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Structure of an entangled heteropolysaccharide from *Pholidota chinensis* Lindl and its antioxidant and anti-cancer properties

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ABSTRACT

A major polysaccharide PCP-I was isolated and purified from *Pholidota chinensis* Lindl. The physicochemical and structural properties of PCP-I were studied using high-performance size-exclusion chromatography (HPSEC), gas chromatography (GC), Fourier transform infrared spectroscopy (FTIR), periodate oxidation-smith degradation, methylation-GC–MS analysis, nuclear magnetic resonance (NMR) spectroscopy and transmission electron microscopy (TEM) analysis. PCP-I was homogeneous with molecular weight (Mw) of 249 kDa and composed of xylose and fucose at a molar ratio of 2.45:1. The repeating structural units of PCP-I were →3)-α-D-Xyl*p*-(1→ and →4)-α-L-Fuc*p*-(1→, the terminal fractions were T-D-Gal*A*p, and TEM further revealed that PCP-I was the entangled microstructure which was composed of four non-branched single chains. Compared with Vitamin C (Vc) and 5 fluorine urine (5-Fu), PCP-I showed scavenging effects of superoxide (EC_{50} = 1.09 mg/mL) and hydroxyl (EC_{50} = 0.11 mg/mL) radicals equivalent to Vc, and PCP-I (IC_{50} = 69.54 μg/mL) also exhibited good anti-proliferation capability for human colon cancer cell line caco-2.

1. Introduction

Pholidota chinensis Lindl, a well-known functional food and a folk medicine, has been used for a long time in southeast of China for alleviating headache and coughing [1]. *P. chinensis* Lindl comprises various classes of compounds, including stilbenoids, terpenoid, phytosterols, alkaloids, phenolic compounds and carbohydrates [1–3]. It has been reported that extracts of *P. chinensis* Lindl have antioxidant, analgesic, anti-inflammatory and antitumor activities [1,3–5]. Polysaccharides from *P. chinensis* Lindl have attracted increasing attention in research due to their potent biological and pharmacological functions [6]. However, previous studies mostly focused on the extraction and purification of *P. chinensis* Lindl polysaccharides [7,8]. Although structural properties and antioxidant activities of *P. chinensis* Lindl polysaccharides from different place of production have been reviewed by Yang (2016) [6], the fine chemical and microcosmic structure of polysaccharides purified from pseudobulbs of *P. chinensis* Lindl have not been investigated, and the structure-function relationship has yet been established.

Fine structural features will help understand the relationship between chemical structures and biological activities of *P. chinensis* Lindl polysaccharides. Therefore, in the present study, a polysaccharide (PCP-I) was isolated and purified from pseudobulbs of *P. chinensis* Lindl, its chemical structure was established using modern techniques including NMR and TEM, and its antioxidant and anti-cancer activities were investigated in order to study structure-function relationships. Our results will benefit the applications of *P. chinensis* Lindl in medical and food industries.

2. Materials and methods

2.1. Materials and chemicals

Pholidota chinensis Lindl was purchased from a local store (Shaoguan, Guangdong Province, China) and dried at room temperature, and the pseudobulbs of *P. chinensis* Lindl were separated for the study. The material was identified by Professor J. F. Liu, and a voucher specimen (pc-20150609-001) has been deposited in Fan-hua experimental building B403, Huaqiao University, Fujian, China. Monosaccharide samples, dextran standard samples, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenazine methosulfate (PMS) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). Deuterium Oxide (D_2O, Cambridge Isotope Laboratories, Inc., Andover, MA, USA), DMEM medium and fetal bovine serum (FBS, Hyclone, Logan, UT) and Penicillin-Streptomycin (MP Biomedicals, CA, USA) were purchased from a local agent (Taijing Co., Xiamen). DEAE Sepharose CL-6B was obtained from Pharmacia, Co, now available from GE-Healthcare. Ascorbic acid (vitamin C, Vc), hydrogen peroxide (H_2O_2), Nitro Blue Tetrazolium (NBT), Nicotinamide Adenine Dinucleotide Hydrogen (NADH) and ferrous sulfate ($FeSO_4$) were

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obtained from Shanghai Macklin Chemical Reagent Company, China. Human colon cancer cell line caco-2 was obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. All reagents were analytical grades.

2.2. Extraction, isolation and purification of polysaccharide

The dry *P. chinensis* Lindl (100 g) was extracted with 3 L of distilled water at 95 °C for 3 h. The extracts were concentrated, 4 volumes of ethanol were used to precipitate crude products, and the free proteins were removed by Sevag reagent (trichloromethane:N-butyl alcohol = 4:1) to obtain 14.85 g dry crude polysaccharides (PC) [9]. The PC (500 mg for each experiment) was dissolved in 10 mL of distilled water and applied to a column of DEAE-Sepharose CL-6B (4.6 cm × 40 cm), followed by elution with distilled water at a flow rate of 50 mL/h (12 min/tube) and gradient elution from 0 to 1 M NaCl aqueous solution at the same flow rate. The solution (12 mL/tube) was collected using a fraction collector and assayed by the phenol sulfuric acid method. The carbohydrate fraction corresponding to a major peak was collected, dialyzed (MWCO 3500) against distilled water for 48 h, and dried in a freeze drier to obtain a purified polysaccharide PCP-I.

2.3. Homogeneity, molecular weight (MW), and monosaccharide composition

Total sugar content was determined using the phenol sulfuric acid method [10]. Total uronic acid was determined using the sulfuric acid carbazole method (Bitter, & Muir, 1962) [11].

