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Elucidation and biological activities of a new polysaccharide from cultured *Cordyceps militaris*

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ABSTRACT

A novel low-molecular-weight polysaccharide (CMP-1) with antioxidant, immunostimulatory and antitumor activities was isolated from the cultured *Cordyceps militaris*. The structure of CMP-1 was elucidated by a combination of physicochemical and instrumental analyses, and its average molecular weight was 4.3 kDa. The backbone of CMP-1 was composed of (1→4)-linked α-D-glucopyranosyl, (1→6)-linked β-D-glucopyranosyl and (1→4)-linked β-D-glucopyranosyl residues which branched at O-6. The branches consisted of (1→3)-linked α-L-rhamnopyranosyl terminated with (1→)-linked α-D-glucopyranosyl residues. The ultrastructure of CMP-1 was further investigated by scanning electron microscope (SEM). The antioxidant assays showed that CMP-1 exhibited free radical-scavenging effects, ferrous ions-chelating ability and reducing power. In vitro test revealed that CMP-1 could significantly stimulate the mouse splenocyte proliferation. The cytotoxicity assay showed that CMP-1 inhibited the proliferation of HT-29, HeLa, HepG2 and K562 cells, with the IC_{50} values of 137.66, 162.59, 176.29 and 364.01 μg/mL, respectively.

1. Introduction

Cordyceps militaris (Fr.) Link is currently available in China and South East Asia for use in the formulation of nutraceuticals and functional foods. *C. militaris* has received considerable attention in recent years due to its excellent biological effects with health-stimulating properties and medicinal applications (Huang, Tsi, Lee, & Mau, 2006; Wang, Lee, Chen, Yu, & Duh, 2012). For instance, the addition of *C. militaris* into chicken essence products makes them more valuable in the market and beneficial for consumer's health (Chu, Chien, & Duh, 2011). *C. militaris* has similar composition and biological activities to the well-known Chinese traditional medicine *C. sinensis*. It has been reported that *C. sinensis* might be substituted by *C. militaris* in its health enhancement and disease prevention and treatment (Das, Masuda, Sakurai, & Sakakibara, 2010; Yu et al., 2007; Zheng & Cai, 2004). Recent investigations have shown that the polysaccharides from *C. militaris* possess various effects including antioxidation (Chen, Wu, & Huang, 2013; Yu et al., 2007, 2009), immunomodulation (Lee & Hong, 2010; Lee, Kwon, & Won, et al., 2010; Lee, Kwon, Yun, et al., 2010; Wang, Meng, et al., 2012), antitumor (Park, Kim, Lee, Yoo, & Cho, 2009; Rao, Fang, Wu, & Tzeng, 2010), and anti-inflammation (Bohn & BeMiller, 1995; Yu, Song, et al., 2004; Yu, Wang, Zhang, Zhou, & Zhao, 2004).

It is well known that the molecular structure of polysaccharides is a very important issue in their biological activity since the bioavailability may be associated to structural patterns. The biological response is closely related to molecular weight, chemical structures, glycosidic bonds, backbone length, polymerization, degrees of branching, and 3D conformation. It has been reported that most bioactive mushroom polysaccharides were β-(1→3), (1→6)-glucans or α-(1→4), (1→4, 6)-glucans. Some of them were heteropolysaccharides (Chen et al., 2011; Fan, Li, Deng, & Ai, 2012; You et al., 2013). The molecular weight of polysaccharides also plays an important role. The investigations have demonstrated that some low-molecular-weight polysaccharides had antioxidant (Liu, Wang, Pang, Yao, & Gao, 2010), antitumor and immunomodulatory activities (Zhao et al., 2013). However, no report was given for the chemical structure and their bioactivities of low-molecular-weight polysaccharides from *C. militaris*.

Detailed characterizations of the polysaccharides are critical to a better understanding of the structural and functional properties for their future nutritional and therapeutic applications. In our previous work, some high-molecular-weight polysaccharides were isolated, purified and identified from the cultured fruiting bodies of *C. militaris* (Yu, Song, et al., 2004; Yu, Wang, et al., 2004; Yu

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et al., 2007, 2009). However, the chemical structures and their biological activities of low-molecular-weight polysaccharides from *C. militaris* have not been reported yet.

In the present paper, we report firstly the extraction, purification, structural characterization and ultrastructure of a novel low-molecular-weight polysaccharide (CMP-1) from the cultured fruiting bodies of *C. militaris*, as well as its in vitro antioxidant, immunostimulatory and antitumor activities.

2. Materials and methods

2.1. Plant material

The cultured fruiting bodies of *C. militaris* (No. 2012010013) were obtained from Jiangmen Honghao Bioscience and Technology Corporation, Jiangmen, China. The material was identified by Professor R.M. Yu, College of Pharmacy, Jinan University, China.

2.2. Experimental reagents and materials

DEAE-52 cellulose and Sephadex G-25 were obtained from Whatman Ltd. Sephacryl S-300 HR were obtained from Amersham Biosciences. XAD-7 macroporous adsorption resin, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), penicillin G and streptomycin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ascorbic acid (Vitamin C, Vc), hydrogen peroxide (H_2O_2), potassium ferricyanide [$K_3Fe(CN)_6$] and ferrous sulfate ($FeSO_4$) were obtained from Guangzhou Chemical Reagent Company, China. The human colorectal adenocarcinoma cell line HT-29, human cervical carcinoma cell line HeLa, human hepatocellular carcinoma cell line HepG2 and human myelogenous leukemia cell line K562 were obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). All reagents were of analytical grade.

2.3. Isolation, and purification of CMP-1

The dried fruiting bodies of *C. militaris* were crushed into powder and pretreated with ethanol (7:1, w/v) to decolor and remove some ethanol-soluble materials, dried at 55 °C, and stored in a dry place.

