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Extraction, structure characterization and biological activity of polysaccharide from coconut peel

Shiyang Zhou[1,2,3] and Gangliang Huang[3*]

Abstract

Taking coconut peel as raw material, the extraction process of coconut peel polysaccharide (CPP) was optimized by boiling water extraction. The coconut peel polysaccharide was characterized by UV, IR, SEM, 1D NMR and 2D NMR spectra. At the same time, the molecular weight and monosaccharide component were analyzed by gel chromatography and ion chromatography, respectively. Antioxidant activity of coconut peel polysaccharide and its derivatives in vitro was evaluated by scavenging ABTS and DPPH radicals and $O_2^{-\cdot}$, and the anti HepG2 proliferation activity in vitro was also carried out. The results showed that the molecular weight of coconut peel polysaccharide was 1.20×10^5 Da, which was mainly composed of arabinose (Ara), galactose (Gal), glucose (Glu), xylose (Xyl) and galacturonic acid (Gal-A). The main chain structure of polysaccharides detected by 1D and 2D NMR spectrum was → 4)-α-D-Gl*cp* (1 → . In vitro antioxidant test showed that coconut peel polysaccharide and its derivatives had a certain scavenging effect on ABTS and DPPH free radical and $O_2^{-\cdot}$. With the increase of polysaccharide concentration, the scavenging ability was gradually increased. In addition, coconut peel polysaccharide and its derivatives showed significant antiproliferative activity against HepG2 cells in vitro.

Keywords Coconut peel, Polysaccharide, Antioxidant, Antiproliferative

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Graphical Abstract

Introduction

The coconut tree (*cocos nucifera L.*) is a large evergreen of the Palmae family and Cocos genus [1]. Coconut trees are suitable for growing in high temperature, rainy and low altitudes, which are mainly distribute in tropical equatorial coastal areas, such as Philippines, India, Malaysia, Sri Lanka and other countries [2]. With high economic value, the whole Coconut plant can be used, especially its fruit [3]. Coconuts that are the fruit of inflorescence after fertilization are common in the tropics and falls off as they ripen [4]. Coconut fruit is one of the largest drupe in plants. Its outer layer is fibrous shell and the inner is thick edible flesh [5–7]. Coconut fruit is the economic part with the highest economic value of the whole plant, the main source of economic income for coconut farmers, and the basic raw material for many coconut products. When the coconut fruit is fresh, it contains juice that is rich in a variety of vitamins, which can improve the vitality of cells and make the peel ruddy and elastic. Coconut peel is a kind of traditional Chinese medicine with great medicinal effect, especially used with other drugs [8]. The main component of coconut peel is fiber, and the hard shell of inside is endocarp [9]. The most effective use of coconut peel was to stop bleeding, and it can also treat diseases, such as heartburn. Studies on the chemical composition of coconut peel show that it contains lignin, cellulose (mainly pentosan), ash (potassium hydroxide), etc. [10, 11].

Polysaccharides was long chain polymers composed of multiple monosaccharide molecules connected by glycosidic bonds, which was a kind of very important macromolecular substances in cells [12]. Polysaccharides were an important component of receptor molecules on cell membrane, a participant in cell recognition and information transmission, a class of non-specific broad-spectrum immunomodulatory agents and important life materials, widely involved in various life activities [13]. Recent studies have shown that polysaccharides show promising application prospects in immunomodulatory, antitumor, antivirus, antioxidation and hypoglycemic aspects, which was the focus of both modern medicine and food functional chemistry [14]. Natural polysaccharides have a wide range of sources and complex chemical composition. Crude polysaccharides were usually accompanied by some proteins, fats, pigments and other substances, which are not conducive to the structure identification and subsequent activity analysis of polysaccharides [15]. Therefore, it was necessary to select suitable polysaccharide extraction methods. At present, the commonly used extraction methods of polysaccharides include water extraction and alcohol precipitation method, enzymatic hydrolysis method, microwave extraction method, ultrasonic method and so on [16, 17]. Usually, when we drink coconut water, we discard the peel, making it a discarded resource. Under the current background of resource reuse and double carbon energy saving, it was a valuable research topic to extract active ingredients from coconut peel and make use of them. At present, there was no literature report on polysaccharide extraction from coconut peel and its biological activity, which also prompted the in-depth study of this topic. In this study, fresh coconut peel was used as material

to extract polysaccharides with boiling water, and the polysaccharides of uniform components was prepared through protein removal, dialysis, column chromatography and other steps. In the column chromatography, DEAE-52 and Sephadex G-200 were successively used as fillers for separation. On this basis, three derivatives of coconut peel polysaccharide, including acetylated (ACPP), phosphorylated (PCPP) and carboxymethylated (CCPP) polysaccharide, were prepared, and the antioxidant activities of the polysaccharide and its derivatives were studied in vitro. This study provided theoretical guidance for the further study of chemical constituents and pharmacological activities of coconut peel, and laid a foundation for the development of antioxidant activities of its polysaccharides.

Materials and methods

Materials and reagents

The remains of a commercially available Hainan coconut after the inner coconut water have been removed, in March 2022. All reagents were purchased from Aladdin (Shanghai, China).

Preparation of polysaccharide from coconut peel

After removing the outermost peel, the fresh coconut peel was thoroughly crushed. The crushed coconut peel of 100 g was placed in a 1000 mL round-bottom flask with 500 mL deionized water, which was extracted by stirring in oil bath at 100 ℃ for 3 h. After extracting for 3 times, the filtrate was collected and concentrated to 200 mL. The polysaccharide concentrate was poured into a 500 mL round-bottomed flask with 60 mL chloroform and 20 mL *n*-butanol, which was stirred for 30 min to remove the protein and nucleic acid by centrifugation at 4000 r/min for 5 min. The supernatant was placed in an ordinary dry dialysis bag (with a retained molecular weight of 3500 Da) and treated with tap water for 24 h, followed by deionized water for 24 h. Then, the dialysate was mixed with anhydrous ethanol to precipitate the polysaccharide. The precipitate was separated by centrifugation at 4000 r/min for 5 min and dried in oven at 50–60 ℃ for 12 h to obtain refined coconut peel polysaccharide.

Purification of polysaccharide from coconut peel

The refined polysaccharides were dissolved in distilled water and purified by DEAE-52 cellulose chromatography column (5.0 cm × 70.0 cm) in the first step. The samples were eluted with gradient of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mol/L NaCl, respectively. Then, the eluate was collected to determine the polysaccharide content by phenol–sulfuric acid method. Samples of the single polysaccharide elution peak were mixed and separated by Sephadex G-200 gel chromatography column, which was eluted with water for the second step of purification. The single elution peak was also determined by phenol–sulfuric acid method to obtain the purified single component of coconut peel polysaccharide.

Preparation of acetylated polysaccharides

The purified polysaccharide of 0.5 g was accurately weighed and dissolved by magnetic stirring at room temperature in a 100 mL round-bottom flask with 10 mL pure water. The pH of polysaccharide solution was adjusted to 9 with 2 mol/L NaOH solution under stirring. Then, 0.6 mL acetic anhydride was slowly added to the solution with an eyedropper. After that, the pH was adjusted to 8–9 with 2 mol/L NaOH solution, and the reaction solution was continue stirred for 1 h. After reaction, the solution pH was adjusted to 7 with 2 mol/L HCl solution, and transferred to a dialysis bag with an interception molecular weight of 3500 Da. The bag was dialyzed first in running tap water for 24 h and then in distilled water for 24 h. After lyophilization of the dialysate, acetylated polysaccharide was obtained.