The homogeneity and molecular weight (MW) of PCP-I were determined using high-performance size-exclusion chromatography (HPSEC) (1100 system, Agilent Technologies, Palo Alto, CA, USA) equipped with a gel-filtration chromatographic column of Shodex Sugar KS-804 (Showa Denko K.K, Japan) and a refractive index detector (RID). PCP-I water solution (0.5 mg/mL) was passed through a 0.22-μm filter prior to injection into the column, and the column was eluted using distilled water at 50 °C with a flow rate of 1 mL/min. MW was calculated using a calibration curve of various dextran standards (Dextran Blue, T10, T40, T70, T500 and Glucose) [12].

Gas chromatography (GC) was used for the identification and quantification of monosaccharide composition of PCP-I. The polysaccharide PCP-I (30 mg) was hydrolyzed with 1 M H_2SO_4 and kept at 100 °C for 8 h and neutralized to pH 7.0 with barium carbonate, then filtered, concentrated and finally freeze-dried to obtain hydrolysis products. Mannitol (3 mg) was added as the internal standard, and derivatization of the products was then carried out using the trimethylsilylation reagent [13]. The derivatization products were injected into a gas chromatography system (6890 system, Agilent Technologies, Palo Alto, CA, USA) fitted with an HP-5 column (30 m × 0.25 mm × 0.25 μm) and a flame-ionization detector (FID). Monosaccharide composition was identified by comparison with the retention times of standard monosaccharide samples, and the molar ratio was calculated according to the peak areas.

2.4. Fourier transform infrared spectroscopy (FTIR) analysis

FTIR spectrum of PCP-I was determined using a Fourier Transform Infrared Spectrometer (Nicolet iS10, Thermo Fisher Scientific, Waltham, MA, USA) with a DTGS detector. The PCP-I (3 mg) was ground with KBr powder (100 mg) and pressed into pellets for FTIR measurement at a frequency range of 4000–400 cm^{-1} with 32 scans.

2.5. Periodate oxidation-smith degradation

PCP-I (25 mg) was dissolved into 15 mmol/L $NaIO_4$ solution and kept in the dark, and an aliquot (0.1 mL) of the solution was diluted to 25 mL with distilled water and used to read a value at 223 nm for 6 h intervals. When the value at 223 nm was stable, glycol (2 mL) was added into the solution to stop the reaction. The remaining solution was dialyzed against tap water and distilled water, each for 24 h. The dialyzed solution was concentrated, reduced with 80 mg $NaBH_4$ at room temperature for 24 h, neutralized to pH 7.0 with 50% acetic acid, dialyzed again as described above, and concentrated to a volume of 10 mL. One-third of this solution was freeze-dried and analyzed with GC (S1), and the remaining solution was hydrolyzed by adding the same volume of 1 M sulfuric acid, heating at 25 °C for 40 h, neutralizing to pH 7.0 with barium carbonate and filtering. The filtrate was dialyzed in distilled water for 24 h, the dialysis solution outside the dialysis tube was freeze-died for GC analysis (S2), and the solution inside the dialysis tube was used for GC analysis (S3) after adding 4 volumes of ethanol into the tube [14].

2.6. Partial hydrolysis with acid

The PCP-I (120 mg) was hydrolyzed with 0.05 M CF_3COOH at 95 °C for 16 h and centrifuged. The sediment (P1) was dried and used for GC analysis, the supernatant was dialyzed with distilled water for 48 h, and the solution outside the tube was dried and used for GC analysis. 4 volumes of ethanol were added into the solution in the tube, and the precipitate (P2) and the supernatant (P3) were freeze-dried, respectively, for GC analysis [15].

2.7. Methylation and GC–MS analysis

The PCP-I (30 mg) was dissolved in 9 mL dimethyl sulfoxide (DMSO), and dry sodium hydroxide powder (360 mg) was also dissolved in DMSO (9 mL) by constant stirring for 24 h. The two DMSO solutions were combined under constant stirring, and then 5.4 mL methyl iodide was added to the DMSO solution for 7 min followed by addition of 6 mL distilled water. The final solution was dialyzed against tap water and distilled water, each for 24 h, and concentrated to 10 mL, and methylated polysaccharides were extracted with 10 mL trichloromethane under constant oscillation for 30 min. The trichloromethane solution (1 mL) was examined by FTIR spectrum, and no absorption peak of hydroxyl groups indicated the complete methylation. The remaining trichloromethane solution was dried, and the desiccated methylated polysaccharides were hydrolyzed by adding formic acid and trifluoroacetic acid (TFA), reduced with $NaBH_4$, and acetylated using acetic anhydride–pyridine (1:1). Preparation of carboxyl-reduced polysaccharide was performed using the literature method [16]. The resulting methylated alditol acetates were subjected to Gas chromatography mass spectrometry (GC–MS) analysis. GC–MS was performed on a HP7890A-5975C instrument (Agilent Technologies, Palo Alto, CA, USA) with an HP-5 ms (30 m × 0.25 mm × 0.25 μm) column at the temperatures programmed from 160 (held for 1 min) to 250 °C (held for 2 min) at 8 °C/min [17,18]. The injector and detector temperatures were set at 250 °C, the ion source temperature was 230 °C, and helium was used as the carrier gas.