C. militaris powder (300 g) was mixed with 3.0 L of distilled water. The extraction was done using an ultrasonic cell disrupter with the ultrasonic power of 250 W for 10 min. Then the solution was placed in boiling water and extracted for 3 h. The water extract was filtered, concentrated under vacuum and centrifuged at 5031 × *g* for 15 min. Anhydrate ethanol was added to reach a final concentration of 80% (v/v), and then the mixture was kept in a beaker overnight at 4 °C and centrifuged at 5031 × *g* for 15 min to precipitate proteins and high-molecular-weight polysaccharides. The supernatant (CM) was collected, and lyophilized.

CM was purified using a column (2.6 cm × 95 cm) packed with XAD-7 macroporous adsorption resin (Yang, Prasad, Xie, Lin, & Jiang, 2011). Distilled water was employed as mobile phase. The flow rate was 2 mL/min. Each fraction (20 mL) was collected and then analyzed with phenol–sulfuric acid reagent at 490 nm using a spectrophotometer. Fractions 8–25, which consisted of the major peak, were collected together, concentrated at 60 °C with a rotary evaporator under vacuum, dialyzed (M_w cut off: 500 Da), and lyophilized. Then CM-1 was obtained.

CM-1 was dissolved in distilled water and was fractionated on a DEAE-52 cellulose column (2.6 cm × 40 cm) equilibrated with distilled water, a linear gradient from 0 to 0.8 M NaCl at a flow rate of 0.5 mL/min. All of the fractions were assayed for carbohydrate content by the phenol–sulfuric acid method, and only one sharp peak was collected, dialyzed, concentrated and further purified by a Sephadex G-25 column (1.6 cm × 70 cm) eluting with distilled water at a flow rate of 0.3 mL/min. Consequently, a polysaccharide, named CMP-1, was obtained. CMP-1, which had $[\alpha]_D^{20} = -55^\circ$ (*c* 1.0, H_2O), was used in the subsequent experiments on its structural elucidation and bioactivity evaluation.

2.4. Analyses of chemical composition

Total sugar content was determined by the phenol-sulfuric acid colorimetric method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Sulfate content was measured according to the method described by Therho and Hartiala (1971). Uronic acid content was estimated by the carbazole-sulfuric acid method using glucuronic acid as the standard (Bitter & Muir, 1962). The homogeneity and molecular weight of CMP-1 were evaluated by gel permeation chromatography (GPC) on a Sephacryl S-300HR column (1.6 cm × 70 cm) with standard dextrans (T-4, T-7, T-10, T-70, T-200 and blue dextran) and glucose. The elution volume was plotted against the logarithm of their respective molecular weights. The elution volume of CMP-1 was plotted in the same graph, and the molecular weight was measured (Luo, 2008). The monosaccharide composition was analyzed by high-performance anion exchange chromatography (HPAEC) after hydrolization and UV detection, coupled with pulsed amperometric detection (PAD), equipped with a Carbo PAC ™PA10 (2.0 mm × 250 mm) column. Gas chromatography (GC) was analyzed on an Agilent 190911J-413 HP-5 equipped with FID, inositol as an internal standard. Gas chromatography–mass spectrometry (GC–MS) was conducted with a Hewlett Packard 5895 instrument, using a fused-silica capillary column (30 mm × 25 mm) coated with a 0.2 mm film of DB-5. The ionization potential was 70 eV and the temperature of the ion source was 220 °C.

2.5. Spectroscopy analysis

Optical rotation was recorded with a Jasco P-1020 polarimeter. The Fourier transform infrared (FT-IR) spectroscopy spectrum was recorded using a Tensor 27 Bruker instrument. The sample was ground with KBr powder and then pressed into pellets for FT-IR measurement in the frequency range of 4000–500 cm^{-1}. 1D and 2D NMR spectra were recorded with a Bruker 500 MHz instrument and the sample was dissolved in D_2O (Yuan et al., 2010).

2.6. Analysis of monosaccharide composition

CMP-1 (5 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA, 2 mL) at 100 °C in a sealed tube for 8 h. Excess acid was removed by evaporation on a water bath at a temperature of 40 °C and co-distilled with MeOH after the hydrolysis was completed. The monosaccharide content was measured by HPAEC-PAD. The hydrolysate (1 mg) was dissolved in pure water (1 mL). The solution was used for the ionic chromatography analysis by HPAEC-PAD on the Dionex ICS-2500 system, eluted with a mixture of water and 200 mM NaOH in the volume ratio of 92:8 (Yu et al., 2007).

2.7. Partial acid hydrolysis

CMP-1 (15 mg) was hydrolyzed with 0.05 M TFA for 6 h at 100 °C, and dialyzed with distilled water for 48 h. The fraction out of the sack was collected. After the excess TFA in the fraction was removed by co-distillation with MeOH (2 mL 3×), the fraction was evaporated to dryness (Fraction 1). The fraction in the sack was dried by evaporation, and then hydrolyzed with 0.5 M TFA. The hydrolysate was dialyzed, and the fraction out of the sack (Fraction 2) and fraction in the sack (Fraction 3) were collected respectively. The

fractions 1, 2 and 3 were hydrolyzed with 2 M TFA and tested by HAPEC-PAD.

2.8. Periodate oxidation-Smith degradation

The polysaccharide (30 mg) was oxidized with 0.015 M sodium periodate ($NaIO_4$, 30 mL) and kept in darkness at 4 °C. Its absorbance was monitored at 223 nm every 6 h (Yuan et al., 2010). The reaction was completed when the absorbance did not descend, and formic acid production was determined by titration with 0.055 M NaOH. The excess $NaIO_4$ was removed by adding 2 mL of ethylene glycol. The solution of oxidation products was extensively dialyzed (M_w cut off: 500 Da) against tap water and distilled water for 48 h, and reduced with sodium borohydride ($NaBH_4$, 80 mg) for 12 h at 25 °C. And then acetic acid was added to neutralize the excess $NaBH_4$. The solution was concentrated and subjected to complete hydrolysis with 2 M TFA. The acid was removed by co-distillation with MeOH under vacuum. Finally, the products were acetylated and analyzed by GC (Das et al., 2010).

