SUPPLEMENTARY MATERIAL

Polysaccharide from *Caulerpa lentillifera*: extraction optimization with response surface methodology, structure and antioxidant activities

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ABSTRACT: Ultrasonic-assisted extraction based on Response Surface Methodology was applied to isolate a polysaccharide (CLP) from *Caulerpa lentillifera* obtained in the South China Sea and the highest yield of the crude polysaccharide was 11.8%. CLP consisted of xylose, galactose, glucose and glucuronic acid in a molar ratio of 2.00: 1.00: 0.26: 0.04, with a molecular weight of 115 kDa. The content of uronic acids and sulfate groups in CLP was 14.10% and 27.40%, respectively. CLP presented good radical scavenging activities against $O_2^{\cdot-}$, DPPH radical, while the scavenging activities against OH· and ABTS radical were not so satisfying.

Keywords: *Caulerpa lentillifera*; polysaccharide; extraction; structure; antioxidant activities

Experimental

Materials and reagents

Caulerpa lentillifera was supplied by Longlou seaweed farm, Wenchang, Hainan province, China. Monosaccharide standards (D-galactose (Gal), L-rhamnose (Rha), L-arabinose (Ara), L-fucose (Fuc), D-glucose (Ghlc), D-xylose (Xyl), D-mannose (Man), and D-glucuronic acid (GlcA)), dextrans (MW: 196 kDa, 43.5 kDa, 9.9 kDa, 1.46 kDa, and 106 Da), 5-methyl-2-phenyl-1,2-dihydropyrazol-3-one (PMP), bovine serum albumin (BSA), Coomassie brilliant blue G-250, 5,5-two methyl-1-pyridine-N-oxide (DMPO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), and 1,2,3-Trihydroxybenzene were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Single factor test

The effects of solution/material ratio, extraction time and extraction temperature on the yield of crude polysaccharide were investigated. The extraction time is the sum of the water bath time and the ultrasound time.

A fresh sample of *Caulerpa lentillifera* was washed with tap water, and then air-dried. Finally, it was pulverized for further extraction. Polysaccharides were isolated through ultrasonic-assisted extraction in dilute NaOH solution (Sun et al. 2018). 5 g of dry seaweed was extracted in 2% NaOH solution under ultrasonication with water bath ultrasonic for 1h, then boiled in a water bath for a period of time (3 h, 4 h, 5 h, 6 h). The supernatant was filtered and concentrated to 100 mL, then deproteinated through the Sevage method (Tian et al. 2015). The resultant liquid was dialysed for 3 days against deionized water in a dialysis bag with a cut-off 3500 Da. The crude polysaccharide was obtained after freeze drying. Different solution/material ratio (30:1, 40:1, 50:1, 60:1), extraction time (4 h, 5 h, 6 h, 7 h), temperature (60℃, 70℃, 80℃, 90℃) were investigated. The yield of the crude polysaccharide was calculated as Eq. (1).

$$\text{Yield (\%, w/w)} = \frac{\text{weight of crude polysacchaside (g)}}{\text{weight of alga powder (g)}} \times 100\% \qquad (1)$$

Experimental design and statistical analysis

Three level RSM (Yin et al. 2018) was employed to optimize the parameters of

ultrasonic-assisted extraction. On the basis of the single factor experiments, a three-factor, three-level Box-Behnken Design (BBD) was employed. Solution/material ratio (X_1), extraction time (X_2), and extraction temperature (X_3) were the independent variables, and the yield of polysaccharide was the dependent variable. Factors and levels used for BBD were listed in Table S1. The model equation for the dependent variable (Y) was showed as Eq. (2).

$$Y = A_0 + \sum A_i X_i + \sum A_{ii} X_i^2 + \sum \sum A_{ij} X_i X_j \quad (2)$$

Where Y was the dependent variable; A_0 was the constant coefficient; A_i was the linear coefficient; A_{ii} was the quadratic coefficient; A_{ij} was the two-factor interaction coefficient; X_i and X_j were the independent variables. Analysis of variance was used to explore the impact of variables on polysaccharide extraction. 3D response surface plot was made using Design-Expert.V8.0.6 software (Kadam et al. 2015).

