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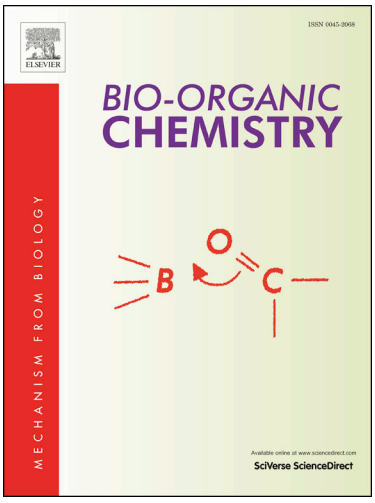

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PII: S0045-2068(21)00652-0
DOI: https://doi.org/10.1016/j.bioorg.2021.105275
Reference: YBIOO 105275

To appear in: *Bioorganic Chemistry*

Received Date: 24 March 2021
Revised Date: 14 July 2021
Accepted Date: 15 August 2021

Please cite this article as: Q. Pan, Y. Sun, X. Li, B. Zeng, D. Chen, Extraction, structural characterization, and antioxidant and immunomodulatory activities of a polysaccharide from *Notarchus leachii freeri* eggs, *Bioorganic Chemistry* (2021), doi: https://doi.org/10.1016/j.bioorg.2021.105275

Extraction, structural characterization, and antioxidant and immunomodulatory activities of a polysaccharide from *Notarchus leachii freeri* eggs

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Abstract

The crude polysaccharides (NLCEP) were extracted from *Notarchus leachii freeri* eggs strings by the salt extraction method. The extraction conditions were optimized using the single-factor experiment method and response surface method (RSM). The results showed that the maximum extraction yield of NLCEP was obtained under the following conditions: NaCl solution concentration of 2.96 %, raw material to liquid ratio of 1: 40 g/mL, extraction time of 2 h and extraction temperature of 69 °C. A new novel pure polysaccharide fraction named as NLCEPs-1 was fractionated from NLCEP by using DEAE-Cellulose 52 and Sephadex G-100. Its structure and immunomodulatory and antioxidant activities were analyzed. The results exhibited that the molecular weight of NLCEPs-1 was 31.4 kDa and it was composed of rhamnose, glucose, galactose, xylose and arabinose in the molar percentage of 11.128: 63.770: 5.439: 6.585: 13.077. The backbone of NLCEPs-1 was mainly consisted of →)4-α-D-Glcp (1→, →6)-α-D-Glcp (1→, →1)-β-D-Galp and β-D-Galp-(1→. NLCEPs-1 exhibited the strong antioxidant activity in scavenging ability of

various free radicals and immunomodulatory activity by the enhancement of the pinocytic capacity, nitric oxide (NO) and cytokines.

Keywords: *Notarchus leachii freeri* eggs, Polysaccharide, Response surface methodology, Structure characterization, Antioxidant activity, Immunomodulatory activity

1. Introduction

The sea hare, belonging to the Aplysiidae family, is a marine opisthobranch gastropod mollusk. There are more than 20 species of the sea hare in China, including *Notarchus leachii freeri*, *Aplysia kurodai, Dolabella auricularia*, *Aplysia dactylomela*, *Aplysia pulmonica,* etc., which are mainly distributed in the southeast coast. The sea hare and its eggs are rich in nutrients and used as the traditional nutritious food source in Asian countries [1-3]. The sea hare eggs have also been used as the traditional medicine in China since Ming Dynasty. The various bioactive substances are found from sea hares, such as peptides, proteins, secondary metabolites, and their structures and biological activities have been widely reported [4-11].

The natural polysaccharides from marine animals have been a hot research topic for their various biological activities, for instance, antioxidant, immunomodulatory, antitumor, anticoagulant and antidiabetes, antibacterial. Many bioactive polysaccharides reported so far have been isolated from sea cucumbers [12, 13], Sepia [14-16], abalone [17, 18] and clams [19]. Although some polysaccharides obtained from sea hare muscle with various biological activities have been reported in recent years [20-22], there was no investigation of polysaccharides from sea hare eggs. The nutritive and medicinal value of sea hare eggs may be related to their polysaccharide activities. Sea hare eggs were a new raw material for active polysaccharide extraction. The biological activities of polysaccharides are determined by their structures. The structure of the polysaccharides from sea hare eggs remains unknown. Therefore, it is important to study the structure and potential biological activities of polysaccharides from sea hare eggs. The enhancement of immune function is an important bioactivity of polysaccharides. One of the most critical members of the immune system is macrophages. Macrophage activity can be regulated by bioactive compounds, possibly leading to the increase of disease resistance [24]. Activated macrophages can produce nitric oxide (NO) and cytokines (IL-1, IL-6, and TNF-α) to against pathogenic microorganisms. The immune responses

of macrophages could be improved by active polysaccharides [25].

Thus, in this study, first the crude polysaccharides from *Notarchus leachii freeri* eggs (NLCEP) were extracted using salt extraction method. The single-factor experiment was utilized combined with response surface methodology to optimize the extraction process of NLCEP. Then, the crude polysaccharides were separated and purified using DEAE-Cellulose 52 chromatography column and Sephadex G-100 chromatography column to obtain the pure polysaccharide fraction named NLCEPs-1. The molecular weight of NLCEPs-1 was measured by high-performance gel permeation chromatography (HPGPC). The structure of NLCEPs-1 was characterized by ultraviolet (UV), fourier transform infrared spectroscopy (FT-IR), high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectra. Furthermore, the immunomodulatory and antioxidant activities of NLCEPs-1 were analyzed by RAW 264.7 cells and the scavenging experiments on different free radicals, respectively.

2. Materials and methods

2.1. Materials and reagents

Notarchus leachii freeri egg strings were obtained from Leizhou, Zhanjiang (latitude 20 degrees 26 minutes 14 seconds north, longitude 109 degrees 56 minutes 40 seconds east, 20 m above sea level, identified by Dr. Chen). DEAE-Cellulose 52, Sephadex G-100, glucose, galactose, rhamnose, mannose, xylose, arabinose, glucuronic acid and galacturonic acid were provided by Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). 1-Phenyl-3-methyl-5-pyrazolone (PMP) and trifluoroacetic acid (TFA) were purchased from Macklin Biochemical Technology Co. (Shanghai, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and lipopolysaccharide (LPS) were purchased from Sigma Company (St. Louis, MO).

RAW 264.7 cells, cell counting kit-8 (CCK-8), neutral red staining solution, Griess assay kit, streptomycin and penicillin were obtained from Beyotime Biotechnology (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were acquired from Gibco Life Technologies (Waltham, MA, USA). Phosphate-buffered saline (PBS, pH 7.4) was purchased from HyClone (Logan, Utah, USA). ELISA kits for mouse TNF-α and IL-6 were purchased from Jiangsu Meimian industrial Co., Ltd (Jiangsu, China).

All the other reagents were of analytical grade.

2.2. Extraction of crude polysaccharides

Notarchus leachii freeri egg strings (200 g) were crushed into homogenate with 500 mL distilled water, and then defatted 3 times with acetone (1500 mL) before being freeze-dried to a powder. The powder was extracted with 3% NaCl solution for 2 h at raw material to liquid ratio of 1:40 g/mL and 69 °C and then centrifuged at 4000 rpm for 15 min. The supernatant was collected and concentrated under vacuum at 55 °C. The concentrated solution was deproteinized by the Sevage method. After adding anhydrous ethanol (3:1, v/v) to the solution, the precipitation was obtained by standing overnight at 4 °C, centrifugation at 4000 rpm for 15 min and collection. It was dialyzed in deionized water for 2 days using dialysis bags (8-10 kDa molecular weight cut off). After that, the crude polysaccharides (NLCEP) from *Notarchus leachii freeri* eggs were gained after lyophilization. The percentage yield of crude polysaccharides were calculated according to the following formula:

$$\text{Crude polysaccharide yield (\%)} = \frac{\text{The weight of polysaccharides extracted}}{\text{The weight of dry powder from sea hare eggs strings}} \times 100\ \%$$

2.3. Experimental design

2.3.1. Single-factor experimental design for crude polysaccharides extraction

Effect of NaCl solution concentration (0, 1, 2, 3, 4 % (w/v)), raw material to liquid ratio (1:10, 1:20, 1:30, 1:40, 1:50 (g /mL)), extraction time (0.5, 1, 1.5, 2, 2.5 h) and extraction temperature (50, 60, 70, 80, 90 °C) on the yield of crude polysaccharides was explored.

2.3.2. Optimized experimental design for crude polysaccharides extraction

Based on the results of single-factor experiment, the effect of the four factors (NaCl solution concentration, raw material to liquid ratio, extraction time and extraction temperature) on the yield of NCLEP were investigated using Box-Behnken (BBD)-RSM. These four factors were represented by A, B, C, D, respectively and divided into three levels (Table 1).

Table 1 Factors and levels for Box-Behnken (BBD)-RSM design

Factors	Coded levels		
	-1	0	1
NaCl solution concentration A (w/v)	2%	3%	4%
raw material to liquid ratio B (g/mL)	1:30	1:40	1:50
extraction time C (h)	1.5	2	2.5
extraction temperature D (°C)	60	70	80

2.4. Purification of crude polysaccharides

500 mL distilled water was used to dissolve the crude polysaccharides (5 g). Then 10 mL solution was loaded into a DEAE-Cellulose 52 chromatography column (2.6 × 60 cm) at a time after being filtered through a 0.45 μm microporous membrane. Then distilled water and different concentrations of NaCl solution (0.2 mol/L, 0.5 mol/L, 1 mol/L) were employed to elute sequentially at a flow rate of 1 mL/min. The fraction eluted by distilled water was collected after being analyzed by the phenol-sulfuric acid method and concentrated at 55 °C, then dialyzed at 4 °C using dialysis bags (8-10 kDa molecular weight cut off) and freeze-dried to gain the polysaccharide (NLCEP-1). In order to further purify NLCEP-1, a Sephadex G-100 chromatography column (1.6 × 100 cm) was employed. The column was eluted with distilled water at a flow rate of 0.5 mL/min for 10 min. According to the elution curve drawn by the phenol-sulfuric acid method, the tubes corresponding the elution peak were collected, then concentrated in a rotary vacuum evaporator, and finally freeze-dried to obtain the pure polysaccharide (NLCEPs-1).

2.5. General physicochemical properties

The total sugar content of NLCEP, NLCEP-1 and NLCEPs-1 was determined by the phenol-sulfuric acid method and glucose acted as the standard [26]. The protein content was determined by Coomassie brilliant blue with bovine serum albumin (BSA) as the standard [27]. The content of glucuronic acid was measured by sulfuric acid-carbazole method using glucuronic acid as standard [28]. The ash content was measured by incineration at 500 °C for 5 h.

