Chemical and Biological Technologies in Agriculture

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Extraction, purification and antioxidant activity of *Juglans regia* shell polysaccharide

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

Abstract

In recent years, the biological activity of plant polysaccharides has attracted more and more attention. *Juglans regia* was one of the four-dry fruits in the world, it has very high value in nutrition and health care value was very high. To better develop the nutritional and health effects of the *J. regia* shell polysaccharide, *J. regia* shell was used as raw material to prepare polysaccharide by the water extraction and alcohol precipitation method. The molecular weight, monosaccharide components, molecular structure characteristics and antioxidant activities of its derivatives were studied. Then, a series of meaningful data information for the study of the *J. regia* shell polysaccharide and its derivatives were obtained. The molecular weight of the polysaccharide was 9.45×10^4 Da after the separation and purification by DEAE-52 and Sephadex G-100. The polysaccharides contained rhamnose, arabinose, galactose, glucose, xylose and galacturonic acid, and the molar ratio of each monosaccharide component was 6.7%: 16.5%: 28.3%: 11.2%: 12.5%: 24.8%. The molecular structure of *J. regia* shell polysaccharide contains β-glucoside bond, its structure may be →4)-*α*-D-Gal*p*-(1 →4)-*β*-D-GalAp-(1 →4)-*α*-D-Xyl*p*-(1 → glycosidic bond). The study of antioxidant activity, *J. regia* shell polysaccharide and its derivatives showed good biological activities, especially the phosphorylated *J. regia* shell polysaccharide. The study of *J. regia* shell polysaccharide has important practical significance for improving the quality and efficiency of the *J. regia* industry chain.

Keywords Polysaccharide from *J. regia* shell, Molecular structure, Antioxidant activity

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Graphical Abstract

Introduction

Polysaccharide is a kind of natural macromolecular compound, which is composed of more than 10 monosaccharides connected by glycosidic bonds. Polysaccharides have various biological activities [1–18]. Chemical modification is one of the important means to study the structure–activity relationship of polysaccharides, which provides the experimental and theoretical basis for the development of polysaccharides [19–30].

In recent years, with the deepening of research and the improvement of purity and yield of formulation methods, polysaccharides have been widely used as antioxidant drugs. Free radicals, mainly including superoxide anion radical ($O_2^{-\cdot}$), hydroxyl radical (·OH) and so on, can interact with many substances in the body, such as fatty acids, proteins and so on, seizing their hydrogen atoms, resulting in the destruction of the structure and function of related cells. More importantly, their oxidation products and intermediate products will harm the function of the biofilms, enzymes, proteins and living cells [31–33]. Therefore, it was of great significance to study the scavenging ability of free radicals and the prevention and treatment of related diseases [34–36].

The *Juglans regia* belongs to the *J. regia* family. It was spherical in shape and has a hard shell with a yellowish-brown surface and irregular markings [37]. *J. regia* naturally loves light and water, which has strong cold, drought and disease resistance, but low requirements for soil fertility. Like cashew, *J. regia* is cultivated almost all over the world, mainly in Europe, America and Asia. *J. regia* is rich in unsaturated fatty acids, which are higher than olives, and contain less saturated fatty acids [38]. The oil squeezed out of *J. regia* is the edible oil. *J. regia* is also rich in vitamin E, which is widely recognized as a good antioxidant and anti-aging fruit. In addition, *J. regia* is rich in high-quality proteins, a variety of minerals, dietary fiber, and carotene, so the nutritional value of *J. regia* is very high. The *J. regia* has high nutritional value, its roots, stems, leaves and fruits have their own uses [39]. For processing, the main products are *J. regia* kernel food, *J. regia* oil, *J. regia* health products, etc., which fully reflects the huge edible value of the *J. regia* [40–42]. The *J. regia* shell who has hard texture is a by-product of the kernel, which is generally discarded or incinerated in the process of processing, resulting in a waste of resources. *J. regia* shell mainly contains phenolic acids, saponins, flavonoids, esters and polysaccharides and other chemical components, which has antioxidant, antibacterial and other effects, which can also be developed masks, antibacterial soaps and other daily chemical products. To better develop the nutritional value of *J. regia* shell polysaccharide, the *J. regia* shell as a raw material was prepared by the hot water extraction, alcohol precipitation, dialysis and other steps. After the separation and purification by DEAE-52 and Sephadex G-100, the single component of *J. regia* shell polysaccharide was obtained, and its molecular weight, monosaccharide component and molecular structure were determined. At the same time, acetylated and phosphorylated *J. regia* shell polysaccharides were prepared by the derivatization of *J. regia* shell polysaccharides. It provided the theoretical guidance for

further study on the structure–activity relationship of *J. regia* shell polysaccharide. This study had important practical significance for improving the quality and efficiency of *J. regia* industry chains.

Materials and methods

Materials

J. regia shell was the hard shell part left after the removal of *J. regia* kernel from the commercially available *J. regia* (Sichuan, China), and all other reagents were from Aladdin company (Shanghai, China).

Preparation of J. regia shell polysaccharide (WSP)

20 g of dried *J. regia* shells are taken and thoroughly crushed in a grinder. The crushed *J. regia* shells were placed into a 250 mL beaker and soaked in 150 mL water at room temperature for 12 h. After soaking, shells were poured into a 1000 mL round-bottomed flask, 450 mL water was added and shells were extracted for 4 h at 90 °C. The aqueous solution was collected by pumping and filtration, and the filter residue was soaked with 600 mL water for 4 h, repeated twice. The filtrate was combined, reduced pressure concentration to 200 mL, then 80 mL chloroform and 20 mL *n*-butanol were added, stirring at room temperature for 30 min for protein removal. After protein removal was completed, the supernatant was centrifuged, collected and placed in a dialysis bag for dialysis treatment. The concentrated solution was dialyzed with tap water for 24 h, and then dialyzed with distilled water for 24 h. After dialysis, 500 mL anhydrous ethanol was added into the *J. regia* shell polysaccharide (WSP) solution, and the alcohol was immersed for 12 h before centrifugation. The precipitation was freeze-dried and refined WSP was obtained.

Purification of J. regia shell polysaccharide

Separation and purification of DEAE-52

The refined WSP was prepared into a solution of 30 mg/mL, and the solution was prepared by DEAE-52 (3×50 cm) column chromatography. The distribution of WSP was detected by phenol–sulfuric acid method, the elution curve was drawn, the peak elution solution was collected, and the purified WSP samples were obtained through concentration and freeze-drying. Column separation conditions: sample loading was 2 mL. After sample loading was completed, gradient elution was performed with distilled water and 0.5 mol/L NaCl. The eluent flow rate was 1 mL/min, and the collector control was 3 min/tube when collecting eluent, and 60 tubes were collected.

