Original Article

DOI: 10.5582/ddt.2015.01023

A novel polysaccharide isolated from *Litchi chinensis* by using a simulated gastric medium and its immunomodulatory activity

Lishan Wang[1,*], Xianjing Hu[2,*], Sixue Bi[2,*], Wensong Tu[1], Yongshuai Jing[1], Liyan Song[2,], Wenjie Lv[1], Rongmin Yu[1,**]**

[1] *Biotechnological Institute of Chinese Materia Medica, Jinan University, Guangzhou, China;*
[2] *Department of Pharmacology, Jinan University, Guangzhou, China.*

Summary **A novel polysaccharide (LCPA50-S1) with immunomodulatory activity was extracted with simulated gastric medium from *Litchi chinensis*, and purified by DEAE-52 cellulose column, Sephadex G-50 column and Sephacryl S-300 HR chromatography. The structural characteristics of LCPA50-S1 were expounded through complete acid hydrolysis, partial acid hydrolysis, methylation and instrumental analysis. The results demonstrated that LCPA50-S1 is a heteropolysaccharide with a molecular weight of 1.58×10^5 Da. The backbone was composed of (1→4)-linked β-D-glucopyranosyl residues, (1→6)-linked β-D-galactopyranosyl, (1→3,6)-linked β-D-galactopyranosyl residues, (1→4,6)-linked α-D-glucopyranosyl residues and branched at O-6. The branches were consisted of (1→2)-linked α-L-rhamnopyranosyl residues, (1→4)-linked β-D-glucopyranosyl residues, and (1→6)-linked β-D-galactopyranosyl, terminated with (1→)-linked α-L-arabinopyranosyl residues and (1→)-linked β-D-galactopyranosyl residues, respectively. The immunoregulatory activity of LCPA50-S1 was evaluated through determination the effect of LCPA50-S1 on nitric oxide (NO) production of RAW264.7 macrophages and spleen lymphocyte proliferation as well as its cytokines secretion level. The results demonstrated that LCPA50-S1 increased NO and TNF-α production in RAW264.7 macrophages significantly, enhanced the proliferation as well as the interleukin-2 (IL-2) production of splenocytes. The data indicated that LCPA50-S1 had the potential to be explored as a novel natural immunomodulator for application in functional foods and medicine.**

Keywords: *Litchi chinensis*, polysaccharide, structure characterization, immunomodulatory activity

1. Introduction

Litchi (*Litchi chinensis* Sonn.), originating from China, is a subtropical fruit belonging to Sapindaceae. As a kind of health-care fruit, litchi contains plenty of nourishments and is considered to be of excellent medicinal value. Recent investigations indicated that the polysaccharides from litchi pulp had antioxidant and immunomolulatory effects *in vitro* (1,2). In addition, litchi has been a kind of favorite fruit from ancient times due to its unique flavor and cultural background.

Polysaccharides are a class of biomacromolecules composed of more than ten monosaccharide units, which generally exist in plants, animals, algae and microorganisms such as fungi and bacteria. Because polysaccharides provide nutritional support for developmental process, they play an important role in the growth of living organisms. Moreover, the interest in natural resources and the regulation of antioxidant, immune ability, anti-tumor effects as well as low toxicity leads to the wide applications of polysaccharides in the medicine and food fields (*3*).

The development of modern science, cytobiology and molecular biology makes people realize that the disorder of the immune system can result in a variety of diseases. Some crude polysaccharides also had effect on the lymphocyte proliferation (*4*). LCPA50W, isolated from *litchi chinensis*, was confirmed to promote the

*These authors contributed equally to this works.
***Address correspondence to:*
Dr. Liyan Song and Dr. Rongmin Yu, Department of Pharmacology, Jinan University, Guangzhou, China.
E-mail: tsly@jnu.edu.cn (Song LY), tyrm@jnu.edu.cn (Yu RM)

proliferation of mouse splenocytes and enhance the cytotoxicity of NK cells (*2*). The production of cytokines is a key event in the initiation and regulation of an immune response. Therefore, we tried to explore the effect of LCPA50-S1 on the secretion of cytokines from macrophages and spleen lymphocytes.

The polysaccharides reported in the research paper mentioned above were extracted by hot water method. Although the traditional extraction is simple, the high temperature may have an impact on the bioactivity of polysaccharides (*5*). Furthermore, different specific structures and biological activity of polysaccharides may be obtained from different extractions (*6*). Therefore, in our study, we chose the stimulated gastric medium to isolate the polysaccharides of litchi, and purify through DEAE-52 cellulose, Sephadex G-50, and Sephacryl S-300 HR column chromatography. Then detailed structure characterization and immunomodulatory activity were also investigated.

2. Materials and Methods

2.1. *Plant materials and reagents*

Litchi chinensis was obtained from Guangzhou Yide Food Market, China. The material was identified to be Feizixiao by Professor R. M. Yu, Biotechnological Institute of Chinese Materia Medica, Jinan University, Guangzhou, China. The seed and peel were removed manually to get the pulp tissues. DEAE-52 cellulose and Sephadex G-50 were purchased from Whatman Ltd. (England). Sephacryl S-300 HR was purchased from Amersham Biosciences (Swenden). Standard monosaccharides, T-series dextrans were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pepsin was purchased from Guangzhou Qiyun Biotechnology Company. All chemicals and reagents were of analytical grade.

Lipopolysaccharides (LPS) were obtained from Sigma Chemical Co. (USA). RPMI 1640 medium, RPMI DMEM medium, Trizol reagent and fetal bovine serum (FBS) were purchased from Gibco Invitrogen Corp. (USA). ELISA test kits for interleukin-2 (IL-2) and tumor necrosis factor (TNF-α) were obtained from RayBiotech Systems (USA). The NO assay kit was obtained from Beyotime Biotech Inc. (China).