2.6. Determination of molecular weight distribution of NLCEPs-1

The molecular weight of NLCEPs-1 was detected by high performance gel permeation chromatography (HPGPC) with various molecular weights of dextran (1.152 kDa to 3693 kDa) as standard. For sample preparation, 5 mg sample was weighed, and then dissolved in 1 mL of 0.05 mol/L NaCl solution. After being centrifuged for 5 min at 12000 rpm, the supernatant was taken and filtered through 0.22 μm microporous filter before analysis. A HPLC system (Shimadzu, Japan) equipped with a BRT105-104102 series gel column (8 × 300 mm) and a RI-10A differential refractive index detectorand (Shimadzu, Japan) was adopted. 20 μL sample was eluted with 0.05 M NaCl solution at a flow rate of 0.6 ml/min at a column temperature of 40 °C. The molecular weight of standards was measured as previously described.

2.7. UV and FT-IR analysis

1 mg/mL NLCEPs-1 aqueous solution was conducted by a UV8453 spectrophotometer (Agilent, USA) in the range of 190 - 400 nm. The dried NLCEPs-1 (2 mg) was ground together with KBr powder and pressed into transparent sheet for testing by using Nicolet 6700 Fourier transform infrared spectrometer (Thermo fisher, USA) in the range of 400-4000 cm^{-1}.

2.8. Monosaccharide composition analysis of NLCEPs-1

Firstly, 1 mL NLCEPs-1 aqueous solution (5 mg/mL) was hydrolyzed by using 1 mL of 4 mol/L trifluoroacetic acid (TFA) at 110 ºC for 4 h in a headspace bottle. With the help of methanol, the excess TFA was removed by rotary evaporation. 1 mL of NaOH solution (0.3 mol/L) and 200 μL of methanol-PMP solution (0.5 mol/L) were added to dissolve and derivatize the hydrolyzed products at 70 ºC for 2 h, respectively. After cooling to room temperature, 1 mL of HCl solution (0.3 mol/L) was added for neutralization. The solution was extracted five times with chloroform, 1 mL each time, then supernatant was filtered through a 0.22 μm microporous membrane. High performance liquid chromatography (HPLC, Shimadzu Corporation, Tokyo, Japan) equipped with a Gemini C18 column (4.6 × 250 mm, 5 μm) was employed to analyze the sample with 8 kinds of monosaccharides as standard at the detection wavelength of 254 nm. The column temperature was maintained at 30 ºC and the flow rate was 1 mL/min. Mobile phase A was 0.1 mol/L phosphate buffer (pH = 6.7), and mobile phase B was acetonitrile with the gradient: 17.8 % B from 0 to 25 min, 18.5% B from 26 to 36 min and 16 % B from 37 to 60 min.

2.9. NMR spectra analysis of NLCEPs-1

About 40 mg NLCEPs-1 was dissolved in 0.55 mL D_2O and transferred to the NMR tube. ^{1}H-NMR, ^{13}C-NMR, DEPT135 NMR and 2D NMR spectra were recorded by a Bruker NMR spectrometer (Avance III 400, Bruker, Switzerland).

2.10. Congo red test

Congo red was used to elucidate the triple helical structure of NLCEPs-1. 2 mL NLCEPs-1 solution (1 mg/mL) was mixed with 2 mL of 100 μmol/L Congo red solution and different gradients of NaOH solution (0, 0.05, 0.1,0.2, 0.3, 0.4 and 0.5 mol/L). The mixed solution was measured in the range of 200-600 nm to obtain the maximum absorption wavelength. Congo red solution without added NLCEPs-1 solution was used as the control.

2.11. Antioxidant activity

2.11.1 DPPH radical scavenging activity

NLCEPs-1 was dissolved in distilled water to prepare sample solution with different concentration gradients (0.5, 2, 4, 6 and 8 mg/mL). The DPPH anhydrous ethanol solution (0.2 mmol/L) was prepared. 2 mL of sample solution was added to 2.0 mL DPPH anhydrous ethanol solution. After 30 min incubation in the dark at room temperature, the absorbance value of the sample solution was measured as A_1 at 517 nm. Ascorbic acid (Vc) served as the positive control. Distilled water served as the control was measured as A_0. The absorbance of the mixture composed of 2 mL sample solution and 2.0 mL anhydrous ethanol was measured as A_2. The DPPH radical scavenging capacity was calculated using the following equation:

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

The DPPH radical scavenging activity of NLCEP was measured as previously described.

2.11.2 Hydroxyl radical scavenging assay

The scavenging ability on hydroxyl radical was determined by Feton method. 1 mL of various concentrations of polysaccharide solution (0.5, 2, 4, 6 and 8 mg/mL) were mixed with 1 mL of 9 mmol/L ferrous sulfate solution, 1 mL of 9 mmol/L alicylic acid-ethanol solution and 1mL of 8.8

mmol/L H_2O_2 solution. The mixed solution was incubated in a water bath at 37 °C for 30 min, then its absorbance was detected at 510 nm. Vc served as the positive control. The hydroxyl radical scavenging capacity was calculated according to the following equation:

$$\text{Scavenging rate (\%)} = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100\%$$

A_0 is the absorbance of the blank (distilled water instead of sample), A_1 is the absorbance of the sample, A_2 is the absorbance of the control (distilled water instead of H_2O_2).

2.11.3 Scavenging ability on superoxide anion radical

1 mL of various concentrations of polysaccharide solution (0.5, 2, 4, 6 and 8 mg/mL) were mixed with 5 mL of Tris-HCl buffer (0.05 mol/L, pH = 8.2), then incubated at 25 °C for 20 min. 0.3 mL of 3 mmol/L pyrogallol-hydrochloric acid solution was preheated in 25 °C water bath. The change in absorbance of the reaction solution was measured at 320 nm every 30 s for 4 min. Vc served as the positive control. The equivalent distilled water served as the control group. The scavenging activity for superoxide anion radical was calculated using the following equation:

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

Where A_0 is auto-oxidation rate of the control group, A_1 is auto-oxidation rate of the sample.

2.11.4 Reducing power

2.5 mL of 0.2 mol/L phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide solution were added into 1 mL of various concentrations of polysaccharide solution (0.5, 2, 4, 6 and 8 mg/mL). When the mixture was incubated in a water bath at 50 °C for 20 min, 2.5 ml of 10% trichloroacetic acid was added into. The mixture was centrifuged for 10 min at 4000 rpm to obtain 2.5 mL of supernatant. The supernatant was mixed with 2.5 ml of distilled water and 1 ml of 0.1% $FeCl_3$ solution, and its absorbance was measured at 700 nm after standing for 10 min. Vc served as the positive control. The equivalent distilled water instead of the polysaccharide solution was used as the control group.

2.12. Immunomodulatory activity assay

2.12.1. Cell culture

Mouse macrophage RAW 264.7 cells were maintained in DMEM supplemented with 10 %

FBS, 1 % penicillin-streptomycin in a humidified atmosphere of 5 % CO_2 at 37 °C.

2.12.2. Cell viability assay

The effect of NLCEP and NLCEPs-1 on cell viability of RAW 264.7 cells was investigated by using the CCK-8 assay. 5×10^4 Cells per well in a 100 μL medium were plated on 96-well plates. After incubation for 4 h, the cells were treated with different concentrations of NLCEPs-1 (100, 250, 500, 1000 μg/mL), 1 μg/mL LPS (positive control) and serum free culture medium (control group), and then incubated for another 24 h at 37 °C and 5% CO_2. After removing the supernatant from each well, 100 μL CCK-8 reagent was added to each well, and the cells were incubated for 4 h, then the absorbance of cells was determined at 450 nm by using an automated microplate reader (Multiskan GO 1510, Thermo Scientific, USA). The cell viability was calculated as follows: cell viability (%) = $A_1/A_2\times100\%$, where A_1 is the absorbance of the test group, and A_2 is the absorbance of the control group. The cell viability of NLCEP was measured in the same manner as that of NLCEPs-1.

2.12.3. Determination of pinocytic activity

The effect of NLCEP and NLCEPs-1 on the pinocytic activity of RAW 264.7 cells was evaluated by using the neutral red staining solution assay. 5×10^5 cells were seeded into each well of 96-well plate and incubated for 4 h at 37 °C and 5% CO_2. Different concentrations of NLCEPs-1 (100, 250, 500, 1000 μg/mL), 1 μg/mL LPS (positive control) and serum-free culture medium (control group) were added into each well. The cells were incubated for 24 h. After removing the medium, the cells were treated with 100 μL neutral red staining solution and incubated for 1 h. When the supernatant was carefully discarded, the cells were washed three times with PBS buffer (100 μL per wash). Then 100 μL cell lysate solution (glacial acetic acid: ethanol = 1: 1) was pipetted into each well. After the cell plate was placed at room temperature for 2 h, the absorbance of cells was measured at 540 nm by using an automated microplate reader (Multiskan GO 1510, Thermo Scientific, USA). The pinocytic activity of NLCEP on RAW 264.7 cells was tested using the same procedure as NLCEPs-1.

2.12.4. Determination of NO, TNF- α and IL-6

5×10^5 cells were seeded into each well of 96-well plate and incubated for 4 h at 37 °C and 5 % CO_2. The supernatant was collected for the subsequent experiments. NO content in the supernatant was measured by the Griess method. The level of TNF- α and IL-6 in the supernatant was evaluated by using ELISA kit according to the instructions.

2.13. Statistical analysis

The results were expressed as the mean ± standard deviation ((SD)) of three repeats, and the difference was tested by analysis of variance ($p < 0.05$). GraphPad Prism 6 software was used for statistical analysis.

3. Results and discussion

3.1. Effect of extraction conditions on the yield of NLCEP

3.1.1. Effect of different concentrations of NaCl solution

The effect of NaCl solution concentration (0%, 1%, 2%, 3%, 4% (w/v)) on the extraction yield of NLCEP was estimated. The raw material to liquid ratio, extraction time and extraction temperature were fixed at 1:40 g/mL, 2 h and 70 °C, respectively. The result was shown in Fig.S1. As shown in Fig.S1, the extraction yield of NLCEP increased significantly when the concentration of NaCl solution rose from 0% to 3%. While the concentration of NaCl solution exceeded 3.0, the yield of NLCEP increased was unconspicuous. Low NaCl concentration can improve the diffusion of NLCEP, which is helpful to increase polysaccharide yield. However, high NaCl concentration induced precipitation of polysaccharide, leading to the decline of polysaccharide yield [29]. Therefore, 3% was the optimal concentration of NaCl solution.

3.1.2. Effect of different raw material to liquid ratios

The influence of different raw material to liquid ratios (1:10, 1:20, 1:30, 1:40, 1:50 (g /mL)) on the extraction yield of NLCEP was investigated. The NaCl solution concentration, extraction time and extraction temperature were set at 3%, 2 h and 70 °C, respectively. The result was illustrated in Fig.S2. As can be seen from Fig.S2, when the raw material to liquid ratio was no less than 1:40 g /mL, the extraction yield of NLCEP increased significantly with the decrease of raw material to

liquid ratio. It may be that the solvent increased the concentration difference of polysaccharides inside and outside the cells of *Notarchus leachii freeri* eggs, which accelerated the release of polysaccharides from the cells of *Notarchus leachii freeri* eggs, so that the extraction yield of NLCEP was enhanced. When the raw material to liquid ratio was less than 1:40 g /mL, the extraction yield of NLCEP decreased. So, the raw material to liquid ratio of 1:40 g /mL was appropriate.