Separation and purification of Sephadex G-100

DEAE-52 purified WSP was prepared into 10 mg/mL solution. The solution was prepared by Sephadex G-100 (2×60 cm) column chromatography, phenol–sulfuric acid method was used to detect the content of WSP, the elution curve was drawn, and the peak eluent of polysaccharide was collected. The homogenized polysaccharide samples of WSP were obtained. Column separation conditions: sample loading was 2 mL, eluted with distilled water after sample loading, eluent flow rate was 1 mL/min, collector control was 3 min/tube when collecting eluent, and 60 tubes were collected.

Preparation of J. regia shell polysaccharide derivatives

Preparation of acetylated J. regia shell polysaccharide (AWSP)

1 g of purified WSP was accurately weighed and placed in a 100 mL round-bottom flask, and 15 mL distilled water was added to completely dissolve it. Adjustment of the WSP solution with 10% sodium hydroxide solution pH value, keep it in 8.5–9.0. A drop of 1.5 mL acetic anhydride was added to the polysaccharides solution, and the reaction solution was stirred continuously by magnetic force. After the acetic anhydride drop was added, the temperature was heated to 45 °C, and the magnetic stirring reaction was continued for 2 h at constant temperature. After the reaction, the pH of the polysaccharides reaction solution was adjusted to 7.0 with 2 mol/L hydrochloric acid. Then, the polysaccharides reaction solution was placed in a dialysis bag and dialyzed with tap water for 24 h, and then with distilled water for 24 h. 200 mL of anhydrous ethanol was added to the dialysed polysaccharides reaction solution, and after 12 h of ethanol sedimentation, the solution was centrifuged. The precipitate was freeze-dried to obtain acetylated *J. regia* shell polysaccharide (AWSP).

Preparation of phosphorylated J. regia shell polysaccharide

5 g sodium tripolyphosphate and 1 g sodium trimetaphosphate were added to a 100 mL round-bottom flask, followed by 10 mL distilled water, which was fully dissolved by magnetic stirring. Under the condition of room temperature, 1 g pure WSP was added in batches and continuously stirred by magnetic force. After the *J. regia* shell polysaccharides was added, the temperature is raised to 60 °C, and the constant temperature magnetic stirring reaction was 5 h. After the reaction, the pH of the polysaccharides reaction solution was adjusted to 7.0 with 2 mol/L hydrochloric acid. Then, the polysaccharides reaction solution was placed in a dialysis bag and dialyzed with tap water for 24 h, and then with distilled water for 24 h. 200 mL of absolute ethanol was added to the dialysed polysaccharide reaction solution, and after 12 h of ethanol sedimentation, the solution was centrifuged. The precipitate was freeze-dried to obtain phosphorylated *J. regia* shell polysaccharide (PWSP).

Ultraviolet spectrum analysis

Purified WSP was prepared as 100 μg/mL aqueous solution and scanned at full wavelength from 200 to 600 nm.

Infrared spectrum analysis

1.0 mg of dried and homogeneous fractions of WSP was mixed and compressed in KBr and scanned on 4000–500 cm^{-1} infrared spectrometer.

Determination of molecular weight

Oligosaccharide with different molecular weights (5, 11.6, 23.8, 48.6, 80.9, 148, 237, 408.8, and 667.8 kDa) were precisely weighed. Distilled water prepared as 5 mg/mL standard solution was added, centrifuged at 12,000 r/min for 10 min, and the clarified liquid filtered by 0.22 μm microporous membrane was taken. The filtrate sample was then transferred to a 1.8 mL injection vial for later use. Standard curves of oligosaccharides with different molecular weights were determined by high performance gel permeation chromatography (HPGPC). The purified WSP solution was prepared according to the same steps, and the concentration was 5 mg/mL polysaccharide solution. The molecular weight and purity of the polysaccharide sample were determined by HPGPC method. Chromatographic 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 °C; injection volume: 20 μL; detector: differential detector RI-10A.

Determination of monosaccharide components

Sixteen kinds of monosaccharide standards (including fucose, rhamnose, arabinose, galactose, glucose, xylose, mannose, fructose, ribose, galacturonic acid, glucuronic acid, galactosamine hydrochloride, glucosamine hydrochloride, *N*-acetyl-*D*-glucosamine, guluronic acid, and manuronic acid) were prepared into mixed standard solutions with certain concentrations. 10 mg of purified WSP sample was accurately weighed and placed in an ampoule, 2 mL of 3 mol/L trifluoroacetic acid solution was added, and hydrolyzed at 120 °C for 3 h after heating. After the hydrolysis of the polysaccharide sample was completed, 5 mL of water was added and mixed. After absorbing 50 uL of the mixed sample solution, 950 uL of deionized water was added and centrifuged at 12,000 r/min for 5 min. The supernatant was used for analysis of monosaccharide components. Chromatographic column: PA20 (3×150 mm); mobile phase: A: H_2O; B: 15 mmol/L NaOH; C: 15 mmol/L NaOH–100 mmol/L NaOAc; flow rate: 0.3 mL/min; injection volume: 5 μL; column temperature: 30 °C; detector: electrochemical detector.

Scanning electron microscopy analysis

10 mg of purified WSP samples were weighed, evenly spread on conductive carbon film, and then placed in the sample chamber of MC1000 ion sputtering instrument for gold spraying 40 s. After removing the WSP samples, they were placed into the observation chamber of the SU8100 scanning electron microscope, and the acceleration voltage was 2 kV to observe the external morphology of the polysaccharide samples.

Determination of total sugar content

The total sugar content of WSP and its derivatives was determined by phenol–sulfuric acid method. The absorbance was measured at 490 nm, the amount of glucose (μg/mL) was used as the abscissa, the absorbance (A) was used as the ordinate, the standard curve was drawn, and the linear regression equation was obtained by regression processing. The absorbance value of the sample polysaccharide at 490 nm was also determined by phenol–sulfuric acid method.

Determination of degree of substitution

Determination of acetyl substitution

Acid–base titration method was used to determine the acetyl group content of polysaccharide sample, and the degree substitution (DS) of acetylation reaction was calculated according to the following equation:

$$\mathrm{DS} = \frac{132A}{4300 - 42A}$$

where A is the percentage of acetyl groups in the sample.

Determination of phosphoric acid substitution

Molybdenum blue colorimetry was used to determine the phosphorus content in polysaccharide sample, and the degree substitution (DS) of phosphorylation reaction was calculated according to the following equation:

$$\mathrm{DS} = \frac{1.62P}{31-0.97P}$$

where P is the percentage of phosphate in the sample.

Nuclear magnetic resonance analysis

The sample was dissolved in 0.5 mL D_2O to a final concentration of 40 mg/mL. 1D-NMR and 2D-NMR (^{13}C-NMR, COSY, DEPT135°, HMBC and HSQC) were recorded at 25 °C with a Bruker AVANCE NEO 500 M spectrometer system (Bruker, Rheinstetten, Germany) operating at 500 MHz by Sanshu Biotech. Co., LTD (Shanghai, China).