2.2. *Animals and cells*

Kunming mice (grade II, SCXK 2011-2015, 20-25 g, 6-8 weeks old) were obtained from the Experimental Animal Center, Sun Yat-sen University, China. Animals were acclimatized for at least 7 days prior to use and maintained in a temperature-controlled environment (22 ± 2°C) with a 12 h light-dark cycle and provided with free access to water and standard rodent chow. Raw264.7 cell line was obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). It was maintained in RPMI DMEM medium supplemented with100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% FBS at 37°C under humidified air with 5% CO_2.

2.3. *Separation of crude polysaccharide LCPA50*

The pulp tissues of L. *chinensis* (500 g) were defatted with 80% ethanol for 6 h at 85°C, and dried in the open air, then immersed in a solution of simulated gastric juice (10 L, pH 2.0). The artificial gastric juice was used to mimic the conditions of the stomach. Simulated gastric juice was formulated from HCl (1.34 g/L), NaCl (2.16 g/L), KH_2PO_4 (0.63 g/L), $CaCl_2$ (0.12 g/L), KCl (0.39 g/L), and pepsin (0.53 g/L). Extraction was performed at 37°C for 4 h and repeated twice (*6*). All the extracts were put together, adjusted to a pH of 7 with NaOH and filtered to remove the insoluble material. Then 95% ethanol was added to a final concentration of 50%, and the solution was stored at 4°C overnight. The precipitate was dissolved in distilled water. To clear off the protein, we used Sevage method ($CHCl_3$/BuOH = 4:1, v/v) for 20 min, and repeated the procedure seven times. The supernatant was concentrated at 40°C by using a vacuum rotary evaporator and then passed through an ultrafiltration cell with membrane pores of 3000 Da. Finally, the crude polysaccharide named LCPA50 was collected after lyophilizing.

2.4. *Preparation and purification of LCPA50-S1*

To purify the crude polysaccharide, anion-exchange DEAE-52 cellulose chromatography was used. LCPA50 was dissolved in distilled water, then centrifuged and filtered through 0.45 μm filters. The filtrate was injected to DEAE-52 cellulose column (5.0 × 70.0 cm). The column was eluted by distilled water, a linear gradient from 0 to 0.5 M NaCl at a flow rate of 0.8 mL/min. Fractions (10 mL) were collected. Phenol-sulfuric acid method was used to monitor the carbohydrate content. Consequently, the two sharp peaks were combined respectively, dialyzed, concentrated and lyophilized (Figure 1). Afterwards, we chose size exclusion Sephadex G-50 column (1.6 × 70 cm) and Sephactyl

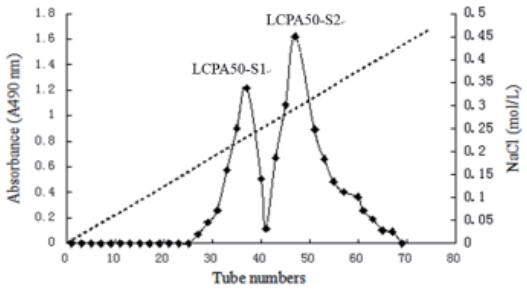

Figure 1. Profile of LCPA50 in DEAE-52 cellulose chromatography.

S-300 HR (1.2 × 90 cm) to purify the polysaccharides. By eluted with distilled water, two polysaccharides of high purity, namely LCPA50-S1, LCPA50-S2 were obtained. LCPA50-S1 was used in the structural analysis and bioactive evaluation.

2.5. *General quantification analysis of LCPA50-S1*

Phenol-sulfuric method was used for the detection of the content of polysaccharide, and glucose was used as the standard (*7*). Uronic acid content was measured by the carbazole-sulfuric acid method using gulcuronic acid as a standard. Protein content was determined by using Bradford reagent and bovine serum albumin was taken as standard.

IR spectra were recorded with a Tensor 27 Bruker instrument. 1 mg LCPA50-S1 was ground with KBr power and pressed into pellets for FT-IR measurement in the frequency range of 4,000-500 cm^{-1} (*8*).

2.6. *Characterization of LCPA50-S1*

2.6.1. *Measurement of molecular weight*

The molecular weight of the LCPA50-S1 was determined by gel permeation chromatography (GPC). A Sephacryl S-300 HR (1.2 × 90 cm) was used, and the mobile phase was distilled water. The LCPA50-S1 molecular weight was measured by comparison to a calibration curve made with T-series Dextran (T-200, T-100, T-70 and T-7), glucose and dextran blue. Then by calculating we get the molecule of LCPA50-S1 (*9*).

2.6.2. *Analysis of monosaccharide composition and partial acid hydrolysis*

LCPA50-S1 (5 mg) was hydrolyzed with 2 mL 2 M trifluoroacetic acid (TFA) at 110°C in a sealed tube for 6 h. After the hydrolysis was completed, azeotropic distillation method was applied to remove the excess TFA by evaporation with methanol. The monosaccharide content was measured by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The hydrolysate (20 μL) was dissolved in distilled water (1 mL) and then passed through a 0.45 μm filter. Finally, the solution was injected into the HPAEC-PAD system (Dionex-5500, Dionex Corp., Canada) (*10*).

LCPA50-S1 (25 mg) was hydrolyzed with 0.05 M TFA for 6 h at 100°C in a sealed tube and then dialyzed with distilled water for 48 h. The distilled water outside the dialysis bag was collected, and concentrated up to dryness. To remove the extra TFA in fraction 1, methanol was added to evaporate with. The fraction in the dialysis bag (3,000 Da) was dried and then hydrolyzed with 0.5 M TFA. After dialyzing with distilled water for 48 h, we got the distilled water containing fraction 2 and the hydrolysate, fraction 3, respectively. Afterwards, fractions 1-3 were hydrolyzed with 2 M TFA and analyzed by HPAEC-PAD.

