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Structure and Inducing Tumor Cell Apoptosis Activity of Polysaccharides Isolated from *Lentinus edodes*

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ABSTRACT: In this study, five novel polysaccharides SLNT1, SLNT2, JLNT1, JLNT2, and JLNT3 were isolated from the fruit body of *Lentinus edodes*. Chemical and physical analyses showed that the five polysaccharides consist of glucose with the structure of β-(1→3)-D-glucose main chains and β-(1→6)-D-glucose side chains. Moreover, all of them had triple-helical conformation and different molecular weight distributions. Animal studies further demonstrated that the antitumor effects were remarkably improved by SLNT1 and JLNT1 treatments with the inhibitory rates of 65.41% and 61.07% in H22-bearing mice, respectively. Additionally, both of them significantly increased the levels of serum IL-2 and TNF-α production and induced the tumor cell apoptosis. Taken together, our findings revealed that the involved antitumor mechanisms possibly in part were mediated not only by enhancing the immunity but also by directly killing the tumor and the induction of tumor cell apoptosis in H22-bearing mice.

KEYWORDS: *Lentinus edodes, polysaccharides, structure, antitumor activity, cytokine, apoptosis*

INTRODUCTION

Cancer is globally the second most life threatening disease in developing countries as people lifestyle changes.[1] So far, surgery and chemotherapy are still the main therapeutic methods of most solid tumors in China.[2] However, the application of therapeutic drugs had some limitations in clinical settings, such as adverse effects, limited efficacy, and drug resistance.[3] Consequently, screening for natural drugs of antitumor activities with low toxicity and high efficacy has been a growing sector in cancer therapy.[4]

Polysaccharides extracted from plants, such as mushrooms and higher plants, are useful resources in food and drug industries. Therefore, mushrooms had been widely studied in the biochemical and medical fields because of their unique bioactivities.[5−8] The largely second popular edible mushrooms in global market was *Lentinus edodes* (*L. edodes*), which was widely cultivated in China and Japan because of its good taste, high nutritional values, and some medicinal attributes, named xianggu and shiitake, respectively.[9] As medicinal food, *L. edodes* has been reported to reduce cancer risks attributed to *L. edodes* polysaccharide lentinan. This polysaccharide was the most active constituent and had become attractive to many researchers because of its low toxicity and multiple pharmacological properties, such as antitumor activity, immunomodulatory, antioxidant, and reducing blood lipid.[10−12] However, much more attention has been paid to its antitumor effects.[13,14] The mechanism of antitumor activity stimulated by lentinan was traditionally only thought to be enhancing the immunity.[6,14] However, our study had surprisingly found that polysaccharides extracted from *L. edodes* could inhibit the growth of tumor by different mechanisms, such as inducing tumor cell apoptosis and directly killing tumor cells. These mechanisms in combination with activating immune system seemed to be more effective and promising when applied to cancer therapy.

Cytokines and apoptosis played significant roles in cancer therapy.[15−17] Apoptosis, a programmed cell death, was a special phenomenon observed in antitumor treatment, which could lead to remarkable morphological changes of tumor cell, such as chromatin condensation, plasma membrane blebbing, and karyorrhexis. Recently, a number of researches have also shown that polysaccharides extracted from mushrooms could induce human cancer cell apoptosis to kill tumor.[4,18] However, to the best of our knowledge, it has not been reported yet whether the antitumor activity could be induced by polysaccharides isolated from *L. edodes* via multiple mechanisms, which may include activating the immune system, directly killing and inducing apoptosis. Besides, interleukin (IL) 2 was involved in regulating immune response and affecting immune cells proliferation, which could improve immunity potentially. Tumor necrosis factor (TNF) α, a proinflammatory cytokine, was a good parameter to show the ability of killing tumor cells by apoptosis without damage to normal cells. The treatment combining apoptosis and directly killing tumor with immunity improvement could result in better antitumor effects. For these reasons, the levels of IL-2 and TNF-α, the morphological changes of tumor tissues, and the apoptosis rate were investigated in the experiments.

Following the views of Chinese scholars, *L. edodes* has been widely used for cancer prevention by people in Asian countries. But the mechanisms of this effect were complicated and remained unclear. So the studies of its antitumor activities and

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the involved mechanisms are of great relevance. Recently, five novel polysaccharides SLNT1, SLNT2, JLNT1, JLNT2, and JLNT3 were isolated from the fruit bodies of *L. edodes* in our laboratory. Their chemical structures, conformation, and antitumor activities in vitro were determined. We have also shown that SLNT1 and JLNT1 possess antitumor activity in vivo with H22-bearing mouse model by different ways, including inducing tumor cell apoptosis and directly killing tumor. This present study laid solid foundations for further research on the underlying mechanisms.

■ MATERIALS AND METHODS

Standards and Reagents. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), cyclophosphamide (CTX), and standard sugars were obtained from Sigma-Aldrich Chemical Co. Fetal calf serum (FCS), RPMI 1640 medium, penicillin, trypsin, and streptomycin (cell culture grade) were purchased from Gibco (Grand Island, NY, USA). Annexin V-FITC apoptosis dectection kit, mouse IL-2, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from KeyGEN Biotech (Nanjing, China). Other regents used were analytical grade.

Extraction, Isolation, and Purification of Samples. The dried fruit body of *L. edodes* was purchased from Xixia, Henan Province, China. It was identified as *L. edodes* by Dr. Jiachun Chen from the Department of Traditional Chinese Medicine, Tongji Medical College at Huazhong University of Science and Technology. The dried fruit bodies (200 g) of *L. edodes* were soaked with water until soft and were cut into small pieces, mixing with 95% alcohol for 24 h to remove lipids. [19] Then they were extracted with boiling water (2000 mL) for 1 h each time and 2 times. All water extracts were gathered and concentrated into one-fifth of the initial volume under 80 °C. The resulting solution was precipitated by adding equal volume of 95% alcohol and then centrifuged at 4000 rpm for 15 min. The precipitates were collected and dried. The fruit body residues after water extraction were soaked with 3000 mL of diluted 2% NaOH and 0.05% $NaBH_4$ solutions for 24 h and filtered through gauze. Then 5% HAc was slowly added into the filtrate to adjust to pH 7, centrifuging to remove the insolubles.[20] The supernatants were concentrated to one-fourth of the initial volume under 80 °C and precipitated by adding equal volume of 95% alcohol overnight. The mixtures were centrifuged to collect the precipitates and dried.