Purification of the crude polysaccharide

The crude CLP (50 mg) was dissolved in distilled water and fractionated on a cellulose DEAE-52 column (2.6×60 cm). The polysaccharides were stepwise eluted with distilled water and NaCl gradient solutions (0.2M, 0.4M, 0.6M, 0.8M, 1.0M). Each fraction (4 mL/tube) was checked at 490 nm using the phenol-sulfuric acid method (Cuesta et al. 2003). A main fraction was collected, concentrated, dialysed against distilled water, and then finally lyophilized to obtain a pure CLP.

Characterization of CLP

Total sugars, protein, uronic acid and sulfate content determination

The total sugar content was determined by the phenol-sulfuric acid method, using D-glucose as the standard (Huang et al. 2011). The protein content was determined by the Bradford's method (Bradford 1976). The content of uronic acid was measured by the sulfuric acid-carbazole method (Blumenkrantz and Asboe-Hansen 1973). The sulfate content of polysaccharides was determined applying the $BaCl_2$-gelation method (Dodgson and Price 1962).

Determination of molecular weight

The molecular weight of CLP was measured by GPC using a Waters 1515 instrument fitted with two connecting columns (Ultra hydrogel TM 120 and Ultra hydrogel TM 500, Waters, USA). The eluent was 0.05% NaN_3 with a flow rate of 0.6 mL/min at 40℃. The measurements were monitored with a Waters 2414 refractive index detector. The GPC system was calibrated before sample analysis with dextran as the standards (M_W: 196 kDa, 43.5 kDa, 9.9 kDa, 1.46 kDa, and 106 Da).

FT-IR and NMR analysis

The FT-IR spectra were measured with KBr method on a Bruker TENSOR27 spectrometer in the scanning range of 4000-400 cm^{-1}. The ^{1}H-NMR spectra were recorded on a Bruker AV 400 NMR spectrometer at 25℃. The polysaccharide sample was dissolved in D_2O with a concentration of 16.7 mg/mL.

Analysis of monosaccharide composition

The monosaccharide composition of CLP was analysed with the 1-phenyl-3-methyl-5-pyrazolone (PMP) derivation method (Lv et al. 2009). 10 mg of polysaccharide was dissolved in 10 mL of 2 mol/L trifluoroacetic acid (TFA) at 120℃ for 8 h to hydrolyse the polysaccharide to monosaccharides in a sealed glass tube. The acid was removed by evaporating with methanol. The hydrolyzed products were modified with PMP. 100 μL standard monosaccharide aqueous solution was mixed with 100 μL of 0.3 mol/L NaOH, and then 120 μL of 0.5 mol/L PMP methanol solution. The reaction was carried out at 70℃ for 1 h. The mixture was cooled to room temperature and then neutralized with 100μL of 0.3 mol/L HCl solution. The resultant solution was extracted with 15 mL chloroform for three times, then the aqueous layer was filtered using a 0.22 μm membrane (Hsu et al. 2013). The obtained sample was measured by HPLC with a ZORBAX Eclipse XDB -C18 column and a UV detector. The analysis conditions of HPLC were: mobile phase, 0.1 mol/L potassium phosphate buffer-acetonitrile (83:17, pH=10) at a flow rate of 1.0 mL/min; column temperature, 30 ^oC; injection volume, 20 μL; UV detector, at 245 nm. The standard sugars (L-rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, D-galactose, D-glucose, D-ribose, D-glucuronic acid, D-galacturonic acid) were determined likewise. The monosaccharide components of the

sample were identified by comparing the HPLC results of the sample and the standard sugars.

Periodate oxidation

Periodate oxidation of CLP was done by using the reported method (Qiao et al. 2010) with slight modification in order to infer the structural information of polysaccharide, such as the position and connection of glycosidic bonds. CLP (50 mg) was oxidized with sodium metaperiodate ($NaIO_4$) solution (15 mM, 50 mL) and stored in refrigerator at 5^{o}C in the dark. Approximately 0.1 mL of the reactive mixture was diluted to 25 mL and the absorption of the diluent at 223 nm was detected. Absorption of the diluent (from 0.1 mL to 25 mL) was monitored at 223 nm every 6 h until the absorption had no more change. Ethylene glycol (1.0 mL) was added to the reaction system with stirring for 30 min to decompose the excess $NaIO_4$. Consumption of $NaIO_4$ was calculated according to the $NaIO_4$ standard curve and the absorption at 223 nm.