2.6.3. *Periodate oxidation and Smith degradation analysis*

LCPA50-S1 (15 mg) was dissolved in 25 mL distilled water, and then 80 mg $NaIO_4$ was added into the solution. The solution was kept in the dark at 4°C, 50 μL aliquots were withdrawn at 6 h intervals, diluted to 10 mL with distilled water and read in a spectrophometer at 223 nm. Consumption of HIO_4 was measured by a spectrophotometric method, and formic acid production was measured by titration with 0.041 M NaOH. The reaction mixture was dialyzed with distilled water. The nondialysate was concentrated and reduced with $NaBH_4$ (30 mg) for 24 h at room temperature. Then add 0.1 M HOAc to neutralize to pH 6.0. After dialysis and concentration to dryness, the mixture product was hydrolyzed with 2 M TFA (2 mL) at 110°C for 6 h and tested by HPAEC-PAD.

2.6.4. *Methylation analysis*

Methylation of LCPA50-S1 was carried out according to the method of Needs and Sevendran with minor modifications (*11*). Eight-hundred mg NaOH and 8 mg dry LCPA50-S1 were precisely weighed, and then dissolved in 8 mL DMSO, dispersed using a blender. The mixture was treated with an ultrasonic wave attached to an ultrasonic cleaner for 30 min. 4.5 mL methyl iodide was added for methylation after incubation for 1h at 25°C. The reaction was kept in darkness at room temperature for 6 h. Distilled water (4 mL) was added to terminate the reaction. The reaction product was extracted with 3 × 2 mL of chloroform and dried on a rotary evaporator at low pressure. The methylated polysaccharide was acid hydrolyzed using TFA and then reduced with $NaBH_4$. Finally, acetic anhydride was used to obtain the acetylated polysaccharide. Alditol acetates were analyzed by GC-MS.

2.6.5. *NMR spectroscopy*

LCPA50-S1 (20 mg) was dissolved in 0.6 mL of D_2O. ^{13}C NMR spectra was recorded on a Bruker AMX 500 FT spectrometer (Bruker, Germany) at 24°C. Chemical shift was expressed in ppm.

2.7. *Splenocyte proliferation assay in vitro (5)*

A spleen was separated from Kunming mice under aseptic condition and kept in phosphate-buffered saline (PBS) solution. The organ was crushed and passed through a steel mesh (200 mesh) to obtain a homogeneous cell suspension. The splenocytes were

collected and washed twice with PBS after removing the red blood cells through adding 0.83% NH_4Cl in 0.01 M Tris-HCl (pH 7.2). Next, the pelleted cells were resuspended and diluted to 5×10^6 cells/mL with RPMI-1640 containing 10% of FBS, and further inoculated into a 96-well microtiter plate (100 μL/well). Different concentrations of LCPA50-S1 was added and the final concentration was 0, 31.3, 62.5, 125, 250, and 500 μg/mL, respectively. ConA (5 μg/mL) was selected as the positive control. The plates were then incubated at 37°C in humidified incubator with 5% CO_2 for 48 h. All the tests were carried out in triplicate and the cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated for another 4 h in dark. The plates were centrifugated to remove the supernate and then 200 μL of DMSO was added into each well to dissolve the MTT-formazan crystals. The plates were shaken for 10 min to dissolve the crystals. Absorbance of each well was detected spectrophotometrically at 570 nm.

2.8. *Determination of IL-2 secretion from splenocytes*

Splenocytes were collected according to the reference (*2*) and was treated with different concentrations of LCPA50-S1 (0, 31.3, 62.5, 125, 250, and 500 μg/mL) for 48 h. The cell supernatant was collected by centrifugation at 1,500 rpm for 10 min at 4°C. IL-2 level in culture supernatant was measured with an ELISA kit according to manufacturer's instruction.

2.9. *Cytotoxicity assay*

MTT assay was selected to evaluate the effect of LCPA50-S1 on RAW264.7 cells. Briefly, RAW264.7 cells were seeded in 96-well microplates at 2×10^4 cells/mL and treated by different concentrations of LCPA50-S1 (0, 31.3, 62.5, 125, 250, and 500 μg/mL) for 48 h. 20 μL of MTT (5 mg/mL) was added into each well and co-incubated for an additional 4 h in dark. Then the supernatant was discarded and 200 μL of DMSO was added to solubilize the MTT-formazan crystals. The absorbance of each well was detected by a microplate reader at 570 nm.

2.10. *Measurement of NO production*

RAW264.7 cells were seeded in a 96-well plate at 2×10^4 cells/well in DMEM medium and incubated at 37°C in a humidified atmosphere with 5% CO_2. After incubation for 24 h, the cells were treated with series of concentrations of LCPA50-S1 (0, 15.6, 31.3, 62.5, 125, and 250 μg/mL) for 24 h. LPS (1 μg/mL) was selected as the positive control. The culture supernatant of RAW264.7 cells was collected after treatment of LCPA50-S1 for 24 h and the NO levels of supernatant were determined by Griess reaction. Briefly, the supernatant was distributed in a 96-well plate at 50 μL/well, and equal volumes of the Griess reaction solutions and Griess reaction solutions II were added. The absorbance of each well in the 96-well plate was detected spectrophotometrically at 540 nm and the contents of NO were calculated through a least squares linear regression analysis of a sodium nitrite standard curve.

2.11. *Determination of TNF-α secretion*

The RAW264.7 cells were treated with LCPA50-S1 (0, 31.3, 62.5, 125, 250, or 500 μg/mL) and LPS (1 μg/mL) for 24 h and then the supernatant was collected for the detection TNF-α secretion. TNF-α levels were detected *via* a commercial ELISA kit. The detailed procedure for determination TNF-α levels was carried out according to the manufacturer's instruction.

3. Results

3.1. *Purity and chemical compositions of polysaccharide*

Crude polysaccharide was purified by a 3,000 Da membrane, and the fraction with a molecular weight over 3,000 Da was collected and lyophilized. The crude polysaccharide (2.23 g) was obtained with a 0.44% yield from the raw plant material. After purification *via* DEAE-52 cellulose, Sephadex G-50 and Sephacryl S-300 HR column chromatography, the profile of LCPA50-S1 appeared as a single and symmetrical sharp peak (Figure 2), which was detected by the phenol-sulfuric acid method. The yield of LCPA50-S1 from crude polysaccharide was 37.7%.