Determination of antioxidant activity

Determination of scavenging rate of DPPH radical

DPPH was dissolved in anhydrous ethanol to prepare 0.2 mmol/L solution, and *J. regia* shell polysaccharide and its derivatives were dissolved in deionized water to prepare 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/mL solutions, respectively. The 2 mL DPPH solution was placed in a test tube, and 2 mL of *J. regia* shell polysaccharide and its derivatives solution of different concentrations were added, respectively (distilled water was used as the control), then the sample solution was oscillated and mixed, and placed at room temperature for 30 min away from light. The absorbance was measured at wavelength 517 nm:

$$\text{DPPH radical scavenging rate (\%)} = [A_0 - A_1)/A_0] \times 100\%$$

where A_0 is the absorbance value of blank control group; A_1 is the absorbance value of sample solution.

Determination of scavenging rate of hydroxyl radical (·OH)

J. regia shell polysaccharides and its derivatives were dissolved in deionized water to prepare mass concentrations of 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/mL, respectively. Take a clean 10 mL Nessler glasses tube and add 1 mL sample solution of different concentrations into it successively (distilled water was used as the control), 1 mL 6 mmoL/L $FeSO_4$, 1 mL 6 mmoL/L salicylic acid, and finally add 1 mL 6 mmoL/L H_2O_2 to start the reaction. Shake and place in 37 °C water bath for 60 min reaction. The absorbance was measured at wavelength at 510 nm:

$$\text{Hydroxyl radical scavenging rate (\%)} = [A_0 - A_1)/A_0] \times 100\%$$

where A_0 is the absorbance value of blank control group; A_1 is the absorbance value of sample solution.

Determination of scavenging rate of superoxide anion radical ($O_2^{-\cdot}$)

J. regia shell polysaccharides and its derivatives were dissolved in deionized water to prepare mass concentrations of 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/mL, respectively. The pyrogallol autoxidation method was adopted to absorb 1 mL of sample solution of different concentrations (distilled water was used as the control). Then, 0.05 mol/L Tris-HC1 buffer 4.0 mL pH 8.0 was added, and the mixture was placed in a 25 °C water bath to preheat for 20 min, and 2.5 mmol/L catechol solution 0.4 mL was added, which was mixed and reacted at 25 °C for 3 min. Finally, 2 mol/L HCl was added 1.0 mL termination reaction. The absorbance was measured at wavelength 325 nm [28]:

$$\text{Superoxide anion radical scavenging rate (\%)} = [A_0 - A_1)/A_0] \times 100\%$$

where A_0 is the absorbance value of blank control group; A_1 is the absorbance value of sample solution.

Determination of reducing power

J. regia shell polysaccharides and its derivatives were dissolved in deionized water to prepare mass concentrations of 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/mL, respectively. Take 1 mL of polysaccharides sample solution with different concentrations (distilled water was used as the control), add 2.5 mL of phosphoric acid buffer solution with pH 6.6 and 5.0 mL of 1% $K_3Fe(CN)_6$ solution and mix evenly. The mixture was held at 50 °C for 20 min, after which 2.5 mL 10% trichloroacetic acid solution was added to terminate the reaction. Centrifuge at 3000 r/min, absorb 2.5 mL supernatant, and add 2.5 mL distilled water and 0.5 mL 0.1% $FeCl_3$ solution, mix evenly, and measure absorbance at 700 nm wavelength 30 min later:

$$\text{Reducing power(\%)} = [A_0 - A_1)/A_0] \times 100\%$$

where A_0 is the absorbance value of blank control group; A_1 is the absorbance value of sample solution.

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 of J. regia shell polysaccharides and determination of the degree of substitution and total sugar of its derivatives

In recent years, the biological activities of plant polysaccharides have attracted much attention, and the research was more and more in-depth, but the research on *J. regia* shell polysaccharides was very few. Each tissue part of *J. regia* contains polysaccharide. To better develop the nutritional and health effects of *J. regia* polysaccharide, especially the extraction and utilization of *J. regia* shell polysaccharide. At present, the *J. regia* polysaccharide was obtained by hot water extraction. To improve the extraction rate of polysaccharides, the previous methods used enzymatic hydrolysis, ultrasonic assisted method and microwave assisted method to extract the polysaccharides. Different tissues of *J. regia* and different extraction methods and processes have different effects on the extraction rate of *J. regia* polysaccharide. In this study, dried *J. regia* shell was used as the raw material for

extracting polysaccharides. After being crushed, *J. regia* shell was soaked in water at room temperature for 12 h, and then crude *J. regia* shell polysaccharide was obtained by hot water extraction. In the process of extracting crude polysaccharides, we optimized the extraction process and finally determined the optimal extraction conditions, that was the ratio of material to liquid was 1:25 (*J. regia* shell: solvent), the extraction temperature was 90 °C, and the extraction time was 4 h. Under the optimal extraction conditions of *J. regia* shell polysaccharide, the extraction rate of polysaccharides can reach 3.51% (Table 1). In the process of refining crude *J. regia* shell polysaccharide, we use the sevage method to remove the protein and nucleic acid components in the crude polysaccharides, and use the dialysis method to remove the small molecular compounds mixed in the crude polysaccharides, so as to obtain the refined *J. regia* shell polysaccharide. The total sugar content of refined *J. regia* shell polysaccharide was determined by phenol–sulfuric acid method, and the total sugar content was 98.51% (Table 1). To further study the structural–activity relationship of *J. regia* shell polysaccharide, we carried out derivatization, including preparation of acetylated *J. regia* shell polysaccharide and phosphorylated *J. regia* shell polysaccharide, and the yields of *J. regia* shell polysaccharide derivatives were 90.36% and 46.93%, respectively (Table 1). From the yield of *J. regia* shell polysaccharide derivatives, the acetylated polysaccharides was significantly higher than the phosphorylation, which was related to the preparation conditions and reaction reagents, which also showed that the phosphorylation of *J. regia* shell polysaccharide was relatively difficult. On this basis, we also tested the total sugar content and substitution degree in *J. regia* shell polysaccharide derivatives, because the total sugar content and substitution degree will affect their biological activity. The results showed that the total sugar content of acetylated *J. regia* shell polysaccharide and phosphorylated *J. regia* shell polysaccharide were 94.29% and 85.37%, respectively (Table 1). Compared with *J. regia* shell polysaccharide, the total sugar content of *J. regia* shell polysaccharide was decreased, but the content was relatively high. In the determination of the degree of substitution of *J. regia* shell polysaccharide derivatives, the substitution pairs of acetylated and phosphorylated *J. regia* shell polysaccharide were 0.89 and 0.44, respectively (Table 1). Among them, the degree of substitution of acetylated *J. regia* shell polysaccharide was relatively high, indicating that in the original *J. regia* shell polysaccharide, more positions are replaced by acetyl groups, which can also reflect the relatively easy acetylation of polysaccharides.

