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ORIGINAL ARTICLE

Structure Identification and Biological Activities of a New Polysaccharide Isolated from *Lyophyllum decastes* (Fr.) Sing

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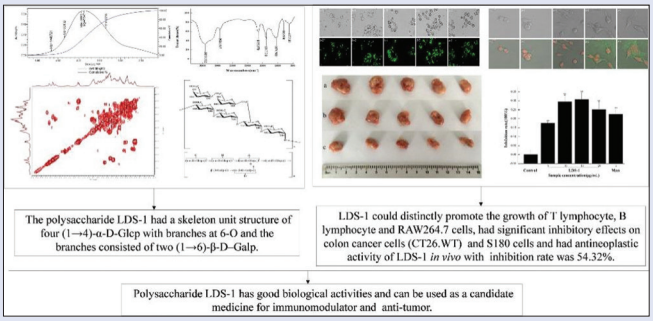
ABSTRACT

Background: Polysaccharide biology, whose core is the structure, function, and edible value of polysaccharides, is regarded as the last great scientific frontier outside the field of protein and nucleic acid research. **Materials and Methods:** In this study, highperformance gelpermeation chromatography, high-performance liquid chromatography, gas chromatography–mass spectrometry, and nuclear magnetic resonance analysis were performed to identify the structure of a new polysaccharide isolated from *Lyophyllum decastes* (Fr.) Sing (LDS-1). The anticancer and immunomodulatory ability of the polysaccharides (LDS-1) was also investigated. **Results:** The results showed that the average molecular weight of LDS-1 was 8681 Da, and the ratio between glucose and galactose was 2:1. Structural analysis revealed four (1 →4)-α-D-Glcp moieties with branches at 6-*O* position, and the branches consisted of two (1 →6)-β-D–Galp moieties. Our results showed that LDS-1 exhibits immunological activity. At a concentration ranging from 2.5 to 25 μg/mL, LDS-1 could distinctly promote the growth of T lymphocyte, B lymphocyte, and RAW264.7 cells; increase the secretion of IgA, IgD, IgE, IgG, IgM, tumor necrosis factor (TNF)-α, TNF-β, and interleukin-2, and enhance the phagocytotic activity of RAW264.7 cells to phagocytize fluorescent microspheres, when compared with the blank control group ($P < 0.01$). Furthermore, under *in vitro* conditions, LDS-1 (2.5-20 μg/mL) exhibited significant antineoplastic activity ($P < 0.01$) against colon cancer cells (CT26.WT) and S180 cells when compared with the blank control group. However, the inhibition effect was different. The research for antineoplastic activity of LDS-1 *in vivo* indicated that the tumor inhibition rate was 54.32%. **Conclusion:** In conclusion, the polysaccharides of LDS-1 exhibited good biological activities, which shows that it can be used as an immunomodulator and an antitumor agent.

Key words: Biological activity, *Lyophyllum decastes* (Fr.) Sing, polysaccharides, structure identification

SUMMARY

- A new polysaccharide (LDS-1) was purified and identified from *Lyophyllum decastes* (Fr.) Sing for the first time
- The polysaccharide (LDS-1) had a skeleton unit structure of four (1 →4)-α-D-Glcp with branches at 6-*O* and the branches consisted of two (1 →6)-β-D–Galp
- LDS-1 could promote the growth of immune cells, promote the secretion of immune factors, and promote phagocytosis of macrophage
- LDS-1 could inhibit the growth of colon cancer cells and S180 cells under *in vitro* conditions and inhibit the growth of S180 tumor under *in vivo* conditions.



Abbreviations used: LDS-1: The polysaccharides from *Lyophyllum decastes* (Fr.) Sing; IR: Infrared spectrum; FTIR: Fourier Transform infrared spectroscopy; HPGPC: Highperformance gelpermeation chromatography; HPLC: Highperformance liquid chromatography; GCMS: Gas chromatography–mass spectrometry; ^{1}H NMR: ^{1}H nuclear magnetic resonance spectroscopy; ^{13}C NMR: ^{13}C nuclear magnetic resonance spectroscopy; 2D NMR: Twodimensional nuclear; DEAE-52 cellulose column: Diethylaminoethyl Cellulose 52 cellulose column; ^{1}H-^{1}H COSY: Nucleus chemical-shift correlation spectroscopy; HMQC: Heteronuclear multiple quantum correlation; HMBC: Heteronuclear multiple bond correlation.

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INTRODUCTION

Lyophyllum decastes (Fr.) Sing is an edible and medicinal mushroom. It belongs to the family *Tricholomataceae*, and in western countries, it is also called "fried chicken mushroom."[1] Ukawa *et al.* isolated 11 different polysaccharides by heat extraction method, among which IV-1 (305,000 Da) is (1 →3)-β-D-glucan, IV-2 (130,000 Da) is (1 → 3,1 → 6)-β-D-glucan, and IV-3 (14,000 Da) is (1 → 6)-β-D-glucan. These three polysaccharides exhibited antitumor effect against S180 tumor cells through host-mediated immune response.[2] They also found that the extract of *L. decastes* exhibited

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antiallergic effect by regulating the secretion of IgE and Th2.[3] Miura *et al*. established the animal model of Type 2 diabetes mellitus and determined the regulation of blood glucose after administering the extract of *L. decastes*. Their results show that the compounds in the extract have antidiabetes effect.[4] The extract of *L. decastes* also has anti-inflammatory, anticancer, antiradiation, and other effects, which has development and application value.[5-10] So far, a detailed chemical structure and mechanism of biological activity of polysaccharides from *L. decastes* have not been reported.

Therefore, in this study, we purified the polysaccharides from *L. decastes* by hot water extraction, sevage method, and by DEAE-52 cellulose chromatography. High-performance gel permeation chromatography (HPGPC), high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and gas chromatography–mass spectroscopy (GC-MS) methods were used to identify the polysaccharide structure. The immunological and antineoplastic activities of the polysaccharides were further investigated, which provided some theoretical support for exploring the mechanism of its immunological and antineoplastic activities.

MATERIALS AND METHODS

Materials

The fruiting bodies of *L. decastes* were collected from Xiaojin County, Aba Tibetan, and Qiang Autonomous Prefecture, Sichuan Province, China. DEAE-52 Cellulose Column was purchased from Shengxing Biotechnology (Nanjing, China) Co., Ltd. Anhydrous sodium sulfate and potassium bromide were purchased from Sangon Biotech (Shanghai, China) Co., Ltd. Trifluoroacetic acid, heavy water, acetonitrile, methanol, iodomethane, chloroform, and anhydrous pyridine were purchased from Maoyang Chemical Reagent (Chongqing, China) Co., Ltd. The experimental reagent is all analytical reagent.