Preparation of phosphorylated polysaccharides

The 5 g sodium polyphosphate and 2 g sodium trimetaphosphate were dissolved in 10 mL distilled water to prepare phosphorylation reagents. The purified coconut peel polysaccharide of 0.5 g was dissolved with 20% 10 mL sodium sulfate in a 100 mL round-bottom flask to prepare polysaccharide solution. Then, the phosphorylation reagent was slowly added to the round bottom flask. The pH of the solution was adjusted to 9 with 2 mol/L NaOH solution and the reaction was carried out at 80℃ for 5 h under magnetic stirring. After reaction, it was cooled to room temperature, and transferred into a dialysis bag with an interception molecular weight of 3500 Da. The bag was dialyzed first in running tap water for 24 h and then in distilled water for 24 h. Phosphorylated coconut peel polysaccharide can be obtained after lyophilization.

Preparation of carboxymethylated polysaccharides

Purified coconut peel polysaccharide of 0.5 g was mixed with 10 mL isopropanol and 10 mL 20% sodium hydroxide solution in a 100 mL round bottom flask, which was stirred at room temperature to prepare a uniform suspension. Chloroacetic acid of 3 g was added to the round bottom flask in batches, and then the mixture was reacted at 60 ℃ for 3 h under magnetic stirring. After the reaction, the solution was cooled to room temperature, and the pH of which was adjusted to 7 with 2 mol/L HCl

solution. The reaction solution was transferred to a dialysis bag with an interception molecular weight of 3500 Da and dialyzed first with running tap water for 24 h and then with distilled water for 24 h. After lyophilization, carboxymethylated coconut peel polysaccharide can be obtained.

Determination of molecular weight of coconut peel polysaccharide

The samples and standards were prepared into 5 mg/mL solution, which was centrifuged at 12000 r/min for 10 min to obtain the supernatant, and then filtered with 0.22 μm microporous filter membrane to obtain an injection volume of 20 μL. Glucans with different relative molecular weights (5000, 11600, 23800, 48600, 80900, 148000, 273000, 409800 and 667800 Da) were used as standard materials to establish standard curves and determine the purity and relative molecular weight of polysaccharides. Chromatographic conditions were: column, BRT105-104–102 tandem gel column (8 × 300 mm); mobile phase, 0.05 mol/L NaCl solution; flow rate, 0.6 mL/min; column temperature, 40 ℃; detector, differential indicator detector RI-10A.

Determination of monosaccharide components of coconut peel polysaccharide

Sample was mixed with 2 mL 3 mol/L trifluoroacetic acid solution in ampoule and hydrolyzed at 120 ℃ for 3 h. The acid hydrolysis solution in a tube was blow dry with nitrogen. The dry matter was dissolved in 5 mL water, and 50 uL of the solution was taken to mix with 950 uL deionized water. The diluent was centrifuged at 12000 r/min for 5 min, and 5 μL supernatant of which was taken for monosaccharide analysis. All monosaccharide standard solutions were mixed accurately as mixing standard. According to the absolute quantitative method, the mass of different monosaccharides was determined, and the molar ratio was calculated according to the molar mass of monosaccharides. Monosaccharides was detected by the chromatographic column of Dionex Carbopac™ PA20 (3 × 150 mm) at 0.3 mL/min flow rate and the temperature of 30 ℃ with the electrochemical detector. The mobile phase was A, H_2O; B, 15 mmol/L NaOH; C, 15 mmol/L NaOH and 100 mmol/L NaOAc.

Scanning electron microscopy of coconut peel polysaccharide

About 5 mg of dried polysaccharide samples were adhered to the conductive carbon film containing double-sided adhesive, which was placed in the sample chamber of ion sputtering apparatus to spray gold for about 40 s. The film with sample was placed into the scanning electron microscope observation chamber, and observed at the accelerating voltage of 2 kV by model SU8100 scanning electron microscope (HITACHI, Japan), MC1000 ion sputtering instrument (HITACHI, Japan).

Determination of total sugar of coconut peel polysaccharide and its derivatives

According the standard curve of glucose, the total sugar content of coconut peel polysaccharide was determined by phenol–sulfuric acid method at the wavelength of 490 nm.

Ultraviolet spectrum analysis of coconut peel polysaccharide

The polysaccharide sample solution was prepared, and full wavelength scanning of distilled water was used as the baseline of blank calibration scan. Using UV-1800 spectrum, absorbance of the sample solution was tested from 200 to 600 nm.

Infrared spectrum analysis of coconut peel polysaccharide

The samples polysaccharide and KBr were mixed and finely grinded in an agate mortar at the ratio of 1:100. Fourier transform infrared spectrometer model 650 (FTIR-650) was used to measure the transmittance at wave number 4000–500 cm^{-1}.

NMR analysis of coconut peel polysaccharide and its derivatives

Coconut peel polysaccharide sample of 25 mg was dissolved in D_2O and characterized at 25 ℃ by the nuclear magnetic resonance (NMR) spectroscopy of JNM-ECZ600R (JEOL, Japan).

Determination of antioxidant activity

Determination of ABTS free radical scavenging rate

The ABTS free radical scavenging ability of coconut peel polysaccharide and its derivatives samples were determined according to reference [18]. The absorbance (734 nm) of ABTS solution was adjusted to 0.70 ± 0.20 with absolute ethanol. ABTS solution of 150 μL and sample solution with different concentrations (0.2, 0.4, 0.8, 1.6, 3.2 mg/mL) of 50 μL was thoroughly mixed by shaking for 10 s. After static standing for 6 min, the absorbance was measured at 734 nm with ethanol (95%, 0.2 mL) as blank control. ABTS free radical scavenging rate was calculated using the following formula:

$$\text{ABST free radical scavenging rate (\%)} = \frac{1-(A_1-A_2)}{A_0} \times 100\%$$

A_0: The absorbance value of blank control solution.
A_1: The absorbance value of the sample.
A_2: The absorbance value of the ABTS solution.

DPPH free radical scavenging rate

DPPH free radical scavenging activity of coconut peel polysaccharide and its derivatives samples was determined according to reference [19]. The new DPPH solution (0.2 mmol/L, 95% ethanol) was prepared for each experiment. Different concentrations of sample solution (100 μL, 0.2, 0.4, 0.8, 1.6, 3.2 mg/mL) and DPPH solution (100 μL, 0.2 mmol/L) were mixed. The absorbance was measured at 517 nm at room temperature for 30 min in the dark with the deionized water as a blank control. After reaction for 30 min at room temperature in the dark, the absorbance was measured at 517 nm. DPPH free radical scavenging rate was calculated using the following formula:

$$\text{DPPH free radical scavenging rate (\%)} = \frac{1-(A_1-A_2)}{A_0} \times 100\%$$

A_0: The absorbance value of blank control solution.
A_1: The absorbance value of the sample.
A_2: The absorbance value of the DPPH solution.

Determination of $O_2^{-\cdot}$ clearance rate

The sample solutions of coconut peel polysaccharide and its derivatives with different concentrations (0.2, 0.4, 0.8, 1.6, 3.2 mg/mL) were prepared. Sample solution of 2.0 mL, 50 mmol/L potassium phosphate buffer (pH 7.8) of 0.5 mL and 10 mmol/L hydroxylamine hydrochloride solution of 0.1 mL were mixed well and kept at 25 ℃ for 20 min. Then, 1 mL 58 mmol/L *p*-aminobenesulfonic acid solution and 1 mL 7 mmol/L *a*-naphthalamine solution was added to the mixture and reacted at 30 ℃ for 30 min. After reaction, equal volume of trichloromethane was added for pigment extraction, followed by centrifugation at 10000 r/min for 3 min. Absorption at 530 nm was measured using the upper pink aqueous phase. The clearance rate of superoxide anion was calculated using the following formula:

$$O_2^- \text{ clearance rate (\%)} = \frac{(A_0-A_1)}{A_0} \times 100\%$$

A_0: The absorbance value of blank control solution.
A_1: The absorbance value of the sample.