Two kinds of precipitates above were dissolved in distilled water and treated with H_2O_2 to decolorize the solution and remove proteins.[21] After precipitation with 95% alcohol, we could obtain two different kinds of crude polysaccharides, SLNT and JLNT, which were water extracts and alkali extracts. An amount of 3.0 g of SLNT (or JLNT) was redissolved in distilled water and intercepted with different molecular weight cut-off membranes including 500, 100, and 10 kDa by ultrafiltration. Two main fractions were collected and lyophilized to get white purified polysaccharides, termed SLNT1 and SLNT2. The other three purified polysaccharides JLNT1, JLNT2, and JLNT3 were received using the same method from JLNT. The whole extraction procedure is shown in Figure 1.

Determination of Purity and Molecular Weight. Total sugar content of the polysaccharides was determined by the phenolsulfuric acid method using D-glucose as standard at 490 nm.[22] A UV spectrophotometer (model UV-1750) was used to detect proteins and nucleic acids in the range of 200−400 nm.

The molecular weights of the five polysaccharides were determined by high performance gel permeation chromatography (HPGPC) on an Agilent-LC 1100 instrument (Agilent, USA). TSK-GEL G-4000PW$_{XL}$ column was maintained at 35 °C, and the mobile phase was a 0.05 M Na_2SO_4 solution at a flow rate of 1.0 mL/min. Detection was by an Agilent refractive index detector. Five samples of 1 mg/mL were passed through a 0.22 μm filter membrane before running. A standard curve linear was calibrated with T-series Dextrans standards (1−2000 kDa). The molecular weights of the samples were determined with the calibration curve equation with the Agilent HPGPC software (Agilent, USA).

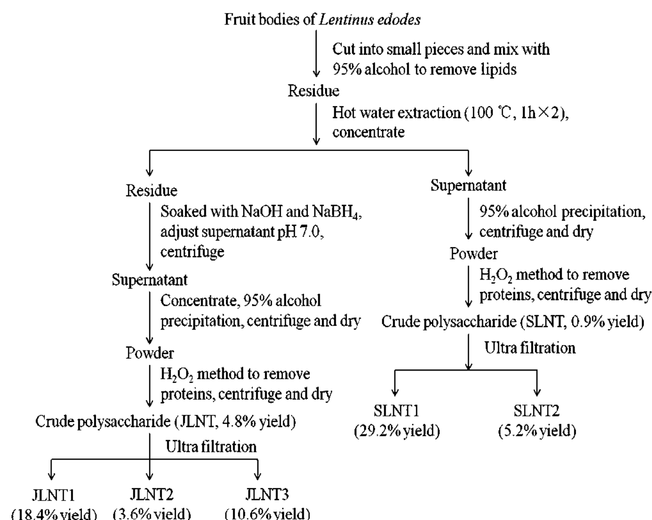

Figure 1. Extraction, isolation, and purification scheme of the five polysaccharides from the fruit bodies of *L. edodes*.

Infrared Spectrum. Samples were prepared by mixtures of polysaccharides with dry KBr and pressed into sheets in a mold, and analysis was with a Bruker-VERTEX 70 Fourier-transform infrared (FT-IR) spectrophotometer in the 4000−400 cm^{-1} region.

Monosaccharide Composition Analysis. Samples (20 mg) were dissolved in 2 M H_2SO_4 (3 mL) and hydrolyzed at 90 °C for 11 h, then neutralized with $BaCO_3$. The supernatants were added to 1 mL of pyridine and 10 mg of hydroxylamine hydrochloride to the hydrolysis products, and the mixture was incubated in a water bath at 90 °C for 30 min. After that, samples were cooled and 1 mL of acetic anhydride was added and the mixture was incubated at 90 °C for 30 min. The hydrolysis products were converted into their acetylated derivatives and analyzed by GC−MS using an Agilent 7890A instrument equipped with a HP-5 column and detected with an Agilent 5975C MS detector. Six monosaccharides (arabinose, mannose, rhamnose, galactose, xylose, and glucose) were used as the external standards to identify the composition of the polysaccharides.

Periodate Oxidation and Smith Degradation. Polysaccharide (20 mg) was dissolved in distilled water. $NaIO_4$ (30 m Mol/L) was then added. The solutions were kept in the darkness at room temperature and read at 223 nm every 4 h using a spectrophotometer until the optical density became stable. Glycol was added to the solutions of periodate products to stop the reaction. Titrating with 0.0198 mol/L NaOH solution was done to calculate the production of formic acid and to further determine the consumption of $NaIO_4$.

The remaining solutions were dialyzed in distilled water and reduced with $NaBH_4$ (40 mg) in the dark at room temperature for 24 h. After neutralization with 0.5 M acetic acid, the solutions were dialyzed again and concentrated. The resulting products were hydrolyzed with 2 M H_2SO_4 followed by neutralization with $BaCO_3$, then filtered, concentrated, acetylated, and analyzed by GC−MS.

Methylation Analysis. The five polysaccharides were methylated by the Needs and Selvendran method.[23] After compete methylation, the premethylated products were depolymerized with HCOOH at 100 °C for 6 h and further hydrolyzed with 2 M CF_3COOH. The residues were reduced with $NaBH_4$, acetylated, and analyzed by GC−MS.

Congo-Red Test. The conformational structures of the five polysaccharides were determined by Congo-red test. Samples (2 mg/mL) were mixed with Congo-red (0.02 mmol/L) and NaOH solution (final concentration of NaOH: 0.00, 0.10, 0.20, 0.30, and 0.40 mol/L). The shift of the maximum absorption wavelength (λ_{max}) of the Congo red−polysaccharide complex was measured in the range from 400 to 600 nm and then compared with λ_{max} of Congo red.

Table 1. Total Sugar Content, Sugar Component, Molecular Weights, Glucosidic Bond Linkage Types, and the Molar Ratios of SLNT1, SLNT2, JLNT1, JLNT2, and JLNT3

sample	SLNT1	SLNT2	JLNT1	JLNT2	JLNT3
sugar content (%)	96.89	102.54	95.12	97.32	101.56
molecular weight (kDa)	617.6	97.57	638.7	273.8	151.3
sugar component	glucose	glucose	glucose	glucose	glucose
linkage type	1,3-linkage or 1,3,6-linkage 1-linkage or 1,6-linkage	1,3-linkage or 1,3,6-linkage 1-linkage or 1,6-linkage	1,3-linkage or 1,3,6-linkage 1-linkage or 1,6-linkage	1,3-linkage or 1,3,6-linkage 1-linkage or 1,6-linkage	1,3-linkage or 1,3,6-linkage 1-linkage or 1,6-linkage
molar ratio	3.4:1	0.9:1	2.8:1	2.7:1	3.0:1

