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Preparation and application of carboxymethylated and phosphatised Melaleuca polysaccharide

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Abstract

Polysaccharides are a class of biological macromolecules made up of sugar chains with multiple glycosidic bonds. To explore the extracted, purified, carboxymethylated and phosphorylated modified to obtained carboxymethylated polysaccharides (HSPS) and phosphorylated polysaccharides (HSPP), and the HSPS and HSPP were characterized and analyzed, and their antioxidant activity measured. Response surface design was used for the synthesis process of HSPS and HSPP, the structure characterization and antioxidant activities of HSPS and HSPP were evaluated. The results showed that the Mw and Mn of HSP were 14.66 kDa and 8.39 kDa, respectively. The monosaccharides were mainly galactose, glucose, xylose, and arabinose in a molar ratio of 37.68%: 24.43%: 13.63%: 9.18%. The optimal synthesis conditions for HSPS were as follows: reaction time of 2.01 h, reaction temperature of 44.61°C and the amount of monochloroacetic acid necessary was 1.18 g. The optimal synthesis conditions for HSPP were: reaction time of 4.28 h, reaction temperature of 80.78°C, and reaction pH of 8.98. HSPS and HSPP have scavenging effects on ABTS, super oxideanion and hydroxyl radicals. The HSPS and HSPP were characterized and analyzed, and their antioxidant activity measured.

Keywords: melaleuca; carboxymethylation; phosphorylation; antioxidant.

Practical Application: Our data revealed that HSPS and HSPP are promising natural antioxidant with potential value as a food supplement.

1 Introduction

Huperzia serrata (HS) is a tibetan plant of the Huperzia genus in the Huperzia family. It is also known as Huperzia (Wang et al., 2017). As a tradition Chinese herbal medicine, HS is often used to treat traumatic injuries, schizophrenia, and inflammation (Zhou et al., 2007) . It has also been used for detoxification. It has a pungent, bitter and flat taste. Melaleuca HS propagates through spores. It is found througout China. Analyses have shown that HS contains huperzine A, terpenoids, flavonoids, and polysaccharides.

Polysaccharides have important physiological functions. The most important biological activity of plant polysaccharides is their immunomodulatory effect of activating lymphocytes (Gan et al., 2021; Ji et al., 2019; Sun et al., 2021; Zhang et al., 2012). After molecular modification, various derivatives of polysaccharides with different structures and biological activities can be obtained (Chen & Huang, 2019; Yu et al., 2022). The biological activity of polysaccharides can be improved by molecular modification. Its water solubility can also be improved to a certain extent. Altering the physical and chemical properties improves their bioavailability and application in different biological systems (Zhang et al., 2017; Xie et al., 2020; Yan et al., 2021, 2022). Therefore, an evaluation index should be established for the molecular modifications of polysaccharides as a guide for choosing the modification methods to obtain the desired biological activity. At present, the standard molecular modification methods for polysaccharides include carboxymethylation, phosphorylation, sulfonation, sulfation, and alkylation. Which enhanced the antioxidant activity and water solubility of native polysaccharides significantly, and also provided structural diversity and even the addition of new bioactivities (Ahmad, 2021; Chakka & Zhou, 2020; Xie et al., 2020; Zhang et al., 2022a; Zhang et al., 2022b). Carboxymethylation is a widely studied modification method for many polysaccharides due to its advantages, such as low cost of reagents, simple operation, safe conversion, and low toxic or nontoxic reaction products. Some carboxymethylated polysaccharides are specifically edible, biocompatible and degradable. Thus, they are widely used in food applications as food auxiliary agents, food packaging, Carrier of food bioactive ingredients,Sensors for analysis of food and functional food. (Xie et al., 2021). Naturally occurring polysaccharides with poor solubility and high viscosity show an improvement in biological activity after phosphorylation. Phosphorylated polysaccharides are known to exert antioxidant, antitumor, antibacterial, hypolipidemic,antiviral, and immunoregulatory effects (Xia et al., 2021). Also have extensive application prospect in food industry.

The antioxidant activity of polysaccharides is often evaluated by its ability to scavenge DPPH, hydroxyl radicals, ABTS cationic

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radicals, superoxide anion radicals, and its ability to chelate metals. Zhang et al. (2014) studied the antioxidant activity of the Tremella fuciformis polysaccharide. The results showed that the degraded polysaccharide samples were obtained with Fe_2^+, ascorbic acid, and H_2O_2 as degradation agents. The degraded samples had lower molecular weights and higher antioxidant activities.

The purpose of this study is to modify Huperzia serrata polysaccharide (HSP) by carboxymethylation and phosphorylation, optimise the synthesis process for carboxymethylated Huperzia serrata polysaccharide (HSPS) and phosphorylated Huperzia serrata polysaccharide (HSPP), characterise the modified polysaccharides by SEM, FT-IR, CD, XRD and NMR, and analyse its antioxidant activity. This paper provides a basis for optimising the carboxymethylation and phosphorylation modification processes for HSP.

2 Materials and methods

2.1 Materials

The *Huperzia serrata* (after identification by Chen Ping) used in this study were planted in the Shennongjia area of China. The reagents used in this study were all analytical grade.

2.2 Extraction and purification of HSP

The polysaccharides were obtained by water extraction and alcohol precipitation, and the crude polysaccharides were purified to HSP following the method published by Feng & Zhang (2020). The obtained supernatant was transferred to a 500 mL conical flask, mixed evenly after adding 95% ethanol (the volume ratio of supernatant to ethanol was 1:4), stored in a refrigerator at 4 °C overnight, and then centrifuged to discard the ethanol and obtain the crude polysaccharide of HSP. The polysaccharides were purified by DEAE-52 and Sephadex gel (Sephadex G---100) columns following the method published by Feng & Zhang (2020) (Zhang et al., 2022c).

2.3 Molecular weight analysis

The purified polysaccharide HSP was detected by with HPSEC-MALLS-RID according to previous reported methods (Yao et al., 2020; Zheng et al., 2022). The HPSEC-MALLS-RID was carried out in a HPSEC columns (TSKgel SuperMultipore PW-M, Tosoh, Japan) coupled with a MALLS detector (DAWN HELEOSII, Wyatt Technology, Santa Barbara, CA, USA), and refractive index detector (2414, Waters, USA) with the refractive index increment (dn/dc) of 0.135 mL/g. The MALLS instrument was equipped with a He-Ne laser (λ = 663.6 nm). The PSPF solution was filtered by a membrane with 0.45 μm pore size before injection and eluted with 0.1 M NaCl (0.5 M/min). The column temperature was kept at 40 °C. Astra software (version 6.0.2, Wyatt Technology Co) was utilized for data acquisition and analysis.