2.9. Methylation analysis

The sample was dried at 50 °C under vacuum overnight and was methylated by the method of Hakomori (Hakomori, 1964). The methylated polysaccharide was treated with 90% formic acid (3 mL) for 10 h at 100 °C in a sealed tube. After removal of the formic acid, the residues were heated with 2 M TFA (2 mL) under the above conditions and the hydrolysate was concentrated to dryness. The methylated sugars were reduced with $NaBH_4$ and then acetylated with acetic anhydride. Alditol acetates were analyzed by GC–MS (Sassaki, Lacomini, & Gorin, 2005).

2.10. Ultrastructure of CMP-1

Scanning electron microscope (SEM) image of CMP-1 was obtained by means of an environmental scanning electron microscope (ESEM, Philips XL-30, Philips-FEI Co., Eindhoven, the Netherlands). The dried powder sample was placed on a specimen holder with the help of double-sided adhesive tapes and then sputtered with gold powder using a sputter coater (Chen, Chen, et al., 2013). The sample was observed with 1000-, 5000-, 10,000- and 30,000-fold magnification at an accelerating potential of 5.0 kV under a high vacuum condition.

2.11. Determination of DPPH radical-scavenging activity

DPPH radical-scavenging activity was determined by the method described by Braca et al. (2001). Briefly, DPPH ethanol solution (190 μL, freshly prepared at a concentration of 0.2 mM) was added to 10 μL of CMP-1 solution of gradient concentrations (0, 25, 50, 100, 200, 400, 800 and 1600 μg/mL) in water. Vc was used as a positive control. The absorbance was measured at 517 nm after 30 min. The lower absorbance of the reaction mixture indicated the higher free radical-scavenging activity. The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\text{scavenging ability}\,(\%) = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

where A_0 was the absorbance of control (without sample) and A_1 was the absorbance in the presence of sample.

2.12. Determination of hydroxyl radical-scavenging activity

The scavenging activity for hydroxyl radicals was measured with Fenton reaction. The reaction was conducted by adding H_2O_2 into the sample and incubating at room temperature for 60 min. Vc was used as a positive control. The absorbance of the mixture at 510 nm was measured. The hydroxyl radicals-scavenging activity was calculated according to the following equation:

$$\text{scavenging ability}\,(\%) = \left[\frac{(A_2 - A_1)}{(A_0 - A_1)}\right] \times 100$$

where A_0 was the absorbance of the control (blank, without of H_2O_2), A_1 was the absorbance in the absence of sample, and A_2 was the absorbance in the presence of sample (Yuan et al., 2010).

2.13. Measurement of reducing power

The reducing power was determined referring to the ferric-reducing antioxidant power (FRAP) assay (Yuan, Carrington, & Walsh, 2005). The different concentrations of samples (0–1600 μg/mL, 2.5 mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 5 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%, w/v). The mixture was incubated at 50 °C for 20 min. Then 2.5 mL of 10% (w/v) trichloroacetic acid was added to the reaction mixture, which was then centrifuged at 1811 *g* for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% (w/v) ferric chloride in a test tube. After a 10 min reaction, the absorbance of the resulting solution was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vc was used to compare the reducing power.

2.14. Measurement of ferrous ion-chelating effects

The chelating activity of sample on ferrous ions was measured by the formation of ferrous iron–ferrozine complex (Dinis, Madeira, & Almeida, 1994). Samples of different concentrations (0–1600 μg/mL) were mixed with 3.7 mL of deionized water, and then reacted with 0.1 mL of 2.0 mM $FeSO_4$. After 0.2 mL of 5.0 mM ferrozine was added, the solution was mixed, left to stand for 10 min at room temperature, and then the absorbance of the mixture was determined at 562 nm. EDTA was co-assayed as a positive control. The chelating activity on ferrous ions was calculated according to the following equation:

$$\text{chelating ability}\,(\%) = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

where A_0 was the absorbance of the control (deionized water, instead of sample), and A_1 was the absorbance of the test sample mixed with reaction solution.

2.15. Splenocyte proliferation assay

Kunming mice (GradeII, 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 with free access to water and standard rodent chow. The splenocyte proliferation assay was performed by MTT method (Chen, Liu, et al., 2011). Briefly, the splenocytes from the mice were isolated by gently pushing RPMI 1640 filled in the syringe into the spleen and then pressing spleen tissue through nylon mesh filters. The removal of erythrocytes was carried out by washing the cells with Tris–NH_4Cl for three times. The viability of isolated splenocytes was more than 90%. The splenocytes were placed into the 96-well flat-bottomed microplates at 5×10^6 cells/well in RPMI 1640 media with or without CMP-1. Final concentrations of CMP-1 were 0.97, 3.9, 15.6, 62.5 and 250 μg/mL, respectively. The positive control was treated with 5 μg/mL ConA, while the negative control with RPMI 1640 only. The cells were incubated at 37 °C under 5% CO_2 for 48 h, and then 20 μL of 5 mg/mL MTT was added to each well, with further incubation for

another 4 h. After removing MTT by centrifugation at 1811 × *g* for 5 min at 4 °C, the formazan precipitate was solubilized in DMSO (200 μL/well). Splenocyte proliferation was measured by determining the absorbance at 570 nm using multifunctional microplate reader (Boteng, USA).