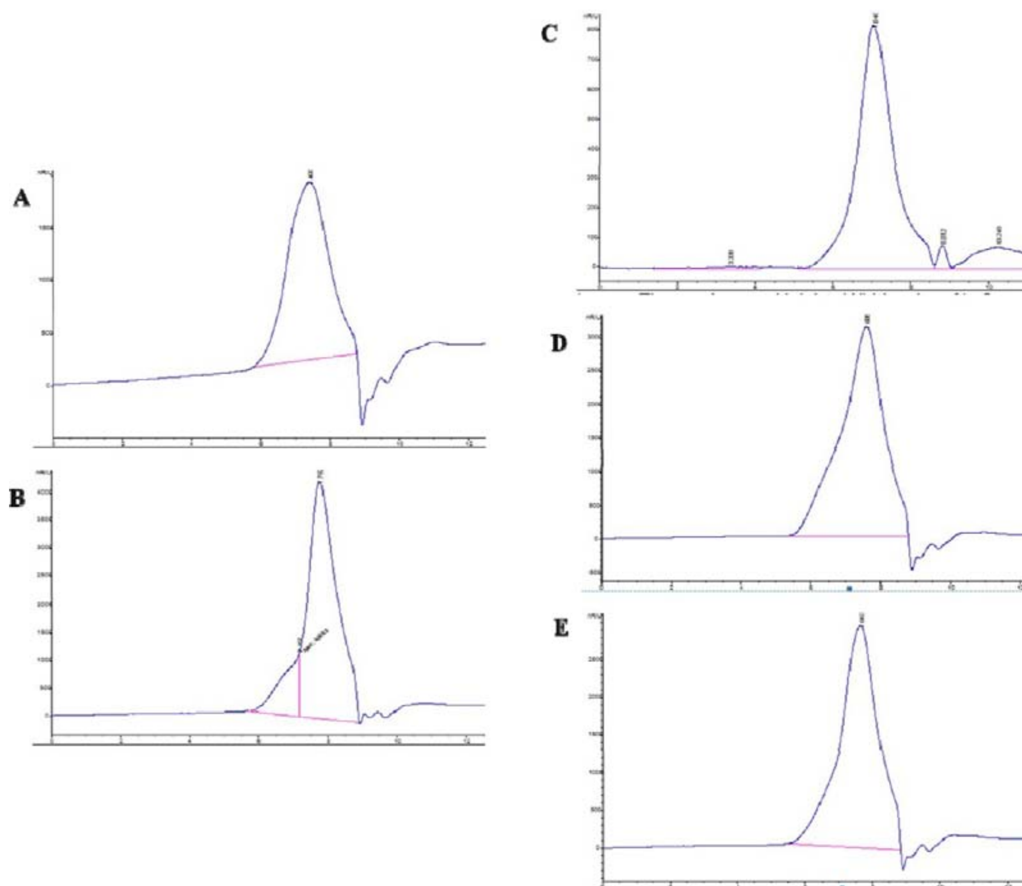

Figure 2. Chromatography of the five polysaccharides by HPGPC. The weight-average molecular weights were the following: (A) SLNT1, 617.6 kDa; (B) SLNT2, 97.57 kDa; (C) JLNT1, 638.7 kDa; (D) JLNT2, 273.8 kDa; (E) JLNT3, 151.3 kDa.

Preparation of SLNT1, SLNT2, JLNT1, JLNT2, and JLNT3 Samples for Cell Culture. Mouse hepatoma cell H22 and human hepatocellular carcinoma HepG2 and SMMC-7721 cell lines were provided by Tongji Medical College and cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. Generally, they were maintained in a humidified incubator of 5% CO_2 at 37 °C. Cells were collected at the exponential growth phase to study the antitumor effects of the five polysaccharides. All samples were dissolved in medium at concentrations of 12.5, 25, 50, 100, 200, 400, 800 μg/mL.

Assay of Inhibition of Tumor Cell Proliferation in Vitro. The inhibition effects in vitro on the three kinds of cancer cells were determined by colorimetric MTT method as previously described with some modifications.[24] Generally, H22, HepG2, and SMMC-7721 cell lines approximately at an initial density of 3×10^5 cells/mL (adjustments according to different cell types) were seeded into the 96-well plates, and subsequently 100 μL of polysaccharide (12.5, 25, 50, 100, 200, 400, and 800 μg/mL, concentration gradient) was added to the wells, culturing for 24 h at 37 °C to determine its effects on cell growth. Cells were cultured with cis-platinum (12.5 μg/mL), which served as the positive control. Then the plates were read at 570 nm using a microplate reader (Multiskan Mk3, Thermo Scientific, USA). All experiments were performed in triplicate. The antitumor effects were assessed by the inhibitory rate which was calculated with the following formula: inhibitory rate (%) = (1 − the A_{570} value for experimental group)/[(A_{570} value of negative control group)] × 100%.

Determination of Effects on SLNT1 and JLNT1 Therapy in H22-Bearing Mice. BALB/c mice (7−8 weeks old, weighting 20 ± 2g) were purchased from the Animal Center of Disease Control and Prevention of Hubei Province, China, and housed under the standard conditions of temperature (22 ± 2 °C), relative humidity (60% ± 5%), and 12 h light/12 h dark cycle. After 3 days of acclimatization, mice were injected with 0.2 mL of H22 cells adjusted to 6×10^6 cells/mL subcutaneously in the right axillary. Normal mice did not have any treatment. The H22-bearing mice were randomly divided into five groups with 10 mice per group as follows: mice intraperitoneally

injected with only 0.9% saline served as negative control group; the experimental groups were intraperitoneally injected with different concentrations of SLNT1 at 50, 100, and 200 mg/kg once daily for 10 days after 48 h of tumor inoculation; CTX dissolved in 0.9% saline (25 mg/kg) was used as positive control group.

On the 11th day, all mice were sacrificed by cervical dislocation. The tumor, spleen, and thymus were cut and weighed. The antitumor activity of SLNT1 was expressed as an inhibition ratio calculated as follows: inhibition ratio (%) = $[(W_a - W_b)/W_a] \times 100$, where W_a and W_b were the average tumor weights of the control and experimental group, respectively. The same method was used to evaluate the effects of JLNT1 therapy in H22-bearing mice.

Measurement of Concentrations of TNF-α and IL-2. The serums collected from different groups were immediately separated by centrifugation at 12 000 rpm at 4 °C for 10 min. ELISA kits were used for detection of TNF-α and IL-2 levels according to instructions of the kits, and absorbance was measured at 450 nm in an ELISA plate reader (Multiskan Mk3, Thermo Scientific, USA). The cytokine levels were calculated by the standard curves of recombinant cytokines using the linear regression method.