Determination of cell viability

The Hcuman hepatoellular carcinomas (HepG2) cells were obtained from Mlbio (Shanghai, China). The cells were maintained in DMEM supplemented with 1.5 g/L $NaHCO_3$, 110 mg/L sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS at 37 ℃ in a 5% CO_2 humidified incubator. Then, the cells were pretreated with different concentration of peel polysaccharide and its derivatives and followed by incubation for 24 h. Finally, cells were conducted for further analysis. Cell counting kit-8 (CCK-8) [20] was used to determine the cell viability of coconut peel polysaccharide. CCK-8 assay was used to measure the ability of living cells to reduce highly water-soluble tetrazolium salt (2-(2-methoxy-4-nitrobenzene)-3-(4-nitrobenzene)-5-(2, 4-disulphyl)-2 h-tetrazolium, monosodium salt) to water-soluble orange methionine by dehydrogenase in the presence of electron-coupled reagents. Cells were maintained in DMEM that was supplemented with 1.5 g/L $NaHCO_3$, 110 mg/L sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS, which were incubated with different concentration of peel polysaccharide and its derivatives at 37 ℃ in a 5% CO_2 humidified incubator for 24 h. After removal of DMEM, the cells were washed with cold PBS and incubated at 37 °C with 10 μL CCK-8 in 100 μL DMEM solution for 1 h. The absorbance was read at 450 nm by a microplate reader (Biotek, USA), and absorbance of the untreated cells was regarded as 100% cell viability.

Statistical analysis

All data were presented as mean ± standard deviation (SD), and the experiment was repeated three times. RSM results were analyzed by ANOVA, and differences were considered significant at $p < 0.05$.

Results and discussion

Extraction, preparation of derivatives and determination of substitution degree of coconut peel polysaccharide

In this study, fresh coconut peel was used as the research material, and refined coconut peel polysaccharide was prepared by grinding and crushing, boiling water extraction, concentration, protein removal, dialysis, alcohol precipitation, drying and other steps. In the process of preparing polysaccharide from refined coconut peel, the temperature and time of water extraction method and the protein removal method were studied. Orthogonal experiment was used to optimize the process of preparing refined coconut polysaccharide, the optimal extraction conditions were obtained [21]. The polysaccharides were extracted with boiling water, the solid–liquid ratio was 1:5, the extraction time was 3 h, and the sevage method was used for protein removal

Table 1 Total sugar content, yield and degree of substitution of coconut peel polysaccharides and its derivatives

	Samples			
	CPP	ACPP	PCPP	CCPP
Yield (%)	4.73 ± 0.23	84.56 ± 0.33	38.34 ± 0.21	81.61 ± 0.37
DS	–	0.86 ± 0.08	0.31 ± 0.07	0.78 ± 0.05
Sugar content (%)	98.33 ± 1.02	95.21 ± 1.22	82.35 ± 1.26	93.14 ± 1.43

(chloroform:*n*-butanol = 4:1, the treatment time was 30 min). Under the optimum conditions, the extraction rate of refined polysaccharide reached up to 4.73% (Table 1). To study the structure–activity relationship of coconut peel polysaccharide, three derivatives, including acetylated, phosphorylated and carboxymethylated coconut peel polysaccharide, were prepared by chemical synthesis. As can be seen from Table 1, the yields of acetylated, phosphorylated and carboxymethylated polysaccharide were 89.05%, 36.22% and 82.49%, respectively. There were differences in yield of derivatives, among which the acetylated products were the highest, while the phosphorylated products were the lowest. This may be due to the reagents and conditions used in the reaction. The reaction activity of acetic anhydride was relatively higher than that of phosphate, so the acetylation reaction was easier. At the same time, phosphorylation and carboxymethylation reactions need to be heated. Under heating conditions, polysaccharides may undergo hydrolysis and other reactions, which increased the loss. Therefore, the yield of phosphorylation and carboxymethylation were lower than that of acetylation products. The degree of substitution could reflect the difficulty of derivatization and affect its activity. According to the data in Table 1, the substitution degree of acetylated, phosphorylated and carboxymethylated coconut peel polysaccharide were 0.86, 0.31 and 0.78, respectively. There was a certain relationship between the substitution degree of polysaccharide derivatives and the preparation yield. The substitution rate of acetylated polysaccharide with higher yield was the highest, which reflects that acetylation of coconut peel polysaccharides was easier than phosphorylation and carboxymethylation. As can be seen from Table 1, the total sugars of coconut peel polysaccharide and its acetylation, phosphorylation and carboxymethylation products were 98.33%, 95.21%, 82.35% and 93.14%, respectively. From the total sugar data, it can be found that the total sugar content of coconut peel polysaccharide and its derivatives was high.

Analysis of coconut peel polysaccharide by ultraviolet spectrum and infrared spectrum

To determine the presence of protein and nucleic acid, the purified coconut peel polysaccharide was usually analyzed by ultraviolet full-wavelength scanning. The maximum absorption wavelength of nucleic acid and protein was 260 and 280 nm, respectively [22]. Therefore, the removal of protein and polysaccharide in coconut peel polysaccharide can be judged by observing whether there was a characteristic absorption peak between 260 and 280 nm by full wavelength UV scanning. As can be seen from Fig. 1a, there was no characteristic absorption peak at 260–280 nm in the full-wavelength ultraviolet spectrum scanning of coconut peel polysaccharide, which can conclude that the protein and nucleic acid in

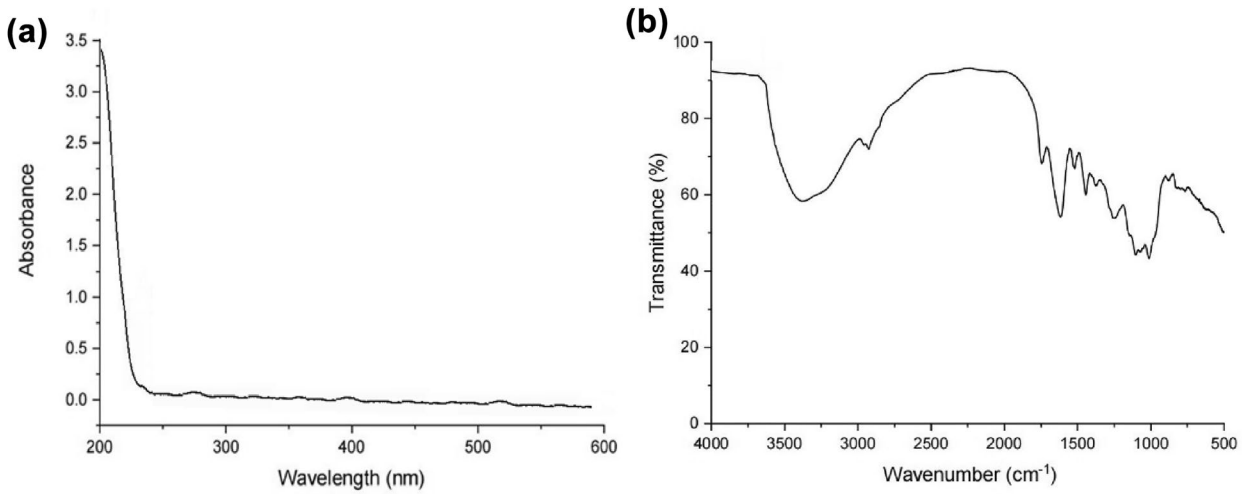

Fig. 1 UV and IR analysis of coconut peel polysaccharide. **a** UV analysis of coconut peel polysaccharide; **b** IR analysis of coconut peel polysaccharide