2.8. Nuclear magnetic resonance (NMR) spectroscopy

For NMR measurements, 30 mg PCP-I was exchanged with deuterium by lyophilizing with D_2O for three times and then dissolved in 0.7 mL D_2O (99.96%). Acetone was used as the internal standard. ^1H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz on an AVANCE-500 NMR spectrometer (Bruker Inc., Rheinstetten, Germany) at 25 °C. 2D NMR experiments were performed using standard Bruker software, and the 2D NMR spectra included $^1H/^1H$ homonuclear correlation spectroscopy ($^1H—^1H$ COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), nuclear overhauser enhancement spectroscopy (NOESY), and heteronuclear multiple-bond coherence (HMBC) [19–21].

2.9. Microscopic analysis

PCP-I aqueous solution (1 mg/mL) was prepared and added into an equal volume of SDS solution (1 mg/mL) at 80 °C for 2 h, diluted to a final concentration of 5 μg/mL with distilled H_2O and heated at 80 °C again for 2 h. After that, a droplet of solution was deposited on a carbon film specimen (200 mesh, Beijing Zhongjingkeyi Technology, Beijing, China) and dried at an ambient temperature and humidity, and observed by transmission electron microscopy (H-7650, Hitachi High-Technologies Corporation, Tokyo, Japan) with an accelerating voltage of 100 kV to visualize PCP-I molecular morphology [22–24].

2.10. Bioactivities assay

Superoxide, hydroxyl and DPPH radicals scavenging activities were measured using the previously described method [16,25,26]. Samples and vitamin C (Vc) were prepared at the final concentrations (0, 0.02, 0.08, 0.16, 0.33, 0.66, 1.3 and 2 mg/mL) for superoxide and hydroxyl radicals scavenging experiments. For the DPPH radical scavenging experiment, the final concentrations were 0, 0.8, 1.6, 3.3, 6.6 and 13 mg/mL. The percentage of the radical scavenging activities was calculated according to the following equation, A_0 was the absorbance of the blank control, and A_1 was the absorbance of the sample.

$$\text{Scavenging activity}\ (\%) = (1 - A_0/A_1) \times 100\%$$

The cytotoxicity of samples (PC, PCP-I and 5-Fu) against Caco-2 cancer cell line was determined using MTT assay. Caco-2 cells were cultured in DMEM medium (high glucose) containing 10% FBS and 100 units/mL penicillin-streptomycin with 5% CO_2 at 37 °C to ~80% confluency, and seeded in 96-well culture dishes at 100 μL/well. The Caco-2 cells were cultured for 20 h, DMEM medium was discarded, and 100 μL DMED medium with samples (25, 50, 250, 500 and 2500 μg/mL) were added to each well of the 96-well culture dishes and the cells were cultured for 48 h. MTT (5 mg/mL) was added (20 μL/well) to cells and incubated for 4 h, the media were removed, DMSO was added (150 μL/well), and the cytotoxicity was determined by measuring the absorbance at 570 nm [25]. The inhibition rate was calculated based on the following equation:

$$\text{Inhibition rate}\ (\%) = \left[1 - \left(A_{570\text{nm,sample}}/A_{570\text{nm,control}}\right)\right] \times 100\%$$

2.11. Statistical analysis

Three independent experiments were conducted, and data were expressed as the mean ± standard deviation (SD). The significance of differences between means was evaluated by one-way analysis of variance (ANOVA), and statistical significance was assigned at $P < 0.05$.

3. Results and discussion

3.1. Extraction, purification and molecular characterization of PCP-I

Crude polysaccharides (PC) were obtained from *P. chinensis* Lindl using hot-water extraction with a yield of 14.85% (Dry to Dry weight ratio, D/D). After fractionation by the DEAE-Sepharose CL-6B, crude PC gave two fractions (Fig. 1a), PCP-I was a major fraction from NaCl aqueous solution (50–60 tube) with a yield of 52.4% (D/D), and PCP-II was another fraction (40–49 tube) with a yield of 1.9% (D/D), which was not analyzed because of the low yield. The elution profile of PCP-I in HPSEC with a Sugar KS-804 column was given in Fig. 1b, which showed that PCP-I was homogeneous with one singular narrow peak in RID (Fig. 1b). PCP-I was composed of 95.72 ± 0.71% (*w*/w) of total carbohydrates and 4.49 ± 0.15% (w/w) of uronic acid.

Using HPSEC coupled with RID, a calibration curve ($y = -4 \times + 6.3$) of standard dextrans was obtained, and the average molecular weight (Mw) of PCP-I was calculated to be 249 kDa. The infrared spectrum of PCP-I displayed characteristic polysaccharide peaks at approximately 3400 cm^{-1} for hydroxyl groups, 2900 cm^{-1} for C—H stretching band, 1665 cm^{-1} for C═O bond, 1400 to 1200 cm^{-1} for C—H variable angle vibration bond, and 1100 cm^{-1} for C—O bond (Fig. 1c). After hydrolysis of PCP-I by 1 M H_2SO_4, GC chromatograms of hydrolyzed PCP-I and standard monosaccharide samples were showed in Fig. 1d and Fig. 1e,