2.4 Monosaccharide composition analysis

The PCD-HPLC method used by Luo et al was used to analyse the monosaccharide components of the Melaleuca polysaccharide (Luo et al., 2005). The monosaccharide standards selected included galacturonic acid (Galua), glucose (GLC), galactose (Gal), arabinose (ARA), mannose (man), xylose (Xyl), rhamnose (RHA), glucuronic acid (GlcUA), and fucose (Fuc). According to the methods published, followed by HPLC analysis (Yao et al., 2020; Zhang et al., 2022c). Standard monosaccharides were subjected to the same conditions described above as a reference. According to the retention time of each chromatographic peak, the composition of monosaccharide sample was determinedand the molar ratios of various monosaccharides were calculated from the peak areas of the respective chromatographic peaks.

2.5 Synthesis of HSPS

The synthesis of HSPS was carried out according to Chen et al. (2019) The single factor experiment was studied by the control variable method. The degree of substitution of HSPS was used as the index. Supplemental Table S2 lists the reaction times (2 h, 3 h, 4 h), amount of monochloroacetic acid (0.5 g, 1.0 g, 1.5 g), and reaction temperatures (40 °C, 45 °C, 50 °C) investigated. The degree of substitution of the carboxymethylation modification of Melaleuca polysaccharide and the optimal amount for the three factors were determined. The dialysed reaction solution was concentrated and precipitated with four times the volume of 95% ethanol. The degree of substitution for the Melaleuca polysaccharide was determined by following the method of Zhang et al. (2013). With slight modifications as follows. Ten milligrams of HSPS powder was placed in a 250 mL conical flask, and 3 ml volume fraction 70% ethanol was added. The solution was mixed, and then 50 ml of 0.5 M NaOH and 10 mL of ultrapure water was added. The solution was mixed with a magnetic stirrer until the sample dissolved. The pH was adjusted to 7.0 with 0.1 mol/l HCl using phenolphthalein as the pH adjustment indicator.

The degree of substitution (DS) of the carboxymethylated Melaleuca polysaccharide is calculated as the regression Equation 1:

$$DS = 0.162A / (1 - 0.058A) \quad (1)$$

where A is equal to the millimoles of HCl required per gram of carboxymethylated Melaleuca polysaccharide.

2.6 Modification of HSPP

The phosphorylated Melaleuca polysaccharide was prepared by response surface design. Using the sodium trimetaphosphate (STMP)/sodium tripolyphosphate (STPP) method, a single factor experiment was done by controlling the other variables. The DS of the phosphorylated Melaleuca polysaccharide was used as the index. In Supplemental Table S5, the reaction pH (8, 9, 10), reaction time (4 h, 5 h, 6 h), and reaction temperature (70 °C, 80 °C, 90 °C) were investigated. The optimal level for each variable was determined.

2.7 Characterisation of HSP, HSPS, and HSPP

Ultraviolet spectrum

An analytical balance was used to weigh 0.1 g of HSP, HSPS, and HSPP powder. The powder was fully dissolved in 1 mL

ultrapure water, and centrifuged at 5000 r/min for 3-5 min to ensure appropriate clarity of the centrifugal supernatant. The ultraviolet spectrum was scanned in the wavelength range of 190-600 nm.

Fourier Transform infrared spectroscopy (FTIR) analysis

A mixture of 3 mg of HSP, HSPS, or HSPP and 100mg potassium bromide was ground thoroughly and put into a circular mould, pressurised (5-10 t/cm^2) for about 1min, and the fine powder pressed into a transparent circular sheet. The samples were characterized by FT-IR spectrometry (PerkinElmer, Spectrum 400, USA) with a scanning wavelength range of 4000-400 cm^{-1}.

Scanning electron microscopy (SEM)

Scanning electron microscope (Hitachi, Japan) was adopted to investigate the morphological features of samples. The samples were dried and evenly dispersed to a copper sheet. The morphologies of the samples were observed using 500, 1000, and 2000× magnification with field emission SEM (Li et al., 2019; Liu et al., 2022).

Monosaccharide composition analysis and determination of molecular weight

The monosaccharide composition of purified HSP was analysed using a Promosil C18 column (250 mm × 4.6 mm × 5 μm). According to previously reported methods, the molecular weights of HSP were measured using HPSEC-MALLS-RID (TSKgel SuperMultipore PW-M, Tosoh, Japan).

Circular dichroism spectral analysis (CD)

The samples were scanned and analysed by a CD spectrometer (Jasco Inc., Easton, MD) at room temperature. The scanning wavelength range was 190-300 nm, the scanning speed was 50 nm/min, the sensitivity was 2 (MB/cm), and the sample concentration was 1 mg/mL.

X-ray diffraction analysis (XRD)

The crystallinity of HSP, HSPS, and HSPP was determined by X-ray diffraction (D2 PHASER, Bruker AXS German). The voltage and current were 40 kV and 40 Ma, respectively, the scanning range was 10°-90°, and the scanning speed was 4°/min.

Nuclear magnetic resonance spectroscopy (NMR)

HSP, HSPS, or HSPP (25 mg) was dissolved in 0.5 mL D_2O, transferred to a nuclear magnetic tube, and detected by NMR (Bruker Instrumental Inc, German) at room temperature.

2.8 Antioxidant activity of HSPS and HSPP

The antioxidant activities of HSPS and HSPP were evaluated by hydroxyl radical scavenging activity, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) (ABTS) radical scavenging activity, and superoxide anion scavenging activity.

3 Results

3.1 Purification of crude polysaccharide

The crude polysaccharide was purified with DEAE 52. Different absorption peaks were obtained after elution with ultrapure water and a gradient concentration of NaCl (0.1, 0.2, 0.3, 0.5 mol/L). As seen in Figure 1A, the large absorption peak was the ultra-pure water washing off. The smaller peaks were elution peaks. Collect the water washing part of the eluent, and obtain the more pure Melaleuca polysaccharide after concentration, dialysis, alcohol precipitation and vacuum freeze-drying.

A purified Sephadex G-100 gel column separated and purified the purified polysaccharide from the previous step. As shown in Figure 1B, only one elution peak appeared. The peak value of the absorption peak was high, and there was no impurity peak. Therefore, the separation effect was good, and the purity was high. The solution in the tube corresponding to the absorption peak was collected and transformed into a white solid after concentration, dialysis, alcohol precipitation, and vacuum freeze-drying. This was HSP and will be used as raw material for carboxymethylation and phosphorylation modifications and subsequent structural identification.