Production of formic acid (HCOOH) was determined by titration. The reaction product (1.0 mL) was mixed with Bromocresol indicator (0.5%, 50 μL) and then NaOH (0.01 mol/L) was added drop by drop until the colour of the reaction mixture changed from colourless to purple red. The amount of produced HCOOH was calculated according to the amount of NaOH.

Antioxidant Activities of CPL

Hydroxyl Radical Scavenging Assay

50 μL CLP solution was mixed with 50 μL of 0.05 M phosphate buffer saline (pH=7.4), 50 μL of 0.3 mol/L 5,5-two methyl-1-pyridine-N-oxide (DMPO) solution and 50 μL of 10 mM ferrous sulphate. The reaction was started by adding 50 μL of 10 mM NaOH solution. The reaction mixture was absorbed into a capillary. After 2.5 min, the electron spin resonance (ESR) pattern was recorded by a Brucker A320 ESR spectrometer (Yoshioka et al. 2001). The test conditions were: central magnetic field (CF), 3512.3 G; scanning width (SW), 200 G; scanning time (ST), 15.36 s; modulation amplitude (MA), 3.0 G; modulation frequency (MF), 100 KHz; microwave frequency (MF), 9.863 GHz; microwave power (MP), 20.0mW; test temperature, room temperature. The hydroxyl radical scavenging ratio of the polysaccharide was calculated according to Eq. (3).

143 $$\text{Hydroxyl radical scavenging ratio (\%)} = \frac{H_0 - H_X}{H_0} \times 100\% \quad (3)$$

144 Where, H_0 and H_x was the second peak signal intensity of the control (deionized
145 water instead of the sample) and sample, respectively.

146 *DPPH Radical Scavenging Assay*

147 The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the
148 polysaccharide was determined to measure the antioxidant activity (Souza et al. 2012),
149 and modified slightly. Briefly, 10 mg/mL CLP solution was diluted into 2 mg/mL, 4
150 mg/mL, 6 mg/mL, and 8 mg/mL. 3 mL of CLP with different concentration was mixed
151 with 5 mL of 0.1 mM DPPH-ethanol solution. The mixed solution reacted at room
152 temperature in the dark for 30 min and then measured by a UV/Vis spectrometer at 517
153 nm. Eq. (4) was used to calculate the DPPH radical scavenging activity.

154 $$\text{DPPH radical scavenging ratio (\%)} = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\% \quad (4)$$

155 Where, A_1 was the absorbance of 3 mL CLP and 5 mL DPPH-ethanol solution;
156 A_2 was the absorbance of 3 mL CLP and 5 mL ethanol; A_0 was the absorbance of 3 mL
157 ethanol and 5 mL DPPH-ethanol solution.

158 *ABTS radical scavenging activity assay*

159 The traditional method (Zhu et al. 2016) was made appropriate modifications. 2,2'-
160 Azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) was dissolved in 2.45 mM
161 potassium persulfate. 7 mM ABTS radical reserve solution was prepared and kept at
162 room temperature in the dark for 16 h. The ABTS radical reserve solution was diluted
163 with 10 mM phosphate buffer solution (pH=7.4) to get an absorbance in the range of
164 0.700 ± 0.020 at 734 nm. 40 μL CLP was added to 4 mL ABTS radical solution. The
165 mixture was oscillated for 30 s, and then the absorbance was measured at 734 nm. The
166 ABTS radical scavenging activity of polysaccharide was calculated by the following
167 equation Eq. (5).

168 $$\text{ABTS radical scavenging ratio (\%)} = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\% \quad (5)$$

Where, A_0 was the absorbance of the control group (deionized water instead of the sample); A_1 was the absorbance of the CLP - ABTS radical solution group; A_2 was the absorbance of the sample only.