Table 1 Total sugar content, yield and degree of substitution of *J. regia* shell polysaccharides and its derivatives

	Samples		
	WSP	AWSP	PWSP
Yield (%)	3.51 ± 0.20	90.36 ± 0.35	46.93 ± 0.22
DS	–	0.89 ± 0.06	0.44 ± 0.07
Sugar content (%)	98.51 ± 0.93	94.29 ± 1.0	85.37 ± 1.21

Purification of *J. regia* shell polysaccharide

After preliminary extraction of plant polysaccharides, there were usually impurities, such as fat, protein, pigment, etc. Proteins were often removed by sevage method or TCA method, small molecular substances were removed by dialysis or ultrafiltration, and a variety of column chromatography purification methods were used for further purification of plant polysaccharides. Cellulose column chromatography, ion exchange column chromatography and gel column chromatography were commonly used in the separation and purification of plant polysaccharides with different molecular weights. Different column chromatography methods have different effects on the separation and purification of plant polysaccharides. To reduce the loss of *J. regia* shell polysaccharide and improve the purity of polysaccharides, we should carefully analyze and design a scientific and appropriate column chromatography separation method when choosing column chromatography separation. In this study, after obtaining the refined *J. regia* shell polysaccharide, we further separated and purified it by DEAE-52 and Sephadex G-100 to obtain a uniform fraction of the polysaccharide. From the DEAE-52 chromatogram in Fig. 1a, it can be seen that two polysaccharide components, WSP-1 and WSP-2, were obtained from the refined *J. regia* shell polysaccharide separated by DEAE-52. Among them, the content of WSP-1 component was significantly higher than that of WSP-2 component, which was an absolute advantage. To further isolate and purify the *J. regia* shell polysaccharide, the WSP-1 fraction was separated by Sephadex G-100. From the Sephadex G-100 chromatogram in Fig. 1b, it can be seen that there was only a single chromatographic peak, which can also indicate that the *J. regia* shell polysaccharide obtained after DEAE-52 and Sephadex G-100 separation was a homogeneous component. From the Sephadex G-100 chromatogram analysis, it can be found that the purity of purified *J. regia* shell polysaccharide is more than 90%.

Molecular weight, monosaccharide composition and SEM analysis of J. regia shell polysaccharide

Plant polysaccharides were a kind of high molecular compound, and its characteristics and biological activity

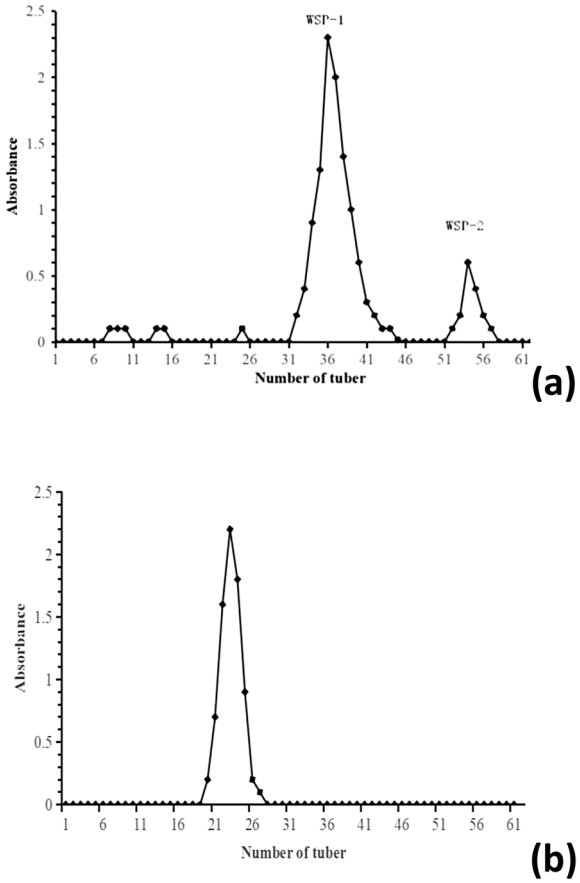
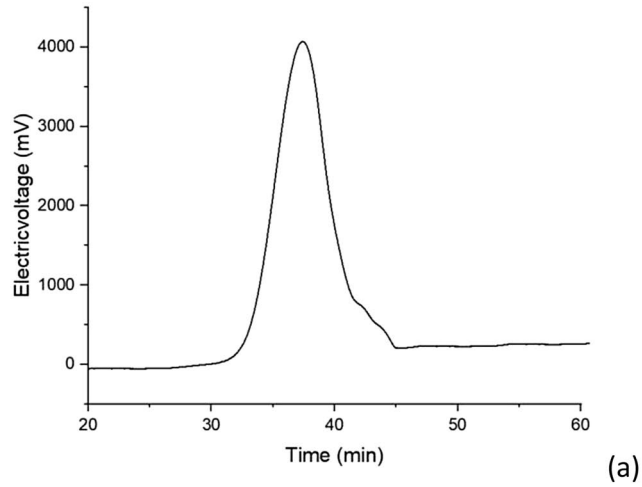


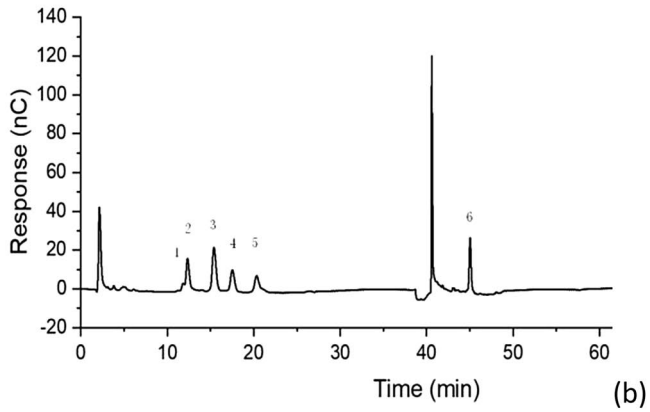
Fig.1 Elution curve of polysaccharide from *J. regia* shell. **a** DEAE-52 column chromatography of *J. regia* shell polysaccharide; **b** Sephadex G-100 column chromatography of WSP-1