3.2 Monosaccharide composition analysis

As shown in Figure 1C, the monosaccharides and corresponding peak time are mannose 12.988 min, rhamnose 17.367 min, glucuronic acid 20.565 min, galacturonic acid 23.562 min, glucose 26.486 min, galactose 30.223 min, xylose 31.343 min, arabinose 33.030 min, and fucose 37.371 min. According to the HPLC performance report, the proportion of each monosaccharide component according to the peak area is mannose: rhamnose: glucuronic acid: galacturonic acid: glucose: galactose: xylose: arabinose: fucose = 6.2%: 1.49%: 2.17%: 0.975:24.43%: 37.68%: 13.63%: 9.18%: 4.23%.

3.3 Molecular weight analysis

High-performance gel chromatography combined with a multi-angle laser scattering detector was used to detect the purified polysaccharide. The results are shown in Figure 1D, the molecular weight of polysaccharide 12422. The Mn and Mw of HSP were 8391 and 14666, respectively. Molecular weight of Melaleuca polysaccharide was shown in Supplemental Table S1.

3.4 Synthesis of HSPS

The Design Expert 8.0 software was used to optimise the synthesis of HSPS. The design scheme is shown in Supplemental Table S3.

The response value is the degree of substitution (DS) of HSPS, and the data in Table S2 are calculated and fitted by the response surface. The regression Equation 2 is as follows:

$$\begin{aligned} Y = {} & +0.023 - 1.000E-003X_1 - 2.625E - \\ & 004X_2 + 9.875E - 004X_3 + 2.000E - 004X_1X_2 - \\ & 1.200E - 003X_1X_3 - 3.750E - 004X_2X_3 - 7.325E - \\ & 004X_1^2 - 3.858E - 003X_2^2 - 3.007E - 003X_3^2 \end{aligned} \quad (2)$$

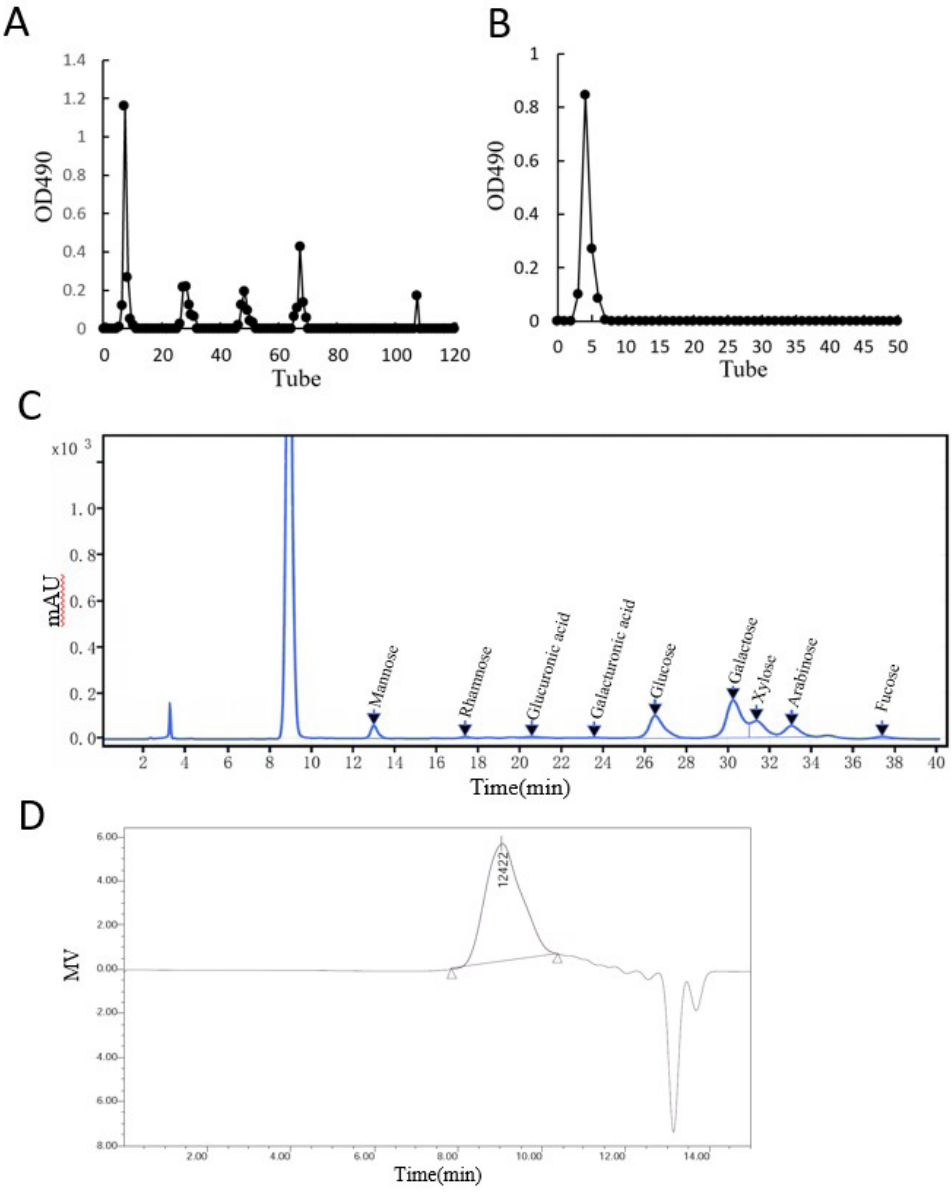

Figure 1. Isolation and analysis of Polysaccharides of Huperzia serrata (HSP). (A) Elution curve of HSP using the DEAE-52 ion-exchange column. The eluents were H_2O and NaCl (0.1, 0.2, 0.3, and 0.5 mol/L) with a flow rate of 0.5 mL/min (10 min/tube). (B) Elution curves of HSP using the Sephadex column. The eluent was H_2O with a flow rate of 0.4 mL/min (20 min/tube). (C) Monosaccharide composition of HSP. (D) Molecular weight determination of HSP for with HPSEC-MALLS-RID system.

The variance analysis of the model is shown in Supplemental Table S4.

According to Supplemental Table S4, X_1 time, X_3 monochloroacetic acid, X_1X_3, X_2^2, and X_3^2 are significant. The model established in the experiment ($P < 0.001$) is significant, and the mismatch term ($P = 0.0519 > 0.05$) is not significant. These data show that the response surface model established in the experiment was correct.

As shown in Figure 2, there is a maximum value at the highest point of the response surface and within the range of the contour line. The highest point in the figure is also the centre point of the smallest ellipse in the contour line. The contour map of carboxylated Melaleuca polysaccharide shows an ellipse, indicating that the interaction between various factors is robust, which significantly impacts the synthesis of the carboxylated Melaleuca polysaccharide. The optimum synthesis conditions obtained by response surface software were: reaction time 2.01 h, reaction temperature 44.61 °C, monochloroacetic acid 1.18 g, and an ideal degree of substitution of 0.239.

