the LDGO-A might serve as an effective healthcare food and source of natural anti-tumor compounds.

CONCLUSIONS

Our results indicated that LDGO-A was composed of D-Glu and D-Xyl, and the average molecular sizes was approximately 945 Da. The inhibitory rate in mice treated with 40 mg/kg LDGO-A can reach 40.02%, being the highest in the three doses, which may be comparable to mannatide. Histology of immune organs shows that the tissues arranged more regular and firmer, but the tumor tissue arranged looser in LDGO-A group than those in the control group. Meanwhile, there is no obvious damage to other organs, such as heart. LDGO-A can also significantly promote the lymphocyte and macrophage cells in the dose range of 100–400 μg/mL *in vitro* and effect the expression of some housekeeping genes mRNA in S180 tumor. Accordingly, the LDGO-A might serve as an effective healthcare food and source of natural anti-tumor compounds.

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REFERENCES

1. Bode L. Human milk oligosaccharides: Prebiotics and beyond. Nutr Rev 2009;67 Suppl 2:S183-91.
2. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, *et al*. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 2010;107:14691-6.
3. Macfarlane GT, Steed H, Macfarlane S. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. J Appl Microbiol 2008;104:305-44.
4. Ayer WA, Trifonov LS. Aromatic compounds from liquid cultures of *Lactarius deliciosus*. J Nat Prod 1994;57:839-41.
5. Bertozzi CR, Kiessling LL. Chemical glycobiology. Science 2001;291:2357-64.
6. Dubois M, Gillis KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350-6.
7. Yamamoto Y, Nunome T, Yamauchi R, Kato K, Sone Y. Structure of an exocellular polysaccharide of *Lactobacillus helveticus* TN-4, a spontaneous mutant strain of *Lactobacillus helveticus* TY1-2. Carbohydr Res 1995;275:319-32.
8. Yu RM, Yin Y, Yang W, Ma WL, Yang L, Chen XJ, *et al*. Structural elucidation and biological activity of a novel polysaccharide by alkaline extraction from cultured *Cordyceps militaris*. Carbohydr Polym 2009;75:166-71.
9. Partridge SM. Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. Nature 1949;164:443.
10. Auddy B, Ferreira M, Blasina F, Lafon L, Arredondo F, Dajas F, *et al*. Screening of antioxidant activity of three Indian medicinal plants, traditionally used for the management of neurodegenerative diseases. J Ethnopharmacol 2003;84:131-8.
11. Cao W, Li XQ, Liu L, Wang M, Fan HT, Li C, *et al*. Structural analysis of water-soluble glucans from the root of *Angelica sinensis* (Oliv.) diels. Carbohydr Res 2006;341:1870-7.
12. Barker SA, Bourne EJ, Stacey M, Whiffen DH. Infra-red spectra of carbohydrates part I. Some derivatives of D-glucopyranose. Chem Commun 1954;171-6.
13. Kim YT, Kim EH, Cheong C, Williams DL, Kim CW, Lim ST. Structural characterization of beta-D-(1−>3, 1−>6)-linked glucans using NMR spectroscopy. Carbohydr Res 2000;328:331-41.

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