Isolation and extraction of polysaccharides from *Lyophyllum decastes*

The fruiting bodies of *L. decastes* were accurately weighed (600 g). After drying and crushing, the powder was boiled for 3 h at 100°C. After centrifugation for 15 min, the supernatant was concentrated to 200 mL. Then, we added anhydrous ethanol (thrice the volume of the concentrate) and stirred which yielded flocculent precipitate (the crude polysaccharide). Then, the proteins in the concentrate were removed by sevage method to yield crude polysaccharide. The crude polysaccharide was dissolved in 50 mL distilled water, which was then loaded onto DEAE-52 cellulose column. The constituents were eluted using 1500 mL of distilled water at a flow rate of 5 mL/min to obtain the eluent. Then, small molecular compounds were removed from the eluent using a dialysis bag and were concentrated to 20 mL and then lyophilized. Finally, the purified polysaccharide of *L. decastes* was obtained and labeled as LDS-1.

Determination of molecular weight of LDS-1 by high-performance gelpermeation chromatography

The molecular weight of LDS-1 was determined by HPGPC. The data were analyzed by GPC software. The standard dextran with known molecular weight was used as the molecular weight reference substance of polysaccharide.

Fourier transform infrared spectroscopy analysis of LDS-1

Briefly, 2 mg LDS-1 and dry potassium bromide powder were thoroughly mixed and ground in an agate mortar. The tablets were pressed by a tablet press and scanned by Fourier transform infrared spectroscopy (FTIR) in the wavenumber range of 4000-400 cm^{-1}.

Analysis of monosaccharide composition of LDS-1

The monosaccharide composition of LDS-1 was analyzed by HPLC. The monosaccharide components of polysaccharides can be determined by comparing the peak time of polysaccharides with the peak time of standard products, and the ratio of monosaccharide components can be determined by the ratio of peak area. The chromatographic conditions were as follows: 4.6 mm × 250 mm, 5 μ column; column temperature: 25°C; mobile phase: 75% acetonitrile; flow rate: 1.4 mL/min; RID temperature: 35°C; injection volume: 10 mL.

Nuclear magnetic resonance analysis

Polysaccharide samples (10 mg for ^{1}H NMR and 50 mg for ^{13}C NMR) were, respectively, taken. After dissolving with 0.5 mL deuterium oxide, the spectra of ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HMQC, and HMBC were measured on NMR spectrometer. We used tetramethylsilane as an internal standard.

Analysis of LDS-1 structure by gas chromatography-mass spectroscopy

Briefly, 25 mg polysaccharide sample was weighed, and 2 mL of anhydrous dimethyl sulfoxide was added to dissolve it completely. Then, 400 mg of predried sodium hydroxide powder was added quickly, dissolved under ultrasound for 10 min, and stirred for 1 h at room temperature. Then, 1.5 mL iodomethane was added to initiate the reaction. Finally, 5 mL distilled water was added to terminate the reaction. The product was extracted with chloroform. Methylated polysaccharide was obtained after drying, which was hydrolyzed by TFA and washed thrice to obtain the products of complete acid hydrolysis. Anhydrous pyridine (2 mL), hexamethyl-disilazane (2 mL), and chlorotrimethylsilane (1 mL) were, respectively, added to the above samples. The reaction time was 20 min at 50°C. After centrifugation (10,000 rpm; 20 min), the upper solution was used for GC-MS analysis. Following chromatographic conditions were applied: DB-5MS quartz capillary column: 30 m × 0.25 nm × 0.25 μm; initial temperature of the column was 80°C and held for 3 min; and the temperature was gradually increased at 10°C/min which lasted for 10 min after reaching 200°C.

Pharmacological evaluation of stimulation of B-, T-, and RAW264.7 cells and inhibition of CT26.WT cells

The pharmacological evaluation of stimulation of B-, T-, and RAW264.7 cells and inhibition of CT26.WT cells was performed using CCK-8 assay. Briefly, cells were cultured in RPMI-1640 medium. When the cells were in the logarithmic growth period, the medium was added to dilute cells. Cells were culture in 96-well plates at a density of 1×10^5 cells/mL (100 μL/well) and incubated in an incubator for 24 h at 37°C with 5% CO_2. For stimulation of B-, T-, and RAW264.7 cells, we used 2.5, 5, 10, 15, 20, and 25 μg/mL LDS-1 prepared in cell culture medium, whereas 5, 10, and 15 μg/mL were used for the inhibition of CT26.WT cells). Then, lipopolysaccharide (LPS) and mannatide were used as a positive control for immune cells, and the cell culture medium without LDS-1 was used as the blank control. After incubation at 37°C for 24 h, 5 μL of CCK-8 reagent was added to each well and the cells were cultured for an additional 2 h. A microplate reader was used to detect the optical density (OD) at 450 nm. The formula used to calculate cell viability (%) was as follows: ([Ac–As]/[Ac – Ab]) × 100%, where Ac is the absorbance of the control group, Ab is the absorbance of the blank group, and As is the absorbance of the experimental groups. The cells were observed under inverted microscope and photographed.

Pharmacological evaluation for macrophage phagocytic activity

Briefly, RAW264.7 cells (1×10^5 cells/mL) were seeded into 96-well plates (100 μL/well) and incubated for 24 h. Subsequently, 100 μL of the cell culture medium (blank control), LPS (final concentration 5 μg/mL, positive control), and LDS-1 solution (5, 10, and 15 μg/mL) was added to the 96-well plates. After incubation at 37°C for 24 h, 100 μL of fluorescent microsphere diluent (preincubated at 37°C for 30 min, the concentration was 1×10^7/mL) was added. After discarding the solution, each well was washed thrice with phosphate-buffered saline. Then, 200 μL cell lysis solution, which was prepared with anhydrous ethanol and glacial acetic acid in the same proportion, was added. After cell lysis for 15 min at 37°C, the OD value was measured by Microplate Reader at 505 nm. The cells were observed under inverted microscope and photographed.

Evaluating cytokine levels

To understand the alterations in the level of cytokines, ELISA kits were used according to the manufacturers' protocols.