3.5 Synthesis of HSPP

The Design Expert 8.0 software was used to optimise the synthesis of HSPP. Taking the phosphate content as the response

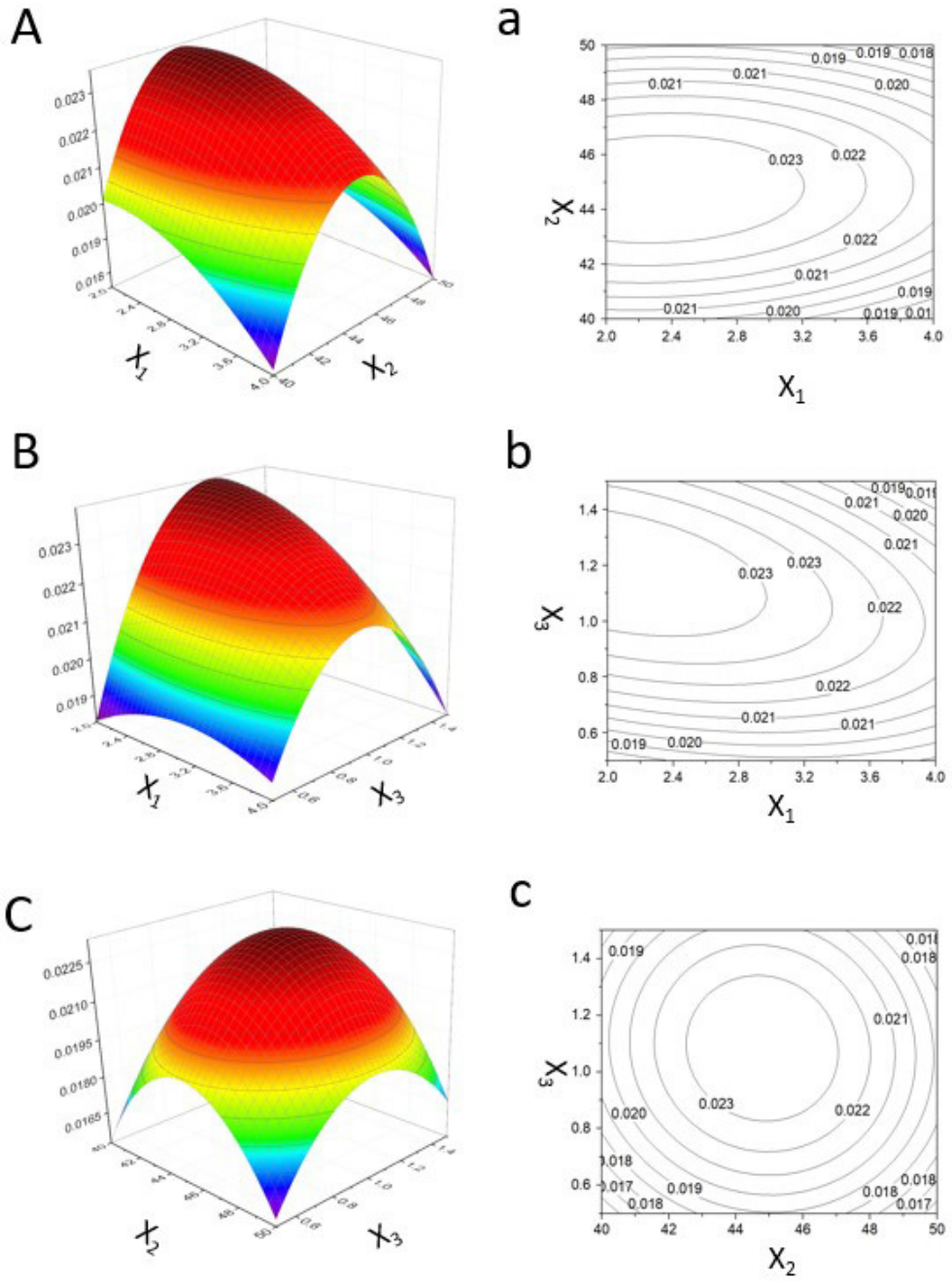

Figure 2. Response surface plots showing the effect of variables on carboxymethylated polysaccharides of Huperzia serrata (HSPS) yield. Effects of (X_1) extraction time, h; (X_2) extraction temperature (°C); and (X_3) monochloroacetic acid (g) on HSPS yield.

value, the data in Supplemental Table S6 were self calculated and fitted through the response surface. The regression Equation 3 is as follows

$$Y = 9.26 - 0.56A + 0.16B + 0.25C + 0.070AB + 0.36AC - 0.11BC - 0.38A2 - 0.69B2 - 0.69C2 \quad (3)$$

Where Y represents the UV absorbance of phosphorylated Melaleuca polysaccharide, A is the reaction time, B is the reaction temperature, and C is the reaction pH. The phosphate content was calculated according to the Formula 4:

$$y = 0.0004x + 0.0001 \quad (4)$$

Where y represents the ultraviolet absorbance of the phosphorylated Melaleuca polysaccharide and x represents the phosphate content in the phosphorylated Melaleuca polysaccharide.

According to Supplemental Table S7, the model established in the experiment ($P < 0.01$) was significant, and the mismatch term ($P = 0.8244 > 0.05$) was not significant. This indicates that the model was established successfully. Adeq precision = 9.748, proving that the model could predict the experimental results.

R^2_{Adj} = 0.8490 (Zhu et al., 2010) . According to the obtained regression equation, the response surface diagram and contour map were drawn using origin. Figure 3 shows a maximum value at the highest point of the response surface and within range of the contour line. The highest point in the figure is also the centre point of the smallest ellipse in the contour line. The results showed that the contour map of phosphorylated Melaleuca polysaccharide showed an ellipse, indicating that the interaction between various factors was robust, which had a significant impact on the synthesis of HSPP. The data optimised by response surface software showed that the optimum synthesis conditions for the synthesis of phosphorylated Melaleuca polysaccharide were as follows: reaction time 4.28 h, reaction temperature 80.78 °C, and pH 8.98.

3.6 Characterisation analysis

UV assay

Figure 4A shows that the sample has no characteristic absorption peaks at 280 nm and 260 nm, proving that HSPS and HSPP do not contain proteins and nucleic acids.