Total carbohydrate content was measured to be 98.2% (w/w), and LCPA50-S1 did not contain any protein. The uronic acid content of LCPA50-S1 was below the detection limit. The optical rotation of LCPA50-S1 was

$$[\alpha]_D^{20} = +131° \text{ (c 1.0, } H_2O)$$

Complete acid hydrolysis of LCPA50-S1 performed by treatment with 2 M TFA allowed for the detection of

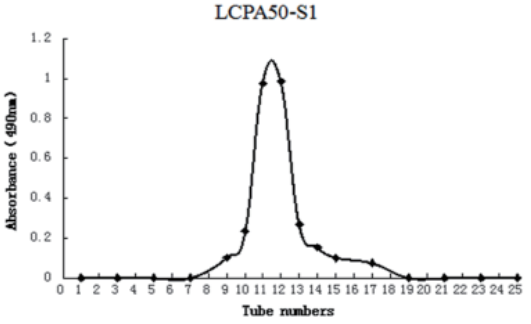

Figure 2. Profile of LCPA50-S1 in Sephacryl S-300 HR column chromatography.

the following monosaccharides in the hydrolysate, which were identified by HPAEC-PAD: rhamnose, arabinose, galactose and glucose with ratio of 1.00:1.37:9.96:8.44. The monosaccharide composition of LCPA50-S1 is presented in Table 1. Fractions 1-3 were obtained through partial acid hydrolysis.

The component of fraction 3 in Table 1 showed that galactose and glucose approximated to be the backbone of the structure of LCPA50-S1. The branched structure of LCPA50-S1 was composed of galactose, glucose and rhamnose. The LCPA50-S1 was terminated with arabinose and galactose from the analysis results of fractions 1 and 2.

3.2. *Molecular weight of LCPA50-S1*

The purified polysaccharide LCPA50-S1 was a white loose powder, odorless, and soluble in water and showed a single and relatively symmetrical peak on GPC, indicating its homogeneity (Figure 2).

From GPC analysis on a Sephacryl S-300 HR column, a calibration curve was obtained by using various Dextran T-series standards of known molecular weights. According to the calibration curve, logMw = 0.9209 − 0.4 Kav (R^2 = 0.991), Kav was the retention volume and the average molecular weight of the polysaccharide was calculated to be 1.58×10^5 Da.

3.3. *Structural Characterization of LCPA50-S1*

The UV absorption spectra of LCPA50-S1 showed no absorption at 280 or 260 nm, implying that there was no protein and nucleic acid in this polysaccharide. The infrared spectrum of LCPA50-S1 showed special absorption bands at 3,428, 2,925, 1,636, 1,422, and 1,076 cm^{-1} (Figure 3). In the FT-IR spectrum, the characteristic bands in the regions of 3,428, 2,925, and 1,636 cm^{-1} belonged to O-H bending, C-H bending, and associated water, respectively. The band at 1,076 cm^{-1} indicated pyranose. The absorption bands at 890 and 810 cm^{-1} showed that LCPA50-S1 contained both α- and β-type glycosidic linkages in its structure (*12*). There was no absorption at 1,740 cm^{-1}, indicating the absence of uronic acid in the polysaccharide structure, which was consistent with the result of the carbazole-sulfuric acid method.

The periodate-oxidized products were fully hydrolyzed and analyzed by HPAEC-PAD after periodate oxidation. The results shown in Table 1 indicated that there was only galactose in the oxidation products. The presence of galactose revealed that residues of galactose had (1→3)-linked, (1→2,3)-linked, (1→2,4)-linked, (1→3,6)-linked, or (1→2,3,4)-linked, which could not be oxidized, respectively. Moreover, the other monosaccharides could be inferred that linkages of were (1→), (1→2), (1→6), (1→4) and (1→4,6), which might be oxidized to produce glycerol (*7,12*).

Table 1. Monosaccharide composition and molar ratio of LCPA50-S1

Items	Molar ratios			
	Rhamnose	Arabinose	Galactose	Glucose
LCPA50-S1	1.00	8.44	9.96	1.37
fraction 1	n.d.	3.02	1.00	n.d.
fraction 2	1.00	n.d.	1.21	2.55
fraction3	n.d.	n.d.	5.62	1.00
Smith degradation	n.d.	n.d.	1.00	n.d.

n.d., not detected.

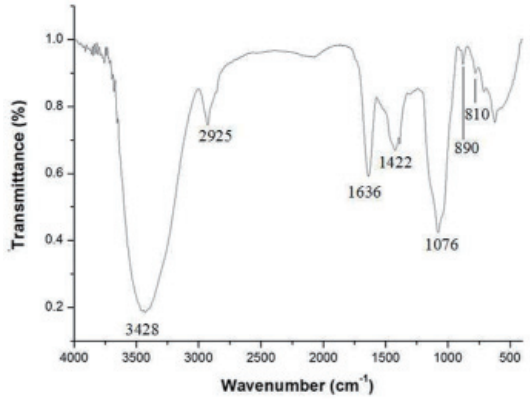

Figure 3. FI-IR spectrum of LCPA50-S1.

3.4. *Methylation and GC-MS*

Methylation analysis offers the information on the positions at which monosaccharide residues are substituted and their relative abundance in the polysaccharide. The polysaccharide was methylated completely, which was confirmed by the IR spectrum.