3.1.3. Effect of different extraction time

The effect of different extraction time (0.5, 1, 1.5, 2, 2.5 h) on the yield of NLCEP was investigated under the fixed conditions of 3% NaCl solution, 1:40 g/mL raw material to liquid ratio and 70 °C extraction temperature. The result was exhibited in Fig.S3. As shown, the extraction yield of NLCEP increased with the extension of extraction time from 0.5 to 2 h, and then decreased when the time exceeded 2 h. Therefore, the extraction time of 2 h was enough to obtain the yield of NLCEP.

3.1.4. Effect of different extraction temperatures

The effect of extraction temperature on the yield of NLCEP was studied under the conditions of 3% NaCl solution, 1:40 g/mL raw material to liquid ratio and 2 h extraction time. The result was shown in Fig.S4. With the increase of extraction temperature from 50 to 70 °C, the yield of NLCEP increased rapidly and reached a maximum value at 70 °C. Then it decreased when the temperature exceeded 70 °C. The decreased extraction yield may be due to the breaking down of the molecular chain of polysaccharide under high temperature [30]. In order to obtain a higher NLCEP extraction yield for further study, 70 °C was considered as an optimal extraction temperature.

3.2. Optimization of extraction process of NLCEP by RSM

According to the results of the singer-factor experiments, the design matrix and corresponding response values of RSM experiments were shown in Table 2. The multivariate quadratic regression model equation is obtained through regression analysis as following:

$$Y=4.27-0.041A+0.074B+0.093C-0.055D-0.028AB-0.049AC+0.013AD+0.16BC-0.11BD-0.029CD-0.66A^2-0.46B^2-0.36C^2-0.33D^2$$

The variance results of the response surface model were shown in Table 3. The P-value and F-value were used to evaluate the significance of the model. As shown in Table 3, the fitness of the model was highly significant because the P value of NLCEP model was less than 0.0001 ($p < 0.001$), and F value was 29.23. The Lack of fit P-value was 0.2136, indicating the suitability of the model for prediction of variations [31]. The R^2 and the R^2_{adj} were 0.9715 and 0.9383, respectively, indicating that the predicted values and experimental values had a high degree of correlation. In addition, the coefficient of the variation (C.V. %) was 2.52%, suggesting that the observed values had a high degree of accuracy and reliability. Additionally, the coefficients (A^2, B^2, C^2, D^2, BC, BD) were significant with very low P-values ($p < 0.05$) while the coefficients (AB, AC, AD, CD) were not significant along with large P-values ($p > 0.05$), indicating that the interactions between AB, AC, AD, CD on the extraction of NLCEP were not significant.

Three-dimensional (3D) response surfaces and two-dimensional (2D) contour plots were drawn by Design expert 8.0.6 software. The steepness of the response surface and the shape and density of the contours were used to analyze the effect of parameters and the interactions between various parameters on the yield of NLCEP. The results were shown in Fig.S5 and S6. As shown in Fig.S6(A-C), the response surfaces were steep, indicating that the effect of NaCl solution concentration on the yield of NLCEP was stronger than that of other factors. The shape of the contour plots (Fig.S6(A-C) and S6F) was almost circular, suggesting that the interactions between AB, AC, AD and CD on the yield of NLCEP were not significant [32], which were consistent with results in the regression model (Table 3).

As shown in Fig.S5(D, E), when two factors were at 0 level, the yield of NLCEP increased first and then decreased with the increase of other two factors. The response surfaces (Fig.S5(D, E)) were steep and the counter plots (Fig.S6(D, E)) were elliptical, suggesting that the interactions between BC and BD were significant, which were in accordance with the results given in Table 3.

By using software, the optimal conditions for NLCEP were NaCl solution concentration of 2.96 %, raw material to liquid ratio of 1: 41.23 g/mL, extraction time of 2.08 h and extraction temperature of 68.90 °C, and the predicted extraction yield was 4.29 %. Considering the practical operability, the optimal conditions were modified to NaCl solution concentration of 3 %, raw material to liquid ratio of 1: 40 g/mL, extraction time of 2 h and extraction temperature of 69 °C.

The actual extraction yield was 4.20 %, which was close to the predicted value, indicating that the model was reliable.

Table 2 Experimental design and results for response surface analysis

Number	A	B	C	D	Y Polysaccharide yield%
1	-1	-1	0	0	3.1724
2	1	-1	0	0	3.038
3	-1	1	0	0	3.2872
4	1	1	0	0	3.0267
5	0	0	-1	-1	3.5673
6	0	0	1	-1	3.7317
7	0	0	-1	1	3.4917
8	0	0	1	1	3.5420
9	-1	0	0	-1	3.3633
10	1	0	0	-1	3.2355
11	-1	0	0	1	3.3161
12	1	0	0	1	3.2012
13	0	-1	-1	0	3.3051
14	0	1	-1	0	3.2094
15	0	-1	1	0	3.4118
16	0	1	1	0	3.8509
17	-1	0	-1	0	3.1202
18	1	0	-1	0	3.2692
19	-1	0	1	0	3.3542
20	1	0	1	0	3.2858

Table 2 Experimental design and results for response surface analysis (Continued)

Number	A	B	C	D	Y Polysaccharide yield%
21	0	-1	0	-1	3.5166
22	0	1	0	-1	3.9016
23	0	-1	0	1	3.1010

24	0	1	0	1	3.3293
25	0	0	0	0	4.2228
26	0	0	0	0	4.2754
27	0	0	0	0	4.3152

Table 3 Analysis of variance of regression model

Source	Sum of squares	df	Mean square	F value	P value
Model	3.12	14	0.22	29.23	< 0.0001***
A	0.02	1	0.02	2.6	0.1331
B	0.066	1	0.066	8.65	0.0124*
C	0.1	1	0.1	13.73	0.003**
D	0.036	1	0.036	4.76	0.0498*
AB	3.24×10^{-3}	1	3.24×10^{-3}	0.42	0.527
AC	9.51×10^{-3}	1	9.51×10^{-4}	1.25	0.2861
AD	6.68×10^{-4}	1	6.68×10^{-4}	0.088	0.7723
BC	0.1	1	0.1	13.16	0.0035**
BD	0.05	1	0.05	6.5	0.0255*
CD	3.26×10^{-35}	1	3.26×10^{-3}	0.43	0.526
A^2	2.32	1	2.32	303.91	< 0.0001***
B^2	1.15	1	1.15	151.07	< 0.0001***
C^2	0.69	1	0.69	91.03	< 0.0001***
D^2	0.59	1	0.59	77.92	< 0.0001***
Residual	0.092	12	7.63×10^{-3}		
Lack of fit	0.087	10	8.73×10^{-3}	4.06	0.2136
Pure error	4.30×10^{-3}	2	2.15×10^{-3}		
Cor total	3.21	26			

R^2=0.9715; R^2_{adj}=0.9383; C.V. %=2.52%; Adeq Precision=18.594

* (p <0.05); ** (p <0.01); ***(p <0.001).

3.3 Purification of NLCEP and characterization

NLCEP were separated on a DEAE-Cellulose 52 chromatography to obtained a main fraction eluted by distilled water, which was named NLCEP-1, and the elution curve of NLCEP on a DEAE-Cellulose 52 ion-exchange chromatography column was shown in Fig.1A. NLCEP-1 was then purified using a Sephadex G-100 column and the elution curve was shown in Fig.1B. As seen from Fig.1B, NLCEPs-1 was a single component. The lyophilized NLCEPs-1 was fluffy white powder and freely soluble in water. The extraction rate was 47.50 %. The physicochemical compositions of

NLCEP, NLCEP-1 and NLCEPs-1 were summarized in Table 4. No protein and ash were found in NLCEPs-1. The total sugar content of NLCEPs-1 was higher than that of NLCEP and NLCEP-1. After purification processes, both NLCEP-1 and NLCEPs-1 contained the low content of uronic acid. The above results demonstrated that the deproteination and purification processes were successful.

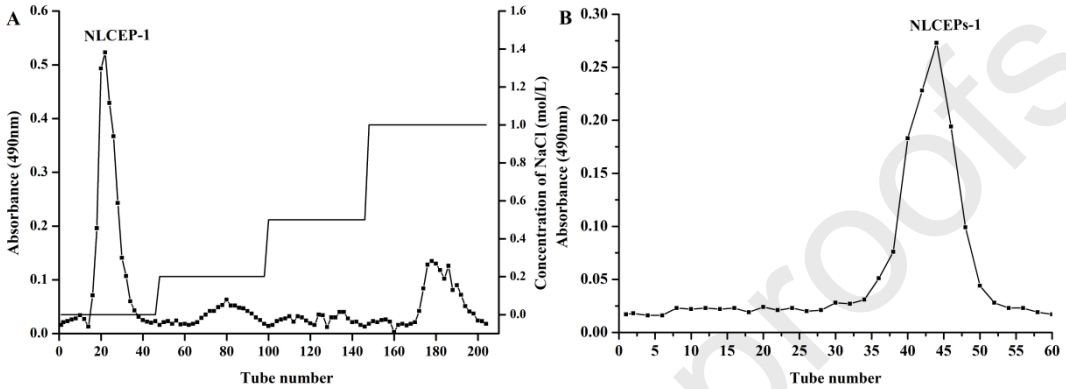


Fig. 1 Elution curve of NLCEP on a DEAE-Cellulose 52 ion-exchange chromatography column (A) and elution curve of NLCEP-1 on a Sephadex G-100 gel chromatography column (B)

Table. 4 Chemical components analysis of NLCEP, NLCEP-1 and NLCEPs-1

Samples	NLCEP	NLCEP-1	NLCEPs-1
Total carbohydrates (%)	85.4312	89.8213	94.2753
Protein (%)	1.5771	ND	ND
Uronic acid (%)	7.9680	0.8869	0.2320
Ash (%)	1.4442	0.2564	ND

Abbreviation: ND, not detected.

3.4. Determination of molecular weight

The molecular weight of NLCEPs-1 was determined by high performance gel permeation chromatography. The single peak on the HPGPC chromatogram (Fig. 2) suggesting that NLCEPs-1 was a homogeneous polysaccharide and was suitable for further study on structural analysis and biological activities. The second peak on the chromatogram was a solvent peak. According to the calibration curve based on the dextran standards (lgMw-RT: $y = -0.224x + 13.402$, $R^2 = 0.9922$, figure of HPGPC chromatogram of dextran standard was shown in supporting information), the molecular weight of NLCEPs-1 was 31.4 kDa.