the sample had been removed by sevage method. Infrared spectroscopy was a molecular spectrum, which has the advantages of high sensitivity, accurate wave number and good repeatability, and was a powerful tool to determine the composition and structure of molecules. Fourier Transform Infrared spectroscopy (FTIR) was the preferred method of Infrared spectrum analysis. It was a powerful tool to analyze the structure of substances, and was widely used to analyze and identify substances, and study the interactions inside and between molecules. The structure of polysaccharides, such as monosaccharide configuration, glycosidic bond type and functional groups, can be analyzed by FTIR scanning. As can be seen from Fig. 1b, coconut peel polysaccharide had an absorption peak at 3380 cm^{-1}, which was the stretching vibration absorption peak of -OH. The absorption peak at 2926 cm^{-1} was the stretching vibration absorption peak of C–H. The absorption peak at 1745 cm^{-1} was the stretching vibration absorption peak of -CO-. The absorption peak at 1618 cm^{-1} was the bending vibration absorption peak of -OH. The absorption peaks at 1445 cm^{-1} and 1375 cm^{-1} were -CH_2- deformation absorption peaks. The absorption peak at 1252 cm^{-1} was the C–H-bending vibration absorption peak. The absorption peak at 1102 cm^{-1} was C–O absorption peak on sugar ring. The absorption peak of 1013 cm^{-1} was the absorption peak of -OH on the sugar chain. There was a characteristic absorption peak of α-type glycosidic linkage at 879 cm^{-1}, indicating that the coconut peel polysaccharide was mainly α-type. However, the weak band around 879 cm^{-1} suggests that the glycoside residues may be of the β-configuration.

Molecular weight, monosaccharide composition and SEM analysis of coconut peel polysaccharide

Coconut peel polysaccharide was separated by column chromatography on DEAE-52 and Sephadex G-200 as filler. The elution curve of column chromatography is shown in Fig. 2a, b. As can be seen from Fig. 2a, the refined coconut peel polysaccharide was separated by DEAE-52 cellulose column to obtain three single components of coconut peel polysaccharide. Which was further separated by Sephadex G-100, the purified coconut peel polysaccharide was obtained single component (Fig. 2b). HPGPC method was an effective method to determine the molecular weight of polysaccharides [23, 24]. First, the lgMw-RT correction curve equation was obtained using different molecular weight glucans as the standard. The correction curve equation of lgMw-RT was $y = -0.1961x + 12.315$ with $R^2 = 0.9934$. The chromatogram of molecular weight determination of coconut peel polysaccharide is shown in Fig. 2c, and the retention time was 36.899 min [25]. The molecular weight of coconut peel polysaccharide was calculated according to the formula of standard curve, namely, $Mw = 1.20 \times 10^5$ Da. According to the difference of pKa value of different monosaccharides and the difference of hydrophobicity between some monosaccharides and anionic resins, ion chromatography realizes the efficient separation of monosaccharides, and then detects the current generated by the oxidation of hydroxyl groups in sugar molecules on the surface of metal electrodes [26]. The monosaccharide composition of coconut peel polysaccharide was determined by ion chromatography, as shown in Fig. 2e, which was mainly composed of arabinose (Ara), galactose (Gal), glucose (Glu), xylose (Xyl) and galacturonic acid (Gal-A) with a molar ratio of 25.5%:26.8%:12.8%:12.7%:15.3%. Figure 2d shows the mixed standard ion chromatograms of the 16 monosaccharide standards. The surface morphology of the coconut peel polysaccharide sample is shown in Fig. 3. It can be seen from the SEM that the coconut peel polysaccharide sample presents granular and massive cluster structure at a magnification of 500 (Fig. 3a). At 2000 magnification, the surface of coconut peel polysaccharide samples was rough, and a large number of fragments were gathered together (Fig. 3b). At 5000 magnifications, it was observed that the surface of the coconut peel polysaccharide sample had granular aggregates packed tightly together, which may be due to the strong intermolecular interaction (Fig. 3c).

The 1D NMR and 2D NMR analysis of coconut peel polysaccharide

1D NMR analysis of coconut peel polysaccharide

Polysaccharides was a kind of complex biological macromolecule. The complexity of its structure brings great difficulties to the process of polysaccharide resolution. The principle of NMR was mainly to make use of nuclear energy level splitting in a strong magnetic field, and the transition of nuclear spin level occurs after absorbing foreign electromagnetic radiation [27]. NMR analysis of polysaccharides was to judge the heterocephalic configuration of polysaccharides, the connection mode and sequence of glycosidic bonds by recording the chemical shifts of protons and carbon atoms under the high-frequency magnetic field [28]. Spectra of 1D NMR mainly include ^{1}H NMR, ^{13}C NMR and DEPT 135. To characterize the structure of purified coconut peel polysaccharide, ^{1}H NMR, ^{13}C NMR and DEPT 135 were tested, and the map is shown in Fig. 4. As can be seen from the ^{1}H NMR spectrum of coconut peel polysaccharide (Fig. 4a), the signals were concentrated at 3–6 ppm in ^{1}H NMR, and there were also signals at 1–2 ppm. In general, the heterocephalic hydrogen signal of α-glycosidic and β-glycosidic bond configuration is mainly distributed in 4.8–5.5 and 4.4–4.9 ppm, respectively. The hydrogen spectrum