2.16. Cell culture and cytotoxicity assay

HT-29, HeLa, HepG2 and K562 cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Experiments were performed when cell growth was approximately 80% confluent. Three independent experiments were performed for each cell line. The cytotoxicity of CMP-1 against four tumor cell lines was determined using the MTT assay (Hu, Song, Huang, Zhen, & Yu, 2012; Shang, Zhang, Wen, Li, & Cui, 2009). Briefly, HT-29, HeLa and HepG2 cells were seeded in 96-well plates, incubated overnight, and then treated with CMP-1 (final concentration: 25, 50, 100, 200, 400 and 800 μg/mL). K562 cells were seeded on 96-well plates at a density of 1×10^4 cells per well and treated with various concentrations of CMP-1. Cisplatin was used as the positive control. After 48 h of treatment, 20 μL of MTT (5 mg/mL) was added to each well and the cells were incubated for another 4 h at 37 °C. After the media were removed, 200 μL of dimethyl sulphoxide (DMSO) was added to each well to dissolve the cellular crystalline deposits and absorbance was measured spectrophotometrically at 570 nm. The inhibition rate (*I*%) was calculated according to the following formula:

$$\text{Inhibition rate}\,(I\%) = \frac{\text{control}\,A_{570\,\text{nm}} - \text{treated}\,A_{570\,\text{nm}}}{\text{control}\,A_{570\,\text{nm}} - \text{blank}\,A_{570\,\text{nm}}} \times 100$$

2.17. Statistical analysis

Data were analyzed using Statistical analysis software SPSS 11.5 and expressed as mean ± standard deviation (SD) for three independent experiments. Significance was determined at $P < 0.05$ by analysis of variance (ANOVA) followed by Dunnett's tests.

3. Results and discussion

3.1. Isolation, purification and chemical composition

Crude low-molecular-weight polysaccharide (10.66 g) was obtained from the cultured fruiting bodies of *C. militaris* by hot water extraction followed by ethanol precipitation. After purification with XAD-7 resin, DEAE-52 cellulose and Sephadex G-25 column, profile CMP-1 appeared as a single and symmertrical sharp peak, which was detected by the phenol-sulfuric acid assay. The yield of CMP-1 from crude low-molecular-weight polysaccharide was 65.26%. The uronic acid content of CMP-1 was below the detection limit. The average molecular weight of CMP-1 was determined as 4.3 kDa by GPC technique on a Sephacryl S-300 HR column. The calibration was performed with dextran molecular weight standards (M_w: 5×10^6, 2×10^5, 7×10^4, 1×10^4 and 3×10^3, Pharmacia). The total carbohydrate content of CMP-1 was measured to be 96.4% (w/w) and *m*-hydroxybiphenyl colorimetric test was shown negative.

Table 1
Results of HAPEC-PAD analysis for CMP-1 degradation products.

	Molar ratios	
	Rhamnose	Glucose
CMP-1	1.00	23.02
Fraction 1	n.d.	1.00
Fraction 2	0.68	2.07
Fraction 3	n.d.	1.00
Smith degradation	1.00	n.d.

n.d.: not detected.

3.2. Structural characterization of CMP-1

The composition of CMP-1 was presented in Table 1. Two monosaccharides, glucose and rhamnose, were identified in the hydrolysate of CMP-1, and their ratios were 23.02:1.00 by HAPEC-PAD analysis. The fractions 1–3 were obtained through partial acid hydrolysis. Each fraction was subjected to HAPEC-PAD analysis and the results were shown in Table 1. Fraction 3, the precipitate in the sack, possessed the biggest size among three fractions. The component of fraction 3 indicated that glucose might be the backbone of the structure of CMP-1. The analysis results of fractions 1 and 2 showed that the branched structure of CMP-1 was composed of rhamnose and glucose, and terminated with glucose.

The periodate-oxidized products were fully hydrolyzed and analyzed by GC after Smith degradation. The results shown in Table 1 demonstrated that there was no glucose in the oxidation products. It could be inferred that linkages of glucose were (1→), (1→2), (1→6), (1→2,6), (1→4) and (1→4,6), which might be oxidized to produce glycerol and erythritol. The presence of rhamnose revealed that some residues of rhamnose were (1→3)-linked, (1→2,3)-linked, (1→2,4)-linked, (1→3,4)-linked or

Table 2
GC–MS results of methylated products of CMP-1.

Methylation product	Retention time (min)	Molar ratio	Mass fragments (m/z)	Linkage type
2,4-Me_2-Rha	12.23	0.89	43, 59, 69, 75, 85, 99, 101, 117, 129	1→3
2,3,4,6-Me_4-Glc	13.02	1.00	45, 71, 87, 101, 129, 161, 205	T→
2,3,6-Me_3-Glc	14.04	8.79	45, 87, 101, 113, 117, 161, 233	1→4
2,3,4-Me_3-Glc	15.20	11.75	87, 99, 101, 117, 129, 161	1→6
2,3-Me_2-Glc	18.33	0.96	43, 101, 117, 261	1→4,6

Table 3
Assignment of ^{13}C NMR and ^{1}H NMR chemical shifts of CMP-1.

Sugar residue	Chemical shifts (ppm)						
	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6	CH_3
A →3)-α-L-Rha(1→	109.30/5.16	79.35/4.04	76.54/3.58	82.07/4.00	74.72/3.62		22.08/1.8
B →4,6)-β-D-Glc(1→	107.57/5.00	76.70/3.76	80.88/4.10	82.32/3.93	75.55/3.38	73.32/3.88	
C →6)-β-D-Glc(1→	103.56/4.42	72.80/3.20	76.01/3.93	69.40/3.32	70.97/3.76	66.09/3.84	
D →4)-α-D-Glc(1→	100.40/5.06	72.36/3.62	74.88/3.87	70.50/3.82	66.05/3.89	62.13/3.55	
E α-D-Glc(1→	99.64/5.31	71.39/3.55	73.38/3.45	69.31/3.98	69.43/3.63	60.73/3.65	