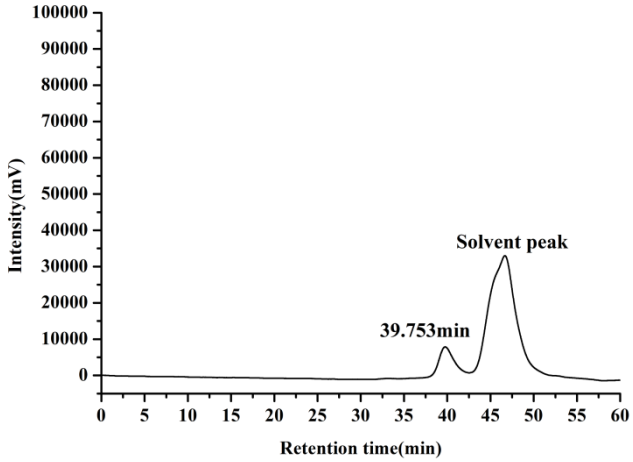


Fig.2 HPGPC chromatogram of NLCEPs-1.

3.5. UV and FT-IR analysis

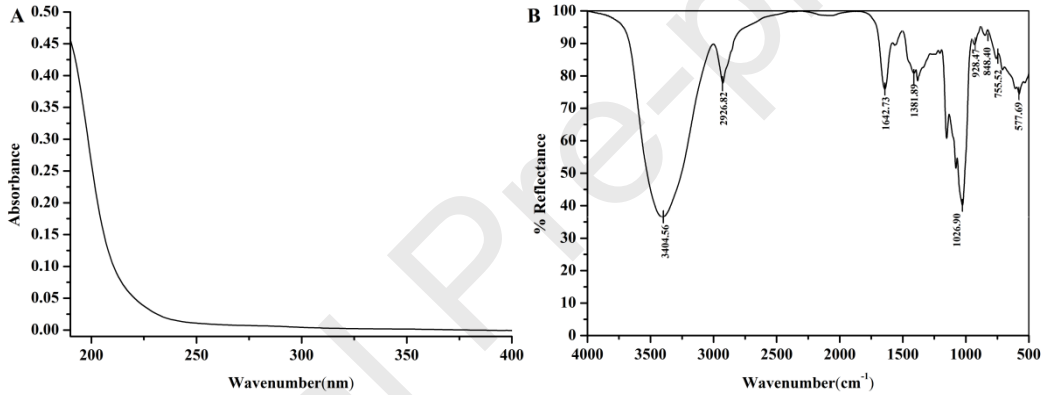


Fig.3 UV (A) and FT-IR spectra of NLCEPs-1 (B).

NLCEPs-1 was scanned by UV spectrometer from 200 to 400 nm, and the result was illustrated in Fig.3A. There were no absorption peaks in the wavelength range of 260 to 280 nm, indicating that NLCEPs-1 contained no nucleic acids and proteins [34]. The infrared spectrum of NLCEPs-1 was shown in Fig.3B. The broad and strong absorption peak at 3404.56 cm^{-1} was contributed to the stretching vibration of the hydroxyl group. The absorption peak at 2926.82 cm^{-1} was caused by C–H stretching vibrations. Both the hydroxyl group and C–H bond belonged to the characteristic peaks of polysaccharides. The absorption peak at 1642.73 cm^{-1} suggested the existence of C=O. The absorption peaks between 1200 and 1000 cm^{-1} were caused by the stretching vibration of the C-O-C ether bond on the pyranose ring and the bending vibration of C-O-H. The appearance of absorption bands at 848.40 and 755.52 cm^{-1} suggested that NLCEPs-1 was a D-glucopyranose derivative [35]. The absorption peaks at 848.40 cm^{-1} and 928.47 cm^{-1} indicated that the sugar linkage

types of NLCEPs-1 were α-type and β-type glycosidic bonds [36].

3.6. Monosaccharide composition

The retention time of eight standard monosaccharides were determined by HPLC and the results were shown in Fig.4A. The monosaccharide composition of NLCEPs-1 was analyzed using HPLC, and the results were shown in Fig.4B. The monosaccharide composition of NLCEPs-1 was determined by comparing with the retention time of standard monosaccharides. The results clarified that NLCEPs-1 was mainly composed of rhamnose, glucose, galactose, xylose and arabinose in the molar percentage of 11.128: 63.770: 5.439: 6.585: 13.077, and glucose was the dominant monosaccharides in NLCEPs-1. Previous work demonstrated that monosaccharide composition was related to the biological activities of polysaccharides. The arabinose, galactose, xylose and mannose may play an important role in the immunomodulatory activity of macrophages [37]. NLCEPs-1 contained these three monosaccharides, which suggested that NLCEPs-1 may have immunological activity.

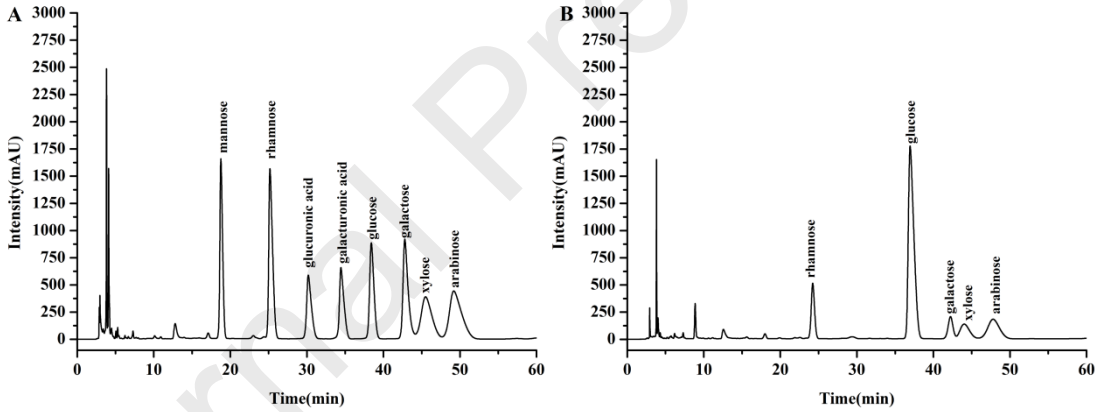


Fig.4 HPLC chromatogram of eight standard monosaccharides (A), HPLC chromatogram of NLCEPs-1 (B).

3.7. NMR analysis

^{1}H NMR and ^{13}C NMR spectra of NLCEPs-1 were shown in Fig.5A and 5B. According to reference [38], the chemical shift of anomeric protons at 4.00-6.00 ppm was usually considered as the sugar residues. The signals in the range of 4.9-6.0 ppm and 4.0-4.9 ppm were separately assigned to α-configuration glycosides and β-configuration glycosides [39-40]. The chemical shifts appeared in the range of 3.60-4.00 ppm in the ^{1}H NMR spectrum showed that NLCEPs-1 was a heteropolysaccharide [41].

In the ^{1}H NMR spectrum, the signal at 5.24 ppm could be determined as the anomeric protons of →)4-α-D-Glcp (1→ (residue A). The cross peak at 5.24/3.51 ppm was obtained in ^{1}H-^{1}H COSY

(Fig.S10A), suggesting that 3.51 ppm was assigned to H2 of residue A. The signals at 3.27, 3.66, 3.55, 3.87 ppm corresponded to H3, H4, H5 and H6 of residue A, respectively. In the HSQC spectrum (Fig.S10E), the cross peak of H1/C1 (5.24/99.98 ppm) revealed close connectivity between H1 and C1. And the carbon peaks of C2(71.41 ppm), C3(69.37 ppm), C4(72.82 ppm), C5(77.70 ppm) and C6(73.28 ppm) of residue A were observed in the HSQC spectrum as well.

The presence of signals of the anomeric proton (5.12 ppm) and anomeric carbon (92.00 ppm) indicated the existence of →6)-α-D-Glcp (1→ (residue B). Furthermore, the cross peaks at 5.12/3.44 ppm (H1/H2), 3.44/3.38 ppm (H2/ H3), 3.38/3.64 ppm (H3/H4), 3.64/3.74 ppm (H4/H5) and 3.74/3.27 ppm (H5/H6) of residue B were detected in the ^{1}H-^{1}H COSY.

In ^{13}C NMR spectrum, the signals at 98.38 ppm could be assigned to the C-1 of →1)-β-D-Galp (residue C) [42]. The other signals of residue B and residue C were also speculated in the same way and were shown in Table 5.

The chemical shifts of the anomeric proton and anomeric carbon of β-D-Galp-(1 → (residue D) appeared at 4.53 ppm and 95.87 ppm . The cross peaks at 3.17/73.28 ppm, 3.38/75.77 ppm, 3.51/74.50 ppm, 3.27/71.24 ppm, 3.12/60.42 ppm were seen from ^{1}H-^{1}H COSY and HSQC spectra.

The HMBC spectrum of NLCEPs-1 was shown in Fig.S10C. An obvious peak was observed in the range of 170-185 ppm, which might be due to the low concentration of uronic acid in NLCEPs-1 [43]. The cross peak signals at 5.24 (residue A, H-1)/72.82 ppm (residue B, C-6), 4.86 (residue C, H-1) /73.28 ppm (residue A, C-6) and 3.51 (residue D, H-4)/99.98 ppm (residue A, C-1) were obtained from the HMBC spectrum, suggesting that the connection between residue A and B, residue A and C, residue A and D were 1,6 glycoside bonds, 1,6 glycoside bonds and 1,4 glycoside bonds, respectively.

The complete ^{1}H and ^{13}C NMR signal assignments for sugar residues were summarized in Table 5. The result demonstrated that NLCEPs-1 consisted of four anomeric residues, including →)4-α-D-Glcp (1→ , →6)-α-D-Glcp (1→, →1)-β-D-Galp and β-D-Galp-(1→, respectively.