Antitumor activity of LDS-1 under *in vitro* conditions

The mouse sarcoma S180 cell line was purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Kunming strain female mice obtained from the Institute of Biochemistry and Molecular Immunology of North Sichuan Medical College (Nanchong, China) were inoculated with 100 μL S180 tumor cells at a concentration of 3×10^6 CFU/mL under the left axillary skin. The Kunming strain mice (aged around 4–6 weeks) weighed 25.0 ± 1.0 g and were divided into three groups of 5 mice in each group. After 7 days, each mouse was treated with 20 mg/kg RF1 (RF1 group) or 20 mg/kg mannatide (positive control group) for 7 consecutive days. The mice were sacrificed and the tumor inhibition rate was calculated as follows: tumor inhibition rate (%) = ([AB]/A) × 100, with A and B being the average tumor weight of the blank control group and treated group, respectively.

Transcriptome sequencing

The collected tumor tissues of control group, mannatide group, and LDS-1 group were used to carry out transcriptome assay. Briefly, the tumor tissues were quickly frozen under liquid nitrogen and were sent to Novogene Bioinformatics Technology Co. Ltd. (Beijing, China). The remaining tumor tissues were stored at −80°C. After the samples were quantified, the library was constructed and checked, and subsequently sequenced using an Illumina Hiseq platform. Genes with an adjusted $P < 0.05$ identified by DESeq2 were classified as differentially expressed. Subsequently, the clusterProfiler R package was used for Gene Ontology analysis and Kyoto Encyclopedia of Genes and Genomes pathway analysis. Differentially expressed genes were analyzed using the edge R program (version 3.11) (bioconductor.org, Cambridge, US).

Statistical methods

All data in this study were analyzed by the way of standard deviation. Methods of data processing were one-way analysis of variance and Student's *t*-test. Moreover, $P < 0.05$ represented a significant difference between the data, and $P < 0.01$ represented a very significant difference between the data.

RESULTS AND DISCUSSION

Molecular weight analysis of LDS-1

As can be seen from Figure 1a, the HPGPC elution curve of LDS-1 has a symmetrical peak, and the weight average molecular weight (Mw) of LDS-1 was 8681 Da, the number average molecular weight (Mn) was 2214 Da, the peak molecular weight (Mp) is 7547 Da, and the polydispersity index (D = Mw/Mn) was 3.92.

Fourier transform infrared spectroscopy analysis of LDS-1

Infrared spectroscopy is a primary method for structural analysis of polysaccharides, which can be used to determine the type and configuration of polysaccharides. The structure of LDS-1 was characterized by FTIR. Figure 1b shows the results. A broad signal peak at 3439.552 cm^{-1} is the hydroxyl stretching vibration of polysaccharides. A sharp signal peak at 2930.597 cm^{-1} is the C-H stretching vibration peak of polysaccharides. The signal peak at 1397.761 cm^{-1} is the C-H angular vibration absorption peak, and the signal peak at 1655.970 cm^{-1} is the absorption peak of bound water in carbohydrate molecule; the signal peak at 1070.900 cm^{-1} is the absorption peak of pyran ring in the range of 1200 cm^{-1}–1000 cm^{-1}, indicating that LDS-1 has pyranose-type monosaccharide residue. In addition, there was no obvious signal peak at 1740 cm^{-1}, indicating that LDS-1 did not contain glucuronic acid and was a neutral polysaccharide.

Analysis of monosaccharide composition of LDS-1

The analysis of monosaccharide composition is a very important step in the study of structure and activity of polysaccharides. In this experiment, LDS-1 was hydrolyzed and its monosaccharide composition was analyzed by HPLC. The retention time of glucose, galactose, and mannose was 14.214 min, 16.704 min, and 18.614 min, respectively. The retention time of monosaccharide of LDS-1 was 14.201 min and 16.693 min, which indicated that LDS-1 was mainly composed of galactose and glucose, and its ratio was about 1:2 according to internal standard method.

Analysis of the gas chromatography-mass spectroscopy data

Methylation analysis is an indispensable step in analyzing the structure of polysaccharides. It can obtain the binding sites of monosaccharide residues and the ratio between various types of bonds in polysaccharides. Table 1 shows the results of methylation analysis. There were three peaks in GC-MS data of LDS-1, which corresponded to 2,3,6-tri-*O*-methyl-1,4-bis-*O*-trimethylsilyl-Glcp; 2,3-di-*O*-methyl-1,4,6-tris-*O*-trimethylsilyl-Glcp; and 3,4-di-*O*-methyl-1,2,6-tris-*O*-trimethylsilyl-Galp. This suggests that galactopyranose is 1,2,6-bonded, whereas glucopyranose is 1,4,6-and 1,4-bonded. Considering that OH on C2 and C3 is not easy to be methylated due to the steric hindrance, which results in incomplete methylation, we concluded that LDS-1 is composed of 1,4-Glcp; 1,4,6-Glcp; and 1,6-Galp. The molar ratio of galactose to glucose is 1:2, which was calculated from the peak area of monosaccharide residue. The result is basically consistent with that of HPLC. In conclusion, the primary repetitive structural unit of LDS-1 is composed of (1 → 4)-α-D-glucopyranose as the main chain and (1 → 6)-β-D-galactopyranose as the side chain, and the 1-*O* of the branched (1 → 6)-β-D-galactopyranose residues are linked to a 6-α-D-glucopyranose.

Analysis of the nuclear magnetic resonance spectra

NMR is one of the most accurate and efficient methods to analyze the structure of polysaccharides. Figure 2c is the hydrogen spectrum of LDS-1. In ^{1}H-NMR, there are four anomeric proton signals, namely δ 5.03, δ 4.96, δ 4.91, and δ 4.40, which indicates that one repetitive unit of LDS-1 contains four types of anomeric protons. Among them, δ 5.03, δ