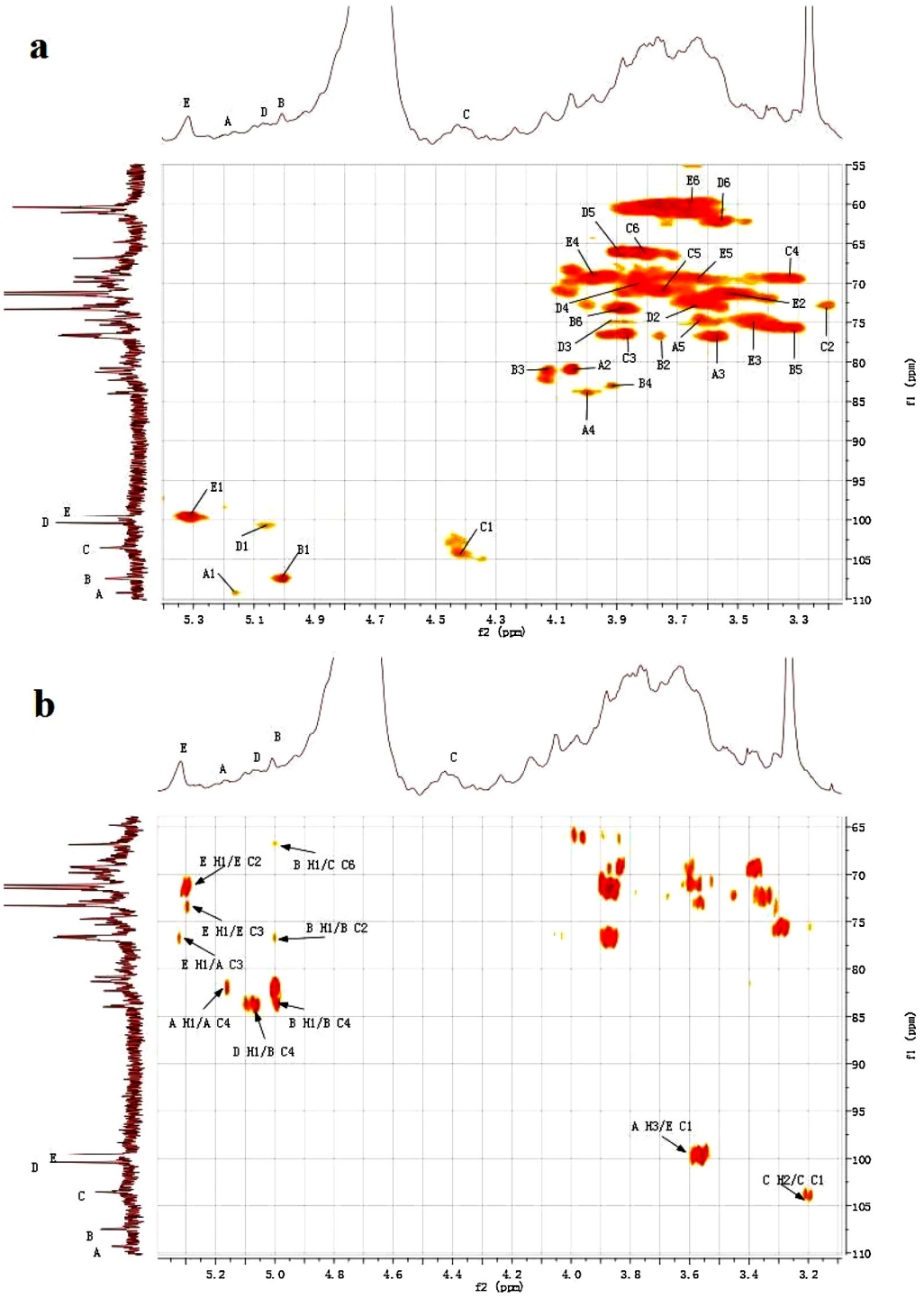

Fig. 1. (a) ^{1}H–^{13}C HSQC spectrum of CMP-1. (b) ^{1}H–^{13}C HMBC spectrum of CMP-1.

(1→2,3,4)-linked, which could not be oxidized, respectively (Abdel-Akher, Hamilton, Montgomeny, & Smith, 1952; Wang, Luo, & Liang, 2004). The fully methylated CMP-1 was hydrolyzed with acid and analyzed by GC-MS. The results showed the presence of five components, namely, 2,4-Me_2-Rha, 2,3,4,6-Me_4-Glc, 2,3,6-Me_3-Glc, 2,3,4-Me_3-Glc, 2,3-Me_2-Glc in a molar ratio of 0.89: 1.00: 8.79: 11.75: 0.96 (Table 2). Based on the standard data in the CCRC Spectral Database for PMAA's, the linkage of rhamnose was deduced as (1→3) while the linkages of glucose as (1→), (1→4), (1→6) and (1→4,6). This result showed a good correlation between terminal and branched residues. In addition, the molar ratios also agreed with the overall monosaccharide composition of CMP-1 described as above.

The infrared spectrum of CMP-1 revealed the purified product's major functional groups and the chemical bonds. The infrared spectrum of CMP-1 showed absorption bands at 3404, 2925, 1641, 1413 and 1077 cm^{-1}. Any signals corresponding to sulfate ester were not found in FT-IR spectrum of CMP-1. The characteristic absorptions at 866 and 896 cm^{-1} indicated that α- and β-configurations were present simultaneously. There was no absorption at 1740 cm^{-1}, indicating the nonexistence of uronic acid in the polysaccharide structure (Sun, Liang, Zhang, Tong, & Liu, 2009; Yuan et al., 2010).