Morphology Observation of Tissues. Tumor was fixed with 4% formalin. After paraffin embedding and sectioning, the metallographs were taken at 400× magnification by an optical microscope (XSP-11CD, Shanghai Caikon Optical Instrument Co. Ltd., China) to observe the H&E staining morphologic changes of the tumor.

Flow Cytometric Analysis of Apoptosis. HepG2 cells in logarithmic growth phase at a density of 4×10^4 cells/mL were seeded in a 12-well plate and treated with different concentrations of SLNT1 (250, 500, and 1000 μg/mL) for 48 h. The control group was treated without drugs. After digestion with trypsin, the cells were washed with PBS twice and resuspended in binding buffer followed by the addition of 5 μL of annexin V-FITC and 5 μL of propidium iodide (PI). The cells were placed in the darkness for 10 min and assayed by flow cytometry (BD, NJ, USA). The experiment was performed in triplicate.

Statistical Analysis. All experimental data were expressed as the mean ± SD (standard deviation). Statistical analysis was performed with Student's *t* test using a statistical analysis system software package (SPSS 17.0), and *p*-values of <0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Isolation, Purification, and Molecular Weight Determination of the Five Polysaccharides. Though hot water extraction method was a traditional way to extract polysaccharide from mushrooms, in our research, we established a new alkali-extractable method, which was a green process. It did not contain any organic solvents and had the advantages of simple procedures, high production, and low cost. Compared with lentinan, the reported yield of which was only 0.12% by the hot water and organic solvent extraction method, [4] our extraction yields of crude polysaccharides for SLNT and JLNT were approximately 0.9% and 4.8%, respectively. The results showed that the solvent of extraction methods could affect the amount of polysaccharides significantly. After decoloration and ultrafiltration, five further purified fractions (SLNT1, SLNT2, JLNT1, JLNT2, and JLNT3) were obtained. Their yields from preliminarily purified SLNT and JLNT were 29.2%, 5.2%, 18.4%, 3.6%, and 10.6%, respectively. Polysaccharides with larger molecular weights (SLNT1 and JLNT1) had higher yields. The total sugar contents and molecular weights were shown in Table 1. According to our results, the total sugar contents reached more than 95%, and HPGPC presented single and symmetrical peaks, basically indicating the homogeneous distributions of the polysaccharides (Figure 2). Besides, there was no absorption at either 280 or 260 nm by UV spectrum (data were not shown), revealing the absence of protein and nucleic acid. All these provided powerful evidence that we obtained five basically purified polysaccharides due to our novel extraction methods. Furthermore, the molecular weights of alkali-extractable polysaccharides (JLNT1, JLNT2, and JLNT3) were higher than those of water-extracted polysaccharides (SLNT1 and SLNT2).

Structure of the Five Polysaccharides. Growing evidence had suggested that the bioactivity of the polysaccharide was related to its structure, such as monosaccharide composition, glycosidic linkage types, and the conformation. The chemical structures and the conformation of the five fractions were therefore investigated to understand the antitumor activities. GC−MS analysis indicated that the five polysaccharides were only composed of glucose (data were not shown). The IR spectrum displayed a broad stretching peak around 3400 cm^{-1} due to the hydroxyl stretching vibration of the polysaccharides. A weak absorption at 2922 cm^{-1} was attributed to the C−H stretching vibration, and the relatively strong absorption peaks at 1424 and 1372 cm^{-1} could be assigned to deforming vibrations of the C−H bond, which were the characteristic absorptions of the polysaccharides. The bands at 1161, 1077, and 1042 cm^{-1} were the skeletal modes of pyranose rings. Moreover, the band at 893 cm^{-1} was ascribed to β-type glycosidic bonds. The IR spectrum showed that the five fractions had the characteristic absorption peaks of polysaccharides containing β-D-glucans with pyranose rings and the similar structures. Polysaccharides extracted by different methods exhibiting similar structures were also found.

Particularly, the types of glucosidic linkages and the molar ratios of the five fractions were also determined by periodate oxidation and methylation. Results were shown in Table 1. The results of periodate oxidation indicated that each of the fraction possibly had (1→3) or (1→3,6) linkages and (1−) or (1→6) linkages. Ratios of these various types of glycosidic bonds were about 3.4:1, 0.9:1, 2.8:1, 2.7:1, and 3:1 of SLNT1, SLNT2, JLNT1, JLNT2, and JLNT3, respectively. The linked ratio of SLNT2 was quite different from others, leading to lower antitumor activities as we found in the below. After Smith degradation, glucose and glycerin were found in GC analysis. The presence of glucose demonstrated that some of the linkages were (1→3)-linked or (1→3,6)-linked; the identification of glycerin showing that (1−) or (1→6)-linked glycosyl residues existed in the five fractions. These results were basically consistent with those of periodate oxidation.

The fully methylated products of the fractions were hydrolyzed with acid and converted into alditol acetates. GC−MS analysis revealed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol, 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-6-deoxy-D-galactitol, and 2,3,6-tri-*O*-methyl-D-glucitol. And the molar ratios were further calculated by the peak area under the curve. The results of methylation analysis (Table 1) were basically in accordance with the conclusions of periodate oxidation and Smith degradation.

The chemical analyses results demonstrated that (1→3)-linked-β-D-glucose was one of the largest amounts of residue of the fractions except for SLNT2, whose (1→6)-linkages were a little more than (1→3)-linkages. Results revealed that (1→3)-linked-β-D-glucose formed the backbone structure of the polysaccharide and (1→6)-linkage or (1→)-linkage formed the side chains, which was crucial to play the role of antitumor activity. What's more important, polysaccharides with this structure had better antitumor activities than others reported

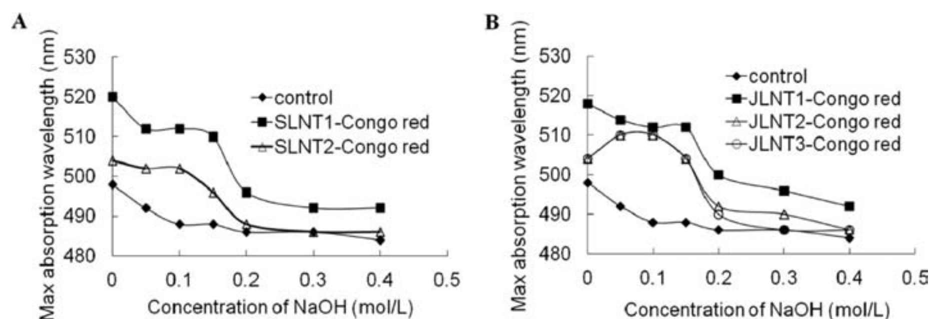

Figure 3. Maximum absorption wavelength of polysaccharide−Congo red complex at different concentrations of NaOH ranging from 0 to 0.4 mol/L: (A) conformation of water-extractable polysaccharides SLNT1 and SLNT2 in NaOH solutions; (B) conformation of alkali-extractable polysaccharides JLNT1, JLNT2, and JLNT3 in NaOH solutions.

whose backbone chain was not (1→3)-linked-β-D-glucose from mushrooms.[4,25] In addition, different ratios of the glucosidic linkages could contribute to different structures.