Superoxide radical scavenging activity assay

Tris-HCl buffer solution (0.05 M, pH=8.2) was placed in a water bath of 25^{o}C for 30 min. Then 0.5 mL CLP solution, 4.5 mL Tris-HCl buffer solution, 0.1 mL of 3 mM 1,2,3-Trihydroxybenzene solution were mixed and reacted at 25^{o}C for 4 min. The reaction was stopped by adding HCl. The mixture was measured by UV/Vis spectroscopy at 325 nm every 30 second in 3 minutes. The control group was 0.5 mL deionized water mixed with 4.5 mL Tris-HCl buffer solution. Each sample was measured for three times, and the average value was taken. The scavenging ratio was calculated with Eq. (6).

$$\text{Superoxide radical scavenging ratio (\%)} = \frac{A_0 - A_1}{A_0} \times 100\% \qquad (6)$$

Where, A_1 was the absorbance of the sample; A_0 was the absorbance of the control group.

Statistical analysis

All assays were measured independently in triplicate. The differences between the means of parameters were determined by One-way analysis of variance (ANOVA). *P* values of <0.05 were assumed to be statistically significant.

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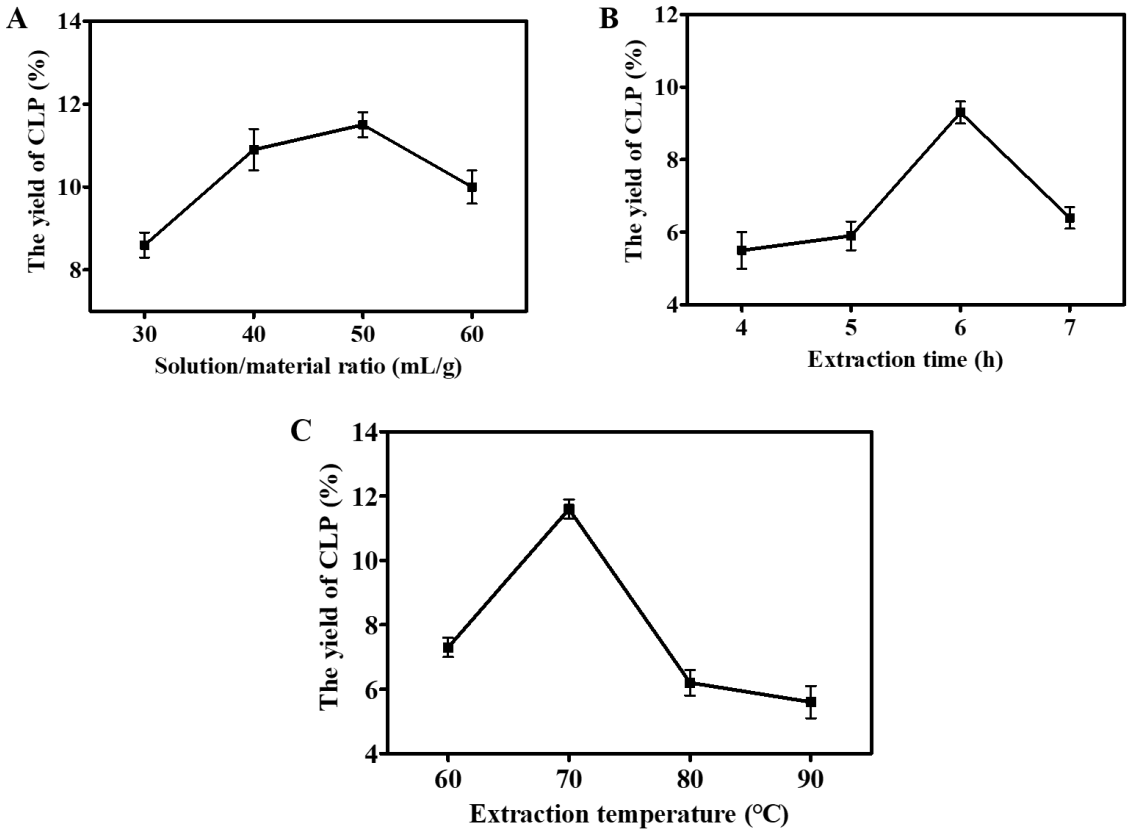

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236 Figure S1. Effect of solution/material ratio (A), extraction time (B), and extraction

237 temperature (C) on the yield of CLP.