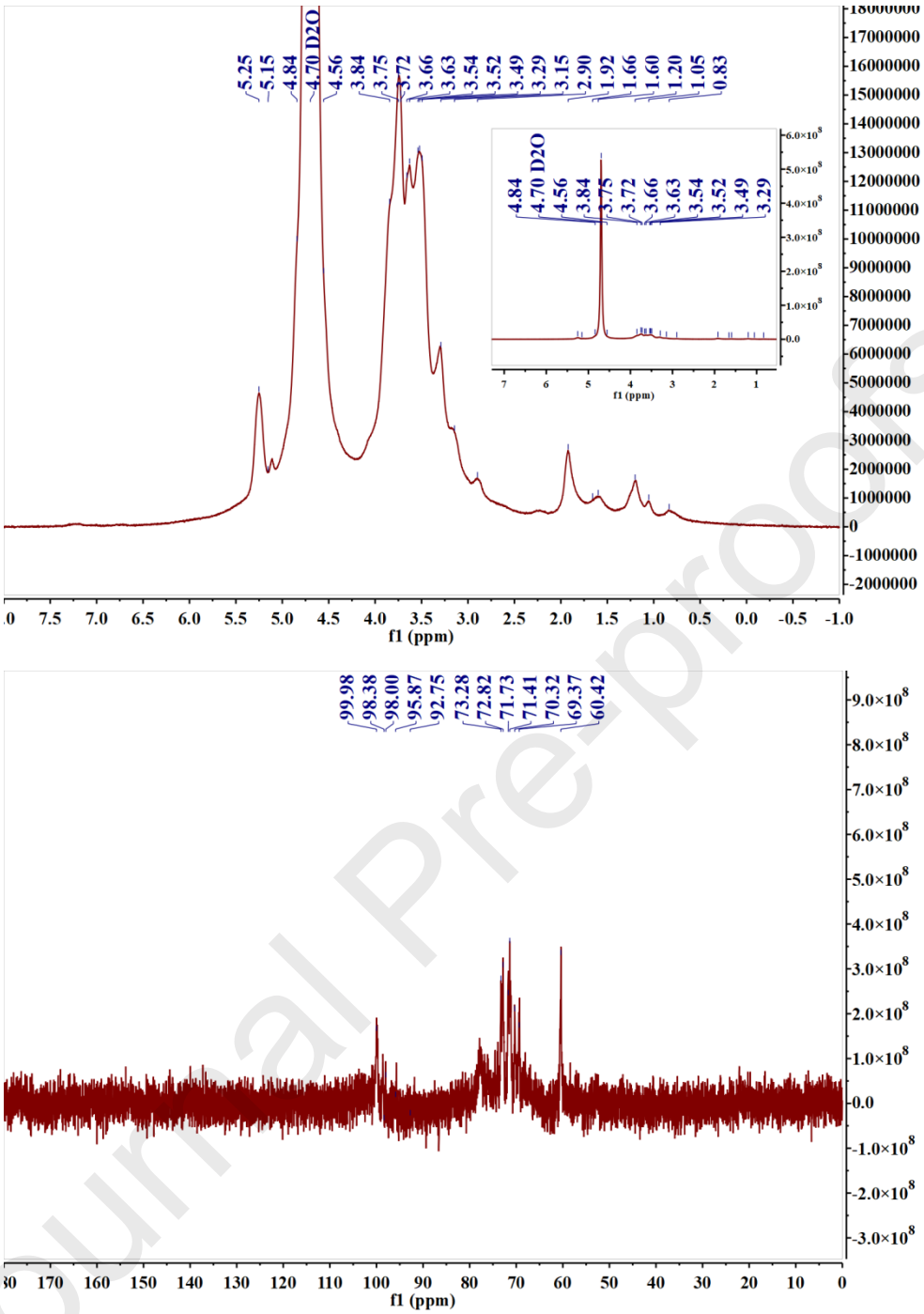


Fig.5 NMR spectra of NLCEPs-1. ^{1}H NMR spectrum (A) .^{13}C NMR spectrum (B).

Table. 5 Chemical shifts of resonances in the ^{1}H and ^{13}C NMR spectra of NLCEPs-1.

Glycosidic linkages		1	2	3	4	5	6
→)4-α-D-Glcp(1→	H	5.24	3.51	3.27	3.66	3.55	3.87
	C	99.98	71.41	69.37	72.82	77.70	73.28
→6)-α-Glcp(1→	H	5.12	3.44	3.38	3.64	3.74	3.27

	C	92.00	77.70	75.77	70.32	71.41	72.82
→1)-β-D-Galp	H	4.86	3.48	3.66	3.38	3.12	-
	C	98.38	75.77	60.42	69.37	71.41	-
β-D-Galp-(1→	H	4.53	3.17	3.38	3.51	3.27	3.12
	C	95.87	73.28	75.77	74.50	71.24	60.42

3.8. Triple helical structure analysis

Cango red is an acid dye and the maximum absorption wavelength will change to the longer wavelength when Congo-red complex forms [46]. The Congo-red complex will form when Cango red interacts with the polysaccharide which has a three-strand helix chain conformation [47]. As shown in Fig.6, as compared to Cango red, the maximum absorption wavelength (λ_{max}) of Congo red-NLCEPs-1 complex was shifted to the longer wavelength, suggesting that NLCEPs-1 had three-helical structure [40, 48]. Based on the previous report, polysaccharides with triple helical structure and hydrophilic groups on the outer surface of the helix are related to the immunomodulatory activity. NLCEPs-1 may have immunomodulatory activity.

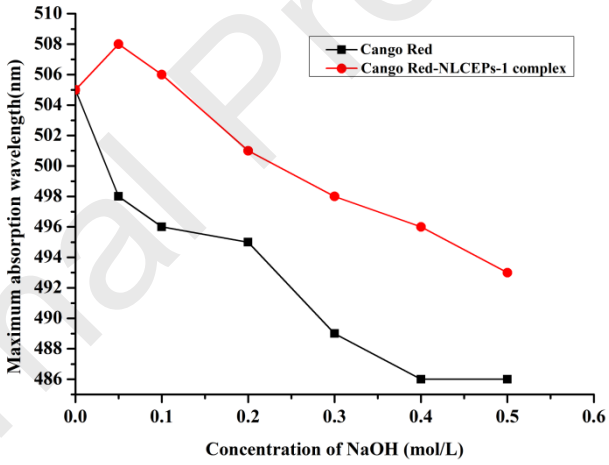


Fig.6 The maximum absorption wavelength of Congo red and Congo red-NLCEPs-1 complex at various concentrations of NaOH.

3.9. Antioxidant activity

3.9.1. DPPH radical scavenging activity

DPPH radical scavenging experiment is a commonly used tool to evaluate the antioxidant effect of natural compounds. The new prepared DPPH anhydrous ethanol solution had a deep purple color with an absorption maximum at 517 nm. The results of DPPH radical scavenging assay of NLCEP and NLCEPs-1 were illustrated in Fig.7A. The scavenging abilities of NLCEP and NLCEPs-1 on DPPH radical increased with the increase of concentration of polysaccharides in the

tested concentration range. Compared with NLCEP and NLCEPs-1, the scavenging activity of V_C was the highest. When the concentration was 8 mg/mL, the DPPH radical scavenging rates of NLCEP and NLCEPs-1 were 63.997 % and 59.420 %, respectively. The IC_{50} values of NLCEP, NLCEPs-1 and V_C on the DPPH radical scavenging ability were 4.113 mg/mL, 5.054 mg/mL and 0.123 mg/mL, respectively. Although the IC_{50} value of NLCEPs-1 scavenging DPPH radical was higher than that of VC and NLCEP, lower than that of the polysaccharides from *Flammulina velutipes* (IC_{50} =8.23 mg/mL) [50], Pumpkin (IC_{50} =6.32 mg/mL) [51] and *Pinus koraiensis* (IC_{50} =122.2 mg/mL) [52].

3.9.2. Hydroxyl radical scavenging assay

Hydroxyl radical is easy to pass through the cell membrane and break the balance in the organism, resulting in cell damage or even death, so the hydroxyl radical scavenging is very important for oxidative damage [53]. The results were shown in Fig.7B. The scavenging abilities of NLCEP and NLCEPs-1 on hydroxyl radicals were lower than that of V_C at the same concentration and displayed a dose-dependent scavenging manner. The active hydroxyl substitutions of NLCEPs-1 might explain the scavenging capacity. The IC_{50} values of NLCEP, NLCEPs-1 and V_C to scavenge hydroxyl radical were 1.446 mg/mL, 4.759 mg/mL and 0.263 mg/mL, respectively. Though the IC_{50} of NLCEPs-1 to scavenge hydroxyl radical was higher than that of V_C and NLCEP, lower than that of the polysaccharides from *Bletilla striata* (IC_{50} =7.634 mg/mL) [54], and motherwort (*Leonurus cardiaca* L.) (IC_{50} =6.98 mg/mL) [55].

3.9.3. Scavenging ability on superoxide anion radical

Superoxide anion radical scavenging ability can be measured by pyrogallol autooxidation experiment. Fig.7C showed the result of superoxide anion radical scavenging assay. The scavenging abilities of NLCEP and NLCEPs-1 towards superoxide anion radical increased in a concentration-dependent manner in the range of 0.5 to 8 mg/mL and reached the maximum value when the concentration of NLCEP and NLCEPs-1 was 8 mg/mL. The IC_{50} values of NLCEP, NLCEPs-1 and V_C on the scavenging ability of superoxide anion radicals were 5.927 mg/mL, 3.952 mg/mL and 0.238 mg/mL, respectively. The IC_{50} value of NLCEPs-1 on scavenging superoxide anion radical

was higher than that of V_C, while lower than that of NLCEP and the polysaccharide from *Auricularia auricula* (IC_{50} =4.092 mg/mL) [56].

3.9.4. Reducing power

Reducing power can be used as an important index to evaluate potential antioxidant capacity. Potassium ferricyanide reduction method was used as the symbol to measure the antioxidant capacity [57]. The test results of reducing power were shown in Fig.7D. The reducing power of NLCEP and NLCEPs-1 increased as the increasing of polysaccharide concentration, and reached the maximum at 8 mg/mL.

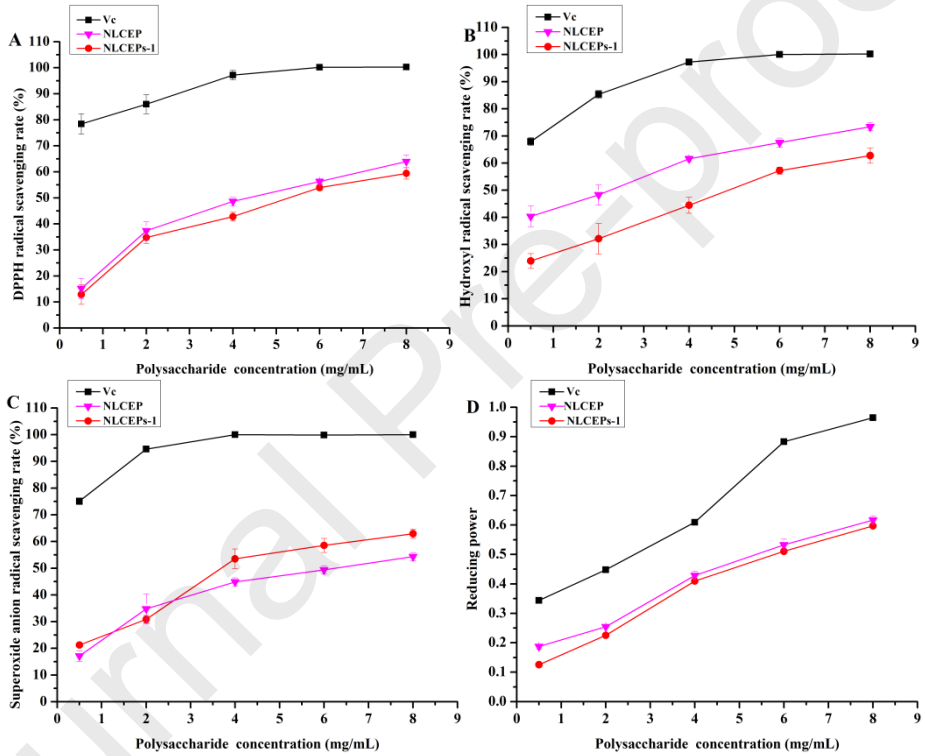


Fig.7 The antioxidant activities of NLCEP and NLCEPs-1. DPPH radical scavenging activity (A), hydroxyl radical scavenging activity (B), superoxide anion radical scavenging activity (C), reducing power assay (D).