Identification of Conformation of the Five Polysaccharides. It has been reported that polysaccharides of β-(1, 3)-D-glucan structure with triple-helix conformation were considered to have better antitumor activities but not as a single flexible chain.[20,26,27] Congo red could form a complex with triple-helix polysaccharides and shifted the maximum absorption wavelength. Therefore, Congo red assay was employed to investigate the triple-helix conformation of the polysaccharides at different concentrations of NaOH ranging from 0 to 0.4 mol/L. As shown in Figure 3, the maximum absorption wavelength (λ_{max}) of Congo red−polysaccharide complex was distinctly shifted in the presence of SLNT1, SLNT2, JLNT1, JLNT2, or JLNT3. λ_{max} increased initially, then decreased sharply in 0.15 M NaOH, and finally kept balance over 0.2 M NaOH where the triple-helix conformation transformed to single coil conformation. From the experimental phenomena above, we could draw a conclusion that all of the five polysaccharides fractions had triple-helix conformation. Moreover, the triple-helical structures were destroyed at higher NaOH concentrations, leading to a decrease in the maximum absorption wavelength.

Inhibition Effects of Tumor Cell Proliferation in Vitro. The activities against the proliferation of H22, HepG2, and SMMC-7721 cells of the five polysaccharides were tested. The results showed that the inhibitory rates displayed a concentration-dependent tendency and are presented in Figure 4. SLNT1 and JLNT1 at 800 μg/mL exhibited the strongest antiproliferation activities on H22 cells with inhibitory rates of 61.48% and 62.32% (Figure 4A), respectively. For HepG2 cells, SLNT1 and JLNT1 had inhibitory rates of 35.85% and 27.99%, respectively (Figure 4B). Meanwhile, both of them could inhibit the growth of SMMC-7721 cells with inhibitory rates of only about 35% at 800 μg/mL (Figure 4C). However, the inhibitory rates against the growth of the three cancer cell lines for the other three polysaccharides (SLNT2, JLNT2, and JLNT3) were lower than those of SLNT1 and JLNT1. The results showed that SLNT2, JLNT2, and JLNT3 presented a relatively higher inhibition on H22 cells with an inhibition ratio of 32−48%, compared to HepG2 and SMMC-7721 cells whose inhibitory rates were below 20% at 800 μg/mL. In particular, as positive control, cis-platinum had the strongest effects against all kinds of cancer cells, reaching to more than 90%. The effects might be caused by its strong cytotoxic activity.[28,29] On the basis of the results above, it could be concluded that the five

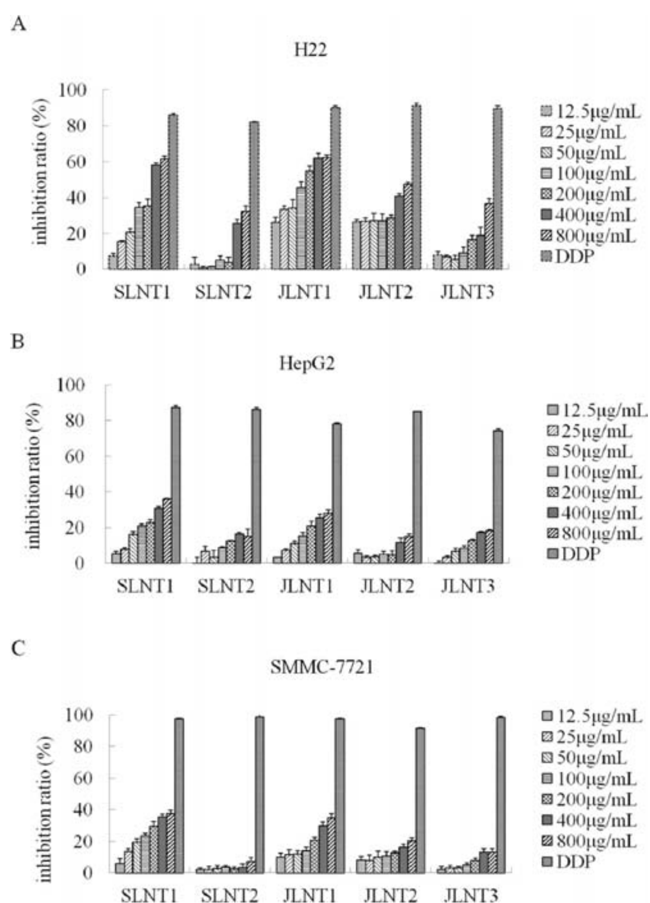

Figure 4. Inhibitory effects of polysaccharides SLNT1, SLNT2, JLNT1, JLNT2, and JLNT3 on three cell lines at different concentrations in vitro: (A) inhibition effects of proliferation on H22 cells; (B) inhibition effects of proliferation on HepG2 cells; (C) inhibition effects of proliferation on SMMC-7721 cells. Data are the mean with error bars representing standard deviations (n = 4).

polysaccharides were potent tumor cell growth inhibitors which showed selectively antiproliferation activities against tumor cells. What's more important, they could inhibit tumor growth by the mechanism of direct killing and are promising as natural antitumor drugs not just because of its immune enhancement effect.[4,30,31]

By correlation of the chemical analyses with the antiproliferation effects in vitro, the differences in antiproliferation of cancer cells among the five polysaccharides might be attributed to their chemical composition and structure. It has been

Table 2. Effects of Polysaccharide SLNT1 on Thymus Index, Spleen Index, and Tumor Growth in H22-Bearing Mice[a]

group	dose (mg/kg)	spleen index (mg/g)	thymus index (mg/g)	tumor weight (g)	inhibition ratio (%)
control		5.73 ± 1.06	2.42 ± 0.43	1.33 ± 0.17	
CTX	25	3.88 ± 0.77**	1.48 ± 0.35**	0.96 ± 0.14**	27.82
SLNT1	50	7.24 ± 0.81*	2.65 ± 0.56##	1.02 ± 0.27	23.31
	100	9.39 ± 0.85***	2.61 ± 0.67##	0.80 ± 0.14***	39.85
	200	10.62 ± 1.07***	2.64 ± 0.68##	0.46 ± 0.22***	65.41

[a]H22-bearing mice were intraperitoneally injected with different doses of SLNT1 for 10 days once daily as experimental groups, and CTX was injected as positive control. The thymus and spleen weights were measured on the 11th day. Thymus and spleen indexes were calculated as the thymus and spleen weight relative to body weight. Data are expressed as the mean ± SD based on eight mice for each group: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ compared to control group; (##) $P < 0.01$ compared to CTX group.