The profile of methylated LCPA50-S1 on GC-MS appeared seven peaks. Methylation analysis of fractions LCPA50-S1 showed the presence of seven components (Table 2), namely 2,3,5-Me_3-Ara, 2,3,4-Me_3-Gal, 2,3,6-Me_3-Glc, 2,3-Me_2-Glc, 3,4-Me_2-Rha, 2,3,4,6-Me_4-Gal, 2,4-Me_2-Gal. On the basis of the standard data in the CCRC Spectral Database for PMAA's, and according to the mass spectrum patterns from the literatures (*2,12-14*), the linkages of L-rhamnose, L-arabinose, and D-galactose were deduced as (1→), (1→2), (1→6), and (1→3,6), whereas the linkages of D-glucose were deduced as (1→4) and (1→4,6). This result showed correlation between terminal and branched residues. In addition, the

Table 2. Methylation Analysis of LCPA50-S1

Methylated sugar	Major mass fragments (m/z)	Linkage pattern
2,3,5-Me_3-Ara	43, 45, 71, 87, 101, 117, 129, 161	T→ linked Ara
2,3,4,6-Me_4-Gal	45, 87, 101, 117, 145, 161, 205	T→ linked Gal
2,3,6-Me_3-Glc	43, 87, 99, 101, 113, 117, 161, 233	1→4 linked Glc
3,4-Me_2-Rha	87, 99, 129, 131, 189	1→2 linked Rha
2,3,4-Me_3-Gal	43, 71, 87, 101, 161, 189, 203, 233	1→6 linked Gal
2,3-Me_2-Glc	43, 58, 101, 117, 203, 261	1→4,6 linked Glc
2,4-Me_2-Gal	43, 87, 101, 189	1→3,6 linked Gal

monosaccharide composition and linkages also agreed with the analysis of LCPA50-S1 described above.

3.5. *NMR analysis of LCPA50-S1*

The spectra of ^{13}C NMR of LCPA50-S1 is shown in Figure 4. Signals of LCPA50-S1 in spectra were analyzed on the basis of the data available in the literatures (*2,13,14,32*). The resonances in the region of 95.0-110.0 ppm in ^{13}C NMR were attributed to the anomeric carbon atoms of D-galactose (Galp), D-glucose (Glcp), L-rhamnose (Rhap), and L-arabinose (Arap). The peaks at 98.6 ppm corresponded to C-1 of (1→2)-linked α-L-Rha unit, 102.7 ppm corresponded to C-1 of (1→3,6)-linked β-D-Gal unit, 103.4 ppm corresponded to C-1 of T→linked β-D-Gal unit, 104.5 ppm corresponded to C-1 of (1→6)-linked β-D-Gal unit, 99.7 ppm corresponded to C-1 of (1→4,6)-linked α-D-Glc unit, 107.9 ppm corresponded to C-1 of (1→4)-linked β-D-Glc unit, and 109.2 ppm corresponded to C-1 of (1→)-linked α-L-Ara unit, respectively. The assignment of the carbon atom signals is shown in Table 3.

On the basis of the results of HPAEC-PAD, GC-MS and NMR, the backbone is composed of (1→4)-linked β-D-glucopyranosyl residues, (1→6)-linked β-D-galactopyranosyl, (1→3,6)-linked β-D-galactopyranosyl residues, and (1→4,6)-linked α-D-glucopyranosyl residues, which both branched at O-6. The branches are consisted of (1→2)-linked α-L-rhamnopyranosyl residues, (1→4)-linked β-D-glucopyranosyl residues, and (1→6)-linked β-D-galactopyranosyl, terminated with (1→)-linked α-L-arabinopyranosyl residues and (1→)-linked β-D-galactopyranosyl residues, respectively. From all above results, the predicated structure of the repeating unit of LCPA50-S1 could be proposed as Figure 5.

3.6. *Effect of LCPA50-S1 on splenocyte proliferation*

Splenocyte proliferation firmly reflects the immunity of biological organism. Therefore, we evaluated the effect of LCPA50-S1 on splenocyte proliferation *via* MTT assay. As shown in Figure 6, LCPA50-S1 significantly promoted splenocyte proliferation in a concentration-dependent manner, indicating that LCPA50-S1 had the potential of immunoregulation.

3.7. *Effect of IL-2 production of spleen lymphocyte cells*

We further detected the effect of LCPA50-S1 on production of IL-2, an important cytokine in the immune responses, *via* an ELISA kit. The results demonstrated that LCPA50-S1 significantly enhanced the IL-2 production of spleen lymphocyte cells in a concentration-dependent manner (Figure 7).

3.8. *Effect of LCPA50-S1 on viability of RAW264.7 cells*

MTT assay was selected to investigate the effect of LCPA50-S1 on the growth of RAW264.7 cells. As shown in Figure 8, LCPA50-S1 (31.5-500 μg/mL) had little cytotoxicity on RAW264.7 cells after treatment for 48 h.

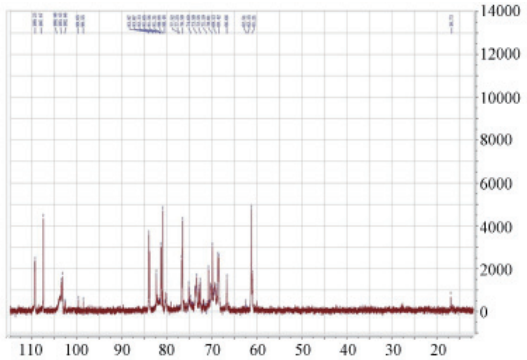

Figure 4. ^{13}C NMR spectrum of LCPA50-S1.

Table 3. Chemical shifts of resonances in the ^{13}C spectra of LCPA50-S1

Sugar residues	Chemical shift (ppm)						
	C1	C2	C3	C4	C5	C6	CH_3
α-L-Ara(1→	109.2	81.6	74.7	83.9	66.7		
β-D-Gal(1→	103.4	69.1	81.3	71.2	74.7	61.2	
→4)-β-D-Glc(1→	107.9	77.5	77.5	82.3	73.5	62.2	
→2)-α-L-Rha(1→	98.6	81.7	68.4	70.0	69.1		16.7
→6)-β-D-Gal(1→	104.5	77.3	73.5	76.6	77.3	62.5	
→4,6)-α-D-Glc(1→	99.7	71.2	72.6	76.5	71.2	74.7	
→3,6)-β-D-Gal(1→	102.7	72.6	80.5	70.0	69.1	70.0	

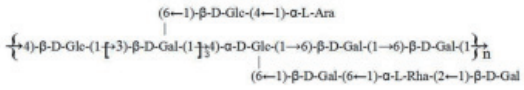

Figure 5. Predicated structure of the repeating unit of LCPA50-S1.