Accordingly, the results indicated that NLCEP and NLCEPs-1 performed their antioxidant activities by scavenging free radicals. The antioxidant activity of polysaccharides was associated with molecular weight and monosaccharide composition. Previous study has reported that the polysaccharide with smaller molecular weight have better antioxidant activity because it has more reductive hydroxyl group terminals [58]. The molecular weight of NLCEPs-1 was smaller than that of polysaccharides from *Pinus koraiensis* (1.83×10^3 kDa), *Bletilla striatas* (230.63 kDa) and *Auricularia auricular* (357 kDa). Monosaccharide composition was also responsible for antioxidant

activity. On the basis of previous report, polysaccharides contained rhamnose, xylose and arabinose showed better antioxidant activity than those without these three monosaccharides [59]. NLCEPs-1 contained these three monosaccharides, suggesting that NLCEPs-1 may have antioxidant activity. Therefore, low molecular weight NLCEPs-1 with special monosaccharide composition in favor of the exposure of the active site, which contributed to the antioxidant activity.

3.10. Immunomodulatory activity assay

3.10.1. Effect of NLCEP and NLCEPs-1 on the cell viability of RAW 264.7 cells

In order to measure the toxic effect of NLCEP and NLCEPs-1 on RAW 264.7 cells, the CCK-8 assay was used to measure cell viability, and the results were exhibited in Fig.8A. When cells were treated with NLCEP and NLCEPs-1 solution at four different concentrations (100, 250, 500, 1000 μg/mL), the changes of cell viability were not significant. Therefore, these four different concentrations of NLCEP and NLCEPs-1 solution were suitable for the subsequent experiments.

3.10.2. Effect of NLCEP and NLCEPs-1 on pinocytic capacity of RAW 264.7 cells

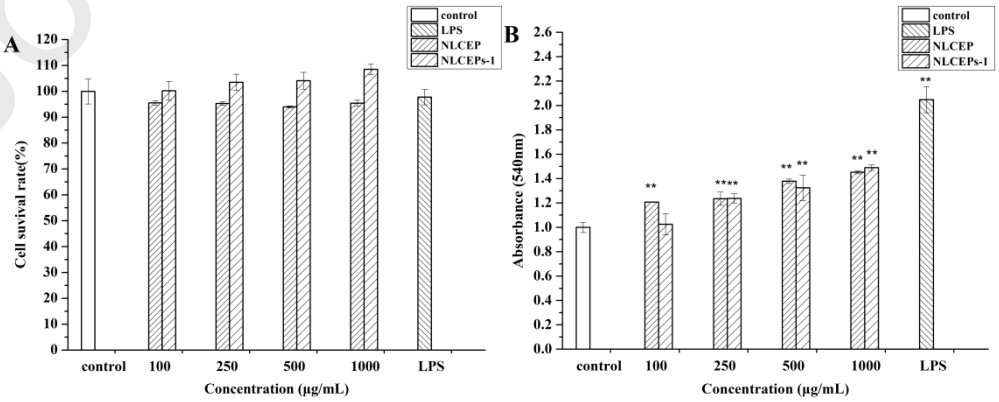
Macrophages activation plays a vital role in immune response, and the capacity of activated macrophage can be characterized by the enhancement in the pinocytic [60]. The effect of NLCEP and NLCEPs-1 on the pinocytic capacity of RAW 264.7 cells were measured by the uptake of neutral red, and Fig.8B illustrated the results. As shown in Fig.8B, compared with the control group, both NLCEP and NLCEPs-1 induced the uptake of neutral red in a dose-dependent manner, and the maximum effect was achieved at 1000 μg/mL. The results showed that NLCEP and NLCEPs-1 promoted the pinocytic capacity of RAW 264.7 cells.

3.10.3. Effect of NLCEP and NLCEPs-1 on NO, TNF- α and IL-6 secretion from RAW 264.7 cells

NO, as an intracellular messenger, mediates various biological responses of activated macrophages [61]. NO is considered as an indicator of classically activated M1 phenotypes [62]. Griess reagent can be used to measure the production of NO for the reason that NO is broken down to nitrite (NO_2^-) in Griess reagent. The test result of the level of NO production were shown in Fig.8C. Compared to the control group, NLCEP and NLCEPs-1 significantly induced the production of NO in a dose-dependent manner ($p < 0.05$). In the maximum concentration of 1000 μg/mL of

NLCEPs-1, the production of NO was 21.15 μmol/L, which was almost equal to the lipopolysaccharide (LPS) group (22.49 μmol/L).

Cytokines are a kind of active protein and glycoprotein molecules produced by immune cells and possess immunomodulatory activity [63]. They play an important role in the immune response by stimulating the proliferation and differentiation of target cells and inducing the expression of a receptor [64]. Tumor necrosis factor is a kind of cytokines produced by activated mononuclear macrophages, NK cells and T lymphocytes. Tumor necrosis factor-α (TNF-α) is the main immunomodulatory factor in tumor necrosis factor, which is able to initiate immune regulation and promote the activity of immune cells. It also has many important biological functions, such as anti-malignant tumor, antivirus, blood coagulation and hematopoiesis [65]. Interleukin (Interleukin, IL) is a kind of cellular molecules, and capable to transmit information between immune cells. Interleukin-6 (IL-6) is a multipotent cytokine in interleukin and plays a significant role in the immune defense of the body. The growth and differentiation of primitive bone marrow-derived cells can be promoted by IL-6 as well [66]. In this study, the effect of NLCEP and NLCEPs-1 on the production of TNF-α and IL-6 were analyzed, and the results were shown in Fig.8(D-E). When the concentrations of NLCEP and NLCEPs-1 were between 100 and 1000 mg/mL, the production of cytokines (IL-6 and TNF-α) produced by cells treated with NLCEP and NLCEPS-1 increased in a dose-dependent manner, and while was lower than that of LPS. These results suggested that NLCEP and NLCEPs-1 boosted immune response through the release of NO and cytokines (IL-6 and TNF-α). On the basis of previous report, moderate molecular weight, triple-helix structure and monosaccharide composition also contributed to the strong immunomodulatory activity [67], which might explain the immunomodulatory activity of NLCEPs-1.



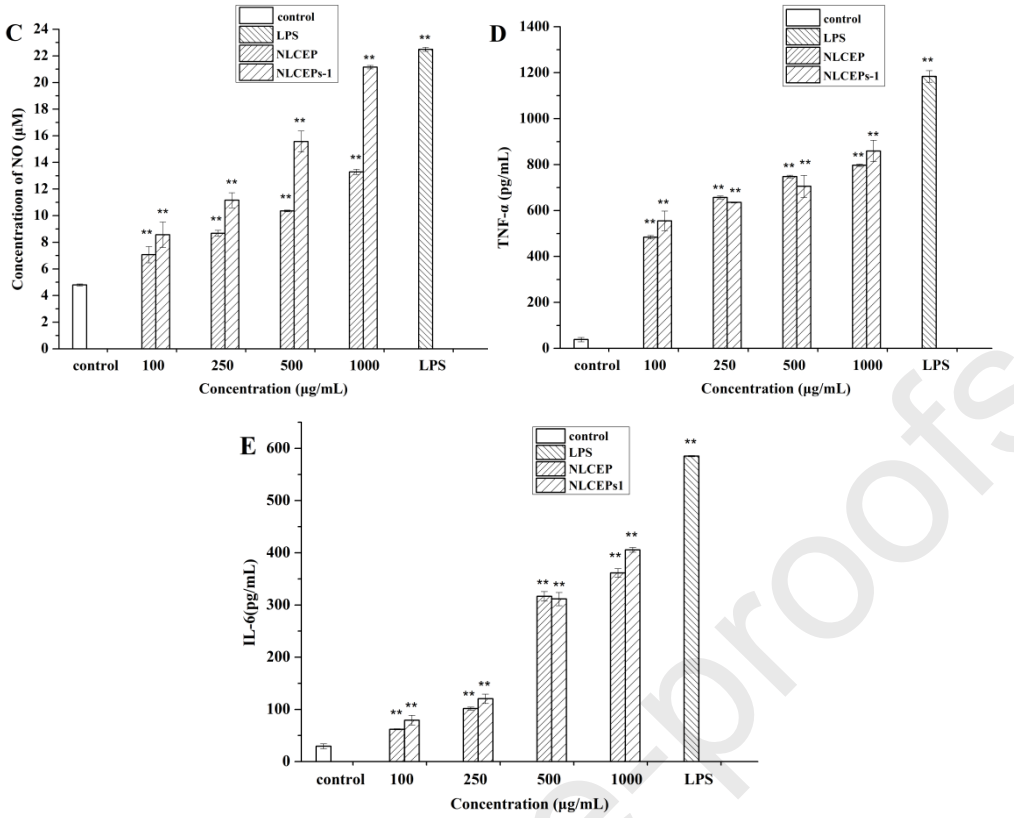


Fig.8 Effect of NLCEP and NLCEP s-1 on cell viability (A) and pinocytic capacity (B) of RAW 264.7 macrophages; Effect of NLCEP and NLCEP s-1 on NO(C), TNF-α (D) and IL-6 (E) secretion from RAW 264.7 macrophages. The data are expressed as means ± SD (n = 3).).

4 Conclusion

In this study, the crude polysaccharides were obtained from *Notarchus leachii freeri* eggs strings by the salt extraction method. The extraction conditions were optimized using the single-factor experiment method and response surface method (RSM). The optimal extraction process conditions of NLCEP were as follows: NaCl solution concentration of 3 %, raw material to liquid ratio of 1: 40 g/mL, extraction time of 2 h and extraction temperature of 69 °C, and the maximum yield was 4.20 %. A new novel pure polysaccharide fraction named NLCEPs-1 was fractionated from NLCEP by using DEAE-Cellulose 52 and Sephadex G-100. The molecular weight of NLCEPs-1 was 31.4 kDa and it mainly composed of glucose. NMR spectra data confirmed that NLCEPs-1 was a heteropolysaccharide and mainly consisted of →)4-α-D-Glcp (1→, →6)-α-D-Glcp (1→, →1)-β-D-Galp and β-D-Galp-(1→. NLCEPs-1 exhibited strong antioxidant and immunomodulatory activities. Therefore, NLCEPs-1 has potential application as the potent antioxidant and immunomodulatory agent in functional foods.

Supporting Information

The following is the supporting information to this article: figures of single-factor experiment; figures of Box-Behnken (BBD)-RSM experiment; figure of HPGPC chromatogram of dextran standard; figures of 2D NMR spectra of NLCEPs-1.