were closely related to its molecular weight and distribution. The size and distribution of molecular weight were important indexes for evaluating the value and quality control of plant polysaccharides. The molecular weight and purity of *J. regia* shell polysaccharide was determined by high performance gel permeation chromatography (HPGPC). The results of molecular weight determination of *J. regia* shell polysaccharide showed that there was only one peak in the chromatograph, and the peak time was 37.4 min (Fig. 2a). The molecular weight of *J. regia* shell polysaccharide was 9.45×10^4 Da by calculation. It can be seen from the molecular weight determination chromatograph that other impurity peaks was less, which can indicate that the purity of purified *J. regia* shell polysaccharide was high, and the purity of the polysaccharide was 94.46% by calculating the peak area ratio. The composition of monosaccharides in plant polysaccharides was often closely related to their biological activities, so understanding the monosaccharide composition of plant polysaccharides was an essential step in polysaccharide research. At present, the determination of monosaccharides in plant polysaccharides mainly uses ion chromatography (IC), high performance capillary electrophoresis (HPCE), high performance liquid chromatography (HPLC), gas chromatography (GC), etc. In this study, the monosaccharide components and content of purified *J. regia* shell polysaccharide was determined by ion chromatography. From the determination of the monosaccharide components of *J. regia* shell polysaccharide, in Fig. 2b, it can be seen that the heteropoly was mainly composed of rhamnose, arabinose, galactose, glucose, xylose and galacturonic acid, and the molar ratio of each monosaccharide component were 6.7%: 16.5%: 28.3%: 11.2%: 12.5%: 24.8%. From the point of view of monosaccharide components and content, galactose and galacturonic acid accounted for a relatively large proportion of *J. regia* shell polysaccharide, the polysaccharide belongs to acid sugar. Scanning electron microscopy (SEM) uses a narrow focused high-energy electron beam to scan the sample. Through the interaction between the beam and the material, the information was excited and collected, amplified and re-imaged to achieve the purpose of characterizing the microscopic morphology of the material. SEM was an observation method between transmission electron microscopy and optical microscopy, which was widely used to observe the morphology and composition of the surface ultrastructure of various solid substances (such as polysaccharides, nanomaterials, metals, etc.). The plant polysaccharide samples were imaged by SEM, and the morphology of granular or aggregated by single particles was obtained. In Fig. 2c, the SEM analysis results of *J. regia* shell polysaccharide showed that the polysaccharide samples presented granular and block structures under 500 magnification. Under the microscope of 2000× magnification, the surface of polysaccharide sample has granular aggregates, which are closely packed together, which may be due to the strong interaction between molecules of the sample. Under the microscope of 5000 times, it can be seen that the surface of the polysaccharide sample was relatively smooth, and a large number of sheets or spheroids were accumulated on the surface.

Ultraviolet and infrared analysis of J. regia shell polysaccharide

Because of its complex structure, it was very difficult to completely analyze the structure of *J. regia* shell polysaccharide by traditional chemical methods, and spectral knowledge must be used to analyze the structure. Ultraviolet spectrophotometer, infrared spectroscopy and nuclear magnetic resonance were commonly used in the polysaccharides of *J. regia* shell. UV method at 280 nm there was no absorption peak to detect whether there was protein and at 260 nm





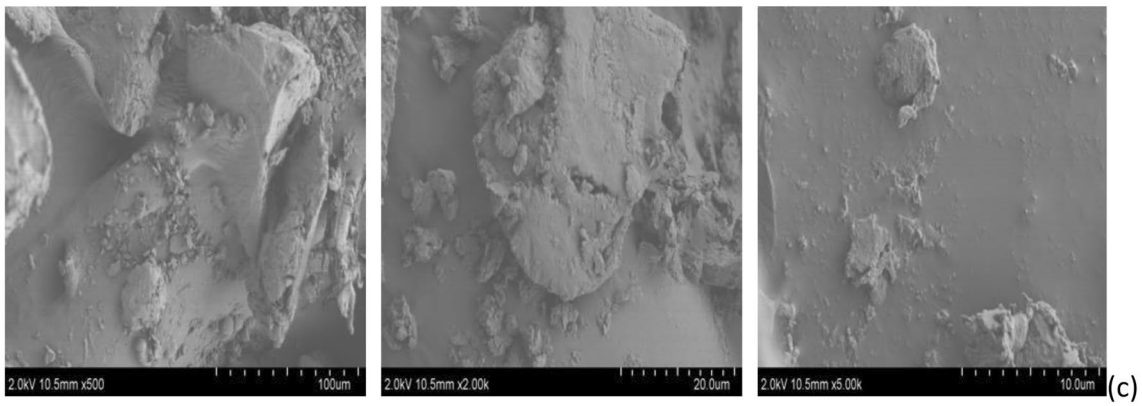


Fig.2 Molecular weight determination, monosaccharide composition and SEM of *J. regia* shell polysaccharide. **a** Chromatograms for determination of molecular weight of *J. regia* shell polysaccharide; **b** ion chromatogram for determination of monosaccharide fraction of *J. regia* shell polysaccharide; **c** SEM of *J. regia* shell polysaccharide

there was no absorption peak to judge whether there was nucleic acid, and at 620 nm there was no absorption peak to judge whether there was pigment. As can be seen from Fig. 3a, the scanning results of full-wavelength (200–800 nm) UV of polysaccharide from *J. regia* shell showed that there was no characteristic peak of nucleic acid at 260 nm, no characteristic peak of protein at 280 nm, and no characteristic peak of pigment at 620 nm. Therefore, it can be inferred that the purified polysaccharide of *J. regia* shell basically does

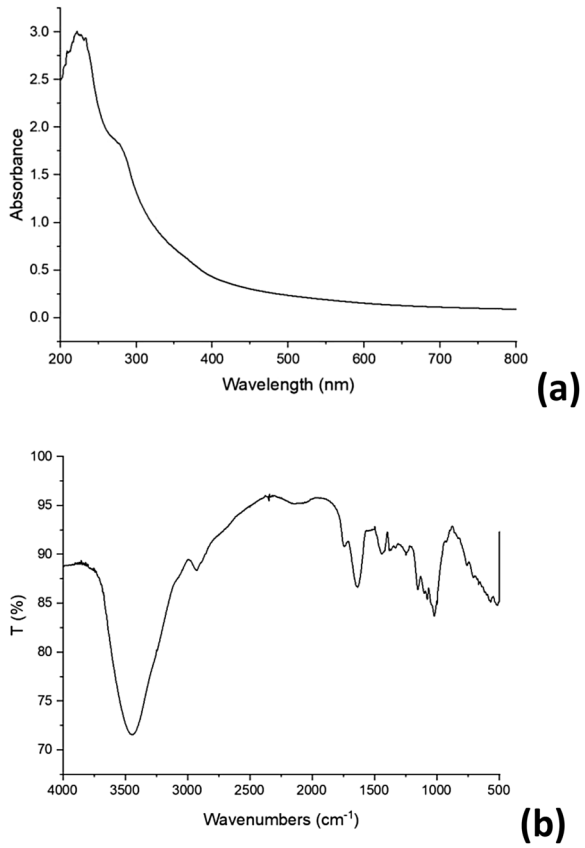


Fig. 3 Ultraviolet and infrared analysis of *J. regia* shell polysaccharide. **a** UV analysis of *J. regia* shell polysaccharide; **b** IR analysis of *J. regia* shell polysaccharide

not contain impurities, such as protein. The structure, functional groups and substituents of polysaccharides can be identified by infrared spectroscopy. Figure 3b shows the results of infrared spectrum analysis of polysaccharide from *J. regia* shell. It can be seen that it has characteristic absorption peak of polysaccharide. At 3448 cm^{-1}, a wide stretching vibration peak was O–H, and the absorption peak was C–H stretching vibration peak at 2932 cm^{-1}, indicating the existence of intramolecular and intermolecular hydrogen bonds in the polysaccharides. At 1637 cm^{-1}, the absorption peak was C = O stretching vibration, indicating the presence of carbonyl group in the polysaccharide of sunflower disk. The absorption peak at 1443 cm^{-1} was the variable angle vibration of C–H, the absorption peak at 1333 cm^{-1} was -COOH, and the absorption peak at 1024 cm^{-1} was the extended vibration peak of C–O. If the absorption peak was found at 894 cm^{-1}, it could be inferred that the polysaccharide configuration was β-glucoside bond. The infrared spectrum analysis of polysaccharide from sunflower disk was consistent with its monosaccharide component analysis.