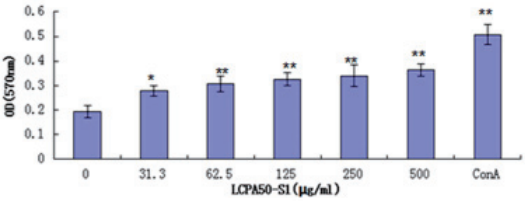

Figure 6. Effect of LCPA50-S1 on splenocyte proliferation.

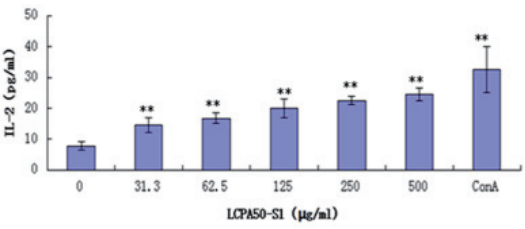

Figure 7. Effect of LCPA50-S1 on the production of IL-2 of spleen lymphocytes.

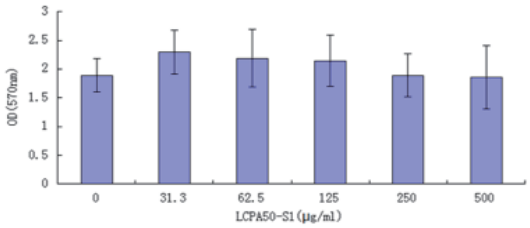

Figure 8. Effect of LCPA50-S1 on cell viability of RAW264.7.

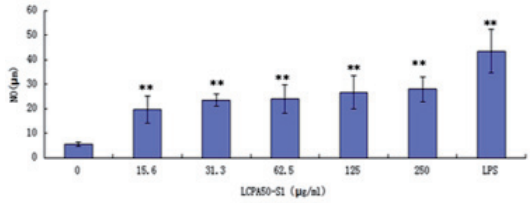

Figure 9. Effect of LCPA50-S1 on the NO secretion from RAW264.7 cells.

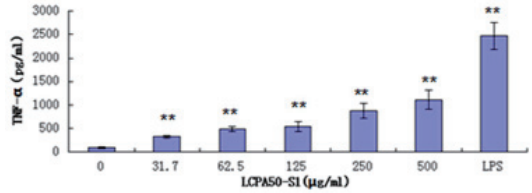

Figure 10. Effect of LCPA50-S1 on the TNF-α secretion from RAW264.7 cells.

3.9. *Effect of LCPA50-S1 on NO production of RAW264.7 cells*

NO is considered to be a major effector molecule produced by macrophages and reflects the activation capacity of macrophage. We subsequently detected the effect of LCPA50-S1 on NO production of RAW264.7 cells. As shown in Figure 9, comparing with the untreated group, the level of NO in the LCPA50-S1 treated group remarkably increased in a concentration-dependent manner.

3.10. *Effect of LCPA50-S1 on TNF-α secretion from RAW264.7 cells*

The effect of LCPA50-S1 on TNF-α secretion of RAW264.7 cells was also evaluated via an ELISA kit. As shown in Figure 10, LCPA50-S1 significantly increased the level of TNF-α in the supernatant of RAW264.7 cells after treatment for 24 h, indicating that LCPA50-S1 could remarkably enhance the TNF-α secretion from RAW264.7 cells.

4. Discussion

Hot water is commonly used to extract the crude polysaccharide of *Litchi chinensis*, and the water-soluble polysaccharide has the backbone substituted with glucose and rhamnose residues (*2,13*). However, to a large extent, the monosaccharide composition and structure of polysaccharide depend on extraction procedure. In the present study, we revealed the composition and structural features of LCPA50-S1, which was extracted from *Litchi chinensis* under conditions closely mimicking the normal fasting human stomach. The pulp tissues were incubated with simulated gastric juice (pH 1.5) at 37°C for 4 h. The pepsin is a protease, whose pH is 1.5-2.5 (*15*). Moreover, suitable dissolution media for simulating the fasted state gastric conditions will have pH values between pH 1.5 and pH 2.0. Therefore, pH 2.0 was chosen in this experiment. The extraction time of 4 h was selected to cover residence time for gastric exposure as most solids are emptied within 4 h (*16*).

Contrast with previous reports, the monosaccharide galactose was identified as the main constituents in the sugar chains of LCPA50-S1. It is generally known that the immunomodulating actions of polysaccharides are related to their molecular weight, chemical composition, glycosidic linkage, conformation, degree of branching and so on. This structural variability could affect the biological activities of these molecules profoundly. Many studies on pharmacology and phytochemistry have demonstrated that the polysaccharides from *Lycium barbarum* (LBP) had the backbones mainly composed of (1→3)-β-Galp, (1→4)-β-Galp, (1→6)-β-Galp, (1→6)-α-glucans, and (1→4)-α-polygalacturonans (*3*). Though the polysaccharides are terminated with different branches or sugar residues, the antioxidant activity and immunomodulatory activity were proved by many experimental results. The LCPA50-S1 monosaccharide ratio showed higher content of galactose than that isolated by hot water. Therefore, the LCPA50-S1 was supposed to have good immunomodulatory activity.

The immune system is the human's ultimate defense against infectious diseases, tumor and cancer growth. Immunostimulation is regarded as an important strategy for improving the body's defense mechanism in elderly people and cancer patients. The immunologic action of polysaccharides may begin with activating major subsets of immune cells such as lymphocytes and macrophages (*17*). Therefore, we tried to explore the effects of LCPA50-S1 on the activation of macrophages and lymphocyte proliferation.