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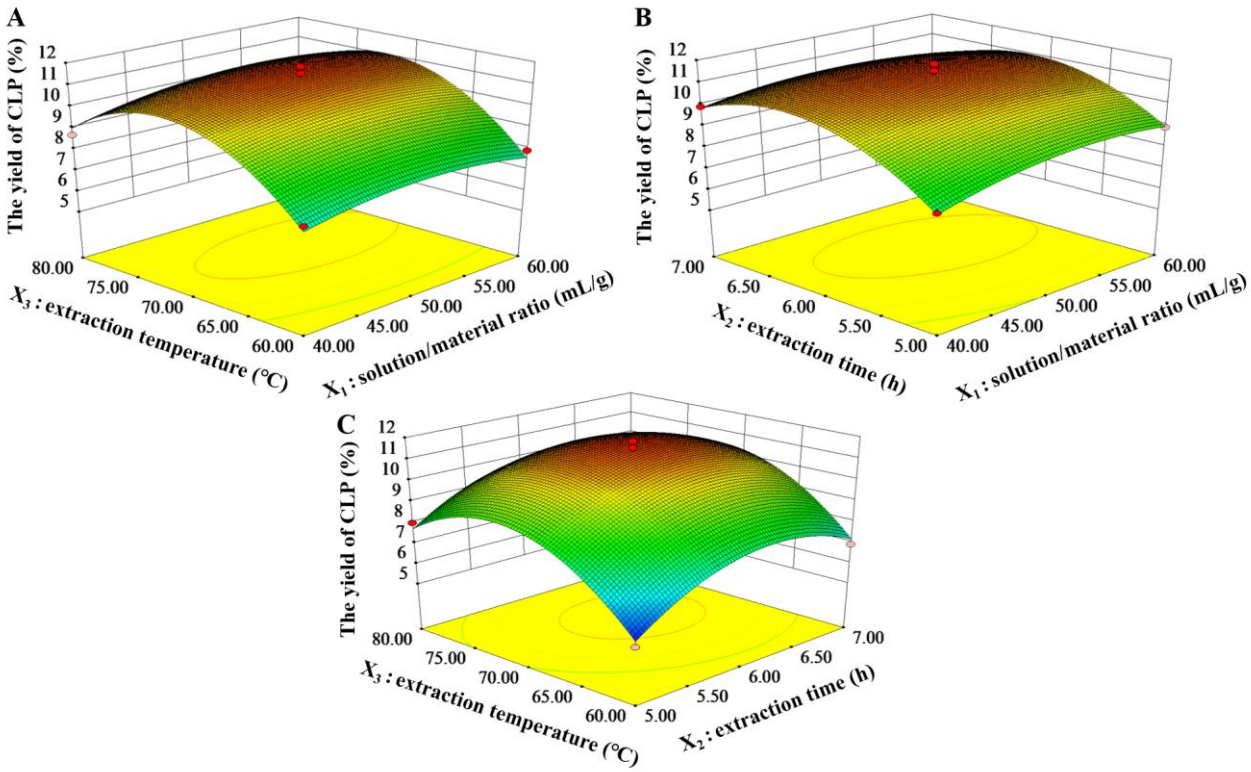

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241 Figure S2. Response surface plots showing the effects of solution/material ratio (X_1),
242 extraction time (X_2), extraction temperature (X_3) on the yield of CLP