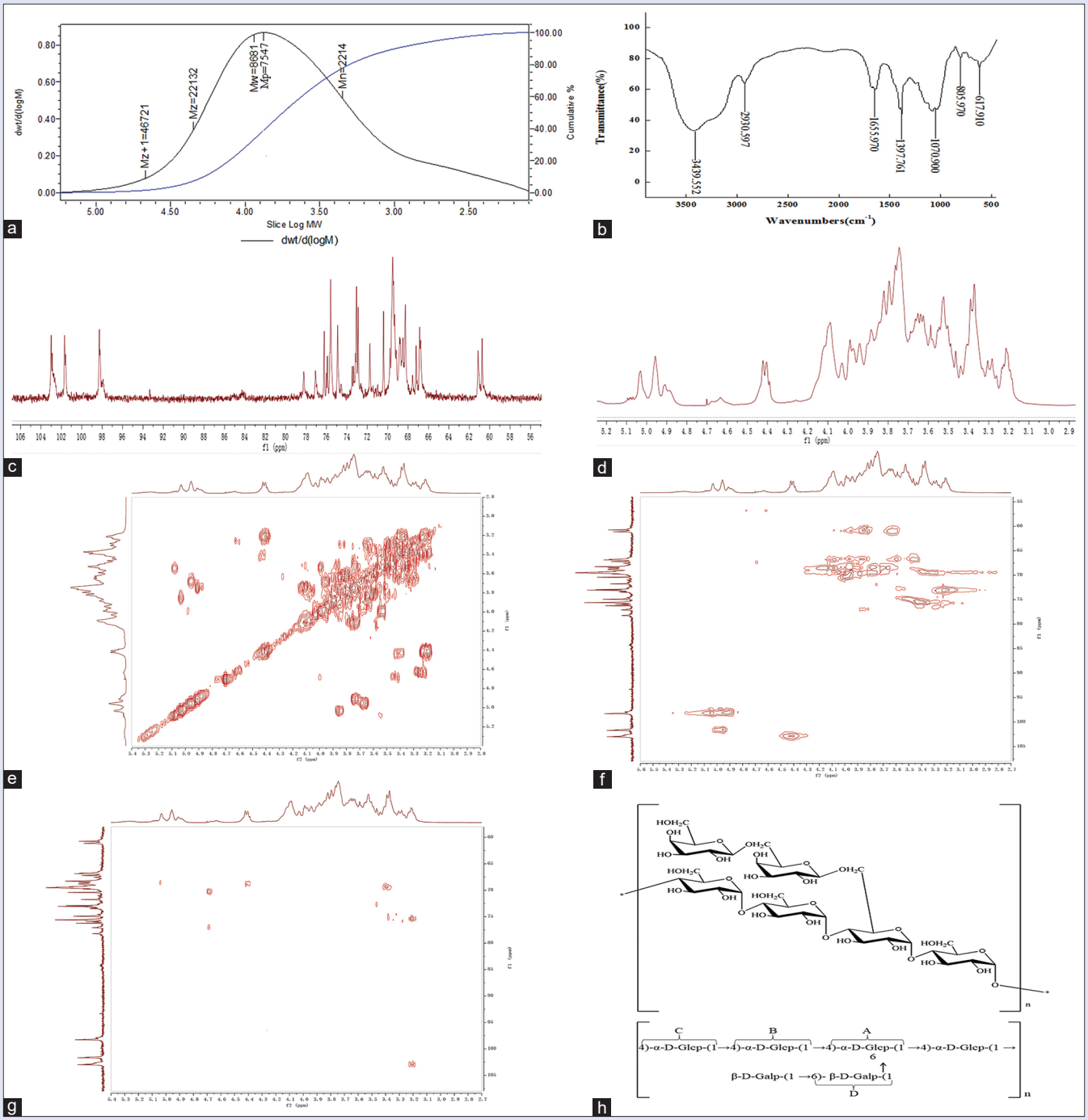

Figure 1: Characterization of the polysaccharide (LDS-1). (a) The molecular weight of LDS-1. (b) Fourier transform infrared spectra of LDS-1.[1]H nuclear magnetic resonance spectra of BRS-X. (d) The [13]C nuclear magnetic resonance spectra of BRS-X. (e) [1]H-[1]H COSY spectrum of BRS-X. (f) HMQC spectrum of BRS-X. (g) HMBC spectrum of BRS-X. (h) Predicted chemical structure of LDS-1

Table 1: Gas chromatography mass spectroscop results of methylation analysis of LDS-1

Methylated sugar	Linkage	m/z
2,3,6-tri-O-methyl-1,4-bis-O-trimethysilyl-Glcp	1, 4-	53 73 88 101 133 146 159 175 201 217 232 261 287 319 351
2,3-di-O-methyl-1,4,6-tris-O-trimethysilyl-Glcp	1, 4, 6-	59 73 88 117 133 147 159 205 232 259 287 319 345 377 409
3,4-di-O-methyl-1,2,6-tris-O-trimethysilyl-Galp	1, 2, 6-	59 73 89 103 133 146 159 173 189 205 232 259 277 317 345 377

LDS: *Lyophyllum decastes* (Fr.) Sing polysaccharide

4.96, and δ 4.91 belong to α-type pyranose, and δ 4.40 belongs to β-type pyranose. Using mestrenova software to analyze the hydrocephalic hydrogen signal in the NMR hydrogen spectrum of polysaccharide LDS-1, it was found that the ratio of δ 5.03, δ 4.91, δ 4.89, and δ 4.40