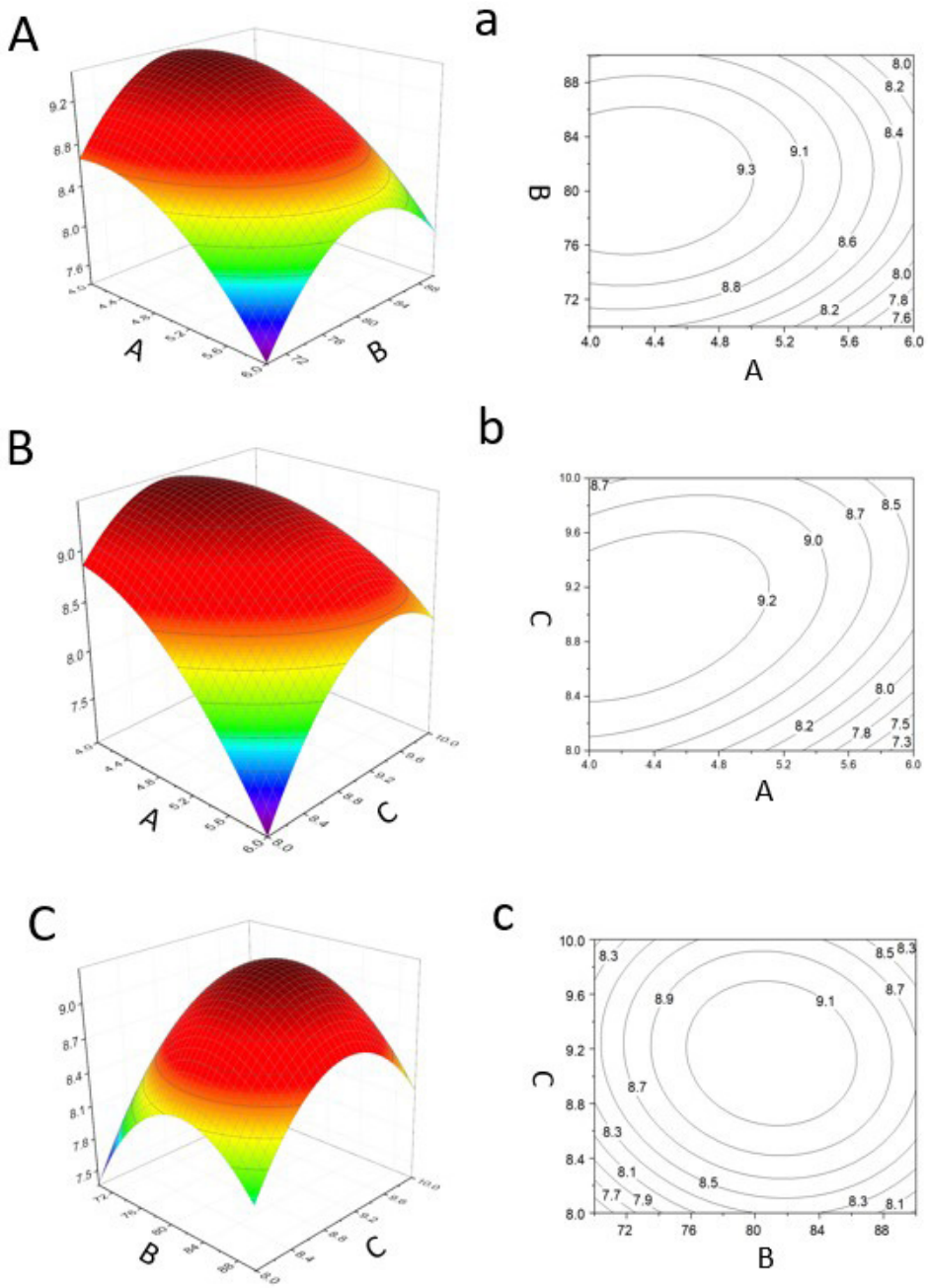

Figure 3. Response surface plots showing the effect of various variables on phosphorylated polysaccharides of Huperzia serrata (HSPP). Effects of (A) reaction time, (B) reaction temperature (°C), and (C) pH on the phosphate radical of HSPP.

FT-IR assay

The infrared scanning spectra of HSP, HSP, and HSPP in the range of 4000 cm^{-1}-400 cm^{-1} by FTIR are shown in Figure 4B. The three polysaccharides have the characteristic absorption peaks of most polysaccharides in the infrared spectrum (the peak around 3400 cm^{-1} is the stretching vibration of O-H or N-H, and the absorption peak near 2931 cm^{-1} is the stretching vibration of C-H). HSPs have strong absorption peaks at 1606.45 cm^{-1}, 1421.00 cm^{-1}, and 1074.40 cm^{-1}. The peak at about 1606 cm^{-1} is the C=O asymmetric vibration absorption peak of carboxyl (-COOH). The peak at 1421.00 cm^{-1} is the vibration of C-H connected with C=O. Compared with HSP, HSPS has a new absorption peak at 1421 cm^{-1}, indicating the successful synthesis of HSPS. Compared with HSP, HSPP has a new absorption peak at 707.25 cm^{-1}, which may be the out of plane bending vibration of O-H. There are no double absorption peaks at 2820 cm^{-1} and 2720 cm^{-1} in HSPP. The P=O stretching vibration may generate the weak absorption peak at about 1250 cm^{-1}. The infrared absorption peak of HSPP at 995 cm^{-1} may be generated by P-O-C stretching vibration, indicating successful HSPP synthesis. In addition, by comparing the infrared spectra of HSPS, HSPP, and HSP, other characteristic absorption peaks changed significantly, indicating that the carboxymethylation modification method used in this experiment successfully modified the carboxymethylation and phosphorylation of the Melaleuca polysaccharide without damaging its molecular structure.

CD analysis

Circular dichroism can analyse the structure of polysaccharides by measuring the conformational changes of polysaccharides in