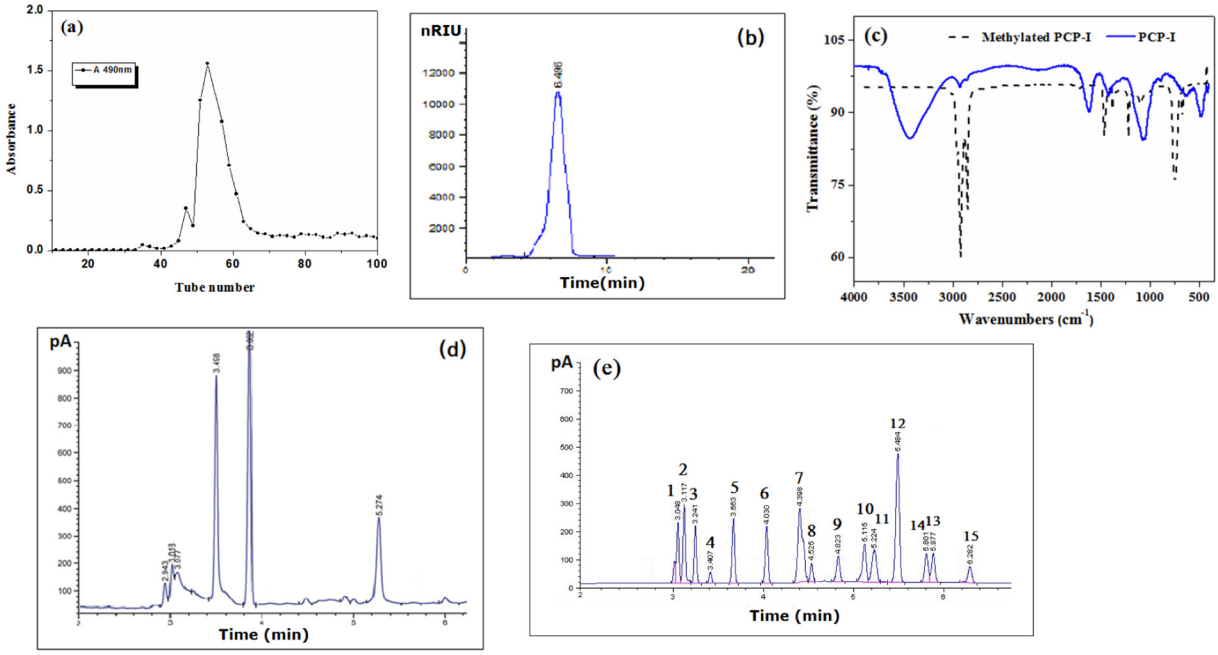

Fig. 1. (a) Sugar curve of PCP-I from NaCl elution of DEAE Sepharose CL-6B column chromatogram, (b) HPSEC chromatogram of PCP-I with a RID, (c) IR spectra of PCP-I and methylated PCP-I, and (d) GC chromatograms of hydrolyzed PCP-I and standard samples (e). In the GC chromatogram of standards, the internal standard mannitol was peak 12, arabinose was peaks 1, 2 and 4, fucose was peaks 2,3 and 5, xylose was peaks 5 and 6, mannose was peaks 7 and 11, galactose was peaks 8, 9 and 11, glucose was peaks 10 and 13, and galacturonic acid was peaks 14 and 15.

Table 1
GC results from fractions of smith degradation and partial acid hydrolysis of PCP-I.

Fractions		Sugar components and molar ratios			
		Fucose	Xylose	Glycerol	Erythritol
Partial acid hydrolysis	Precipitation (P1)	−	+		
	Precipitation in the sack (P2)	−	+		
	Supernatant in the sack (P3)	3.09	1		
Smith degradation	Full acid hydrolysis (S1)	−	+	−	+
	Out of sack (S2)	−	−	−	−
	Supernatant in the sack (S3)	−	+	−	−

−: Undetectable; +: Detectable.

respectively. Calculating the ratio of retention times (each peak/the internal standard mannitol) and referring to the ratios of standards (Fig. 1e), we concluded that PCP-I was composed of xylose and fucose. The molar ratio was 2.45:1 by calculating a ratio of the corresponding peak areas.

3.2. Periodate oxidation-smith degradation

The polysaccharide PCP-I showed HIO_4 uptake while it was oxidized. The periodate-oxidized products were hydrolyzed in 1 M H_2SO_4, and the hydrolysate was examined by GC (Table 1). Xylose and erythritol were identified in the fraction of full acid hydrolysis. The presence of xylose indicated that its linkages, namely (1 → 3)-glycosidic linkages, could not be oxidized. Fucose was absent, suggesting that fucose was oxidized. In addition, erythritol was identified, further suggesting that linkages of fucose in PCP-I were (1 → 4)-glycosidic linkages or (1 → 4,6)-glycosidic linkages. The conclusions were further confirmed by the NMR results.

3.3. Partial hydrolysis with acid

After partial acid hydrolysis of PCP-I, the fractions were subjected to GC analysis (Table 1). Both hydrolysis precipitation and precipitation in the sack contained xylose, indicating that xylose was the major component of backbone structure in PCP-I. Due to high percentage of fucose in the supernatant of the sack (Fuc:Xyl = 3.09:1) and by comparison with the previous analysis of monosaccharide composition (Fuc:Xyl = 1:2.45) of PCP-I, it was concluded that fucose was mainly existed in the position of edges in PCP-I.

3.4. Methylation–GC–MS analysis of PCP-I

Comparing the FTIR spectrum of PCP-I with that of methylated PCP-I (Fig. 1c), methylated PCP-I lacked an absorption peak at approximately 3400 cm^{-1} for hydroxyl group, indicating that PCP-I was completely methylated. GC–MS analysis showed the presence of two components, namely 1,4,5-*O*-Ac3–2,3-*O*– Me2–6-deoxy-L-galactiol and 1,3,5-O-Ac3–2,4-O-Me2-D-xylitol (Table 2). The results of methylation-GC–MS analysis showed that fucose and xylose were (1 → 4)-glycosidic linkage and (1 → 3)-glycosidic linkage, respectively, which were in consistent with the results of periodate oxidation and smith degradation.