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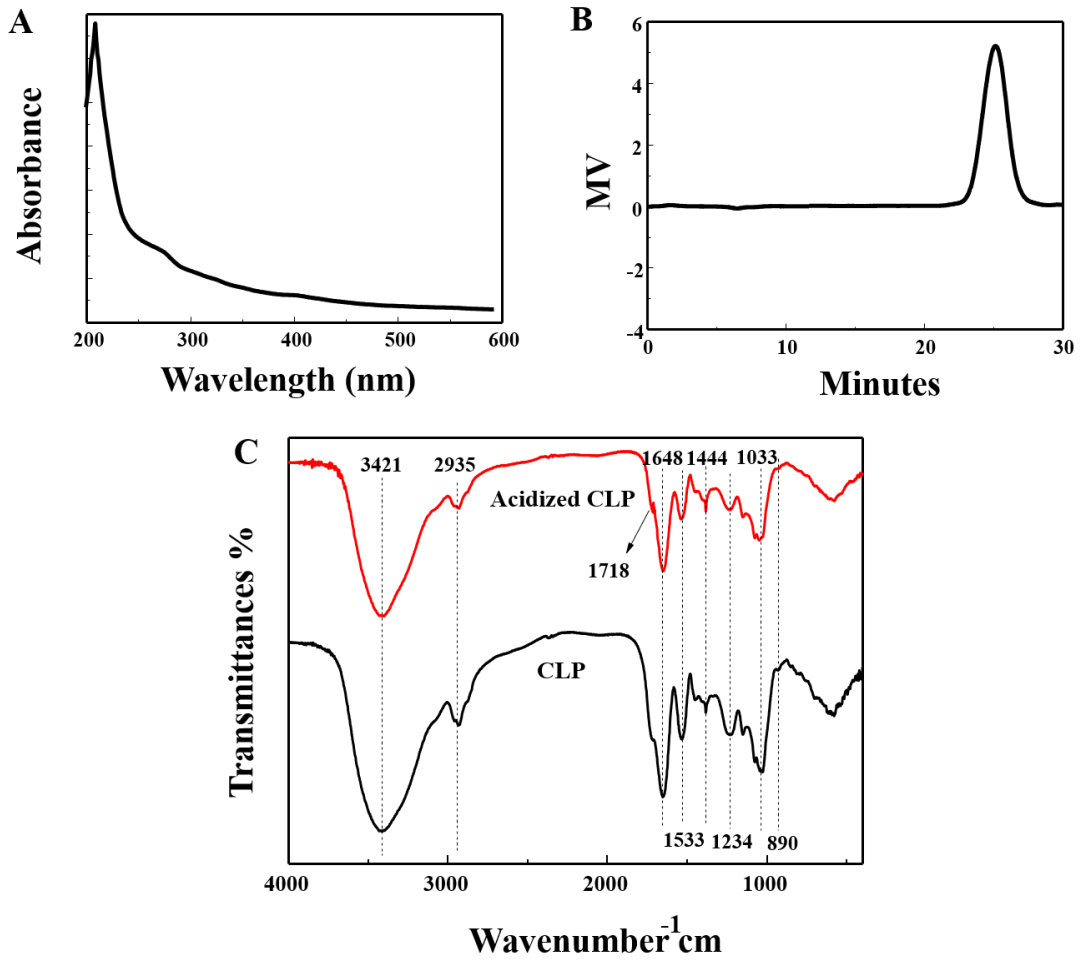

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256 Figure S3. The UV/vis spectrum (A) , GPC distribution (B), and FT-IR spectra (C) of
257 CLP