1D NMR and 2D NMR analysis of J. regia shell polysaccharide

Polysaccharides have a more complex macromolecular structure than proteins. It was difficult to identify the structure of monosaccharides because of their diversity, linkage methods and complexity of branch chains. At present, the main structure was the target of polysaccharide structure identification, mainly to analyze the molecular weight range of polysaccharide, monosaccharide type, proportion and binding order, as well as the configuration of glycosidic bond. Nuclear magnetic resonance (NMR) was mainly based on the principle of nuclear energy splitting in a strong magnetic field, absorption of external electromagnetic radiation after the nuclear spin level transition, that was, nuclear magnetic resonance. NMR analysis was used to determine the heterohead configuration, glycosidic bond connection mode and connection order of polysaccharides by recording the proton chemical shift under high frequency magnetic field. 1D NMR spectrum mainly includes ^{13}C NMR and DEPT135°, which can be used to determine the chemical shift of carbon and hydrogen in sugar residues. Due to the serious signal overlap of the same atoms in 1D NMR, the 2D NMR technology was also needed to analyze the molecular structure of polysaccharides, such as COSY, HSQC and HMBC. To study the molecular structure of *J. regia* shell polysaccharide, we characterized it by 1D and 2D NMR. The results of 1D NMR characterization of *J. regia* shell polysaccharide showed that there was an obvious signal peak at 170.8 ppm of ^{13}C NMR chemical shift in Fig. 4a, which was the carbonyl signal peak (C6). Combined with the data of *J. regia* shell monosaccharides, it could be inferred that the peak was the carboxyl signal peak of galacturonic acid. Among the three heterocapsid C1 signals of 99.7 ppm, 100.4 ppm and 104.6 ppm in the ^{13}C NMR spectra, there were two strong absorption peaks above 100 ppm, and only one weak absorption peak below 100 ppm, indicating that the heterocapsid carbon configurations in the polysaccharides were β-type. One was α-type, indicating that the sugar contains three sugar residues. In the ^{13}C NMR spectra of *J. regia* shell polysaccharides, the chemical shift of 60.8–78.7 ppm was the carbon signal peak of C2–C5. In the ^{13}C NMR spectrum, carbon signal appeared at 16.7 ppm of chemical shift, indicating the presence of rhamnose in *J. regia* shell polysaccharide. The analysis results were consistent with the analysis results of monosaccharide components. At the same time, the DEPT135° results of the characterization of pecan shell polysaccharide 1D showed that the positive signal peak appeared at the chemical

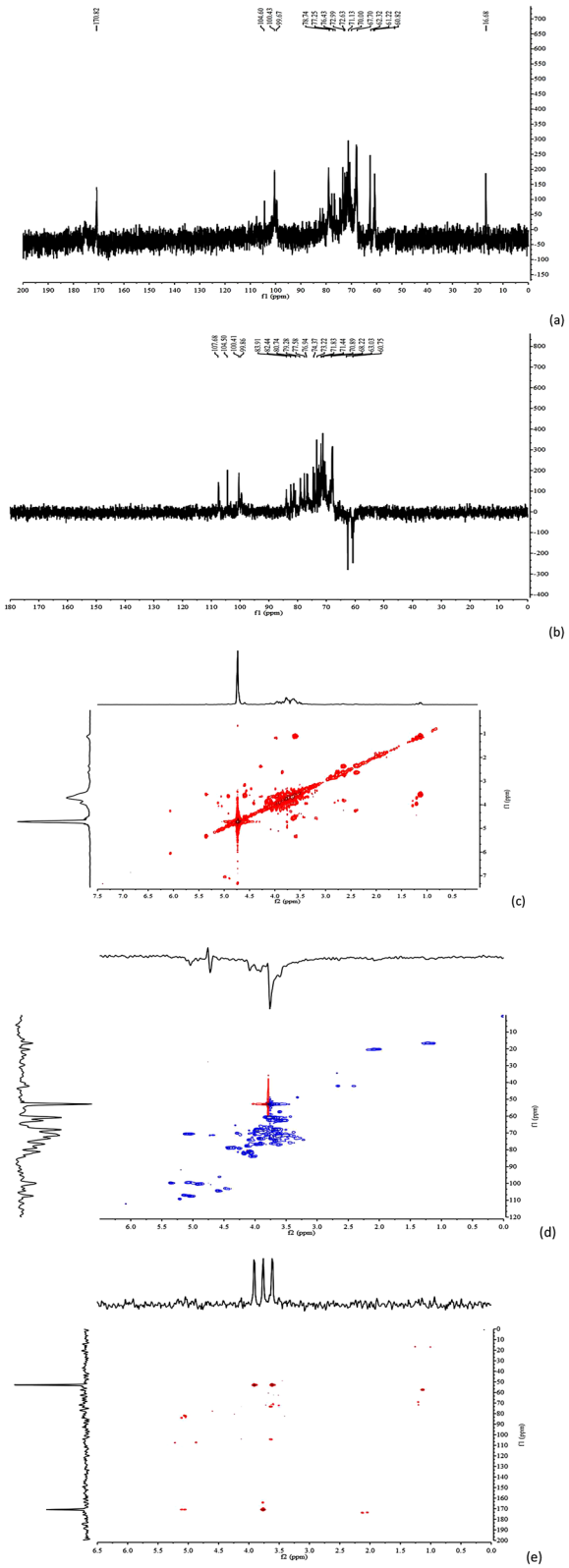


Fig.4 1D and 2D NMR characterization of *J. regia* shell polysaccharide. **a** ^{13}C NMR of *J. regia* shellpolysaccharide; **b** DEPT135° of *J. regia* shell polysaccharides; **c** COSY spectrum of *J. regia* shell polysaccharide; **d** HSQC spectrum of *J. regia* shell polysaccharide; **e** HMBC spectrum of *J. regia* shell polysaccharide