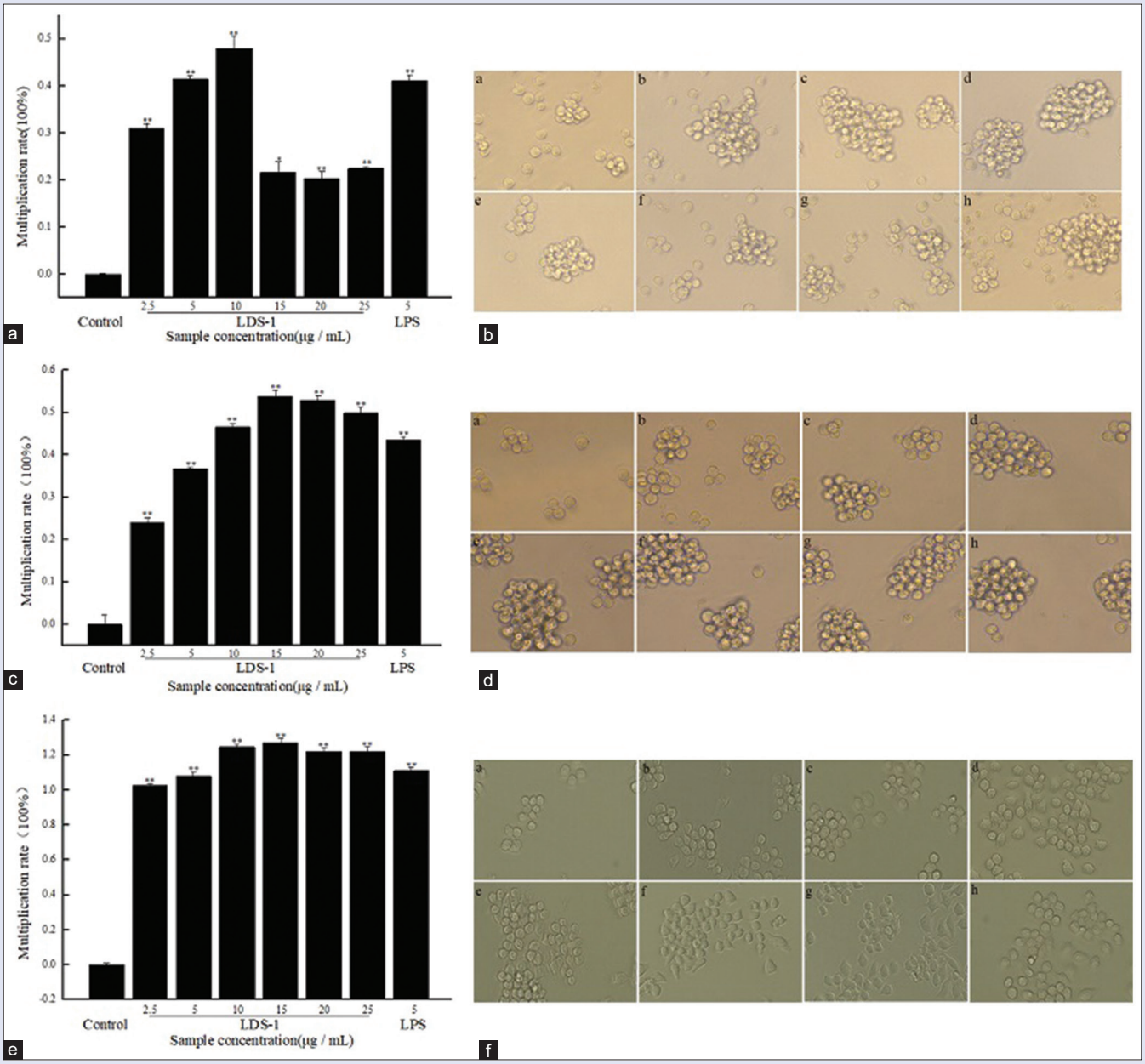

Figure 2: (a) Effect on the proliferation of T-cells by LDS-1. (b) Effect on T-cells morphology by LDS-1 (10 × 20). (c) Effect on the proliferation of B-cells by LDS-1. (d) Effect on B-cells morphology by LDS-1 (10 × 20). (e) Effect on the proliferation of RAW264.7 cells by LDS-1. (f) Effect on RAW264.7 cells morphology by LDS-1 (10 × 20). Note: (a) was the control group, b-g was the experimental group, and the mass concentrations were 2.5, 5, 10, 15, 20, 25 μg/mL, and h was the LPS positive control group (5 μg/mL). Compared with the control group, the difference was significant ($P < 0.05$) expressed by * and very significant ($P < 0.01$) expressed by **

was 1:2:1:2, and the ratio of α-pyranose to β-pyranose was 2:1. The ratio of glucose to galactose was 2:1 by GC-MS and HPLC, which indicated that glucose was α-pyranose and galactose was β-pyranose. Signals at δ 4.11–δ 3.18 were the overlapping hydrogen signals of H2–H6.

Figure 2d is the one-dimensional carbon spectrum of LDS-1. There are three kinds of hydrocephalic carbon signals in the polysaccharide, namely δ 103.00, δ 101.69, and δ 98.28. The chemical shifts of α-and β-glycosyl groups were at δ 90–δ 102 and δ 102–δ 112, respectively. In the range of δ 90–δ 102, signal at δ 101.69 and δ 98.28 were assigned to α-glycosyl groups and δ 103.00 were assigned to β-glycosyl groups. It is speculated that the anomeric carbon chemical shift of α-glucose is δ 101.69 and δ 98.28 and that of β-galactose is δ 103.00. Signals at δ 78.23–δ 60.75 were the carbon signal of C2–C6.

The ^{1}H-^{1}H COSY data can provide the coupling relationship between protons on adjacent carbon atoms in the polysaccharide ring. Figure 1e shows the ^{1}H-^{1}H COSY spectrum of LDS-1. Four signals (δ 5.03/δ 3.85, δ 4.97/δ 3.68, δ 4.91/δ 3.73, and δ 4.40/δ 3.21) were identified as the coupled signals of H1/H2 in the monosaccharide residues of A, B, C, and D, respectively. This indicates that the chemical shifts of H2 of A, B, C, D, and E residues were δ3.85, δ3.68, δ3.73, and δ3.21, respectively. The chemical shifts of all protons of monosaccharide residues can be found in ^{1}H-^{1}H COSY spectra. Table 2 shows the assignment of protons in LDS-1.

The HMQC spectrum reflects the coupling relationship of direct bonded ^{1}H-^{13}C. Figure 1f shows the direct bonding of ^{1}H-^{13}C coupling signal on LDS-1 monosaccharide ring. Combined with ^{1}H-^{1}H COSY spectra, the

resonance peaks of H1-C1 on LDS-1 monosaccharide ring are at δ 5.03/δ 98.28, δ 4.96/δ 101.69, δ 4.91/δ 98.28, and δ 4.40/δ 103.00, respectively. The chemical shifts are consistent with the results of proton and carbon spectral analysis, suggesting that the C1 signal peak were at δ 98.28, δ101.69, δ 98.28, and δ103.00, respectively. Similarly, the chemical shifts of the C2–C6 can be identified. Table 2 shows the chemical shifts of C1–C6 of all monosaccharide residues.

HMBC spectrum reflects the skeleton information of polysaccharide molecule, which were consistent with those of ^{1}H-^{1}H COSY and HMQC. Based on the analysis of monosaccharide composition, methylation, and NMR, we concluded that LDS-1 was composed of galactose and glucose in a ratio of 1:2. The main repetitive structural units of LDS-1 are composed of (1 → 4)-α-D-glucopyranose as the main chain and (1 → 6)-β-D-galactopyranose as the side chains, and the 1-*O* of the branched (1 → 6)-β-D-galactopyranose residues are linked to a 6-α-D-glucopyranose. The SCI Finder novelty search results showed that the LDS-1 was a novel polysaccharide as shown in Figure 1 h.