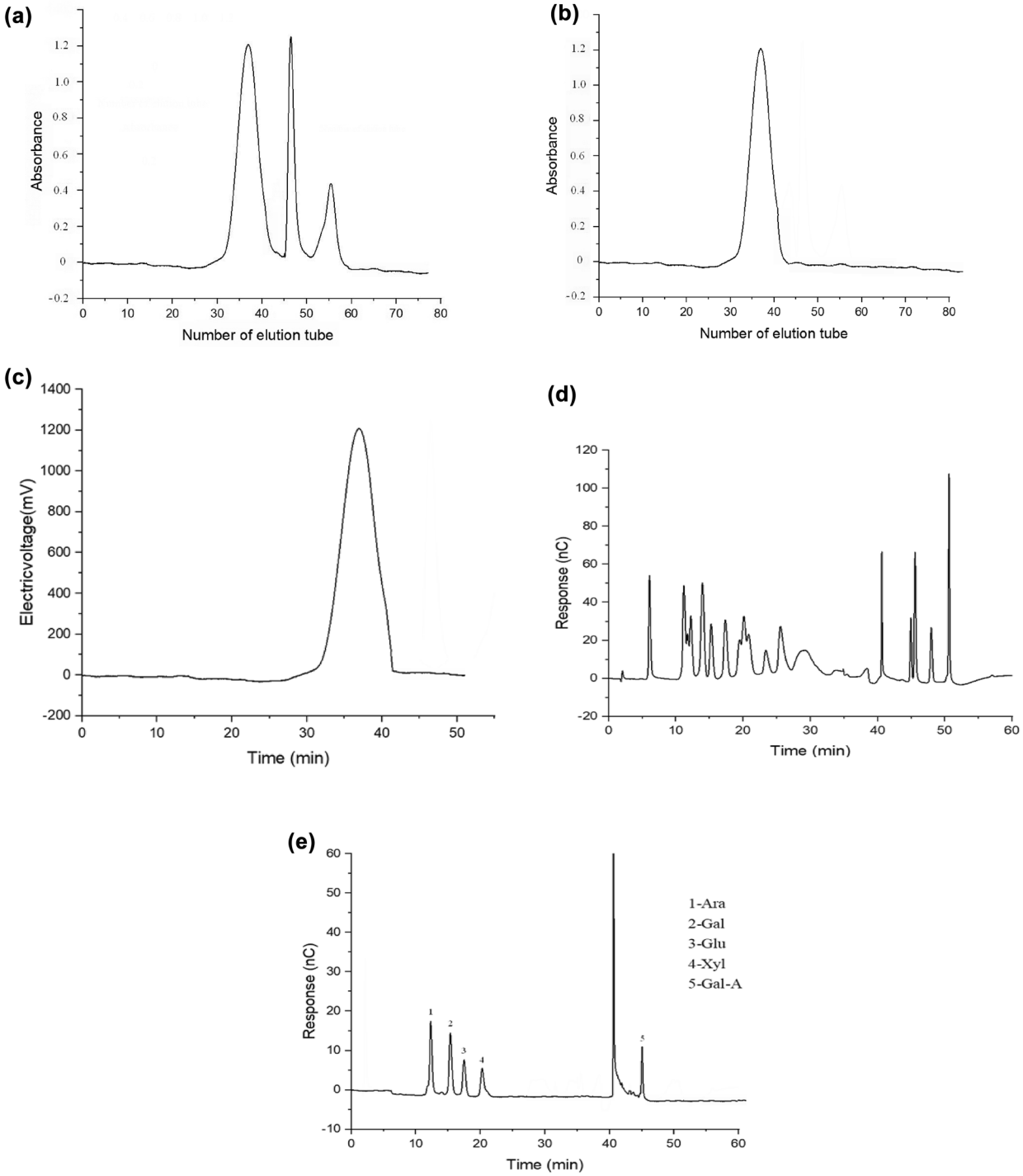

Fig. 2 Molecular weight determination and monosaccharide composition of coconut peel polysaccharide. **a** DEAE-52 column chromatography of coconut peel polysaccharide; **b** Sephadex G-200 column chromatography of coconut peel polysaccharide; **c** chromatograms for determination of molecular weight of coconut peel polysaccharide; **d** ion chromatogram for determination of monosaccharide fraction of mixed standards; **e** ion chromatogram for determination of monosaccharide fraction of coconut peel polysaccharide

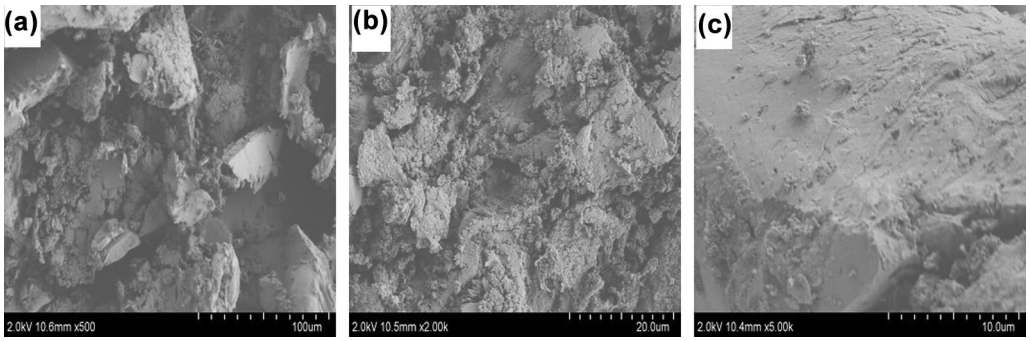

Fig. 3 SEM of coconut peel polysaccharide. **a** 500 magnification; **b** 2000 magnification; **c** 5000 magnification

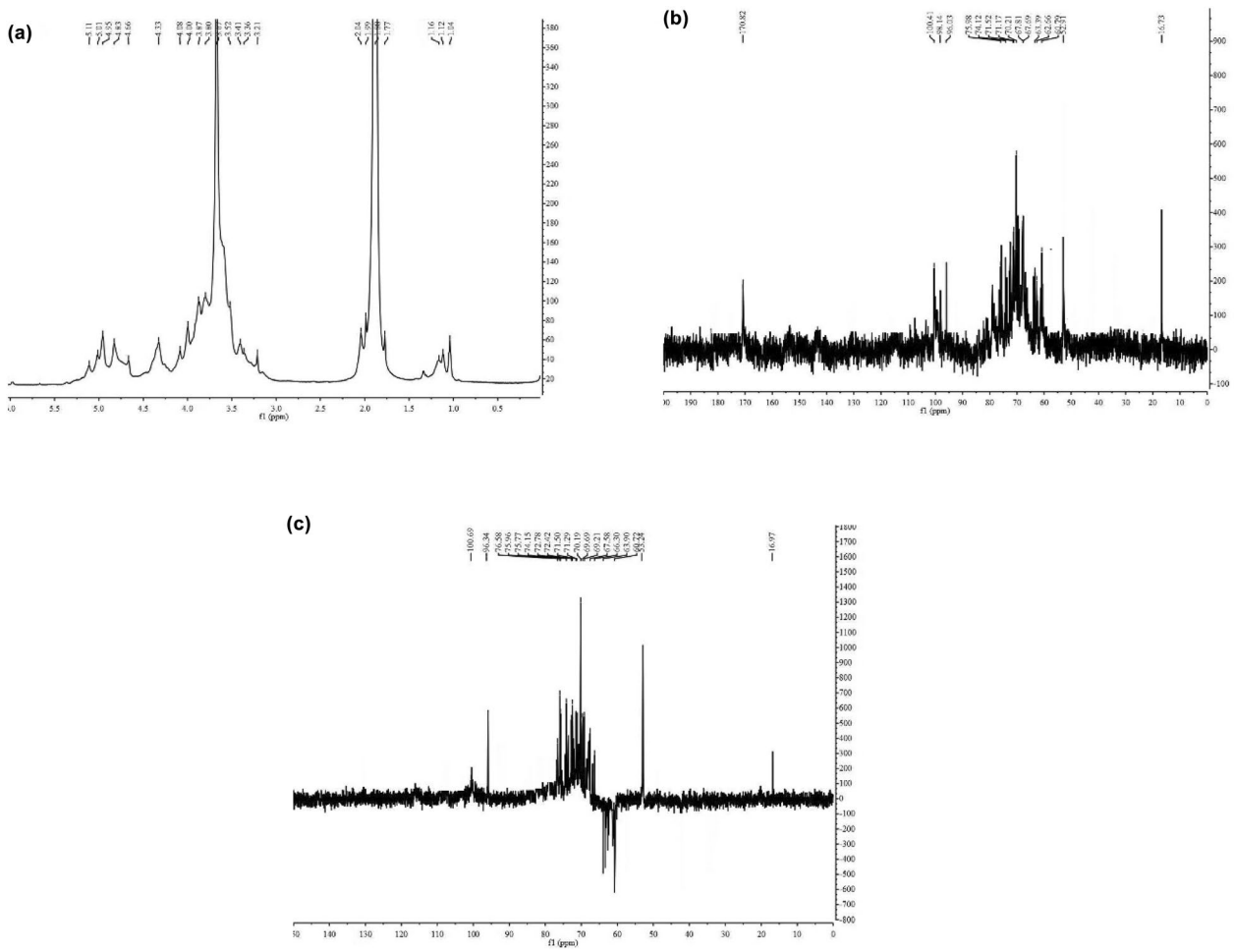

Fig. 4 1D NMR characterization of coconut peel polysaccharide. **a** ^{1}H NMR spectrum of coconut peel polysaccharide; **b** ^{13}C NMR spectrum of coconut peel polysaccharide; **c** DEPT 135 of coconut peel polysaccharide

signal of coconut peel polysaccharide samples was mainly concentrated in the range of 3.2–5.1 ppm, which was α-glycosidic bond configuration. There were four coupling signal peaks in the 4.6–5.1 ppm heteroheaded signal region, indicating that the sample contained at least four sugar residues. The non-heterocephalic hydrogen signals were all concentrated in the 3.2–4.3 ppm region, and some signals were difficult to be assigned due to low content and serious overlap. In addition, there were signal peaks at 1.0–1.2 ppm and 1.7–2.0 ppm, which

are usually characteristic peaks of methyl groups, indicating that there may be two methyl groups in polysaccharide. As can be seen from the ^{13}C NMR spectrum of coconut peel polysaccharide (Fig. 4b), 170.82 ppm was the chemical shift of carbonyl group (-CO-) on the sugar ring. The chemical shift of C1 was 96.03–100.41 ppm and less than 103 ppm. Combining with infrared spectrum analysis, it could be inferred that the glycosidic bond in coconut peel polysaccharide was α-D type. Peaks of 60.79–75.98 ppm were the chemical shift of C2–C6 in coconut peel polysaccharide. In ^{13}C NMR spectra, absorption signals at 52.91 ppm and 16.73 ppm were showed to be the two methyl signal peaks by analysis. Peak at 52.91 ppm was the methoxyl signal that may be methoxylated at sugar chain C6, while 16.73 ppm was the methyl signal peak in acetyl group. According to the results of DEPT 135, Fig. 4c shows that the signal was positive at 96.34–100.69 ppm, which was consistent with C1 in sugar ring. The positive peaks in 66.30–76.58 ppm were corresponding to C2–C5, while the negative in 60.72–63.90 ppm were corresponding to C6. The positive signals of 53.24 ppm and 16.97 ppm are corresponded to $-OCH_3$ and $-COCH_3$, respectively.