Acknowledgement

This work was supported by Technology Service Platform for Sustainable Utilization and Protection of Zhanjiang Seafood Ingredient Resources of China (2014B040404071). The Natural Science Foundation of Guangdong Province, China (No. 2021A1515011398). The Public Service Platform of Biomedical Resources Research and Development of South China Sea (2017C8B2).

References

[1] T. Braga, M.J. Rodrigues, H. Pereira, J. Varela, L. Barreira, M. González-Wangüemert, L. Custódio, Bursatella leachiifrom Mar Menor as a source of bioactive molecules: preliminary evaluation of the nutritional profile,in vitro biological activities, and fatty acids contents, Journal of Aquatic Food Product Technology 26(10) (2017) 1337-1350.

[2] R.B. Pereira, A.A. Almeida, D.M. Pereira, O. Silva, P.B. Andrade, E. Pinto, P. Valentao, Trace elements in wild edible Aplysia species: Relationship with the desaturation-elongation indexes of fatty acids, Chemosphere 208 (2018) 682-690.

[3] D.M. Pereira, P. Valentao, N. Teixeira, P.B. Andrade, Amino acids, fatty acids and sterols profile of some marine organisms from Portuguese waters, Food Chemistry 141(3) (2013) 2412-2417.

[4] J. Kisugi, H. Kamiya, M. Yamazaki, Purification and characterization of aplysianin E, an antitumor factor from sea hare eggs, Cancer research 47(21) (1987) 5649-53.

[5] K. Miyazaki, M. Kobayashi, T. Natsume, M. Gondo, T. Mikami, K. Sakakibara, S. Tsukagoshi, Synthesis and antitumor-activity of novel Dolastatin-10 analoge, Chemical & Pharmaceutical Bulletin 43(10) (1995) 1706-1718.

[6] J. Rajaganapathi, K. Kathiresan, T.P. Singh, Purification of anti-HIV protein from purple fluid of the sea hare Bursatella leachii de Blainville, Marine Biotechnology 4(5) (2002) 447-453.

[7] K.-C. Ko, B. Wang, P.C. Tai, C.D. Derby, Identification of potent bactericidal compounds produced by Escapin, an L-Amino Acid Oxidase in the ink of the sea hare Aplysia californica, Antimicrobial Agents and Chemotherapy 52(12) (2008) 4455-4462.

[8] S.M.A. Kawsar, R. Matsumoto, Y. Fujii, H. Matsuoka, N. Masuda, I. Chihiro, H. Yasumitsu, R.A. Kanaly, S. Sugawara, M. Hosono, K. Nitta, N. Ishizaki, C. Dogasaki, J. Hamako, T. Matsui, Y. Ozeki, Cytotoxicity and glycan-binding profile of a d-galactose-binding lectin from the eggs of a Japanese Sea Hare (Aplysia kurodai), Protein Journal 30(7) (2011) 509-519.

[9] T.C.L. Tavares, V.L.R. Nogueira, I.M. Vasconcelos, V.M. Gomes, M. da Cunha, A.F.U. Carvalho, V.M.M. Melo, Further characterization and mode of action of dactylomelin-P, an antibacterial protein isolated from the ink of the sea hare Aplysia dactylomela (Rang, 1828), Journal of Experimental Marine Biology and Ecology 407(2) (2011) 200-206.

[10] R.B. Pereira, P.B. Andrade, P. Valentao, Chemical diversity and biological properties of secondary metabolites from Sea Hares of Aplysia Genus, Marine Drugs 14(2) (2016) 39.

[11] R.F. Carneiro, R.C. Farias Torres, R.P. Chaves, M.A. de Vasconcelos, B.L. de Sousa, A.C. Rodrigues Goveia, F.V. Arruda, M.N. Carneiro Matos, H. Matthews-Cascon, V.N. Freire, E.H. Teixeira, C.S. Nagano, A.H. Sampaio, Purification, biochemical characterization, and amino acid sequence of a novel type of lectin from Aplysia dactylomela eggs with antibacterial/antibiofilm potential, Marine Biotechnology 19(1) (2017) 49-64.

[12] N. Gao, R. Chen, R. Mou, J. Xiang, K. Zhou, Z. Li, J. Zhao, Purification, structural characterization and anticoagulant activities of four sulfated polysaccharides from sea cucumber Holothuria fuscopunctata, International Journal of Biological Macromolecules 164 (2020) 3421-3428.

[13] M. Wu, L. Xu, L. Zhao, C. Xiao, N. Gao, L. Luo, L. Yang, Z. Li, L. Chen, J. Zhao, Structural analysis and anticoagulant activities of the novel sulfated fucan possessing a regular well-defined repeating unit from sea cucumber, Marine Drugs 13(4) (2015) 2063-2084.

[14] M. Jridi, R. Nasri, Z. Marzougui, O. Abdelhedi, M. Hamdi, M. Nasri, Characterization and assessment of antioxidant and antibacterial activities of sulfated polysaccharides extracted from cuttlefish skin and muscle, International Journal of Biological Macromolecules 123 (2019) 1221-1228.

[15] H. Liu, F. Li, P. Luo, Effect of carboxymethylation and phosphorylation on the properties of polysaccharides from Sepia esculenta ink: antioxidation and anticoagulation in vitro, Marine Drugs 17(11) (2019) 626.

[16] H. Liu, Y. Zhang, M. Li, P. Luo, Beneficial effect of Sepia esculenta ink polysaccharide on cyclophosphamide-induced immunosuppression and ovarian failure in mice, International Journal of Biological Macromolecules 140 (2019) 1098-1105.

[17] S. Guo, J. Wang, C. He, H. Wei, Y. Ma, H. Xiong, Preparation and antioxidant activities of polysaccharides obtained from abalone viscera by combination of enzymolysis and multiple separation methods, Journal of Food Science 85(12) (2020) 4260-4270.

[18] S. Song, B. Zhang, S. Wu, L. Huang, C. Ai, J. Pan, Y.-C. Su, Z. Wang, C. Wen, Structural characterization and osteogenic bioactivity of a sulfated polysaccharide from pacific abalone (Haliotis discus hannai Ino), Carbohydrate Polymers 182 (2018) 207-214.

[19] Z. Du, X. Jia, J. Chen, S. Zhou, J. Chen, X. Liu, X. Cao, S. Zhong, P. Hong, Isolation and characterization of a heparin-like compound with potent anticoagulant and fibrinolytic activity from the clam coelomactra antiquata, Marine Drugs 18(1) (2020) 6.

[20] M. Dhahri, S. Sioud, R. Dridi, M. Hassine, N.A. Boughattas, F. Almulhim, Z. Al Talla, M. Jaremko, A.M. Emwas, Extraction, characterization, and anticoagulant activity of a sulfated polysaccharide from Bursatella leachii Viscera, ACS Omega 5(24) (2020) 14786-14795.

[21] B.-Y. Yoon, B.-D. Choi, YeungjoonChoi, Screen of functional activity of polysaccharide and glycosaminoglycan from sea hare (Aplysia kurodai) by cell line, Journal of the Korean Society of Food Science and Nutrition 40(1) (2011) 14-19.

[22] J.H. Ryu, J. Sung, C. Xie, M.-K. Shin, C.-W. Kim, N.-G. Kim, Y.J. Choi, B.D. Choi, S.S. Kang, D. Kang, Aplysia kurodai-derived glycosaminoglycans increase the phagocytic ability of macrophages via the activation of AMP-activated, protein kinase and, cytoskeletal reorganization in RAW264.7 cells, Journal of Functional Foods 27 (2016) 122-130.

[23] C.S. Young, YeungjoonChoi, 박시향, Immune regulating effect of polysaccharide fraction from sea hare (Aplysia kurodai), Journal of the Korean Society of Food Science and Nutrition 40(3) (2011) 372-378.

[24] Q. Zhan, Q. Wang, R. Lin, P. He, F. Lai, M. Zhang, H. Wu, Structural characterization and immunomodulatory activity of a novel acid polysaccharide isolated from the pulp of Rosa laevigata Michx fruit, International Journal of Biological Macromolecules 145 (2020) 1080-1090.

[25] I.A. Schepetkin, C.L. Faulkner, L.K. Nelson-Overton, J.A. Wiley, M.T. Quinn, Macrophage immunomodulatory activity of polysaccharides isolated from Juniperus scopolorum, International Immunopharmacology 5(13-14) (2005) 1783-1799.

[26] M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Analytical Chemistry 28(3) (1956) 350-356.

[27] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical biochemistry 72 (1976) 248-54.

[28] D. Gupta, M. Silva, K. Radziun, D.C. Martinez, C.J. Hill, J. Marshall, V. Hearnden, M.A. Puertas-Mejia, G.C. Reilly, Fucoidan inhibition of osteosarcoma cells is species and molecular weight dependent, Marine Drugs 18(2) (2020) 104.

[29] Z. Ruan, Q.C. Zhou, Z.Y. Deng, Y.L. Yin, Study on optimization of extraction conditions of platycodon grandiflorum A. DC polysaccharide using Response Surface Methodology, Food Science 29(12)(2008), 283-287.

[30]X. Shu, Y. Zhang, J. Jia, X. Ren, Y. Wang, Extraction, purification and properties of water-soluble polysaccharides from mushroom Lepista nuda, International Journal of Biological Macromolecules 128 (2019) 858-869.

[31]Y. Ka-Man, X. Jun, T. Wing-Sum, Z. Shan-Shan, Y. Tao, Z. Zhong-Zhen, C.J.M. Hu-Biao, Ultrasound-assisted extraction may not be a better alternative approach than conventional boiling for extracting polysaccharides from herbal medicines, Molecules 21(11) (2016) 1569.

[32] R.V. Muralidhar, R.R. Chirumamila, R. Marchant, P. Nigam, A response surface approach for the comparison of lipase production by Candida cylindracea using two different carbon sources, Biochemical Engineering Journal 9(1) (2001) 17-23.

[33] Y.-K. Liu, E. Yan, H.-Y. Zhan, Z.-Q. Zhang, Response surface optimization of microwave-assisted extraction for HPLC-fluorescence determination of puerarin and daidzein in Radix Puerariae thomsonii, Journal of pharmaceutical analysis 1(1) (2011) 13-19.

[34] Y. Chen, J. Zhang, Q. Li, J. Wu, F. Sun, Z. Liu, C. Zhao, S. Liang, Response surface methodology for optimizing the ultrasound-assisted extraction of polysaccharides from Acanthopanax giraldii, Chemical & Pharmaceutical Bulletin 66(8) (2018) 785-793.

[35] Q. Yuan, L. Zhao, Q. Cha, Y. Sun, H. Ye, X. Zeng, Structural characterization and immunostirnulatory activity of a homogeneous polysaccharide from Sinonovacula constricta, Journal of Agricultural and Food Chemistry 63(36) (2015) 7986-7994.