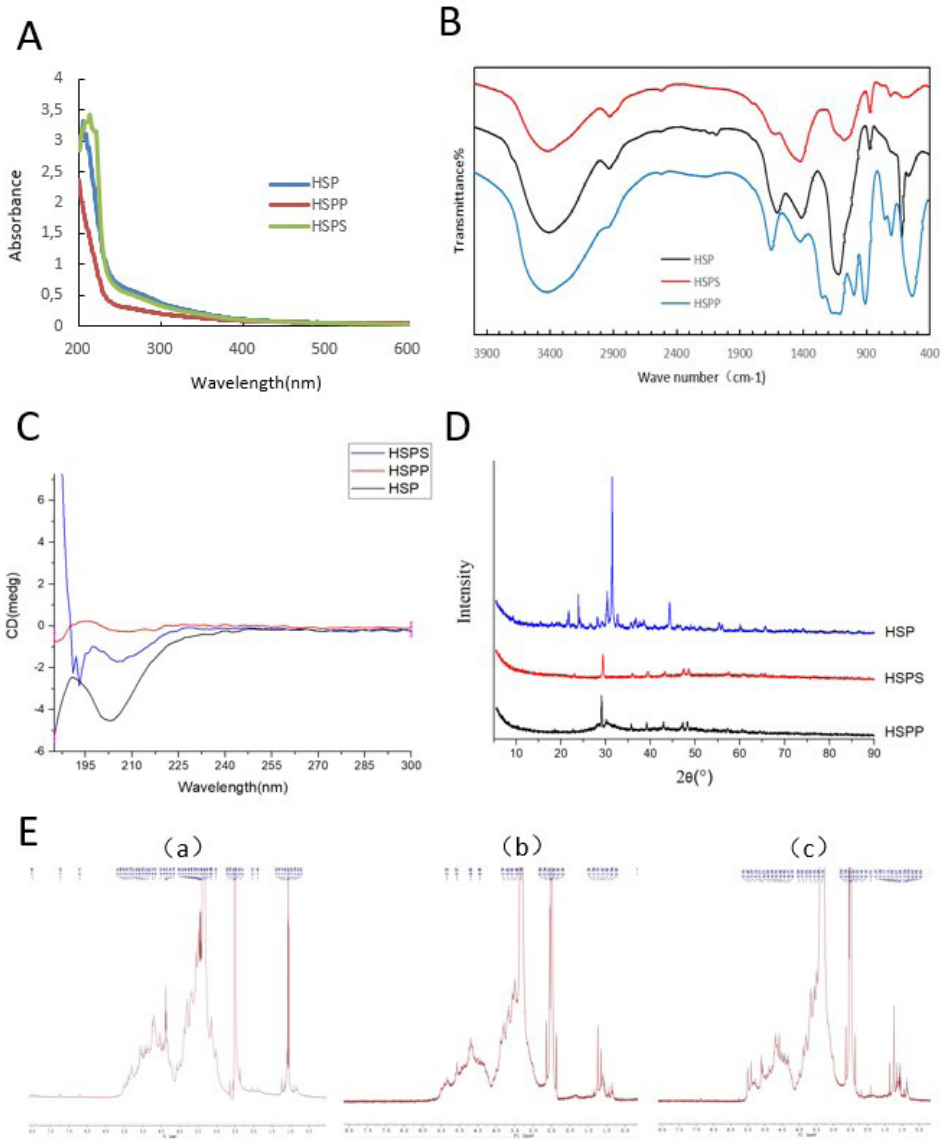

Figure 4. Characterisation analysis of HSP, HSPS, and HSPP. (A) Ultraviolet spectra. (B) Fourier Transform infrared spectroscopy (FTIR) spectra. (C) Circular dichroism spectral (CD) analysis. (D) X-ray diffraction (XRD) analysis. (E) Nuclear magnetic resonance (NMR) analysis.

solution. In general, due to the characteristics of polysaccharides, there is no significant Cotton effect on circular dichroism. However, polysaccharide molecules will fold and entangle in an aqueous solution due to their interactions, resulting in asymmetry and causing a Cotton effect.

HSP, HSPs, and HSPP were analysed by CD spectrometer. The scanning results are shown in Figure 4C. After carboxymethylation modifications, the negative Cotton effect of HSPs was weakened, and the helical structure of the polysaccharide was weakened. After phosphorylation modifications, the Cotton effect of HSPP was significantly weakened. HSP had a negative absorption peak at about 200 nm wavelength. It is speculated that HSP may have a helical structure.

XRD assay

The XRD analysis results are shown in Figure 4D. There is a narrow and high absorption peak at 2 θ= 32°, which indicates that it has high crystallinity. However, there are small heteropeaks at at 2 θ = 20°, 2 θ= 25°, 2 θ = 45°, and 2 θ = 55 °. HSP may be rod structure or have a variety of crystallisation modes. There are no diffraction peak at 2 θ=32° in HSPS and HSPP, which may be due to the destruction of the intramolecular and intermolecular hydrogen bonds and hydroxyl groups of the Melaleuca polysaccharide, resulting in different degrees of changes in the crystal structure of the two modified Melaleuca polysaccharides. In addition, there are two small peaks at about 2 θ=47° in HSPS and HSPP, which further shows that carboxymethylation and phosphorylation modified the structure of the Melaleuca polysaccharide. The crystallinity of HSP was better than HSPs and HSPP. Bai et al found that the cations of EmimAc can penetrate into the network structure of polysaccharides and change the crystal structure by destroying hydrogen bonds, which is similar to the results of this study (Bai et al., 2019).

NMR assay

The chemical shift values of δ5.02, 5.07, 5.14, 5.30, 5.38, 5.44, and 5.61 may be the hydrogen signal of the rhamnose terminal matrix. The chemical shifts of δ3.02, δ 3.35, δ3.41, δ3.42, δ3.43, δ3.44, δ 3.45, δ 3.47, and δ 3.49, δ 4.34, δ 4.35, δ 4.37, δ 4.38 may be the proton hydrogen signal at positions C-2-C-5. The chemical shifts of δ 1.23, δ 1.18, δ1.15, δ 1.09, δ 1.07, δ 1.06, δ 1.04, δ 0.93, δ 0.85 may be galacturonic acid, glucose, galactose, arabinose, mannose, xylose, glucuronic acid and fucose. As shown in Figure 4E, the chemical shifts for HSPS and HSPP are δ 1.23, δ 1.18, δ1.15, δ 1.09, δ 1.07, δ 1.06, δ 1.04, δ 0.93, and δ 0.85. A decrease of 0.85 was seen in the signal intensity, which may be because the polysaccharide carboxymethylation modification reaction site is usually the alcohol hydroxyl group on the sugar residue. After carboxymethylation modification, it is replaced by a -CH_2COO group, weakening the signal.

In the nuclear magnetic spectrum of HSPP, chemical shifts are seen at δ5.33, δ4.69, δ4.45, δ3.79, δ3.66, and a new signal appeared at 3.56, which may be the introduction of a phosphate group. This data further proved that the carboxymethylation and phosphorylation modifications of the Melaleuca polysaccharide were successfully.

SEM

The SEM observation results of HSP, HSPs, and HSPP are shown in Figure 5. According to the SEM images, the HSP, modified HSPs, and HSPP are relatively fluffy, consistent with the morphology of polysaccharides after being freeze-dried. The morphology of HSP was primarily rod-shaped, with folding to varying degrees. Compared with HSP, the modified polysaccharide particles of HSPs and HSPP are larger and more uniform.