3.3. NMR spectroscopy analysis

Signals of CMP-1 in 1D NMR (^{1}H NMR, ^{13}C NMR) and 2D NMR (HSQC and HMBC) spectra were assigned based on the monosaccharide analysis, linkage analysis and chemical shifts (Lee & Hong, 2010; Chu, Chien, & Duh, 2011; Niu et al., 2011; Song & Du, 2012; Mandal et al., 2012). ^{1}H NMR spectrum contained five signals at δ 5.16, 5.00, 4.42, 5.06 and 5.31 ppm for the anomeric protons, indicating that five residues, designated as A, B, C, D and E, respectively, were present in CMP-1 (Table 3). In ^{13}C NMR spectrum, there were five anomeric carbon signals at δ 109.30, 107.57, 103.56, 100.40 and 99.64 ppm (Table 3). Furthermore, the cross-peaks of δ 5.16/109.30, 5.00/107.57, 4.42/103.56, 5.06/100.40 and 5.31/99.64 ppm were observed in 2D NMR (HSQC) spectrum (Fig. 1a), indicating that the anomeric carbon signals at δ 109.30, 107.57, 103.56, 100.40 and 99.64 ppm corresponded to the anomeric proton signals at δ 5.16, 5.00, 4.42, 5.06 and 5.31 ppm, respectively. The cross-peaks of protons and carbons of each of the sugar moieties were also examined in the HSQC spectrum (Fig. 1a).

The chemical shifts larger than 5.0 ppm and their small J-coupling constants of $^3J_{H\text{-}1,H\text{-}2}$ for the anomeric protons of residues A, D and E suggested that these residues were α-linked. And the chemical shifts values smaller than 5.0 ppm and relatively large J-coupling constant of $^3J_{H\text{-}1,H\text{-}2}$ for the anomeric protons of residues B and C proposed that residues B and C were β-linked (Pan et al., 2012). Presence of both α- and β-linked residues was identical with the results from FT-IR.

The HMBC spectrum (Fig. 1b) of CMP-1 showed strong cross-peaks between ^{1}H and ^{13}C peaks in different residues and could be assigned as follows: A H-3 (δ 3.58) and E C-1 (δ 99.64), C H-2 (δ 3.20) and C C-1 (δ 103.56), D H-1 (δ 5.06) and B C-4 (δ 82.32), B H-1 (δ 5.00) and B C-4 (δ 82.32), A H-1 (δ 5.16) and A C-4 (δ 82.07), E H-1 (δ 5.31) and A C-3 (δ 76.54), B H-1 (δ 5.00) and B C-2 (δ 76.70), E H-1 (δ 5.31) and E C-3 (δ 73.38), E H-1 (δ 5.31) and E C-2 (δ 73.39), B H-1 (δ 5.00) and C C-6 (δ 66.09). By the means of 1D and 2D NMR spectra, the entire assignment of the ^{1}H and ^{13}C chemical shifts of CMP-1 was achieved and presented in Table 3.

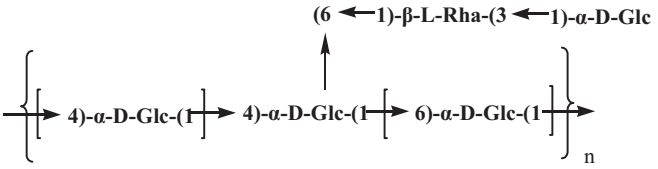

Fig. 2. Predicted structure of CMP-1.

Based on the results of HAPEC-PAD, FT-IR, GC, GC-MS as well as 1D and 2D NMR, the backbone of CMP-1 was composed of (1→4)-linked α-D-glucopyranosyl residues, (1→6)-linked β-D-glucopyranosyl residues and (1→4)-linked β-D-glucopyranosyl residues which branched at O-6. The branch consisted of (1→3)-linked α-L-rhamnopyranosyl residues terminated with (1→)-linked α-D-glucopyranosyl residues. From the aforementioned results, the repeating structural unit of CMP-1 could be as shown in Fig. 2.

3.4. Ultrastructure

Fig. 3 presented the scanning electron micrographs (SEM) of CMP-1 at magnifications of 1000, 5000, 10,000 and 30,000. SEM observations revealed that the ultrastructure of CMP-1 was mainly composed of a surface with a sheet-like appearance and randomly distributed ovoid-shape particles, with diameters of approximately