2D NMR analysis of coconut peel polysaccharide

Two-dimensional nuclear magnetic resonance (2D NMR) technology, including COSY, HSQC, NOESY and HMBC, plays a very important role in the resolution of polysaccharides structure. To further analyze the molecular structure of coconut peel polysaccharide, 2D NMR analysis was performed [29]. Hydrogen–hydrogen correlation spectrum (COSY) was the most commonly used homonuclear displacement correlation spectrum. As can be seen from Fig. 5a, the ^{1}H signal was mainly concentrated on the diagonal, and $-OCH_3$ and $-COCH_3$ were also found on the diagonal, indicating that -OCH3 and $-COCH_3$ may be located on one residue. In addition, it was found from the COSY diagram that there were three

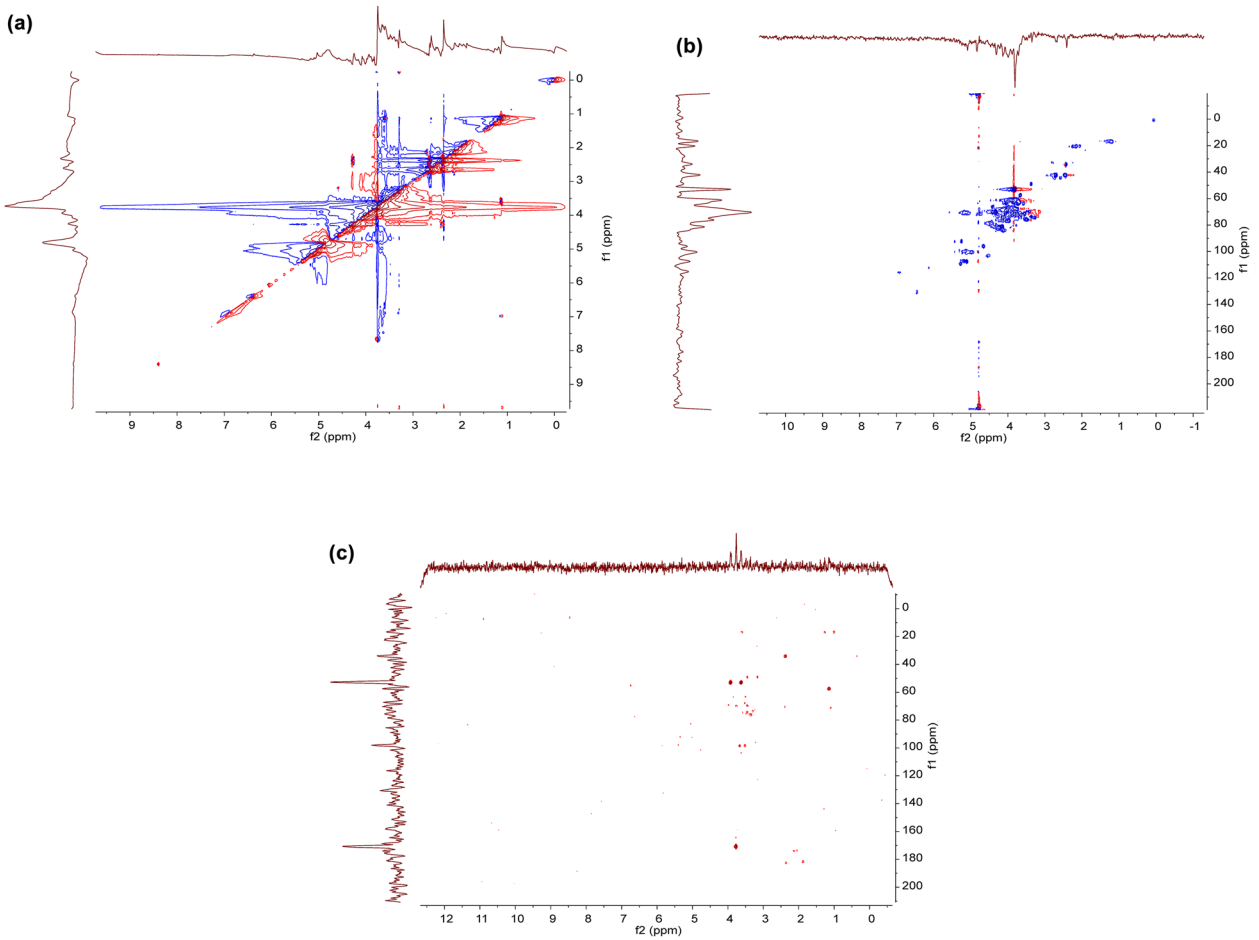

Fig. 5 2D NMR characterization of coconut peel polysaccharide. **a** COSY spectrum of coconut peel polysaccharide; **b** HSQC spectrum of coconut peel polysaccharide; **c** HMBC spectrum of coconut peel polysaccharide

glycosidic bonds formed by the linkage of different residues. HSQC spectrum was a C–H heteronuclear single quantum correlation spectrum, which can associate ^{1}H nucleus with its directly connected ^{13}C nucleus and solve the connection relationship between C–H. It can be found from Fig. 5b that there were 3 C–H connection relationships at C1, so it can be inferred that there may be 3 different residues here. In HSQC spectrum, -OCH_3 and -$COCH_3$ were also found and linked to C6 and C2, respectively. The HMBC was a C–H heteronuclear multi-bond correlation spectrum. HMBC can detect the remote coupling signal of carbon and hydrogen with high sensitivity, that was, the coupling of C and H between two adjacent sugar residues. It can be found from Fig. 5c that glycosidic bonds were formed between sugar residues through 1 → 4 linkage. Based on the analysis of 1D NMR, 2D NMR and monosaccharide components, the possible structure of coconut peel polysaccharide was inferred as follows.