shift of 99.9–107.7 ppm, 68.2–83.9 ppm, and the negative signal peak appeared at 60.8–63.0 ppm (Fig. 4b). To obtain detailed molecular structure information of *J. regia* shell polysaccharide, two-dimensional NMR spectra including COSY, HSQC and HMBC were analyzed (Fig. 4c–e). 2D NMR of *J. regia* shell polysaccharide showed that the signals of H1/C1 (5.08/99.5 ppm), H2 (3.75 ppm), H3 (4.10 ppm), H4/C4 (4.23/79.21 ppm), H5 (3.67 ppm), H6 (3.61 ppm) indicated the existence of the A → 4)-*α*-D-Gal*p*-(1 → was detected. The presence of residue B → 4)-*β*-D-GalAp-(1 → unit was confirmed based on characteristic signals at H1/C1(5.06/107.5 ppm), H2 (3.778 ppm), H3 (3.91 ppm), H4/C4 (4.34 ppm/78.7 ppm), H5 (3.73 ppm) and a carbon signal at 172.26 ppm. Chemical shifts of H1/C1 (4.91/100.3 ppm), H2 (4.30 ppm), H3 (4.08 ppm), H4/C4 (4.10 ppm/81.0 ppm), H5 (3.74 ppm) and H6 (3.65 ppm) were assigned to the sugar residue C → 4)-*α*-D-Xyl*p*-(1 →). Based on 1D NMR and 2D NMR information analysis, it was concluded that the main chain structure of *J. regia* shell polysaccharide might be → 4)-*α*-D-Gal*p*-(1 → 4)-*β*-D-GalAp-(1 → 4)-*α*-D-Xyl*p*-(1 →).

^{13}C NMR and ^{31}P NMR analysis of J. regia shell polysaccharide derivatives

There were many methods for polysaccharide derivatization, and it was possible to improve the activity of polysaccharide using the hydroxyl group, carboxyl group, amino group and other groups on the sugar residues for chemical derivatization. To improve the biological activity of *J. regia* shell polysaccharide, the molecular modification and structural modification of *J. regia* shell polysaccharide was important. In this study, we carried out derivative of *J. regia* shell polysaccharides, including acetylation and phosphorylation of *J. regia* shell polysaccharide, to improve the antioxidant activity of *J. regia* shell polysaccharide through structural modification. To confirm the successful derivatization of *J. regia* shell polysaccharides, the acetylated and phosphorylated *J. regia* shell polysaccharide were characterized by ^{13}C NMR and ^{31}P NMR. It can be seen from Fig. 5a, compared with ^{13}C NMR of *J. regia* shell polysaccharide, there was an obvious peak at the chemical shift of 181.2 ppm, which was the characteristic peak of carbonyl group in the acetyl group. The peak at the chemical shift of 52.9 ppm was the characteristic peak of methyl in the acetyl group. After

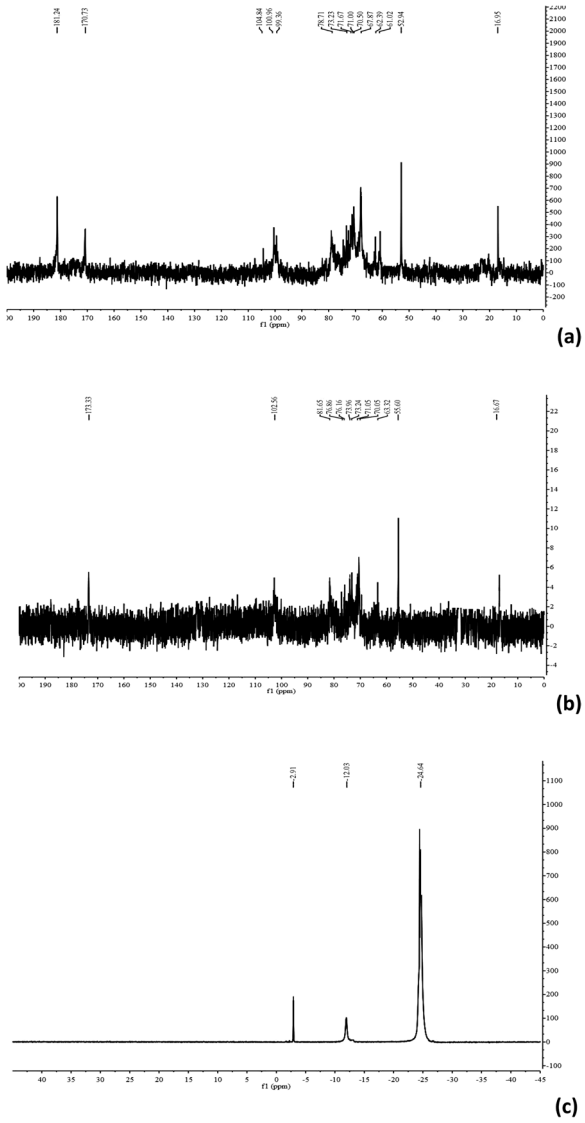


Fig. 5 ^{13}C NMR and ^{31}P NMR characterization of *J. regia* shell polysaccharide derivatives. **a** ^{13}C NMR spectrum of acetylated *J. regia* shell polysaccharide; **b** ^{13}C NMR spectrum of phosphorylated *J. regia* shell polysaccharide; **c** ^{31}P NMR spectrum of phosphorylated *J. regia* shell polysaccharide

acetylation, the chemical shift of other carbon atoms remained unchanged. Figure 5b, shows ^{13}C NMR and ^{31}P NMR of phosphorylated *J. regia* shell polysaccharide, respectively. As can be seen from Fig. 5b, compared with ^{13}C NMR of *J. regia* shell polysaccharide, its chemical shift changes little. The difference was that the carbonyl peak tends to a higher field, with a chemical shift of 173.3 ppm, while the chemical shift of C1 also slightly tends to a lower field, with a chemical shift of 102.6 ppm. To verify phosphorylation, ^{31}P NMR characterization of phosphorylated *J. regia* shell polysaccharides was performed. As shown in Fig. 5c, phosphorylated *J. regia* shell polysaccharide were replaced by phosphoric acid at three sites, and there was an obvious peak at − 24.6 ppm, indicating that phosphoric acid groups were most easily modified at this site, while the other two sites were difficult to modify. The results from the derivatization of *J. regia* shell polysaccharide showed that acetylation and phosphorylation were successfully modified.