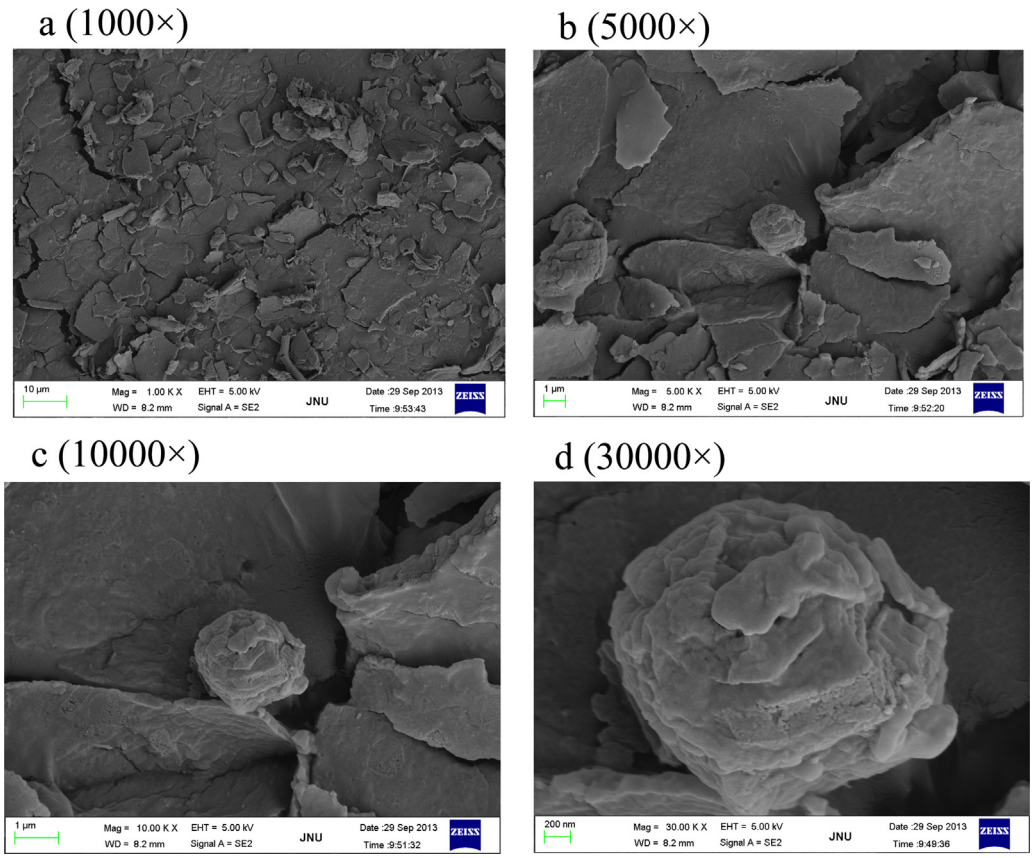

Fig. 3. SEM images of CMP-1. (a) The morphology of CMP-1 at 1000× scalebar is 10 µm. (b) The morphology of CMP-1 at 5000× scalebar is 1 µm. (c) The morphology of CMP-1 at 10,000× scalebar is 1 µm. (d) The morphology of CMP-1 at 30,000× scalebar is 200 nm.

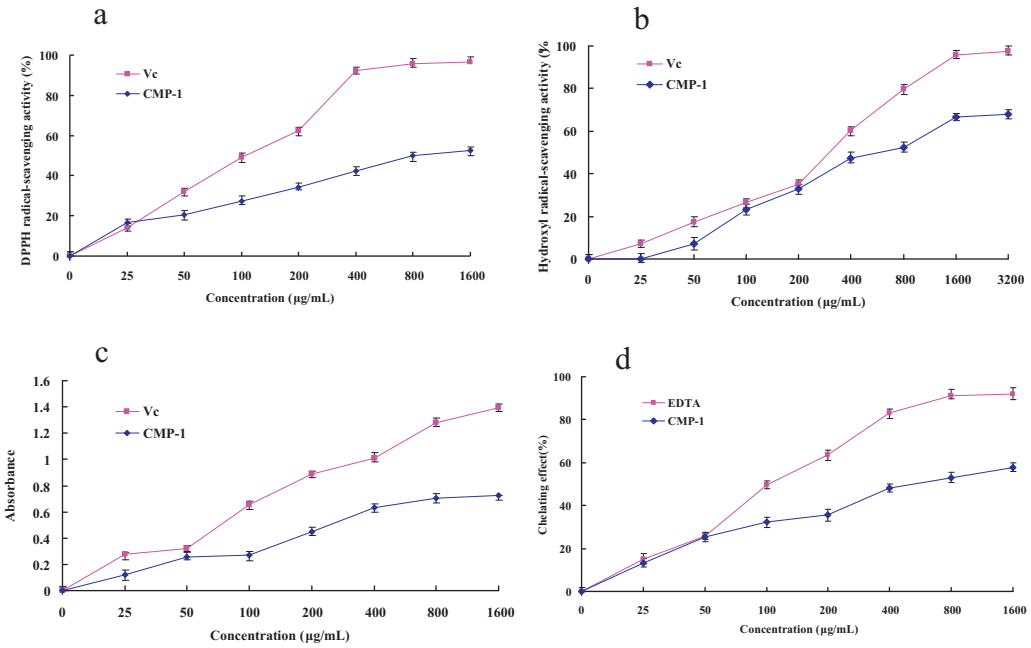

Fig. 4. Antioxidant activities of CMP-1. (a) DPPH radical-scavenging activity of CMP-1 and ascorbic acid. (b) Hydroxyl radical-scavenging activity of CMP-1 and ascorbic acid. (c) Reducing power of CMP-1 and ascorbic acid. (d) Ferrous ions chelating effect of CMP-1 and EDTA. Values are means ± SD of three separate experiments.

2 μm. These might be due to the branches and network structures of the polysaccharide.

3.5. Scavenging activity of CMP-1 for DPPH radicals

DPPH as a stable free radical compound which shows maximum absorbance at 517 nm has been widely used to test the free radical-scavenging ability of various antioxidative samples (Benvenuti, Pellati, Melegari, & Bertelli, 2004). As shown in Fig. 4a, both CMP-1 and Vc reacted directly with and quenched DPPH radicals to different degrees as the concentrations increased. The IC_{50} values of CMP-1 and Vc were 1150 and 105 μg/mL, respectively.

3.6. Scavenging activity of CMP-1 for hydroxyl radicals

The hydroxyl radical, known to be generated through the Fenton reaction in this system, was scavenged by samples. The scavenging effect of CMP-1 for hydroxyl radicals was in a concentration-dependent manner (Fig. 4b). The IC_{50} values of CMP-1 and Vc were 650 and 330 μg/mL, respectively. In our previous research, two polysaccharides (P70-1 and CBP-1) were isolated from the cultured fruiting bodies of *C. militaris*, which exhibited the scavenging activity toward hydroxyl radicals with an IC_{50} value of 0.548 mg/mL and 0.638 mg/mL (Yu et al., 2007, 2009).

3.7. Reducing power of CMP-1

Fig. 4c depicted the reducing power of tested samples. Higher absorbance value means stronger reducing power of samples. In this assay, the reducing power of the tested polysaccharide steadily increased with increasing sample concentration. The reducing ability of CMP-1 at 400 μg/mL was 0.62. The reducing properties were generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free-radical chain which is done by donating a hydrogen atom. The data showed that the reducing power of CMP-1 might play a role in the antioxidation of *C. militaris*.

3.8. Ferrous ions chelating effect of CMP-1

The chelating abilities of CMP-1 and EDTA on Fe^{2+} were shown in Fig. 4d. CMP-1 and EDTA in the range of concentrations from 25 to 1600 μg/mL were found to have more potent chelating ability on Fe^{2+} in a concentration-dependent manner. At the concentration of 1600 μg/mL, CMP-1 chelated ferrous ions by 57.9%. The EC_{50} values of CMP-1 and EDTA were 550 and 110 μg/mL, respectively.