Effect of LDS-1 on T-lymphocyte proliferation

Mature T-cells can specifically bind to target cells, directly kill or even kill target cells, or enhance the immune effect by releasing lymphokines, which is mainly involved in cellular immunity. The effect of LDS-1 on T-lymphocyte proliferation was detected via *in vitro* CCK-8 method. Figure 2a showed that LDS-1 could significantly stimulate T-lymphocyte proliferation in the concentration range of 2.5–25 μg/mL. The highest proliferation rate was 47.95% at the concentration of 10 μg/mL. Figure 2b shows the cell morphology of T-lymphocyte stimulated by LDS-1. With the increase in the concentration of polysaccharide of LDS-1, the number of T-lymphocyte increased and the number of T-lymphocyte clusters increased significantly. In conclusion, LDS-1 has the effect of enhancing cellular immunity.

Effect of LDS-1 on B-lymphocyte proliferation

Immunoregulation is one of the important biological functions of natural polysaccharides. The effect of LDS-1 on B-lymphocyte proliferation was shown in Figure 2c. The results showed that LDS-1 with different concentrations (2.5–25 μg/mL) could significantly promote B-lymphocyte proliferation compared with the control group ($P < 0.01$). When the concentration of LDS-1 was 15 μg/mL, the growth-promoting rate of B-lymphocyte reached 53.86%, and the effect of LDS-1 stimulation was better than that of LPS (5 μg/mL). It is hypothesized that LDS-1 directly promotes the proliferation of B-lymphocyte, and the effect of LDS-1 on B-lymphocyte proliferation is better at higher concentration. Figure 2d shows the morphological results of B-lymphocyte. In the blank control group, the number of cells is small and the number of clumps is also small, while the number of single cells is large. Compared with the control group, the number of cells in LDS-1 group increased significantly, and the agglomeration became larger. Increasing the concentration of LDS-1 can accelerate B-lymphocyte division and promote cell clumping. When the concentration of LDS-1 was 15 μg/mL, the effect of B-lymphocyte aggregation was the best. In conclusion, LDS-1 can enhance humoral immunity by stimulating B-lymphocyte proliferation.

Effect of LDS-1 on the proliferation of RAW264.7 cells

Macrophages are multifunctional immune cells, which participate in the humoral and cellular immunity of the body. Their main functions include phagocyte cell debris and pathogens, activation of lymphocytes or other immune cells, anti-infection and anti-tumor effects. Figure 2e shows the effect of LDS-1 on the proliferation of RAW264.7 cells. LDS-1 can significantly promote the proliferation of RAW264.7 cells in a certain concentration range ($P < 0.01$). At 15 μg/mL, LDS-1 showed the strongest proliferative effect (127.15%), and the increment rate was higher than that of LPS (5 μg/mL). These results suggest that LDS-1 with high concentration can promote the proliferation of RAW264.7 cells. Morphological analysis [Figure 2f] showed that the cell morphology and number changed significantly under LDS-1 and LPS stimulation.

Effect of LDS-1 on the secretion of immunoglobulin by B-lymphocyte

B-lymphocytes are derived from bone marrow. After receiving antigen stimulation, a large number of cells proliferate and differentiate into plasma cells, which can synthesize and secrete immune-related proteins and circulate in the blood and participate in humoral immune response. Figure 3a-e are the results of the effect of LDS-1 on the secretion of IgA, IgD, IgE, IgG, and IgM by B-lymphocyte. Compared with the blank control group, 5 μg/mL LDS-1 showed secretion of IgA (18.60%) and IgM (10.86%), which was significantly different ($P < 0.01$); 15 μg/mL LDS-1 showed the secretion rates of IgE (5.63%) and IgM (11.53%), which was significantly different ($P < 0.01$); and the secretion rate of IgG (7.50%) was significantly different ($P < 0.05$). When the concentration of LDS-1 was 20 μg/mL, the secretion rates of IgE (46.22%), IgG (20.80%), and IgM (16.76%) were very significantly different ($P < 0.01$), and the secretion of IgA (11.69%) and IgD (9.13%) were significantly different ($P < 0.05$). The proliferation effect of B cells was the best when the concentration of LDS-1 was 15 μg/mL, which was higher than that of 5 μg/mL LPS. In the results of promoting the secretion of IgA, IgD, IgE, IgG, and IgM by B cells, when the concentration of LSD-1 was 5 μg/mL, the secretion effect of IgA by B cells was the best, and when the concentration of LDS-1 was 20 μg/mL, the secretion effect of IgD, IgE, IgG, and IgM by B cells was the best. These results suggested that different concentrations of LSD-1 have different stimulating effects on the secretion of cytokines by B-cells.

Effect of LDS-1 on the secretion of immunoglobulin by RAW264.7 cells

Compared with the blank control group, when the concentration of LDS-1 was 5 μg/mL, there was a very significant difference in the amount of secretion of tumor necrosis factor (TNF)-α ($P < 0.01$), and

Table 2: Chemical shift data of the polysaccharide LDS-1

Sugar residues	Chemical shift, δ (ppm)					
	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
A: 1,4,6-α-D-Glcp	5.03/δ 98.28	3.85/δ 60.70	3.50/74.96	3.27/73.05	3.63/74.57	3.36/75.55
B: 1,4-α-D-Glcp	4.96/δ 101.69	3.67/δ 74.36	3.39/69.37	3.21/66.52	3.38/75.56	3.29/76.58
C: 1,4-α-D-Glcp	4.91/δ 98.28	3.73/δ 68.42	3.80/69.58	3.35/75.42	3.58/68.64	3.50/75.60
D: 1,6-β-D-Galp	4.40/δ 103.00	3.21/δ 73.14	3.20/69.03	3.38/75.43	3.40/76.21	3.60/69.48

LDS: *Lyophyllum decastes* (Fr.) Sing polysaccharide

the secretion rate was 10.31%. There was a significant difference in the amount of secretion of TNF1-β ($P < 0.05$), and the secretion rate was 6.61%. When the concentration of LDS-1 was 15 and 20 μg/mL, there was a significant difference in the amount of secretion of TNF-α, TNF1-β, and interleukin (IL)-2 ($P < 0.01$), and the secretion rate were 23.55%, 25.64%, and 21.18%, and 23.59%, 16.12%, and 18.90%, respectively [Figure 3f-h]. The proliferation of RAW264.7 cells and the secretion rates of TNF1-β and IL-2 were the highest when the concentration of LDS-1 was 15 μg/mL, and the secretion effect was almost the same when the concentration was 15 and 20 μg/mL.