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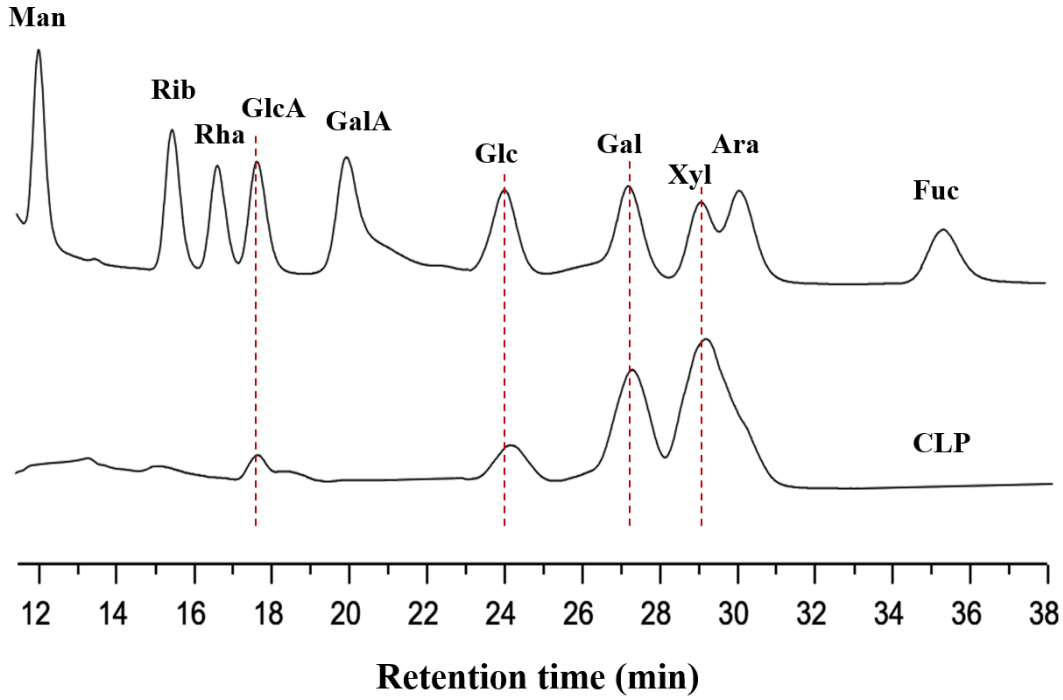