The carboxyl-reduced polysaccharide was methylated, and the methylated sugars were identified by GC–MS analysis, which showed the presence of 1,4,5-*O*-Ac3–2,3-*O*-Me2–6-deoxy-L-galactiol, 1,3,5-O-Ac3–2,4-O-Me2-D-xylitol and a new trace peak 1,5-*O*-Ac2–2,3,4,6-*O*-Me4-D-galactiol. It could be confirmed that 1,5-*O*-Ac2–2,3,4,6-*O*-Me4-D-galactiol was the carboxyl-group reduced product of galacturonic acid, and the terminal fractions of PCP-I were T-D-Gal*A*p.

The branched fractions of PCP-I were not identified in the methylation-GC–MS analysis, probably because their contents were the least among the residues for detection or PCP-I was a non-branched polysaccharide. PCP-I was further confirmed to be a non-branched polysaccharide by the NMR and TEM results.

3.5. NMR spectroscopy

NMR experiments were performed to identify sugar residues of PCP-I. 1D NMR included ^1H (Fig. 2a), ^{13}C (Fig. 2b) and DEPT135 (Fig. 2c) spectra. The ^1H NMR spectrum showed two signals in anomeric regions of δ 4.6 and δ 5.29 ppm, and the anomeric carbon signals appeared at δ 102 and δ 98.3 ppm in the ^{13}C NMR and DEPT135 spectra. One signal at high field (δ 1.27 ppm) was assigned to the proton of methyl ($-CH_3$) group, which is likely derived from fucose (C-6). The two sugar moieties were designated as A and B according to their increasing chemical shifts (Fig. 2, Table 3).

Because 1D NMR did not provide enough information, 2D NMR experiments were also performed, including ^1H—^1H COSY (Fig. 2d), TOCSY (Fig. 2e), HSQC (Fig. 2f), NOESY (Fig. 2g) and HMBC (Fig. 2h). In the ^1H NMR spectrum of PCP-I, the chemical shift at δ 4.6 ppm was assigned to residue A. C-1 chemical shift of the residue A was obtained by HSQC at δ 102 ppm. Through ^1H—^1H COSY spectroscopy, the H-2, H-3, H-4 and H-5 chemical shifts of the residue A were determined at δ 3.28, δ 3.43, δ 3.55 and δ 3.29 ppm, respectively. By the TOCSY spectrum, the H-3, H-4 and H-5 chemical shifts of the residue A were confirmed. The corresponding ^{13}C chemical shifts of residue A could be obtained through the data in the HSQC spectrum, and ^1H and ^{13}C chemical shifts of residue A were listed in Table 3. According to ^{13}C chemical shifts, the residue A was identified as →3)-α-D-Xyl*p*-(1→, and this assignment was consistent with the result reported in the literature [27], and the results were in accordance with the results of monosaccharide composition and GC–MS analysis.

The anomeric chemical shift for residue B was at δ 5.29 ppm, and the anomeric carbon signal was observed at δ 98.3 ppm in the HSQC spectrum. ^1H assignments (H-2, H-3, H-4 and H-5) of residue B were obtained through the ^1H—^1H COSY and TOCSY spectra. The carbon signals of residue B were identified from the HSQC spectrum. The cross peak in HSQC with the chemical shift of δ 1.27 ppm and δ 19.9 ppm confirmed that the $-CH_3$ group was derived from residue B. ^1H and ^{13}C chemical shifts of residue B were listed in Table 3. According to the chemical shifts, it was confirmed that residue B was →4)-α-L-Fuc*p*-(1→, and this assignment was consistent with the results of Periodate oxidation-Smith degradation and GC–MS analysis.

NOESY correlates nuclei through space and reveals the glycosyl linkages between sugar residues, and intra- and inter-residue connectivities are also obtained in NOESY spectrum (Fig. 3g) [28,29]. By the NOESY experiment, the sequence of glycosyl residues in PCP-I was determined.

Table 2
GC–MS chromatogram of methylated PCP-I products.

Methylation product	Molar ratio	Mass fragment (*m*/*z*)	Linkage type
1,4,5-*O*-Ac3-2,3-*O*-Me2-6-deoxy-L-galactiol	1	43,87,101,117,129,189	1,4-L-Fuc*p*
1,3,5-*O*-Ac3-2,4-*O*-Me2-D-xylitol	2.16	43,117	1,3-D- Xyl*p*