Antioxidant activity of J. regia shell polysaccharide and its derivatives

In recent years, many plant polysaccharides have been found to have anti-tumor, anti-bacterial, enhance immune function, reduce blood sugar, reduce blood lipids, antioxidant and other health effects, and polysaccharide-related research has become a research hotspot in recent years. It was of practical significance to study the biological activity of *J. regia* shell polysaccharide for further understanding the nutritional and health effects of *J. regia*. In this study, the antioxidant activities of *J. regia* shell polysaccharides and their derivatives (acetylated polysaccharides and phosphorylated polysaccharides) were investigated, including the scavenging capacity of superoxide anions, hydroxyl radicals, DPPH radicals and reducing capacity. The results of antioxidant activity showed that *J. regia* shell polysaccharide and its derivatives had a good biological activity. As can be seen from Fig. 6a, the superoxide anion clearance rate of *J. regia* shell polysaccharide and its derivatives increased with the increase of the concentration. When the sample reached 3.2 mg/mL, the superoxide anion clearance rate of phosphorylated *J. regia* shell polysaccharide could reach 92.3%, basically reaching the level of positive control product V_c. Figure 6b shows the change curve of hydroxyl radical scavenging rate of *J. regia* shell polysaccharide and its derivatives with concentration. It can be seen that the concentration dependence of *J. regia* shell polysaccharide and its derivatives on hydroxyl free radical exists, and the scavenging ability increases with the increase of sample concentration, when the sample concentration was 3.2 mg/mL, phosphorylated polysaccharide had the highest scavenging rate of hydroxyl radical (98.2%), which was better than the positive control substance V_c (96.5%). As can be seen from Fig. 6c, the scavenging rate of Vc on DPPH free radical changes little with the increase of concentration, while the scavenging rate of *J. regia* shell polysaccharide and its derivatives on DPPH free radical has a certain concentration dependence, and its scavenging ability will continue to improve when the concentration continues to increase. When the sample concentration reaches 3.2 mg/mL, The DPPH free radical scavenging rate of phosphorylated *J. regia* polysaccharide was close to that of positive control substance

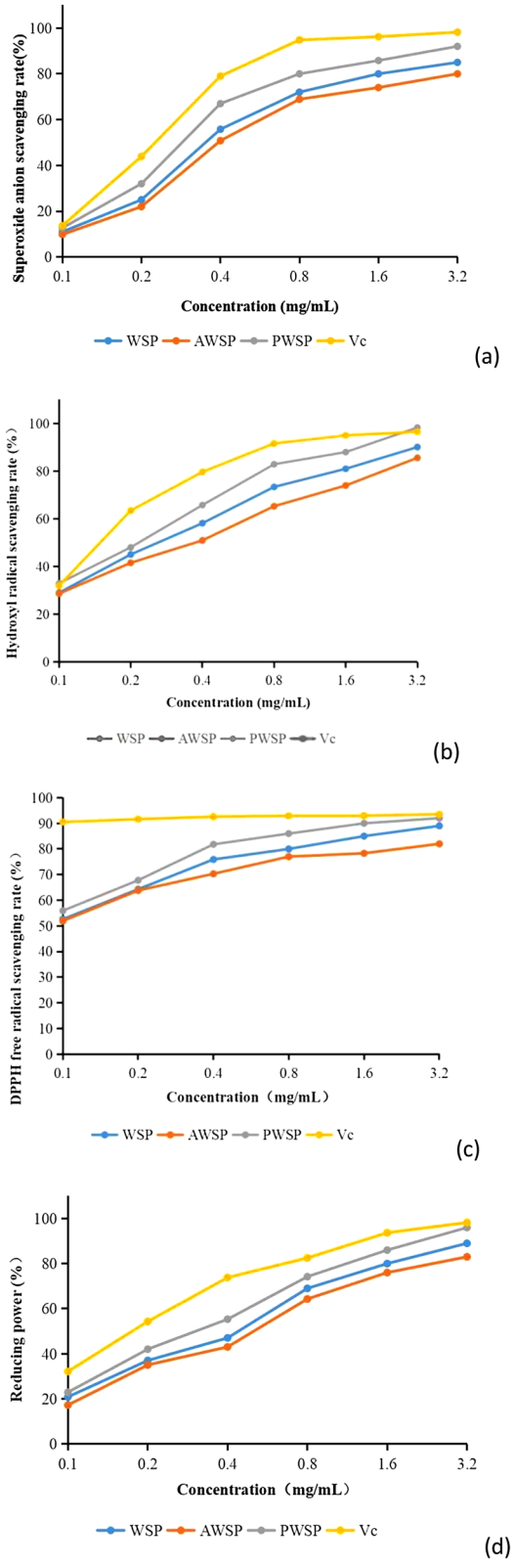


Fig. 6 Antioxidant activities of *J. regia* shell polysaccharide and its derivatives. **a** Superoxide anion radical scavenging rate of *J. regia* shell polysaccharide and its derivatives; **b** hydroxyl radical scavenging rate of *J. regia* shell polysaccharide and its derivatives; **c** DPPH free radical scavenging rate of *J. regia* shell polysaccharide and its derivatives; **d** reducing power of coconut *J. regia* shell polysaccharide and its derivatives

V_c. Figure 6d shows the curve of the influence of reducing power and concentration of *J. regia* shell polysaccharide and its derivatives. It can be seen that when the sample concentration reaches the maximum, the reducing power of phosphorylated *J. regia* shell polysaccharide was basically at the same level as that of positive control substance V_c. It can be seen from the results of antioxidant activity that phosphorylation of *J. regia* shell polysaccharide was helpful to improve its activity, which can also provide a theoretical basis for the derivation of other polysaccharides and provide a reference for other polysaccharide researchers. In addition, existing data on antioxidant activity of *J. regia* shell polysaccharide and its derivatives can provide a basis for further studies activity and mechanism of action.

Conclusion

Using the *J. regia* shell as the raw material, the *J. regia* shell polysaccharide was prepared by hot water extraction, alcohol precipitation and dialysis. DEAE-52 and Sephadex G-100 were used to separate the polysaccharide from the *J. regia* shell. The molecular weight of the *J. regia* shell polysaccharide was 9.45×10^4 Da, and the monosaccharide component showed that the main polysaccharides from *J. regia* shell are acidic heteropolysaccharides. It contains rhamnose, arabinose, galactose, glucose, xylose and galacturonic acid, and the molar ratios of each monosaccharide component was 6.7%: 16.5%: 28.3%: 11.2%: 12.5%: 24.8%. The results of the molecular structure determination of *J. regia* shell polysaccharide show that the polysaccharide contains β-glucoside bond, the structure of which may be →4)-α-D-Gal*p*-(1→4)-β-D-GalA*p*-(1→4)-α-D-Xyl*p*-(1→glycosidic bond). To study the antioxidant activity of the *J. regia* shell polysaccharide and its derivatives, the scavenging capacity and the reducing capacity of superoxide anion, hydroxyl radical and DPPH radical are used as indicators. The antioxidant activity of the *J. regia* shell polysaccharide and its derivatives show good biological activities. In general, this study can provide some theoretical guidance for the further research on the structure–activity relationship of the *J. regia* shell polysaccharide. In addition, to better develop the nutritional and health effects of the *J. regia* shell polysaccharide, it has important practical significance for

improving the quality and efficiency of the *J. regia* industry chain.

Acknowledgements
Not applicable.

Author contributions
SZ wrote the manuscript. GH reviewed and edited the manuscript.

Funding
Not applicable.

Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 17 May 2023 Accepted: 27 July 2023
Published online: 10 August 2023

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