Oxidation phenomena have been implicated in many illnesses, such as diabetes mellitus, arteriosclerosis, nephritis, Alzheimer's disease and cancer. Antioxidants have the potential to prevent and treat diseases associated with reactive oxygen species, especially some forms of cancer (Fleischauer, Simonsen, & Arab, 2003; Yuan, Zhang, Fan, & Yang, 2008). The presence of excessive reactive oxygen species can lead to damage or destruction of a variety of tissues. Thus, the free radical-scavenging activity, ferrous ion-chelating ability and reducing power were evaluated in this study. The results showed that CMP-1 had antioxidant activity, which may protect the organs from free radicals and retard the progress of the diseases.

3.9. Immunostimulatory activity of CMP-1 on splenocyte proliferation

Lymphocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses. As shown in Fig. 5a, CMP-1 was found to significantly stimulate the proliferation of splenocytes compared with the control ($P < 0.01$). At the concentrations from 0.97 to 62.5 μg/mL, CMP-1 stimulated the proliferation of mouse splenocytes in a concentration-dependent manner. The immune system plays an important role in anti-tumor defense. Anti-tumor activity of the polysaccharides was also mediated through enhancing the immune response (Chen et al., 2012). Splenocyte proliferation was an indicator of immunoenhancement. The immunologic action of polysaccharides might begin with activating effect of cells such as lymphocytes and macrophages.

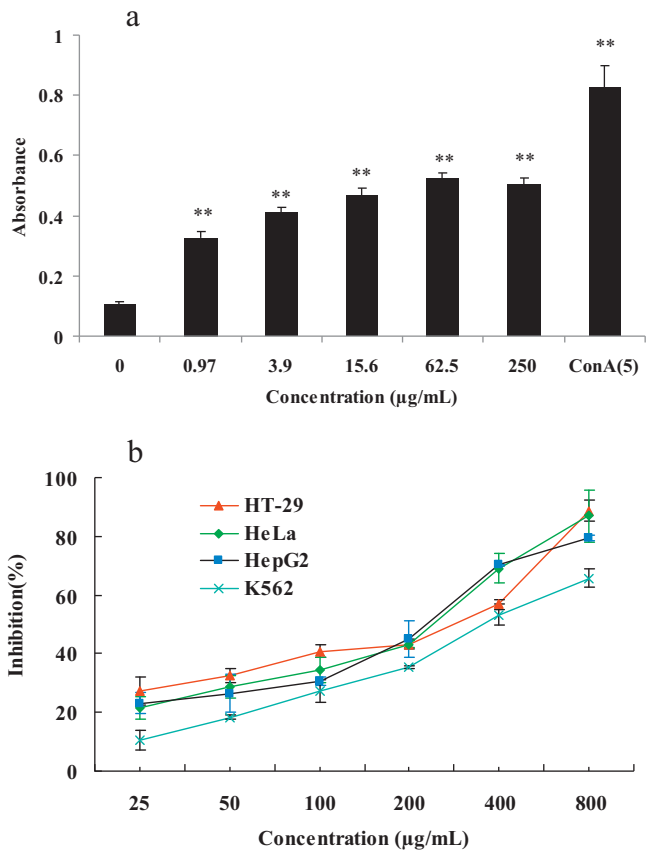

Fig. 5. (a) Effect of CMP-1 on mouse splenocyte proliferation. $^{**}P < 0.01$ compared with negative control group. (b) Effects of CMP-1 on human tumor cell growth. HepG2, HT-29, HeLa and K562 were treated by CMP-1 at different concentrations for 48 h in 96-well plates. The absorbance of the culture was measured at 570 nm. Data are presented as mean ± SD of three separated experiments.

3.10. Cytotoxicity of CMP-1 against four human tumor cell lines

The cytotoxicity induced by CMP-1 was investigated in four human cancer cell lines using MTT assay. All tested cells were treated with CMP-1 at various concentrations for 48 h. As shown in Fig. 5b, CMP-1 exhibited the capability of anti-proliferation against HT-29, HeLa, HepG2 and K562 cells, with the IC_{50} values of 137.66, 162.59, 176.29 and 364.01 μg/mL, respectively. Cisplatin exhibited the capability of anti-proliferation against HT-29, HeLa, HepG2 and K562 cells, with the IC_{50} values of 5.06, 0.63, 0.58 and 1.74 μg/mL, respectively. In recent years, more and more polysaccharides have been reported to exhibit antiproliferative activities. A polysaccharide fraction (TM-P2) was purified from *Tricholoma matsutake*. The antiproliferative activities of TM-P2 (4.0 mg/mL) on the growth of HepG2 and A549 cells were 67.98% and 59.04%, respectively (You et al., 2013). A polysaccharide (CPS) was isolated and purified from the mycelia of *Cordyceps gunnii*. The tumor inhibition ratio on K562 cell by CPS (400 μg/mL) was 56.65% (Zhu et al., 2012). Compared with the mentioned polysaccharides, CMP-1 exhibited a strong inhibition effect on the growth of HT-29, HeLa, HepG2 and K562 cells.

4. Conclusion

In conclusion, the present paper clearly demonstrated that a novel low-molecular-weight polysaccharide (CMP-1) isolated from the cultured fruiting bodies of *C. militaris* contains predominantly D-glucose and L-rhamnose. This natural water-soluble polysaccharide had appreciable antioxidant activity via multi-mechanisms, which was proved by evaluating its free radical-scavenging activity, ferrous ion-chelating ability and reducing power. CMP-1 possessed the cytotoxicity against HT-29, HeLa, HepG2 and K562 cells, as well as the stimulatory effect on the proliferation of mouse splenocytes, which provide the experimental evidence and scientific explanation for the folkloric uses of *C. militaris* as a substitute for *C. sinensis*. Therefore, *C. militaris* would have broad application prospects in exploiting new functional foods as well as therapeutic agent.

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