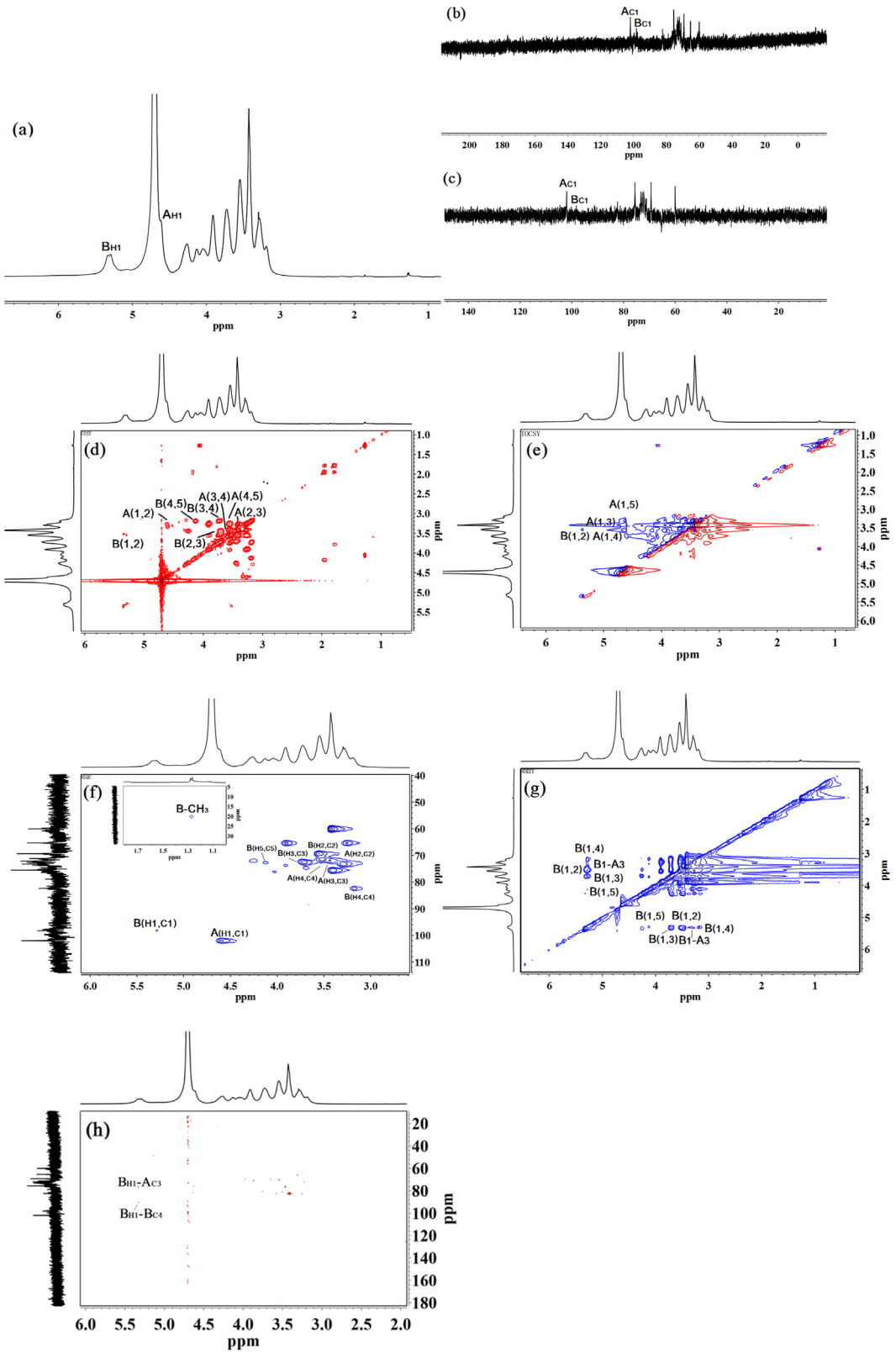

Fig. 2. ^{1}H NMR (a), ^{13}C NMR (b), DEPT135 (c), ^{1}H—^{1}H COSY (d), TOCSY (e), HSQC (f), NOESY (g) and HMBC (h) spectra of PCP-I polysaccharide. A_{H1} and A_{C1} represent the anomeric protons of residue A. B (1,2) represents the correlation or the NOE contact between H-1 and H-2 of residue B, etc. In the NOESY, B1-A3 represents the NOE contact between H-1 of residue B and H-3 of residue A. A (H1-C1) means the correlation between H-1 and C-1 of residue A, etc.

Residue B had an NOE contact from H-1 to H-3 of residue A, in addition to intra-residue NOE contacts with H-2, H-3, H-4 and H-5. Long-range ^{13}C—^{1}H correlations were obtained from HMBC. Cross-peaks were observed between the H-1 (δ 5.29) of B and the C-3 (δ 76.3) of A. The above result indicated that residue B was linked to the 3-position of residue A, and the above NOE result suggested the following sequence

Table 3
^{1}H and ^{13}C chemical shifts of the PCP-I recorded in D_2O at 25 °C.

Label	Glycosyl residues	Chemical shifts (δ)						
			1	2	3	4	5	6
A	→3)-α-D-Xyl*p*-(1→	H	4.6	3.28	3.43	3.55	3.29	
		C	102.0	73.1	76.3	71.4	–	
B	→4)-α-L-Fuc*p*-(1→	H	5.29	3.55	3.72	3.19	4.11	1.27
		C	98.3	69.4	71.9	82.4	72.4	19.9

–: Not obtained.

between residues A and B.

→4)−α−L−Fuc*p*−(1→3)−α−D−Xyl*p*−(1→

Based on the result of monosaccharide composition of PCP-I (Fuc:Xyl = 1:2.45), residue A (→3)-α-D-Xyl*p*-(1→) corresponded to three times of residue B (→4)-α-L-Fuc*p*-(1→). The results of partial acid hydrolysis of PCP-I showed that the percentage of residue B obviously increased in the supernatant of the sack (Fuc:Xyl = 3.09:1) compared to the monosaccharide composition of PCP-I, indicating that residues A and B are not alternating copolymerization (ABAB).