Figure S4. HPLC chromatograms of PMP modified sugars

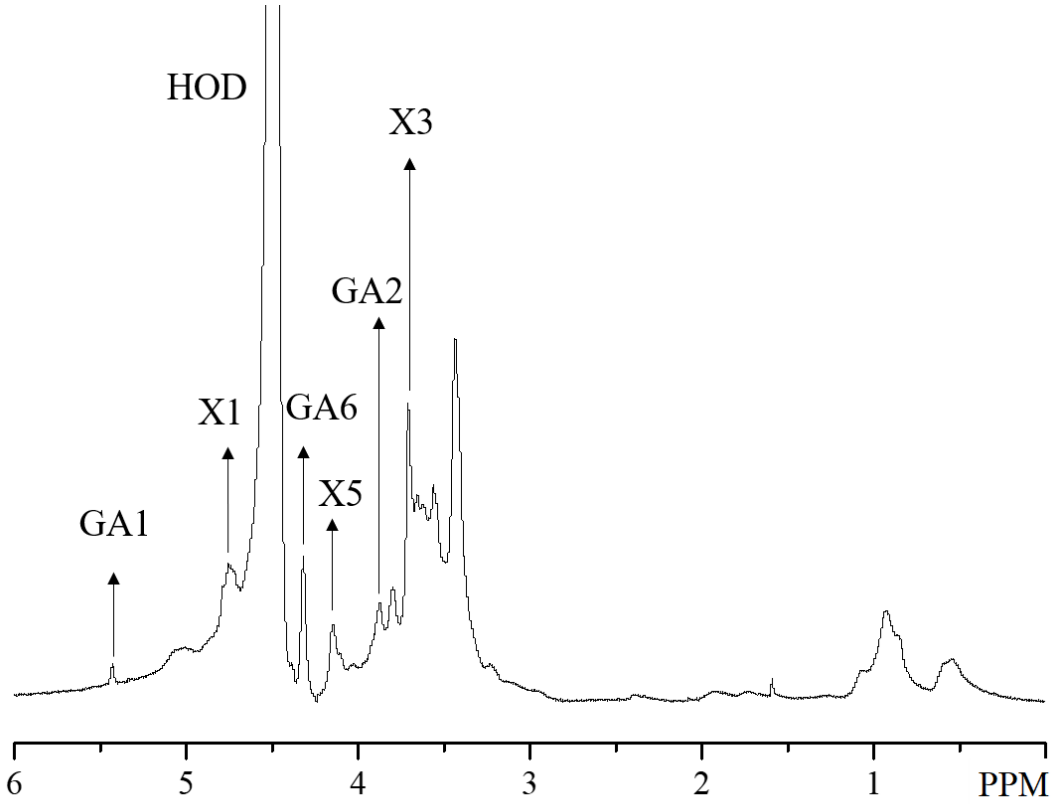

Figure S5. The ^{1}H-NMR spectrum of CLP

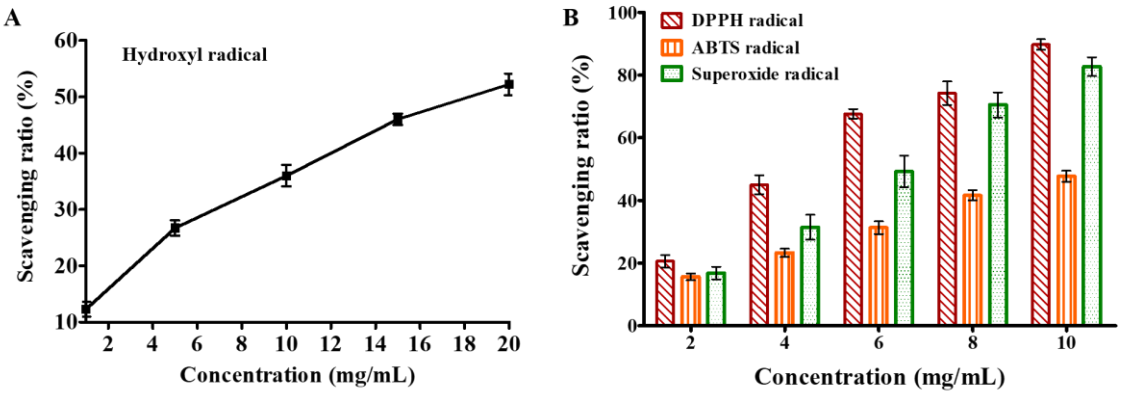

Figure S6. (A) Scavenging activity of CLP against hydroxyl radical; (B) Scavenging activity of CLP against DPPH radical, ABTS radical and superoxide radical.

Table S1. Factors and levels used for BBD

Factors	Coded variables	Levels		
		-1	0	1
solution/material ratio（mL/g）	X_1	40	50	60
extraction time（h）	X_2	5	6	7
extraction temperature（℃）	X_3	60	70	80

Table S2. Experiment design and results of RSM analysis

No.	X_1 (mL/g) solution/material ratio	X_2 (h) extraction time	X_3 (℃) extraction temperature	yield (%)
1	0	0	0	10.9
2	-1	0	1	8.7
3	0	0	0	10.9
4	0	1	-1	6.9
5	-1	1	0	9.9
6	0	-1	1	8.0
7	0	0	0	11.8
8	1	0	-1	7.9
9	0	0	0	11.5
10	1	-1	0	8.9
11	1	0	1	9.4
12	1	1	0	9.7
13	0	-1	-1	5.4
14	-1	0	-1	7.6
15	-1	-1	0	8.1
16	0	1	1	9.1
17	0	0	0	11.5

Table S3. Analysis of variance for the established regression model

Variables	Sum of squares	DF	Mean square	*F* value	*P* value	Significant
Model	50.15	9	5.57	30.56	<0.0001	**
X_1- solution/material ratio	0.30	1	0.30	1.67	0.2375	
X_2-extraction time	3.34	1	3.34	18.32	0.0037	*
X_3-extraction temperature	7.24	1	7.24	39.70	<0.0001	**
X_1X_2	0.28	1	0.28	1.51	0.2586	
X_1X_3	0.03	1	0.03	0.17	0.6942	
X_2X_3	0.04	1	0.04	0.22	0.6537	
X_1^2	1.31	1	1.31	7.16	0.0317	
X_2^2	10.64	1	10.64	58.35	0.0001	*
X_3^2	23.94	1	23.94	131.30	<0.0001	**
Residual error	1.28	7	0.18			
Lack of fit	0.61	3	0.20	1.21	0.4149	
Pure error	0.67	4	0.17			

R^2 =0.9752. * Significant at $p \leq 0.05$. ** Significant at $p \leq 0.0001$.

Table S4. The chemical compositions of CLP (%)

Fraction	monosaccharide composition				Sulfate content	Uronic acid content
	Xyl	Gal	Glc	GlcA		
CLP	59.80	29.80	7.80	2.50	27.40	14.10