The ^{13}C NMR and ^{31}P NMR analysis of coconut peel polysaccharide derivatives

To study the structure–activity relationship (SAR) of single component coconut peel polysaccharide, acetylated, phosphorylated and carboxymethylated coconut peel polysaccharide were further prepared. To determine whether polysaccharide derivatization was successful, ^{13}C NMR and ^{31}P NMR analysis was performed [30]. It can be seen from Fig. 6a that after acetylation of coconut peel polysaccharide, an obvious carbonyl signal fraction appeared at 174.96 ppm. The peak at 171.13 ppm was the carbonyl signal of coconut peel polysaccharide itself. Compared with the ^{13}C NMR spectrum of coconut peel polysaccharide, the chemical shift of C1–C6 did not change much, and a new methyl signal peak appeared at 20.51 ppm, which was the methyl characteristic peak in acetylation. As can be seen from Fig. 6b, compared with the original polysaccharide, the chemical shift of phosphorylated coconut peel polysaccharide in ^{13}C NMR spectrum shifted to high field on the whole, but the magnitude was not large, which may be because the configuration

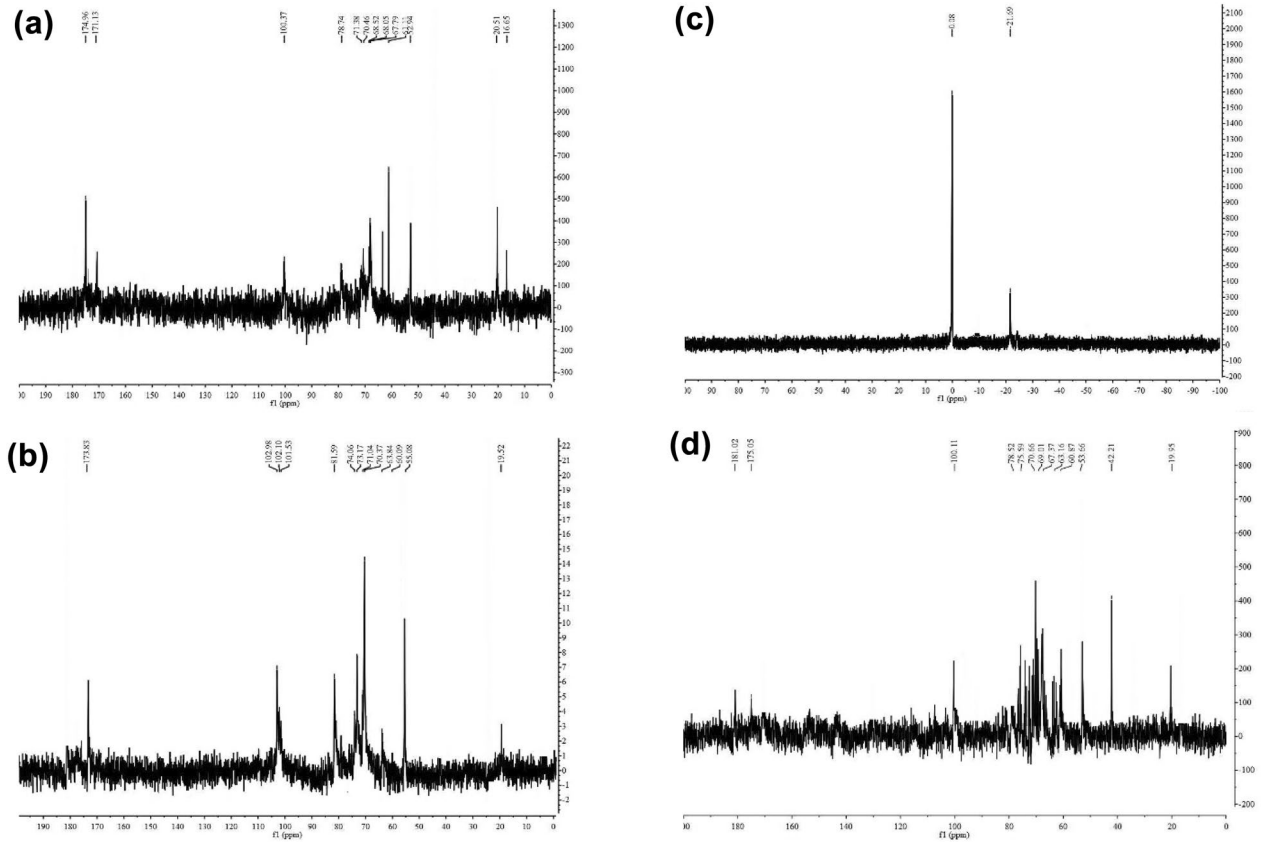

Fig. 6 ^{13}C NMR and ^{31}P NMR characterization of coconut peel polysaccharide derivatives. **a** ^{13}C NMR spectrum of acetylated coconut peel polysaccharide; **b** ^{13}C NMR spectrum of phosphorylated coconut peel polysaccharide; **c** ^{31}P NMR spectrum of phosphorylated coconut peel polysaccharide; **d** ^{13}C NMR spectrum of carboxymethylated coconut peel polysaccharide

of coconut peel polysaccharide changed after phosphorylation. To confirm the phosphorylation of coconut peel polysaccharide, ^{31}P NMR spectroscopy was further performed. As can be seen from Fig. 6c, there were obvious signal peaks at 0.08 ppm and -21.69 ppm, indicating that coconut peel polysaccharide was phosphorylated and easier to be phosphorylated at these two positions. Figure 6d shows the.^{13}C NMR spectrum after carboxymethylation of coconut peel polysaccharide. It can be seen from the figure that a new carbonyl peak appears at 181.02 ppm. Under the influence of carboxymethylation, the chemical shift of the original carbonyl group of coconut peel polysaccharide also changed and became 175.05 ppm. The chemical shifts of other places do not change much, and the signal peak appears at 42.21 ppm was the characteristic of $-CH_2-$.

Antioxidant and cell viability activity in vitro

To investigate the biological activities of coconut peel polysaccharides and its derivatives, we evaluated their antioxidant and antiproliferative activities in vitro. The antioxidant activity in vitro included ASBT and DPPH free radical scavenging rate and $O_2^{-\cdot}$ scavenging rate. The anti-proliferation activity in vitro was determined by inhibiting HepG2 cell activity [31]. The results of ABTS free radical scavenging by coconut peel polysaccharide and its derivatives are shown in Fig. 7a. As can be seen from Fig. 7a, coconut peel polysaccharide and its derivatives had a concentration-dependent inhibitory effect on ABTS radical scavenging rate, and its scavenging capacity increased with the increase of concentration. Compared to vitamin C (V_c), the ABTS radical scavenging ability of PCPP was significantly different from that of CPP ($p < 0.05$). At 3.2 mg/mL, the ability of phosphorylated coconut peel polysaccharide to eliminate ABTS free radicals was comparable to that of V_c, and its scavenging rate was 94.2%. The results of DPPH free radical scavenging by coconut peel polysaccharide and its derivatives are shown in Fig. 7b. As can be seen from Fig. 7b, the scavenging ability of coconut