[36] T. Chen, M. Zhang, J. Li, M.M. Surhio, B. Li, M. Ye, Structural characterization and hypoglycemic activity of Trichosanthes peel polysaccharide, Lwt-Food Science and Technology 70 (2016) 55-62.

[37] T.C.T. Lo, Y.H. Jiang, A.L.J. Chao, C.A. Chang, Use of statistical methods to find the polysaccharide structural characteristics and the relationships between monosaccharide composition ratio and macrophage stimulatory activity of regionally different strains of Lentinula edodes, Analytica Chimica Acta 584(1) (2007) 50-56.

[38] M. Wang, Y. Liu, M. Qiang, J. Wang, Structural elucidation of a pectin–type polysaccharide from Hovenia dulcis peduncles and its proliferative activity on RAW264.7 cells, International Journal of Biological Macromolecules 104 (2017) 1246-1253.

[39] S. Zou, X. Zhang, W. Yao, Y. Niu, X. Gao, Structure characterization and hypoglycemic activity of a polysaccharide isolated from the fruit of Lycium barbarum L, Carbohydrate Polymers 80(4) (2010) 1161-1167.

[40] M. Zhu, R. Huang, P. Wen, Y. Song, B. He, J. Tan, H. Hao, H. Wang, Structural characterization and immunological activity of pectin polysaccharide from kiwano (Cucumis metuliferus) peels, Carbohydrate Polymers 254 (2021) 117371.

[41] Y. Ren, G. Zheng, L. You, L. Wen, C. Li, X. Fu, L. Zhou, Structural characterization and macrophage immunomodulatory activity of a polysaccharide isolated from Gracilaria lemaneiformis, Journal of Functional Foods 33 (2017) 286-296.

[42] Y. Song, M. Zhu, H. Hao, J. Deng, M. Li, Y. Sun, R. Yang, H. Wang, R. Huang, Structure characterization of a novel polysaccharide from Chinese wild fruits (Passiflora foetida) and its immune-enhancing activity, International Journal of Biological Macromolecules 136 (2019) 324-331.

[43] O. Gutiérrez de G, M. Martínez, L. Sanabria, G.L. de Pinto, J.M. Igartuburu, 1D- and 2D-NMR spectroscopy studies of the polysaccharide gum from Spondias purpurea var. lutea, Food Hydrocolloids 19(1) (2005) 37-43.

[44] L. Wang, T. Li, F. Liu, D. Liu, Y. Xu, Y. Yang, Y. Zhao, H. Wei, Ultrasonic-assisted enzymatic extraction and characterization of polysaccharides from dandelion (Taraxacum officinale) leaves, International Journal of Biological Macromolecules 126 (2019) 846-856.

[45] J.-S. Ma, H. Liu, C.-R. Han, S.-J. Zeng, X.-J. Xu, D.-J. Lu, H.-J. He, Extraction, characterization and antioxidant activity of polysaccharide from Pouteria campechiana seed, Carbohydrate Polymers 229 (2020) 115409.

[46] C. Deng, H. Fu, L. Teng, Z. Hu, X. Xu, J. Chen, T. Ren, Anti-tumor activity of the regenerated triple-helical polysaccharide from Dictyophora indusiata, International Journal of Biological Macromolecules 61 (2013) 453-458.

[47] J. Liu, C. Zhang, Y. Wang, H. Yu, H. Liu, L. Wang, X. Yang, Z. Liu, X. Wen, Y. Sun, C. Yu, L. Liu, Structural elucidation of a heteroglycan from the fruiting bodies of Agaricus blazei Murill, International Journal of Biological Macromolecules 49(4) (2011) 716-720.

[48] W. Chen, X. Zhu, J. Ma, M. Zhang, H. Wu, Structural Elucidation of a Novel Pectin-Polysaccharide from the Petal of Saussurea laniceps and the Mechanism of its Anti-HBV Activity, Carbohydrate Polymers 223 (2019) 115077.

[49] X. Meng, H. Liang, L. Luo, Antitumor polysaccharides from mushrooms: a review on the structural characteristics, antitumor mechanisms and immunomodulating activities, Carbohydrate Research 424 (2016) 30-41.

[50] Y. Liu, B. Zhang, S.A. Ibrahim, S.S. Gao, H. Yang, W. Huang, Purification, characterization and antioxidant activity of polysaccharides from Flammulina velutipes residue, Carbohydrate Polymers 145(2016) 71-77.

[51] G. Yu, J. Zhao, Y. Wei, L. Huang, F. Li, Y. Zhang, Q. Li, Physicochemical Properties and Antioxidant Activity of Pumpkin Polysaccharide (*Cucurbita moschata* Duchesne ex Poiret) Modified by Subcritical Water, Foods 10(1) (2021) 197.

[52] H. Zhang, P. Zou, H. Zhao, J. Qiu, J. M. Regenstein, X. Yang, Isolation, purification, structure and antioxidant activity of polysaccharide from pinecones of *Pinus koraiensis*, Carbohydrate Polymers 251(2021) 117078.

[53]Y. Sun, Y. Li, M. Li, H. Tong, X. Yang, J. Liu, Optimization of extraction technology of the Anemone raddeana polysaccharides (ARP) by orthogonal test design and evaluation of its anti-tumor activity, Carbohydrate Polymers 75(4) (2009) 575-579.

[54] H. Chen, J. Zeng, B. Wang, Z. Cheng, J. Xu, W. Gao, K. Chen, Structural characterization and antioxidant activities of *Bletilla striata* polysaccharide extracted by different methods, Carbohydrate Polymers 266 (2021) 118149.

[55] S. Tahmouzi and M. Ghodsi, Optimum extraction of polysaccharides from motherwort leaf and its antioxidant and antimicrobial activities, Carbohydrate Polymers 112 (2014) 396-403.

[56] Q. Wu, D. Qin, H. Cao, Y. Bai, Enzymatic hydrolysis of polysaccharide from *Auricularia auricula* and characterization of the degradation product, International Journal of Biological Macromolecules, 162 (2020) 127-135.

[57] H.-H. Sun, W.-J. Mao, Y. Chen, S.-D. Guo, H.-Y. Li, X.-H. Qi, Y.-L. Chen, J. Xu, Isolation, chemical characteristics and antioxidant properties of the polysaccharides from marine fungus Penicillium sp. F23-2, Carbohydrate Polymers 78(1) (2009) 117-124.

[58] J. Wang, S. Hu, S. Nie, Q. Yu, M. Xie, Reviews on mechanisms of in vitro antioxidant activity of polysaccharides, Oxidative Medicine and Cellular Longevity 2016 (2016) 5692852.

[59] H. Zhang, P. Zou, H. Zhao, J. Qiu, J. Mac Regenstein, X. Yang, Isolation, purification, structure and antioxidant activity of polysaccharide from pinecones of Pinus koraiensis, Carbohydrate Polymers 251 (2021) 117078.

[60] S. Gordon, Phagocytosis: an immunobiologic process, Immunity 44(3) (2016) 463-475.

[61] Z. Lin, W. Liao, J. Ren, Physicochemical characterization of a polysaccharide fraction from Platycladus orientalis (L.) Franco and its macrophage immunomodulatory and anti-Hepatitis B virus activities, Journal of Agricultural and Food Chemistry 64(29) (2016) 5813-5823.

[62] R. Ghosh, S.A. Smith, E.E. Nwangwa, B.A. Arivett, D.L. Bryant, M.L. Fuller, D. Hayes, J.L. Bowling, D.E. Nelson, J.D. DuBois, E. Altman, P.C. Kline, A.L. Farone, Panax quinquefolius (North American ginseng) cell suspension culture as a source of bioactive polysaccharides: Immunostimulatory activity and characterization of a neutral polysaccharide AGC1, International Journal of Biological Macromolecules 139 (2019) 221-232.

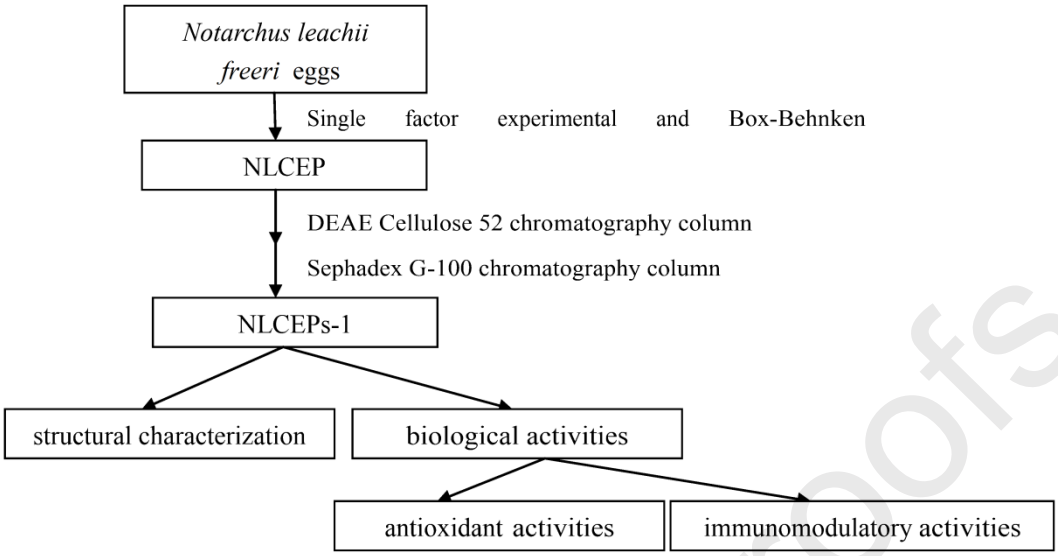
[63] H.M.S. Akhtar, Z. Ye, M. Abdin, Y.S. Hamed, G. Chen, X. Zeng, Immunomodulatory activity in vitro and in vivo of polysaccharides from Kabuli Chickpea (Cicer arietinum L.) Hull, Food Technology and Echnology and Biotechnology 58(4) (2020) 370-380.

[64] I.A. Schepetkin, M.T. Quinn, Botanical polysaccharides: macrophage immunomodulation and therapeutic potential, International Immunopharmacology 6(3) (2006) 317-33.

[65] F. Balkwill, Tumour necrosis factor and cancer, Nature Reviews Cancer 9(5) (2009) 361-371.

[66] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 124(4) (2006) 783-801.

[67] C. Li, Q. Huang, X. Fu, X.-J. Yue, R.H. Liu, L.-J. You, Characterization, antioxidant and immunomodulatory activities of polysaccharides from Prunella vulgaris Linn, International Journal of Biological Macromolecules 75 (2015) 298-305.



Highlight

•A novel polysaccharide(NLCEPs-1) was obtained from Notarchus leachii freeri eggs.

•The structure of NLCEPs-1 was investigated.

•NLCEPs-1 showed potential antioxidant and immunomodulatory activities.

Conflicts of interest

We confirm that the manuscript has been read and approved by all named authors and
that there are no other persons who satisfied the criteria for authorship but are not listed.We further confirm that the order of authors listed in the manuscript has been approved by all of us.