Table 3. Effects of Polysaccharide JLNT1 on Thymus Index, Spleen Index, and Tumor Growth in H22-Bearing Mice[a]

group	dose (mg/kg)	spleen index (mg/g)	thymus index (mg/g)	tumor weight (g)	inhibition ratio (%)
control		5.69 ± 0.85	2.14 ± 0.59	1.31 ± 0.21	
CTX	25	3.78 ± 0.55***	0.95 ± 0.31***	0.81 ± 0.40*	38.17
JLNT1	50	9.66 ± 0.93***	2.01 ± 0.25###	0.81 ± 0.31**	38.17
	100	13.75 ± 0.83***	1.92 ± 0.39###	0.51 ± 0.20***	61.07
	200	9.93 ± 1.13***	2.22 ± 0.65###	0.93 ± 0.31*	29.01

[a]H22-bearing mice were intraperitoneally injected with different doses of JLNT1 for 10 days once daily as experimental groups. Other methods used were the same as the animal study of SLNT1 as mentioned in the text. Data are expressed as the mean ± SD based on eight mice for each group: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ compared to control group; (##) $P < 0.01$ compared to CTX group.

reported that polysaccharide bioactivities were closely related to several factors, such as the main chain structure, branching degree, helical conformation, molecular weight, and the monosaccharide composition.[32,33] Generally, polysaccharides of molecular weights between 400 and 800 kDa appeared to be more effective than those of low molecular weight in suppressing tumor cell growth.[32,34] Our results suggested that fractions (SLNT1 and JLNT1) had relatively higher molecular weights resulting in stronger antiproliferation effects than others (SLNT2, JLNT2, and JLNT3) as reported, and therefore, they were selected for animal tests below. Besides, some polysaccharides isolated from *L. edodes* were found to be composed of glucose, galactose, and mannose. But they had lower molecular weights (4−26 kDa) and weaker inhibiting tumor cells proliferation abilities (inhibition ratio of 27−45%) at 5 mg/mL.[13] Compared with them, our polysaccharides had better antiproliferation effects for cancer cells due to their simple monosaccharide composition, which only consists of β-glucose. In addition, SLNT2 exhibited the lowest antiproliferation activity because of its different structures from others, demonstrating that (1→3)-linked-β-D-glucose backbone chain was an essential structural feature for antiproliferation effects, which was in good agreement with the report that β-glucose containing mainly 1→6 linkages exhibits weaker activities.[35] Moreover, according to the report, lentinan with triple-helix conformation exerted a relatively higher inhibition ratio against sarcoma 180 tumor. [20] In this study, all polysaccharides had triple-helix structure and excellent inhibitory effects against cancer cells as reported above.

Effects of SLNT1 and JLNT1 Therapy in H22 Tumor-Bearing Mice. SLNT1 and JLNT1 exhibiting the strongest antitumor activities in vitro were chosen to further test their antitumor activities in vivo against H22 solid tumor growth. The results are summarized in Table 2 and Table 3.

During the experiments, mice in model control group and CTX treatment group gradually exhibited a series of weak appearance, such as loss of appetite, activity reduction, and dim hair. But no signs of toxicity and side effects were observed in the mice treated with SLNT1 and JLNT1. After 10 days of treatment, the growth of H22 tumor in mice was significantly suppressed by treating with SLNT1 and JLNT1, compared with the negative control. The inhibition ratios of SLNT1 were 23.31%, 39.85%, and 65.41% at various doses of 50, 100, and 200 mg/kg, compared with the negative control. Nevertheless, it was noteworthy that the inhibition effect of JLNT1 was obviously the greatest at a middle dose of 100 mg/kg with the inhibitory rate of 61.07%. The inhibition ratio decreased instead with the highest concentration of 200 mg/kg, possibly because high molecular weights resulted in bad solubility and inadequate absorption. Moreover, it was notable that the spleen index of the mice treated with both SLNT1 and JLNT1 was higher than that of the model mice or CTX treatment, but no significant changes were observed in the thymus index. Reasons might be that SLNT1 and JLNT1 could significantly stimulate the proliferation of spleen cells instead of thymus cells. However, both spleen and thymus indexes were dramatically lower than the model mice with CTX treatment because CTX often resulted in immunosuppressive effects in antitumor chemotherapy. From this point of view, our polysaccharides SLNT1 and JLNT1 had better antitumor effects than CTX. Thus, according to the results both in vitro and in vivo, immunostimulation and direct killing of tumor by SLNT1 and JLNT1 treatments may be possible ways to inhibit H22 tumors.

Effects of SLNT1 and JLNT1 Therapy on Serum IL-2 and TNF-α Production. IL-2 and TNF-α are two important cytokines involved in antitumor therapy. Studies have shown that application of lentinan could prolong the survival time and improve the life quality in patients with cancers by activating effector cells like microphages, T cells, and NK cells to secret cytokines IL-2, TNF-α, and IFN-g, which had characteristics of antiproliferation, apoptosis, and differentiation in tumor cells.[32,36] According to our knowledge, IL-2 played a prominent role in regulating immune response and was capable of inducing T cells proliferation with distribution in T cells, B cells, NK cells, and monocytes. TNF-α has been regarded as an