3.7 Antioxidant activities

Polysaccharides are one of the main objects food research. They have a wide range of sources and have a variety of biological activities (Huang & Huang, 2020; Zhang et al., 2019). Among them, the antioxidant activity of polysaccharides is the most widely studied (Chen et al., 2019; Zhong et al., 2019). Polysaccharides have a scavenging effect on various reactive oxygen species (ROS), can reduce the production of the lipid peroxidation product malondialdehyde (MDA), increase the activity of antioxidant enzymes, and exhibit a variety of antioxidant effects. Antioxidants can decrease the signs of ageing, reduce tumour growth, and lower the blood sugar and blood fat of some polysaccharides.

As shown in Figure 6A, polysaccharide solutions of various concentrations (1 mg/mL, 2 mg/mL, 4 mg/mL, 6 mg/mL) prepared from HSPS and HSPP all can scavenge ABTS free radicals. The removal rate of ABTS by the aqueous solutions showed an upward trend as the concentration of the polysaccharide solution increased. At 6 mg/ml, HSPS removed 99.47% and HSPP removed 53% of ABTS. Compared with HSP, carboxymethylation enhanced the scavenge ABTS free radicals of polysaccharide, while phosphorylation decreased the scavenge ABTS free radicals of HSP.

As a prerequisite substance for other active oxygen in the organism, superoxide anion can cause cell death, DNA degradation, and inactivation of other active substances (Jambunathan, 2010). The scavenging results of HSPS and HSPP on superoxide ions are shown in Figure 6B. At 4 mg/mL, the super oxideanion radical scavenging rate of HSPP reached 44.44%. After modification, the super oxideanion radical scavenging rate of polysaccharides were decreased. Hydroxyl radicals can react with almost all cell components. Therefore, it is common to study antioxidant efficiency by measuring the ability of compounds to scavenge hydroxyl radicals. The various concentrations of polysaccharide solutions (1 mg/mL, 2 mg/mL, 4 mg/mL, and 6 mg/mL) prepared from the carboxymethylated melaleuca polysaccharide and phosphorylated melaleuca polysaccharide could scavenge hydroxyl free radicals. As shown in Figure 6C, when the concentration of HSPS was 6 mg/mL, the scavenging efficiency of hydroxyl radicals reached 94.84%. When the concentration of HSPP was 4 mg/ml, the scavenging efficiency of hydroxyl radicals reached 92.57%. In general, the Hydroxyl radical scavenging rate of HSP did not change significantly after the modification of carboxymethylation and phosphorylation.

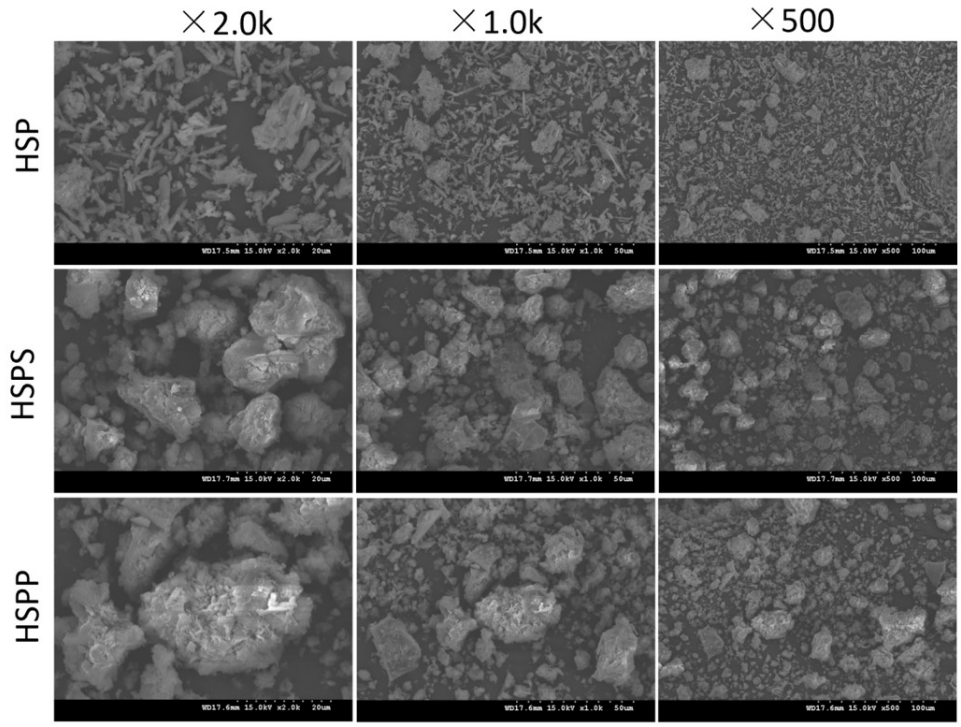

Figure 5. Scanning electron microscopy (SEM) analysis.