The terminal galacturonic acid of PCP-I was not identified in GC and NMR, probably because the content was the least among the residues for detection.

According to the results of the monosaccharide composition, partial acid hydrolysis and NMR analysis, it was concluded that the residues A were first connected to each other, and residues B was linked to them later. The repeating units present in polysaccharide PCP-I was determined to be the following:

→[4)−α−L−Fuc*p*−(1]$_n$→[3)−α−D−Xyl*p*−(1]$_{3n}$→

The terminal fractions of PCP-I were T-D-Gal*Ap* according to the results of GC–MS.

3.6. Molecular morphology of PCP-I

To determine fine structure of macromolecules, TEM was performed to illustrate the morphological information [27]. Sodium dodecyl sulfate (SDS) was added as a surfactant to disperse PCP-I polysaccharides [24], and then the microstructure of PCP-I was observed. A portion of PCP-I molecule with extended conformations was shown in Fig. 3. Non-branched conformation was observed from the TEM image of PCP-I in its fully extended state, which was in agreement with the results of methylation-GC–MS and NMR analyses, thus PCP-I was a non-branched polysaccharide. In addition, PCP-I molecules tended to form entangled chains under the experimental conditions, which were composed of four signal chains as shown in Fig. 3. It has been reported that entangled chains and helix conformations were beneficial for improving antioxidant and anti-cancer activities of the polysaccharides [30], and PCP-I showed strong inhibitory effect on the growth of colon cancer cell Caco-2.

3.7. Antioxidant and anti-proliferative activities of PCP-I

To test antioxidant activity of PCP-I, scavenging assays for superoxide, hydroxyl and DPPH radicals were performed, EC_{50} values were calculated, and Vc was used as the positive control. As shown in Fig. 4, the scavenging effects of PCP-I on the radicals increased with increasing PCP-I concentrations. The EC_{50} values of PCP-I for superoxide, hydroxyl and DPPH radicals were 1.09, 0.11 and 2.22 mg/mL, respectively, and EC_{50} values of Vc for the three radicals were 1.667 mg/mL, 0.505 mg/mL and 2.72 μg/mL, respectively. The results of the EC_{50} values showed that PCP-I had scavenging effects on superoxide and hydroxyl radicals, which was equivalent to Vc according to their EC_{50} values ($P > 0.05$).

The cytotoxicity induced by PCP-I and PC was investigated in human colon cancer cells caco-2 using the MTT assay, and 5-Fu was used as the positive control. As shown in Fig. 4d, PCP-I could inhibit proliferation of cancer cells caco-2 at 25–2500 μg/ml concentration range, and the anti-proliferation capability increased with the increasing PCP-I concentrations. The IC_{50} values of PCP-I, PC and 5-Fu for caco-2 cells were 69.54, 677.95 and 37.95 μg/mL, respectively, indicating that PCP-I exhibited good anti-proliferation capability for caco-2 cells. Comparing with the chemical 5-Fu (IC_{50} = 37.95 μg/mL), PCP-I (IC_{50} = 69.54 μg/mL) from the natural food can also effectively inhibit cancer cell growth, which is not as good as 5-Fu ($P < 0.05$).

Compared with the polysaccharide previously found from *P. chinensis* Lindl [6], PCP-I shows the different structural characteristics and more noticeable antioxidant activity. The bioactivities of polysaccharides might be influenced by molecule weight, glycosidic linkages and chain conformation, the low MW exhibited stronger antioxidant activity [31], and 1 → 3 glycosidic linkage showed a better promoting effect on anti-cancer activity [32]. Notably, chain conformation might play an important role on the bioactivity of polysaccharides [30], and entangled chain conformations may be related to good antioxidant and anti-cancer activities [22,27].

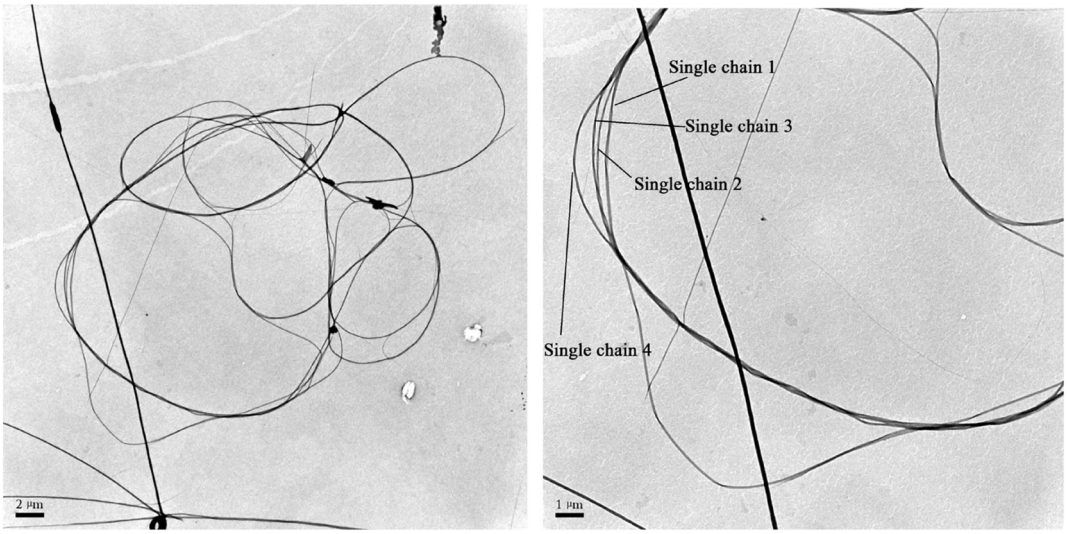

Fig. 3. TEM image of PCP-I in sodium dodecyl sulfate (SDS) solution (left) and the local amplification of the left image (right).