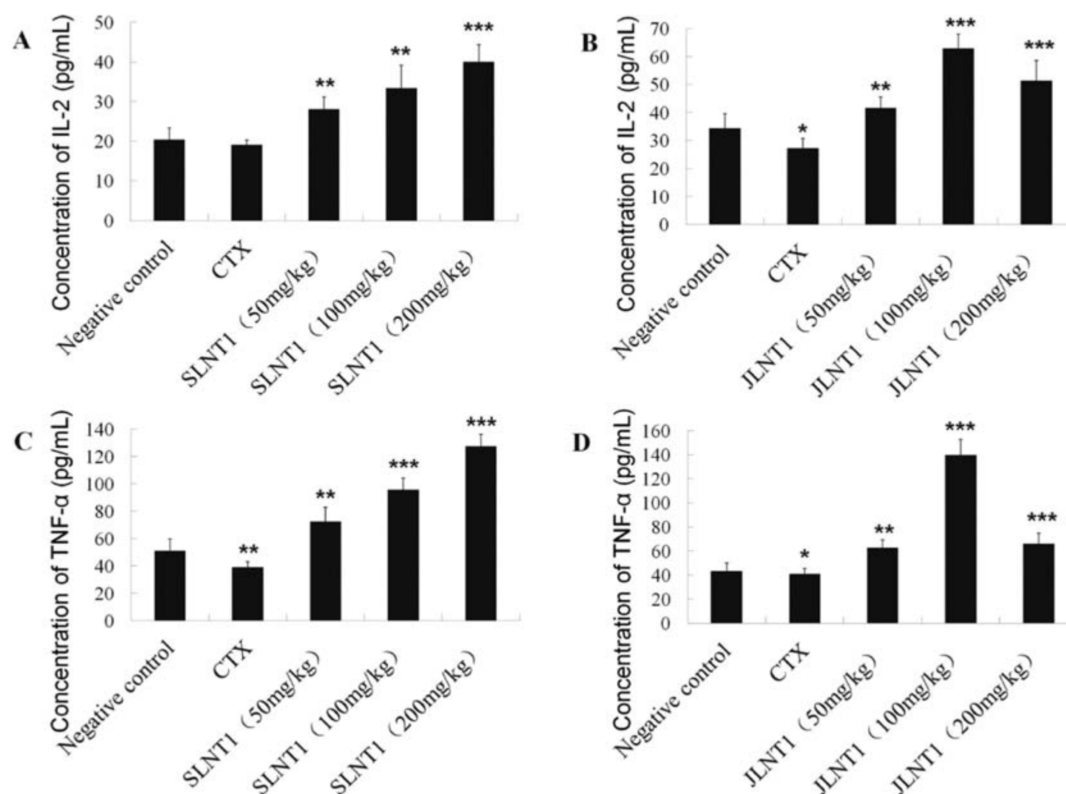

Figure 5. Effects of various doses of SLNT1 and JLNT1 on (A, C) IL-2 and (B, D) TNF-α production in serum of H22-bearing mice: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ compared to control group (without SLNT1 and JLNT1). CTX (2.5 mg/mL) served as the positive control. The data shown are the mean ± SDs (n = 8).

effective anticancer agent in vitro and in vivo in preclinical studies by inducing tumor cell apoptosis.[16] Besides, it was one of the strongest biological activity factors, which presented no obvious toxicity to normal cells when killing tumor.[37−39] What is more important, it has been reported the cytokine stimulating activity of (1→3)-β-D-glucans was dependent on the triple-helix conformation. Polysaccharides with single-helix state were identified as having less TNF-α stimulation efficiency compared with the triplex.[40] Therefore, SLNT1 and JLNT1 with triple-helix conformation were investigated for the effects of IL-2 and TNF-α production in serum by ELISA kits. The results are shown in Figure 5. The levels of IL-2 and TNF-α were decreased to some degree in CTX-treated mice compared to those of the model group, which was attributed to the immunosuppression of CTX.[41] On the contrary, the treatments of both SLNT1 and JLNT1 could obviously promote IL-2 and TNF-α production in serum and could not lead to immune dysfunction by comparison with CTX treatment. The production of IL-2 and TNF-α induced by SLNT1 reached 40.03 and 127.36 pg/mL at 200 mg/kg, respectively. Similarly, JLNT1 at a middle dose showed the strongest effects with levels of 62.85 and 139.7 pg/mL for IL-2 and TNF-α, which were in accordance with the inhibition ratios of the in vivo antitumor activity. On the basis of the results obtained, the effects of SLNT1 and JLNT1 on TNF-α production were much more prominent than that of IL-2. Moreover, our polysaccharides showed more advantages on stimulating TNF-α production compared to other polysaccharides from *Ganoderma atrum*.[41] It seemed that the treatments of SLNT1 and JLNT1 could greatly increase the release of TNF-α to kill tumor by the mechanism of inducing tumor cell apoptosis. The increase of IL-2 showed the effects of immunity. All of the results indicated that both SLNT1 and JLNT1 could present prominent antitumor activity in H22-bearing mice by releasing effective cytokines to inhibit H22 tumor growth and induce tumor cell apoptosis as reported.

Effects of SLNT1 and JLNT1 Therapy on Apoptosis. The death of tumor cells during antitumor therapy could be caused by apoptosis. Apoptosis was a kind of gene-mediated programmed cell death and a key phenomenon that might be induced by drugs in antitumor treatment.[42,43] In recent years, there were many reports about polysaccharides extracted from different kinds of mushrooms presenting excellent antitumor activities by activating the cellular apoptotic response in cancer.[44−46] Nevertheless, few reports of polysaccharide from *L. edodes* inducing tumor cell apoptosis were found. Moreover, morphologic analysis of cells also indicated that mushroom extracts might initiate apoptotic mechanisms. [47] In this study, we have observed the existence of apoptosis cells in tumor tissues of H22 tumor-bearing mice unexpectedly. As shown in Figure 6, after SLNT1, JLNT1, and CTX treatment, marked morphological changes in nucleus morphology such as karyorrhexis, nucleus fragmentation, and irregular arrangement of karyomorphism, which were typical characteristics of apoptosis cells, were observed indicating the apoptosis mechanism. Conversely, no obvious morphological changes occurred in the negative control (Figure 6A and Figure 6F) in which cells kept good original grown status and the karyomorphism was well-regulated, completed, and clearly visible. From the phenomena, we preliminarily concluded that the treatments of SLNT1 and JLNT1 could possibly lead to apoptosis in H22 tumor-bearing mice. From the effects of