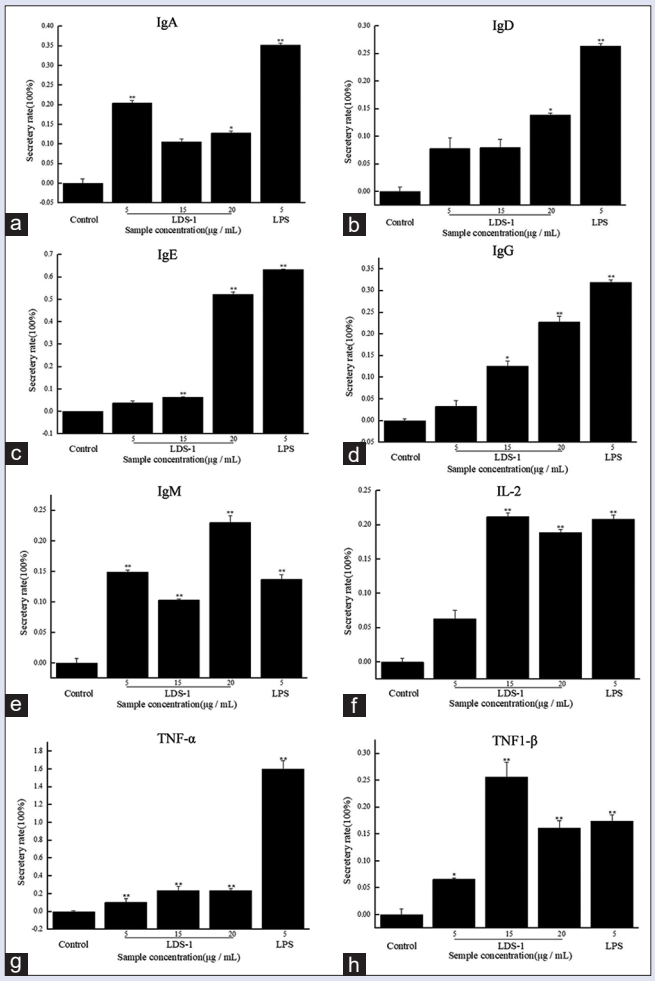

Figure 3: (a) The secretion of IgA in B cells stimulated by LDS-1. (b) The secretion of IgD in B cells stimulated by LDS-1.(c) The secretion of IgE in B cells stimulated by LDS-1. (d) The secretion of IgG in B cells stimulated by LDS-1. (e) The secretion of IgM in B cells stimulated by LDS-1. (f) The secretion of interleukin-2 in RAW264.7 cells stimulated by LDS-1. (g) The secretion of tumor necrosis factor-alpha in RAW264.7 cells stimulated by LDS-1. (h) The secretion of tumor necrosis factor 1 beta in RAW264.7 cells stimulated by LDS-1. Note: Compared with the control group, the difference was significant ($P < 0.05$) expressed by * and very significant ($P < 0.01$) expressed by **

Effect of LDS-1 on phagocytic function of RAW264.7 cells

Macrophages belong to phagocytes, and their ability to phagocytose reflects the level of the immune system. Fluorescent microsphere assay was used to evaluate whether LDS-1 enhances the phagocytic activity of RAW264.7 cells. Under LDS-1 stimulation, the phagocytosis of RAW264.7 cells was significantly increased [Figure 4a-b]. When the concentration of LDS-1 was between 5, 10, and 15 μg/mL, the phagocytosis ability of RAW264.7 cells increased gradually, which were 35.49%, 37.54%, and 48.46%, respectively. In conclusion, LDS-1 can improve the nonspecific immune level of macrophages.

Inhibitory effect of LDS-1 on proliferation of CT26.WT cells

The changes of A450 in CT26.WT cells stimulated by LDS-1 with different mass gradients (5, 10, 15, and 20 μg/mL) were measured to evaluate the viability of CT26.WT cells. As shown in Figure 4c-d, the proliferation of CT26.WT cells was significantly inhibited by LDS-1 at a certain concentration ($P < 0.01$). With the increase of concentration, the inhibitory effect showed an upward trend, which were 16.87%, 24.44%, 32.13%, and 39.11%, respectively. These results show that LDS-1 had the effect of inhibiting CT26.WT colon cancer cells.

In vivo and *in vitro* antitumor activity of LDS-1

LDS-1 5, 10, 15, and 20 μg/mL inhibited the growth of S180 tumor at a rate of 17.52%, 29.48%, 30.81% and 25.16%, respectively [Figure 4e]. The results of antitumor activity of LDS-1 against S180 tumor in mice showed that the tumor size of LDS-1 polysaccharide group and mannatide group was smaller than that of the control group; however, there was no significant difference between the mannatide group and LDS-1 group (3.81% and 54.32%, respectively). After 8 days of administration, there was no significant change in liver weight between LDS-1 group and control group, indicating that LDS-1 was nontoxic to the liver. However, the total weight (27.13 g), thymus weight (0.09 g), and spleen weight (0.16 g) of mice in LDS-1 group (20 mg/kg) were slightly lower than those in mannatide group and control group [Figure 4f and Table 3].

CONCLUSION

In this study, HPGPC, HPLC, GC-MS, and NMR analysis were performed to identify the structure of the new polysaccharide isolated from *L. decastes*. The anticancer and immunomodulatory ability of the polysaccharides (LDS-1) was also investigated. These results showed that the weight-average molecular weight of LDS-1 was 8681 Da, and the monosaccharide composition of LDS-1 was glucose and

Table 3: Antitumor activities of LDS-1 on S180 tumor

Group	Total weight (g)	Liver index (mg/g)	Spleen index (mg/g)	Thymus index (mg/g)	Average tumor weight (g)	Tumor inhibition rate (%)
S180 control	29.33±1.93	1.99±0.24	0.20±0.02	0.15±0.02	0.96±0.18	--
LDS-1 (20 mg/kg)	27.13±1.32	1.89±0.13	0.16±0.04	0.09±0.03	0.44±0.05**	54.32
Mannatide (20 mg/kg)	30.69±2.75	2.25±0.28	0.26±0.25	0.18±0.09	0.45±0.14**	53.81