The immune response including cellular and humoral immunity, characterized by T cells and B cells respectively, plays an important role in the host defense system such as anti-tumor and infectious defenses (*18*). The capacity to elicit an effective T and B cells immunity could be shown by the stimulation of lymphocyte proliferation response (*19*). The splenocyte proliferation is an indicator of immunoenhancement and related to immunity improvement of T-lymphocytes or B-lymphocytes (*19,20*). The splenic lymphocytes proliferation was considered as an important indicator

for studying immune response and evaluating cellular immune function. After stimulated by antigenic properties, the T and B lymphocytes, which are considered as the most important immune effector cells, can proliferate and differentiate, generate specific immune response, and produce lymphokine and antibody (*23*). To investigate the immunoregulatory function, we detected the effect of LCPA50-S1 on splenic lymphocyte proliferation. As shown in Figure 6, the A570 values of LCPA50-S1-treated cells were remarkably higher than those of the LCPA50-S1-untreated cells. It demonstrated that both T and B cells may be activated by LCPA50-S1.

The overall immune response elicited within each individual tumor results from the balance of the type of cells and cytokines secreted. Some cytokines may help to reject tumor cells whereas others promote tumor growth. Previous studies showed that polysaccharides from *Coriolus versicolor* can induce T cell proliferation and production of interfero-γ (INF-γ) as well as IL-2 in experimental animals (*24*). It is reported that Th1 cytokine IL-2 supports CTL reaction (*25*). IL-2 and TNF-α are two important cytokines in immune response in human body (*26*). In our present investigation, LCPA50-S1 was verified to increase the IL-2 level of spleen lymphocyte cells in a concentration-dependent manner remarkably, demonstrating that LCPA50-S1 had the potential of enhancing immune function.

NO is a gaseous molecule synthesized from L arginine by nitric oxide synthase (NOS) and acts as an inducer of immunoregulation (*27*). It is reported that NO is a major effector molecule produced by macrophages (*28,29*) and recognized as a quantitative index of macrophage activation (*30*). Several polysaccharides were reported to have the function of macrophage activation (*31*). An increased level of NO secretion reflects phagocytic stimulation. In our present study, LCPA50-S1 could increase the NO production of RAW264.7 cells after 24 h stimulation quickly, suggesting that LCPA50-S1 could act as an immunostimulant of innate immunity. Indeed, there are various surface receptors on macrophages, which are known as pattern recognition molecules such as polysaccharides and can recognize foreign ligands during initial phases of the immune response.

TNF-α is a cytokine mainly produced by the activated macrophages, which are exceedingly important for killing and degrading tumor cells. To investigate whether LCPA50-S1 could enhance TNF-α secretion of macrophages, RAW264.7 cells were incubated with LCPA50-S1 for 24 h. The result showed that LCPA50-S1 highly enhanced TNF-α secretion of RAW264.7 cells, demonstrating that LCPA50-S1 can enhance phagocytosis function of RAW264.7 cells. The results indicated that LCPA50-S1-treated macrophages could enhance LCPA50-S1 uptake capacity and TNF-α secretion, suggesting that this might be the mechanism by which litchi polysaccharide acts to stimulate the killing and degradation of invading pathogens and tumor cells.

In conclusion, the present experimental results indicated that LCPA50-S1 significantly enhanced NO and TNF-α production of RAW264.7 macrophages, promoted splenocyte proliferation as well as IL-2 production of splenocytes. These findings indicated that LCPA50-S1 had the potential to be an immunomodulator and applying in treating series of diseases associated with immunity disorder. Further research on the stereochemical structure, the bioactive effects on human subjects and the structure-bioactivity relationship are in progress, which could provide a better understanding of the functional effects about polysaccharide.

References

1. Kong FL, Zhang MW, Liao ST, Yu SJ, Chi JW, Wei ZC. Antioxident activity of polysaccharide-enriched fractions extracted from pulp tissue of *Litchi chinensis* sonn. Molecules. 2010; 15:2152-2165.
2. Jing YS, Huang LJ, Lv WJ, Tong H, Song LY, Hu XQ, Yu RM. Structural characterization of a novel polysaccharide from pulp tissues of *Lichi chinensis* and its immunomodulatory activity. J Agric Food Chem. 2014; 62:902-911.
3. Jin ML, Huang QS, Zhao K, Shang P. Biological activities and potential health benefit effects of polysaccharides isolated from *Lycium barbarum* L. Int J Biol Macromol. 2013; 54:16-23.
4. Li XH, Li FS, Wei W, Wu NN. Studies on the immunomodulatory effects of Litchi polysaccharides. Shizhen Guoyi Guoyao 2008; 19:2119-2120.
5. Jiang YY, Wang L, Zhang L,Wang T, Yu L, Ding CB, Yang RW, Wang XL, Zhou YH. Characterization, antioxidant and antitumor activities of polysaccharides from *Salvia miltiorrhiza* Bunge. Int J Biol Macromol. 2014; 70:92-99.
6. Golovchenko VV, Khramova DS, Ovodova GR, Shashkov AS, Ovodov YS. Structure of pectic polysaccharides isolated from onion *Allium cepa* L. using a simulated gastric medium and their effect on intestinal absorption. Food Chem. 2012; 134:1813-1822.
7. Yuan F, Yu RM, Yin Y, Shen JR, Dong QF, Zhong L, Song LY. Structure characterization and antioxidant activity of a novel polysaccharide isolated from *Ginkgo biloba*. Int J Biol Macromol. 2010; 46:436-439.
8. Yu RM, Yang W, Song LY, Yan CY, Zhang Z, Zhao Y. Structural characterization and antioxidant activity of a polysaccharide from the fruiting bodies of cultured *Cordyceps militaris*. Carbohydr Polym. 2007; 70:430-436.
9. Xie JH, Liu X, Shen YM, Nie SP, Zhang H, Li C, Gong DM, Xie MY. Purification, physicochemical characterisation and anticancer activity of a polysaccaride from *Cyclocarya paliurus* leaves. Food Chem. 2013; 136:1453-1460.
10. Yu RM, Yin Y, Yang W, Ma WL, Yang L, Chen XJ, Zhang Z, Ye B, Song LY. Structural elucidation and biological activity of a novel polysaccharide by alkaline extraction from cultured *Cordyceps militaris*. Carbohydr Polym. 2009; 75:166-171.
11. Bao Y, Jiang YM, Zhao MM, Chen F, Wang R, Chen YL, Zhang DD. Structural characterisation of polysaccharides purified from longan (*Dimocarpus longan* Lour.) fruit pericarp. Food Chem. 2009; 115:609-614.