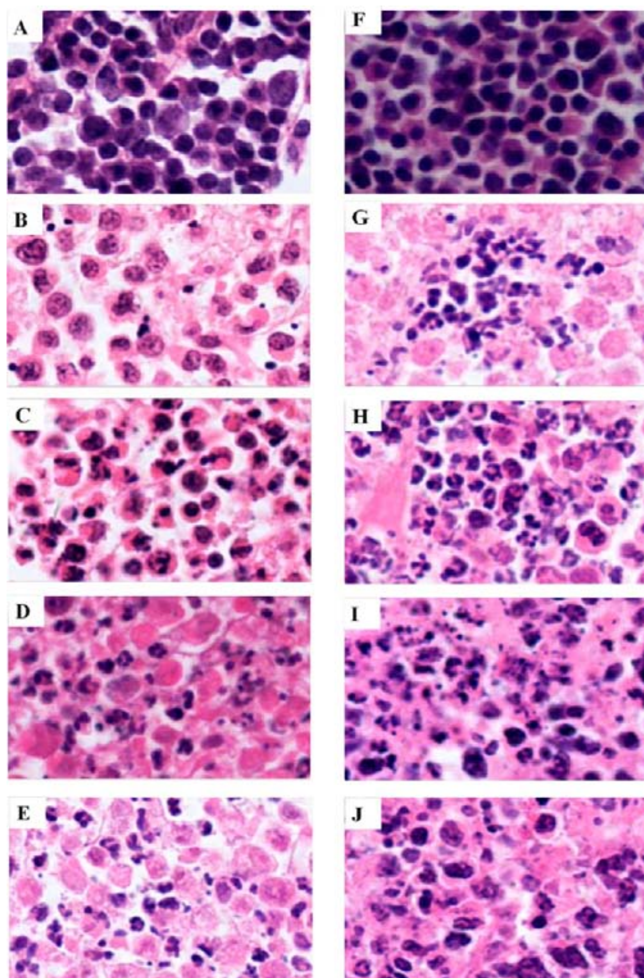

Figure 6. H&E staining analysis of histological sections of tumor tissues applied with SLNT1 (A−E) and JLNT1 (F−J), with magnification ×400: (A) negative control group in SLNT1; (B) dose of 50 mg/kg SLNT1-treated mice; (C) dose of 100 mg/kg SLNT1-treated mice; (D) dose of 200 mg/kg SLNT1-treated mice; (E) dose of 25 mg/kg CTX-treated mice serving as the positive control; (F) negative control group in JLNT1 treatment; (G) dose of 50 mg/kg JLNT1-treated mice; (H) dose of 100 mg/kg JLNT1-treated mice; (I) dose of 200 mg/kg JLNT1-treated mice; (J) dose of 25 mg/kg CTX-treated mice serving as the positive control.

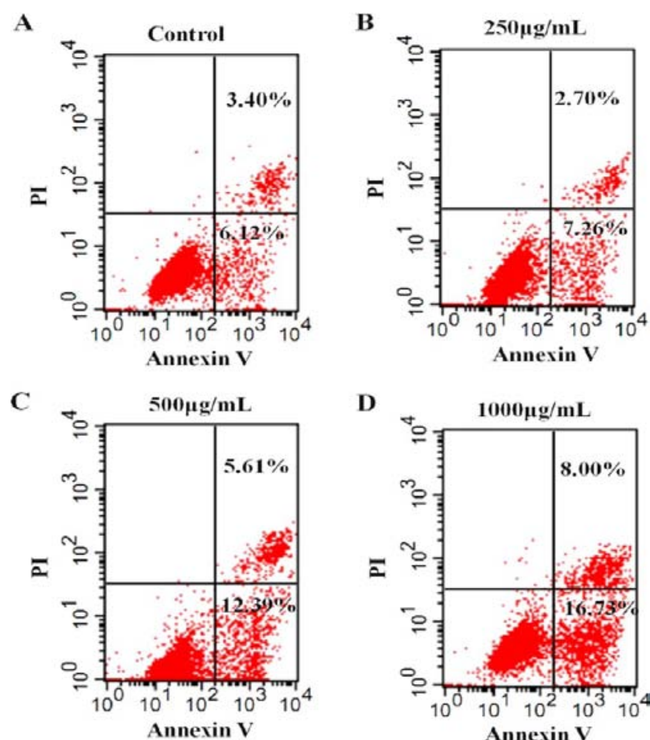

Figure 7. HepG2 cells treated with different concentrations of SLNT1 (B−D) stained with annexin V-FITC and PI. Part A served as the control. Also shown are the flow cytometric analysis results of the apoptosis rates on HepG2 cells.

animal tests, SLNT1 with good solubility and adequate absorption was selected to quantitatively analyze the apoptosis rate on HepG2 cells, which was close to human disease. The results of flow cytometry indicated that there was an increase of apoptosis rate with the treatment of SLNT1 and the apoptosis rates increased in a concentration-dependent manner (Figure 7). The highest apoptosis rate of SLNT1 reached 15.21% (Figure 7D). Consequently, morphologic analysis of cells and determination of the apoptosis rate illustrated that the antitumor activity of SLNT1 may be associated with inducing tumor cell apoptosis mechanism and further molecular mechanisms possibly related to apoptosis proteins, such as Bcl-2, Bax, and caspase3.

All results in animal studies showed that the best inhibition effects of both SLNT1 and JLNT1 in H22-bearing mice were higher than that of reported lentinan, which was only 25.2% in S180-bearing mice by enhancing immunity.[48] Furthermore, compared with the heteropolysaccharide isolated from *L. edodes*, whose inhibition ratio just was 45.9% on S180-bearing mice,[9] our polysaccharides exhibited higher antitumor activity distinctly by more than one mechanism. Apart from enhancing immunity, both inducing tumor cell apoptosis and directly killing tumor could also participate in antitumor therapy so that the best inhibition ratio on H22-bearing mice almost reached 65.41%. SLNT1 and JLNT1 showed good advantages on antitumor effects compared with other similar polysaccharides, such as *Ganoderma lucidum* polysaccharide extracted from fungi, which did not show any direct inhibitory effect against the proliferation and apoptosis of S180.[49] They were the most potent antitumor agents based on their high inhibition ratios.

At present, the proposed mechanism of LNT exerting antitumor effects was generally accepted by enhancing the immunity. However, different mechanisms were put forward based on our study, especially for inducing tumor cell apoptosis, the molecular mechanisms of which were complicated and could be regulated by multiple signaling pathways, such as mitochondria-mediated apoptotic pathway and death receptor-mediated apoptotic pathway. Therefore, the apoptosis proteins and some possible signaling pathways, like activating MAPK pathway and inhibiting PI3K-Akt/NF-κB pathway, are worthy to be conducted in our subsequent work.[50−52]

In summary, five novel polysaccharides were isolated from *L. edodes* using two different methods. All of them only consist of glucose and had similar structures of (1→3)-linked-β-D-glucan. Besides, they had triple-helix conformation and exhibited significant antitumor activity in vitro involving directly killing tumor. Two polysaccharides (SLNT1, JLNT1) with the highest molecular weights and the best antitumor activities in vitro were chosen to test their effects in H22-bearing mice. Then SLNT1 with good solubility and adequate absorption was further selected to detect the apoptosis rate on human HepG2

cells. Our results demonstrated that the antitumor activities of SLNT1 and JLNT1 could be resulted from inducing tumor cell apoptosis and directly killing tumor besides improving immunity. Therefore, our studies could provide new evidence for future human solid tumor therapy by the treatments of SLNT1 and JLNT1, which could be used for potential application in cancer therapy as antitumor functional components in combination with other drugs.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

L. edodes, *Lentinus edodes*; TNF-α, tumor necrosis factor α; IL-2, interleukin 2; HPGPC, high performance gel permeation chromatography; CTX, cyclophosphamide; ELISA, enzyme-linked immunosorbent assay; PI, propidium iodide

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