Significant differences from negative control group and positive control group were evaluated using Student's *t*-test: As compared with control group, *$P<0.05$; **$P<0.01$. S180-Control: Negative control group; LDS-1: Polysaccharide LDS-1 group, 20 mg/kg; Mannatide: Positive control group of mannatide, 20 mg/kg. LDS: *Lyophyllum decastes* (Fr.) Sing polysaccharide

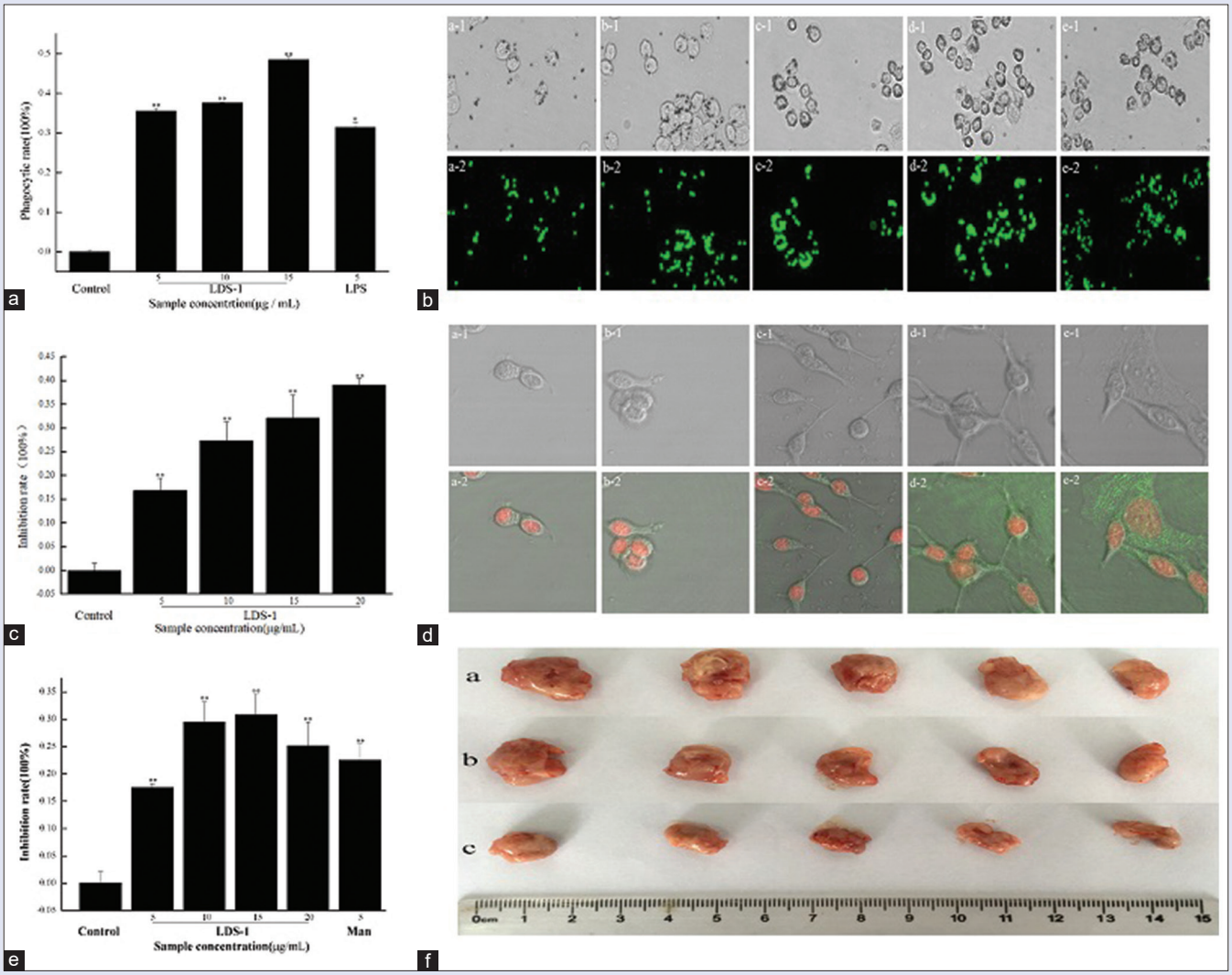

Figure 4: (a) Phagocytosis rate of fluorescent microspheres by LDS-1 stimulated RAW264.7 cells. (b) Morphology of RAW264.7 cells phagocytosis of fluorescent microspheres by LDS-1stimulated RAW264.7 cells (10 × 20). Note: a-1 and a-2 were the blank control group, b-1, b-2, c-1, c-2, d-1, d-2 were the experimental group, and the mass concentrations were 5, 10, 15 μg/mL, and e-1, e-2 were the LPS positive control group (5 μg/mL). (c) Effect on the inhibition of CT26.WT cells by LDS-1. (d) Morphology of CT26.WT cells stimulated by LDS-1 (10 × 40). Note: a-1and a-2 were the blank control group, b-1, b-2, c-1, c-2, d-1, d-2, e-1, e-2 were the experimental group, and the mass concentrations were 5, 10, 15, 20 μg/mL. (e) Effect on the inhibition of S180 cells by LDS-1. (f) Anti-tumor activity of LDS-1 *in vivo*. Note: a: Model control group, b: LDS-1 group of 20 mg/kg, c: Positive control group

galactose (2:1). It had a skeleton unit structure of four (1 → 4)-α-D-Glcp with branches at 6-*O* and the branches consisted of two (1 → 6)-β-D–Galp. The research for immunological activity under *in vitro* conditions revealed that LDS-1 showed immunological activity. At a concentration range of 2.5–25 μg/mL, it promoted the growth of T-lymphocyte, B-lymphocyte, and RAW264.7 cells; it also promoted the secretion of IgA, IgD, IgE, IgG, IgM, TNF-α, TNF-β, and IL^{-2}; finally, it promoted the phagocytosis of fluorescent microspheres by RAW264.7 cells compared with the blank control group ($P < 0.01$). Antitumor activity of LDS-1 showed (2.5–20 μg/mL; $P < 0.01$) it had significant inhibitory effects on colon cancer cells (CT26.WT) and S180 cells compared with the blank control group, but the degree of inhibition was different. The tumor inhibition rate was 54.32%. These data show a new way for the high-quality utilization of *L. decastes*. It also provides theoretical basis and technical support for the further development and utilization of *L. decastes* polysaccharides.

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Conflicts of interest

There are no conflicts of interest.

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