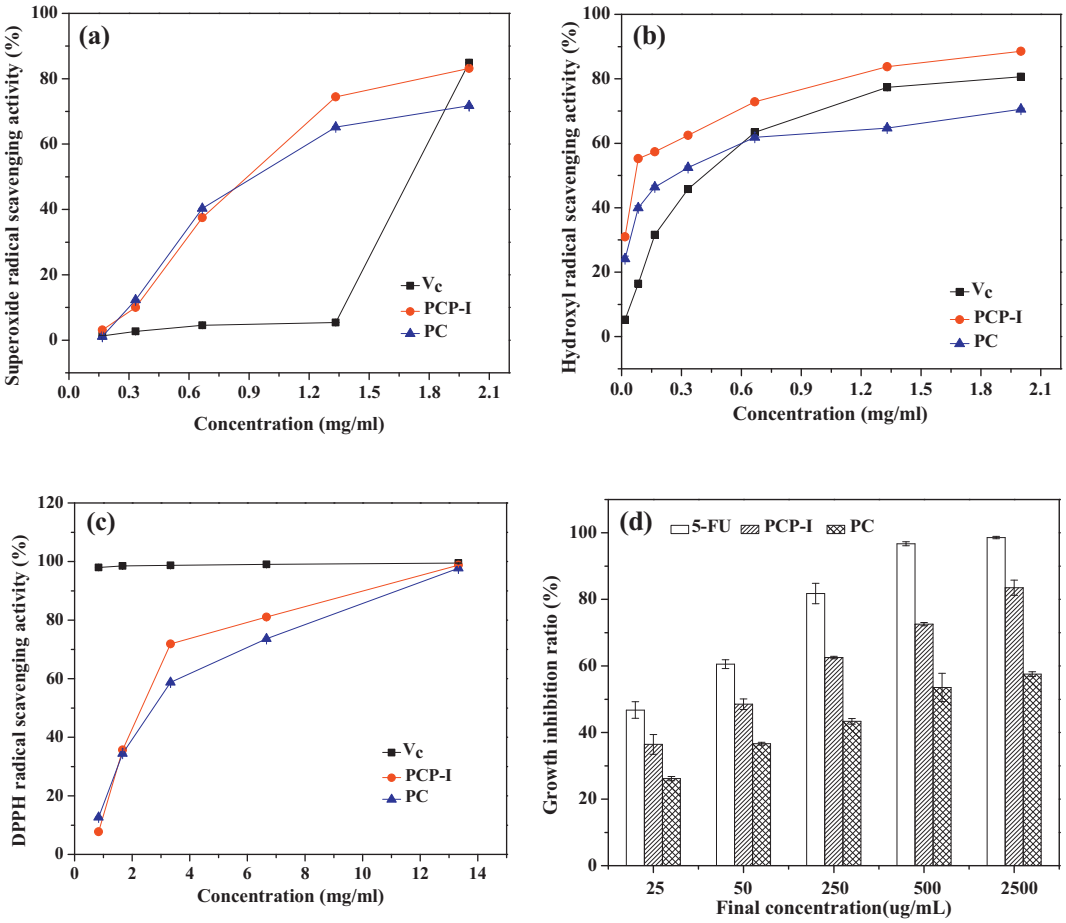

Fig. 4. Bioactivities of PCP-I: (a) superoxide radical scavenging activity, (b) hydroxyl radical scavenging activity, (c) DPPH radical scavenging activity, and (d) inhibitory effects on the growth of colon cancer cell Caco-2. PC and PCP-I were crude polysaccharides and the purified polysaccharide from *P. chinensis* Lindl, respectively. Each data *point* represents the mean ± SD ($n = 3$).

4. Conclusion

According to our results, we concluded that the crude polysaccharide PC from *P. chinensis* Lindl contained major polysaccharide PCP-I, which was mainly purified using DEAE Sepharose CL-6B column chromatography with a yield of 52.4%. Using HPSEC coupled with a Sugar KS-804 column and RID, PCP-I was purified to homogeneous and Mw of PCP-I was 249 kDa. FTIR of PCP-I displayed the characteristic polysaccharide peaks. PCP-I consisted of xylose and fucose with a molar ratio 2.45:1 by GC analysis. The results of periodate oxidation-smith degradation, partial hydrolysis with acid, methylation-GC–MS and NMR indicated that →3)-α-D-Xyl*p*-(1→ and →4)-α-L-Fuc*p*-(1→ is the major component of the main-chain structure of PCP-I, the terminal fractions of PCP-I were T-D-GalA*p*, and PCP-I was a non-branched heteropolysaccharide. TEM analysis revealed the entangled microstructure of PCP-I that was composed of four non-branched single chains. Comparing the antioxidant activities of PCP-I with Vc, PCP-I showed equal scavenging effects on superoxide and hydroxyl radicals, and the EC_{50} values of PCP-I for superoxide and hydroxyl radicals were 1.09 and 0.11 mg/mL, respectively. The cytotoxicity experiment against human colon cancer cells caco-2 showed that PCP-I and 5-Fu displayed the IC_{50} values of 69.54 and 37.95 μg/mL, respectively.

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