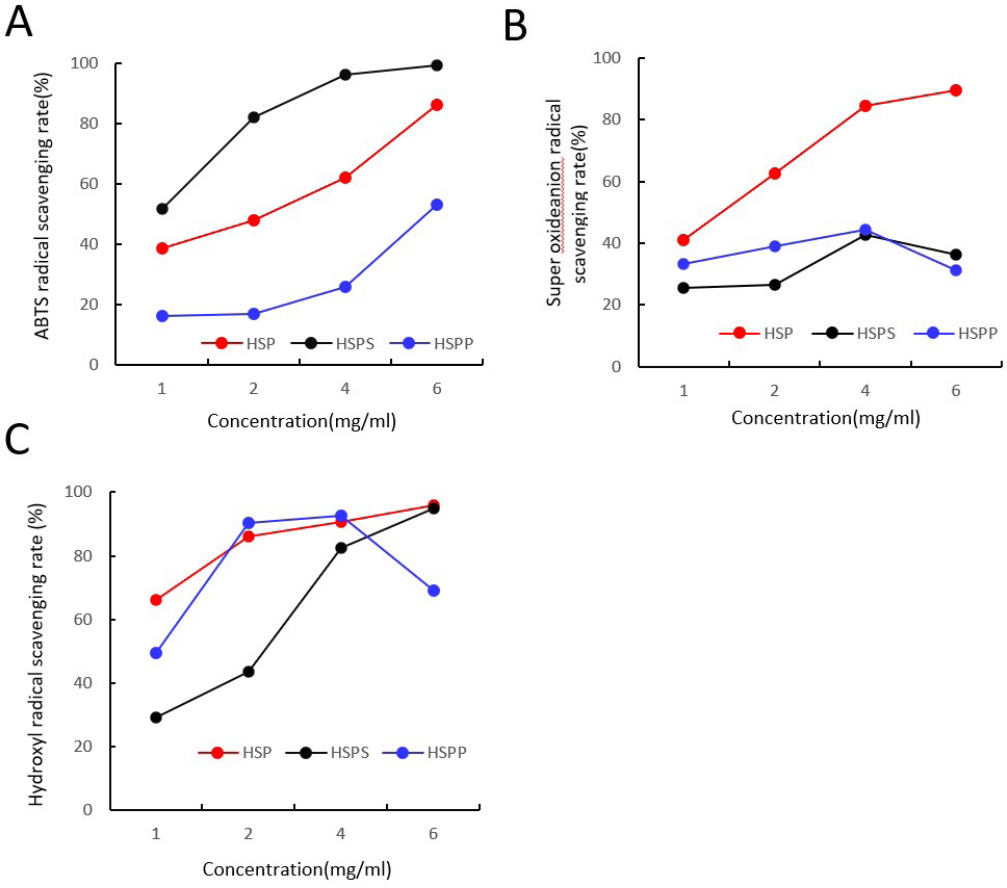

Figure 6. Antioxidant capacity of HSPS and HSPP. (A) Scavenging of ABTS radicals by HSPS and HSPP. (B) Superoxide anion radical scavenging ingrate by HSPS and HSPP. (C) Hydroxyl radical scavenging rate by HSPS and HSPP.

4 Discussion

Polysaccharides exist widely in herbaceous species and are the main components of herbaceous plants, which are one of the four basic substances that constitute life activities and are closely related to various physiological functions (Chen & Huang, 2018a). In recent years, polysaccharides isolated from different kinds of Chinese herbal medicines have attracted much attention because of their significant physiological activities and low toxicity, such as anti-tumor, anti-oxidation, anti-diabetes, anti-radiation, anti-virus, reducing blood lipids and immune regulation (Zeng et al., 2019). Polysaccharides have attracted much attention in the preparation of nano-vaccines and nano-drugs because of their inherent immune regulation, biocompatibility, biodegradability, low toxicity and safety, and polysaccharides have a good immune-promoting effect, and polysaccharides can enhance humoral, cellular and mucosal immunity (Sun et al., 2018). Hericium Erinaceus (H.erinaceus) is a kind of edible fungus with medicinal value. Polysaccharide is one of the main bioactive compounds in H.erinaceus. It has the activities of immune regulation, anti-cancer, antioxidation, gastric protection and intestinal health promotion, nerve protection, liver protection, hypoglycemia and hypolipidemia (Wang et al., 2019b). As a kind of natural product with unique structure and biological activity, the polysaccharide of Tremella has a wide range of activities and applications in food industry, daily chemical industry and pharmaceutical industry (Ma et al., 2021). Ganoderma lucidum polysaccharides are used in drugs because of their unique structural characteristics, properties and biological activity (Zhang et al., 2019). Marine polysaccharides and algal polysaccharides are widely used in the field of biomedical materials, such as drug delivery, tissue engineering, wound dressings and sensors (Sun et al., 2021). And With its excellent gel properties, it has been used in the research and application of nano-drug delivery systems, targeted and controlled drug delivery systems (Rahmati et al., 2019). Algal polysaccharides play an important role in cancer treatment by inducing apoptosis, cell cycle arrest, regulating transduction signal pathways, inhibiting migration and angiogenesis, and activating immune response and antioxidant system. Which may be considered as the next frontier of drug research (Sajadimajd et al., 2019). The extracts or preparation pages of animal polysaccharides also in molluscs can be used in anticoagulant, anti-atherosclerotic, antioxidant, immunomodulatory, antiviral and anti-tumor medical fields (Wang et al., 2019a).

Sulfation, carboxymethylation, phosphorylation and acetylation are common methods of polysaccharide modification,which can improve the biological activity of polysaccharides (Chen & Huang, 2018b; Huang & Huang, 2017). Sulfated polysaccharides from macroalgae have exceptional properties for Bone Tissue Regeneration and may be promising biomaterials in bone tissue repair and regeneration (Venkatesan et al., 2019). Sulfated polysaccharides have already proved to possess a high level and broad-spectrum antiviral activity (Ray et al., 2021). Sulfated modification of AbM polysaccharides can increase their anti-HIV pharmacological activity, which makes them promising alternative candidates as bioactive macromolecules for biomedical applications in HIV/AIDS (Zhao et al., 2021). The carboxymethyl modification of Ganoderma lucidum polysaccharide showed strong anti hydroxyl radical activity in vitro (Xu et al., 2009). Liu et al reported the carboxymethylated cushaw polysaccharide had better ability to scavenge superoxide anions and hydroxyl radicals (Liu & Huang, 2019). And carboxymethyl polysaccharide had significant water solubility significantly (Chakka & Zhou, 2020; Zhang et al., 2022b). Also Phosphorylated polysaccharides improved the physicochemical properties of native polysaccharides and enhanced their biological activity, or even imparting novel biological activity (Xia et al., 2021). Acetylated polysaccharides were suitable for natural food for prevention or remission in acute lung injury (Song et al., 2020).

In this study, polysaccharides were extracted and purified, and the synthesis process of carboxymethyl polysaccharides and phosphorylated polysaccharides was established. SEM, CD, XRD, NMR analysis of HSP, HSPS and HSPP were used. Structural characterisation analyses were performed. Synthetic HSPS and HSPP have different degrees of antioxidant capacity, which change with concentration. HSPS is significantly better than HSPP in scavenging ABTS, superoxide ions, and hydroxyl radicals. Though present research data suggested that carboxymethylated polysaccharides were generally nontoxic in cell and animal models (Santos & Garcia-Rojas, 2021; Wang et al., 2015). carboxymethylation and phosphorylation are chemical modification, and are still worth considering whether all carboxymethylated and phosphorylated polysaccharides together with their products are nontoxic and safe. More clinical evidence is required to support the safety and nontoxicity in the further research.

Conflict of interests

The authors have declared that no competing interest exists.

Data Availability

The data used to support the findings of this study are included in the article.

Author Contributions

Dr. Zhang XF designed the study. Yan YY, Xu CY, Yuan S, Sun LY, and Zhang XF collected data. All authors agreed to the final version.

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Supplementary Material

Supplementary material accompanies this paper.

Supplemental Table S1. Molecular weight of Melaleuca polysaccharide.

Supplemental Table S2. Levels and code of extraction variables used in Box-Behnken design.

Supplemental Table S3. Box-Behnken experimental design and the results for HSPS.

Supplemental Table S4. Analysis of variance of the experimental results of the BBD.

Supplemental Table S5. Levels and code of extraction variables used in Box-Behnken design.

Supplemental Table S6. Box-Behnken experimental design and the results for HSPP.

Supplemental Table S7. Analysis of variance of the experimental results of the BBD.

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