12. Zhu ZY, Liu N, Si CL, Liu Y, Ding LN, Jing C, Liu AJ, Zhang YM. Structure and anti-tumor activity of a high-molecular-weight polysaccharide from cultured mycelium of *Cordyceps gunnii*. Carbohydr Polym. 2012; 88:1072-1076.
13. Hu, XQ, Huang YY, Dong QF, Song LY, Yuan F, Yu RM. Structure characterization and antioxidant activity of a novel polysaccharide isolated from pulp tissues of *Litchi chinensis*. J Agric Food Chem. 2011; 59:11548-11552.
14. Niu YG, Yan W, Lv JL, Yao WB, Yu LL. Characterization of a novel polysaccharide from tetraploid *Gynostemma pentaphyllum* Makino. J Agric Food Chem. 2013; 61:4882-4889.
15. Dressman JB, Amidon GL, Reppas C, Shah VP. Dissolution testing as a prognostic tool for oral drug absorption: Immediate release dosage forms. Pharmaceut Res. 1998; 15:11-22.
16. Kong FB, Singh RP. Solid loss of carrots during simulated gastric digestion. Food Biophysics. 2011; 6:84-93.
17. Chen XM, Nie WJ, Fan SR, Zhang JF, Wang YX, Lu JX, Jin LQ. A polysaccharide from *Sargassum fusiforme* protects against immunosuppression in cyclophosphamide-treated mice. Carbohydr Polym. 2012; 90:1114-1119.
18. Jeong SC, Koyyalamudi SR, Jeong YT, Song CH, Pang G. Macrophage immunomodulating and antitumor activities of polysaccharides isolated from *Agaricus bisporus* white button mushrooms. J Med Food. 2012; 15:58-65.
19. Chen XM, Nie WJ, Yu GQ, Li YL, Hu YS, Lu JX, Jin LQ. Antitumor and immunomodulatory activity of polysaccharides from *Sargassum fusiforme*. Food Chem Toxicol. 2012; 50:695-700.
20. Marciani DJ, Press JB, Reynolds RC, Pathak AK, Pathak V, Gundy LE, Farmer JT, Koratich MS, May RD. Development of semisynthetic triterpenoid saponin derivatives with immunestimulating activity. Vaccine. 2000; 18:3141-3151.
21. Methacanon P, Madla S, Kirtikara K, Prasitsil M. Structural elucidation of bioactive fungi-derived polymers. Carbohydr Polym. 2005; 60:199-203.
22. Desai VR, Ramkrishnan R, Chintalwar GJ,Sainis KB. G1-4A, an immunomodulatory polysaccharide from *Tinospora cordifolia*, modulates macrophage responses and protects mice against lipopolysaccharide induced endotoxic shock. Int Immunopharmacol. 2007; 7:1375-1386.
23. Minatoa KI, Kawakami S, Nomura K, Tsuchida H, Mizuno M. An exo β-1,3-glucanase synthesized de novo degrades lentinan during storage of *Lentinule edodes* and diminishes immunomodulating activity of the mushroom. Carbohydr Polym. 2004; 56:279-286.
24. Ho CY, Laua BS, Kim CF, Leung KN, Fung KP, Tse TF, Chan HL, Chow SS. Differential effect of *Coriolus versicolor* (Yunzhi) extract on cytokine production by murine lymphocytes *in vitro*. Int Immunopharmacol. 2004; 4:1549-1557.
25. Bos R, Sherman LA. $CD4^+$ T-cell help in the tumor milieu is required for recruitment and cytolytic function of $CD8^+$T lymphocytes. Cancer Res. 2010; 21:8368-8377.
26. Cai ZB, Li W, Wang HT, Yan WQ, Zhou YL, Wang GJ, Cui JW, Wang F. Antitumor effects of a purified polysaccharide from *Rhodiola rosea* and its action mechanism. Carbohydr Polym. 2012; 90:296-300.
27. Coleman JW. Nitric oxide in immunity and inflammation. Int Immunopharmacol. 2001; 1:1397-1406.
28. Hibbs JB, JR, Taintor RR, VavrinZ. Macrophage cytotoxicity: Role for L-arginine deiminase and imino nitrogen oxidation to nitrite. Science 1987; 235:473-476.
29. Schepetkin IA, Faulkner CL, Nelson-Overton LK, Wiley JA, Quinn MT. Macrophage immunomodulatory activity of polysaccharides isolated from *Juniperus scopolorum*. Int Immunopharmacol. 2005; 5:1783-1799.
30. Li XQ, Xu W. TLR4-mediated activation of macrophages by the polysaccharide fraction from *Polyporus umbellatus* (*pers*.) Fries. J Ethnopharmacol. 2011; 135:1-6.
31. Xia LJ, Liu XF, Guo HY, Zhang H, Zhu J, Ren FZ. Partial characterization and immunomodulatory activity of polysaccharides from the stem of *Dendrobium officinale* (*Tiepishihu*) *in vitro*. J Funct Foods. 2012; 4:294-301.
32. Corrêa-Ferreira ML, Noleto GR, Petkowicz CLO. *Artemisia absinthium* and *Artemisia vulgaris*: A comparative study of infusion polysaccharides. Carbohydr Polym. 2014; 102:738-745.

(Received April 12, 2015; Accepted April 28, 2015)