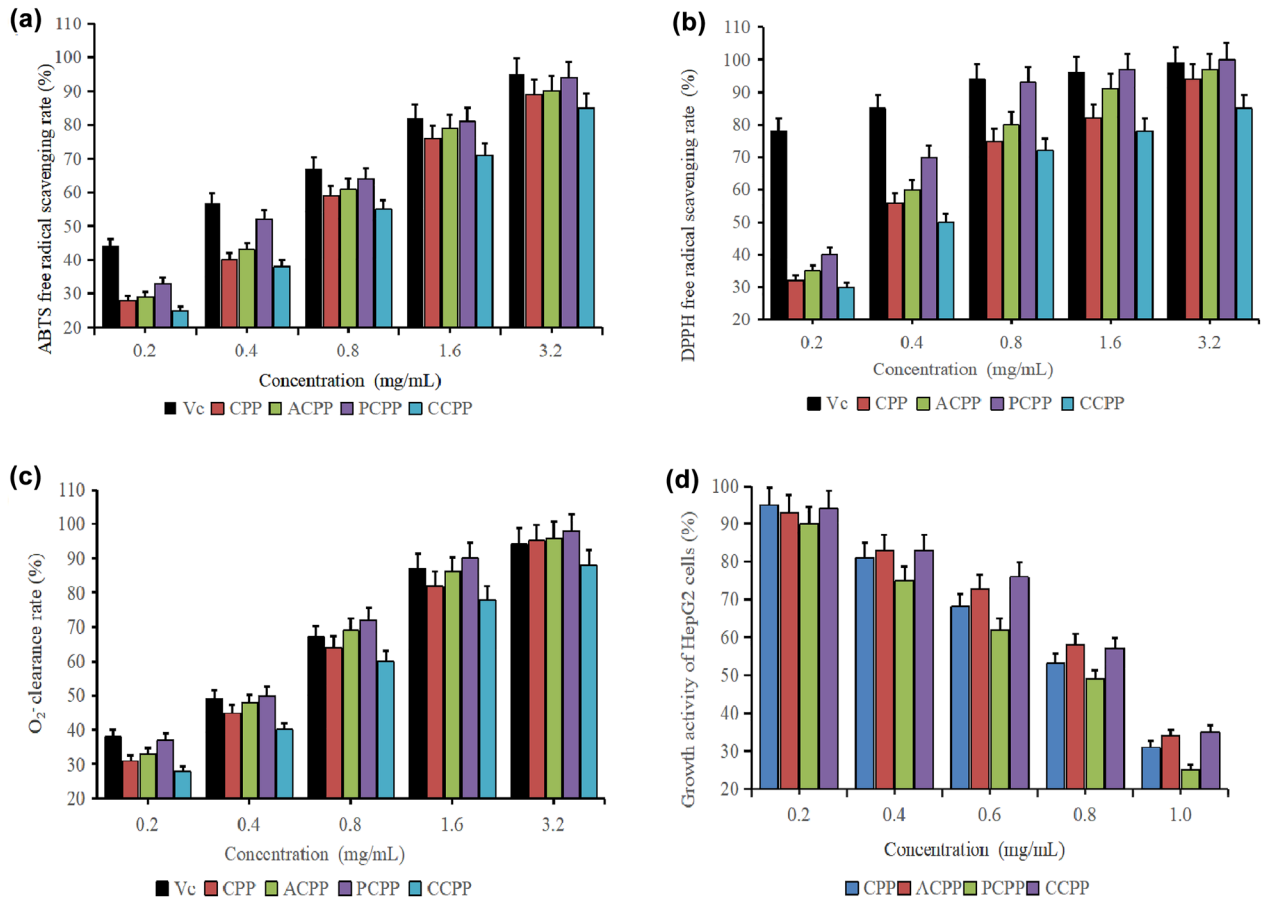

Fig. 7 Antioxidant and anti-proliferative activities of coconut peel polysaccharide and its derivatives. **a** ASBT free radical scavenging rate of coconut peel polysaccharide and its derivatives; **b** DPPH free radical scavenging rate of coconut peel polysaccharide and its derivatives; **c** $O_2^{-\cdot}$ scavenging rate of coconut peel polysaccharide and its derivatives; **d** inhibitory activity of coconut peel polysaccharide and its derivatives on HepG2 cells

peel polysaccharide and its derivatives on DPPH free radical increased with the increase of concentration. Compared to vitamin Vc, he DPPH free radical scavenging ability of PCPP was significantly different from that of CPP ($p < 0.05$), which at 3.2 mg/mL was comparable to the positive control V_c, and its scavenging rate was 99.8%. The results of $O_2^{-\cdot}$ scavenging by coconut peel polysaccharide and its derivatives are shown in Fig. 7c. As can be seen from Fig. 7c, compared to vitamin V_c, the $O_2^{-\cdot}$ scavenging ability of PCPP was significantly different from that of CPP ($p < 0.05$), at the same concentration, its ability of phosphorylated coconut peel polysaccharide to remove $O_2^{-\cdot}$ basically kept the same level as the positive control V_c, and even better at the higher concentration. In terms of SAR, the antioxidant activity of coconut peel polysaccharide was improved to a certain extent after phosphorylation. In the evaluation of antioxidant activity index in vitro, the antioxidant activity of phosphorylated coconut peel polysaccharide could basically maintain the same level as V_c, and even better at high concentration. Compared with coconut peel polysaccharide, the antioxidant activity of acetylated coconut peel polysaccharide increased less, while the antioxidant activity of carboxymethylated coconut peel polysaccharide decreased. Combined with the results of antioxidant activity, SAR of coconut peel polysaccharide was obtained. The SAR analysis showed that the activity of phosphorylated coconut peel polysaccharide was better than that of underived coconut peel polysaccharide, and the antioxidant activity of acetylated and carboxymethylated coconut peel polysaccharide was basically at the same level as that of coconut peel polysaccharide. The activity of phosphorylated coconut peel polysaccharide was better than that of V_c at the same concentration. The results of the preliminary SAR could provide some theoretical guidance for further modification of coconut peel polysaccharide. Combined with the data of molecular weight, monosaccharide component and NMR, the antioxidant activity of coconut peel polysaccharide was different compared with that of polysaccharides extracted from other materials [32–34]. On the whole, the antioxidant activity of coconut peel polysaccharide was better than that of other types of polysaccharides, and the derivative products were also improved. The reason for the improved antioxidant activity may be the difference between the extraction and purification process of coconut peel polysaccharide. In the extraction process, fresh coconut peel was used to obtain polysaccharides by water extraction, which could maximize the original polysaccharide and facilitate the realization of small-scale production in the future. In addition, DEAE-52 cellulose chromatography column and Sephadex G-200 gel chromatography column were used in the purification process of coconut peel polysaccharide, so that the purity of polysaccharides obtained from the process was higher than that of other extracted polysaccharides. At the same time, there may also be structural differences with other extracted polysaccharides, which was also the reason for the difference in antioxidant activity [13, 15, 35–59]. In the structure analysis of coconut peel polysaccharide, we found that C2 may be acetylated and C6 methylated. Compared with other types of polysaccharide, such structure was different. Generally speaking, once the sugar ring or sugar chain was replaced, its spatial structure and biological activity will change. This may also be one of the reasons for the differences in antioxidant properties of coconut peel polysaccharide and their derivatives. On the basis of antioxidant activity in vitro, the anti-proliferative activity of coconut peel polysaccharide and its derivatives was further studied. CCK-8 assay was used to evaluate the inhibitory activity of coconut peel polysaccharide and its derivatives on HepG2 cells. As shown in Fig. 7d, samples of coconut peel polysaccharide and its derivatives showed significant anti-proliferative activity against HepG2 cells in vitro. The inhibiting HepG2 cell activity of PCPP was significantly different from that of CPP ($p < 0.05$). At the same time, the inhibiting HepG2 cell activity of the derived coconut peel polysaccharide was also changed, and the phosphorylation inhibition activity was the best. Compared with other polysaccharides, coconut peel polysaccharide has certain structural differences, so it can also show a good activity in anti-HepG2 cell proliferation. The results provided a new idea for the study of the anti HepG2 proliferation activity of coconut peel polysaccharide and its derivatives. Analysis of available activity data indicates that the coconut peel polysaccharide and its derivatives had the significance for further study.

Conclusion

In this study, the extraction conditions of coconut peel polysaccharide by boiling water method were optimized. Under the optimal extraction conditions, the extraction rate of coconut peel polysaccharide was 4.73%, and the extraction process had a good stability. Results showed that the molecular weight of coconut peel polysaccharide was 1.20×10^5 Da, which was mainly composed of Ara, Gal, Glu, Xyl and Gal-A with the proportions of 25.5%, 26.8%, 12.8%, 12.7% and 15.3%, respectively. The main chain structure of polysaccharide detected by 1D and 2D NMR spectrum was →4)-α-D-Glc*p* (1→. In vitro antioxidant test showed that coconut peel polysaccharide and its derivatives had a good scavenging activities

of ABTS and DPPH radical and $O_2^{-\cdot}$. At the same time, tests of in vitro showed that coconut peel polysaccharide and its derivatives had obvious antiproliferation activity on HepG2 cells. In addition, phosphorylation could improve the biological activity of polysaccharide in vitro. The results of this study provide theoretical support for the industrial production of coconut peel polysaccharide and its derivatives as medicines and health food.

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