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Extraction, characterization and bioactivities of novel purified polysaccharides from Baphicacanthis Cusiae Rhizoma et Radix

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ABSTRACT

The purpose of this study was to investigate the extraction, characterization and bioactivities of purified water-soluble polysaccharides (BCP) from Baphicacanthis Cusiae Rhizoma et Radix. Based on the response surface methodology, the optimal extraction parameters were obtained as follows: extraction temperature of 60.0 °C, extraction time of 35.0 min, and ratio of water to raw material of 24.5 ml/g. Then, BCP was separated and purified by chromatography of DEAE-52 and Sephadex G-100, and obtained two purified fractions, named as BCP-1 and BCP-2. Their molecular weights were respectively 11.6 and 26.7 KDa with mainly composed of glucose, arabinose and galactose. BCP-2 had higher contents of sulfuric radical and uronic acid than BCP-1. Finally, their antioxidant and anti-inflammatory activities were evaluated. Both of BCP-1 and BCP-2 exhibited strong antioxidant activity *in vitro*, and the antioxidant of BCP-2 was better. Besides, they showed ideal anti-inflammatory activity *in vitro* and *in vivo*.

1. Introduction

Baphicacanthis Cusiae Rhizoma et Radix (short as Baphicacanthis Cusiae) belongs to the family Acanthaceae, is a traditional Chinese medicine produced from the dry rhizomes and roots of *Baphicacanthus cusia* (Nees) Bremek. It is recorded in the Chinese Pharmacopoeia [1], and has mainly been used for the treatment of pestilence, fever, sore throat, *etc* [2]. Also, its potential in the prevention and treatment of severe acute respiratory syndrome (SARS) has been discovered in some studies since 2003 [3], then it became more widely used in China. Current researches have showed that Baphicacanthis Cusiae has antiviral, anticancer, antibacterial and anti-inflammatory bioactivities, and the function for liver protection [4]. Its antiviral activity might be related to the components of indigo, tryptanthrin or indirubin [5], but there is no direct evidence to support other activities due to these experiments were based on water extract as the experimental material. It has reported that the water extract of Baphicacanthis Cusiae is rich in water-soluble polysaccharides, and the polysaccharide has antibacterial activity [6]. However, there was little report devoted to thoroughly studying the characterization, antioxidant and anti-inflammatory activities of purified polysaccharide from Baphicacanthis Cusiae (BCP). Therefore, this study was aimed to update the data and discover potential bioactivities of purified BCP.

In the present study, Box-Behnken Design was employed to optimize the extraction of BCP. Then, the crude BCP was separated and purified by chromatography of DEAE-52 and Sephadex G-100. The purified fractions of BCP were characterized by chemical analysis, gas chromatography (GC), high-performance liquid chromatography (HPLC) and Fourier transform-infrared spectroscopy (FT-IR). At last, the antioxidant activity *in vitro* and anti-inflammatory activity *in vitro* and *in vivo* of purified fractions of BCP were evaluated

2. Materials and methods

2.1. Materials and reagents

Baphicacanthis Cusiae was purchased from Guangzhou Caizhilin Pharmaceutical Co. Ltd (Guangzhou, China). RAW264.7

Abbreviations: BC, Ppolysaccharides from Baphicacanthis Cusiae Rhizoma et Radix; NO, nitric oxide; NBT, nitrotetrazolium; PMS, phenazine methyl sulfate; NADH, nicotinamde adenine dinucleotide reduced; LPS, lipopolysaccharide; IL-6, interleukin-6; IL-8, interleukin-8; FT-IR, Fourier transform infrared; BW, body weight.

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Table 1
The response value and the actual value of Box-Behnken Design.

No.	Extraction temperature (°C)		Extraction time (min)		Water to raw material ratio (ml/g)		Yield (%)
	X_1	Code x_1	X_2	Code x_2	X_3	Code x_3	
1	60	−1	25	−1	25	0	6.70
2	80	1	25	−1	25	0	8.43
3	60	−1	35	1	25	0	7.77
4	80	1	35	1	25	0	7.70
5	60	−1	30	0	20	−1	6.70
6	80	1	30	0	20	−1	8.10
7	60	−1	30	0	30	1	7.10
8	80	1	30	0	30	1	7.54
9	70	0	25	−1	20	−1	6.40
10	70	0	35	1	20	−1	6.90
11	70	0	25	−1	30	1	6.94
12	70	0	35	1	30	1	7.00
13	70	0	30	0	25	0	7.14
14	70	0	30	0	25	0	6.72
15	70	0	30	0	25	0	7.20
16	70	0	30	0	25	0	7.39
17	70	0	30	0	25	0	7.26

Table 2
Analysis of variance of Box-Behnken Design.

Variables	Sum of Squares	DF[a]	Mean Square	*F*–Value	p-value Prob.>F
Model	4.267244	9	0.474138	9.565298	<0.01
A	1.53125	1	1.53125	30.89155	<0.01
B	0.10125	1	0.10125	2.042625	0.1960
C	0.0288	1	0.0288	0.581013	0.4708
AB	0.81	1	0.81	16.341	<0.01
AC	0.2304	1	0.2304	4.648107	0.0680
BC	0.0484	1	0.0484	0.976425	0.3560
A^2	1.178278	1	1.178278	23.77066	<0.01
B^2	0.001857	1	0.001857	0.03746	0.8520
C^2	0.407246	1	0.407246	8.215817	<0.05
Residual	0.34698	7	0.049569		
Lack of Fit	0.0901	3	0.030033	0.467663	0.7207
Pure Error	0.25688	4	0.06422		
Correlation total	4.614224	16			

[a] Degree of freedom.

cells (mouse macrophage cell line) were obtained from the Shanghai Cell Biology Research Institute (Shanghai, China). DEAE-52 cellulose and Sephadex G100 were purchased from Beijing Solarbio Technology Co., Ltd (Beijing, China). Nitrotetrazolium (NBT), phenazine methyl sulfate (PMS), nicotinamde adenine dinucleotide reduced (NADH) and lipopolysaccharide (LPS) were obtained from Sigma Chemical Co., Ltd. (Sigma-Aldrich, St. Louis, MO, USA). Ibuprofen suspension was purchased from Jiangsu Hengrui Medicine Co. Ltd (Lianyungang, China). Acetaminophen was purchased from Bayer Medicine Health Care Co., Ltd Guangzhou Branch (Guangzhou, China). RPMI 1640 culture medium was purchased from Gibco Corporation (USA). Enzyme linked immunosorbent assay (ELISA) kits of tumor necrosis factor-α, interleukin-6 (IL-6) and interleukin-8 (IL-8) were purchased from Wuhan Boster Company (Wuhan, China). All the other reagents used in this study were analytical grade and purchased from Aladdin Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of crude BCP

Baphicacanthis Cusiae was dried at 60 °C for 24 h and crushed to obtain fine powder, all powder were passed through a 60-mesh sieve (particle diameter less than 0.25 mm). After treatment with petroleum ether, the powder was treated with 95% ethanol. The pretreatment powder (5.0 g) was extracted by distilled water in an ultrasonic cleaner (KQ-250B, Kunshan Ultrasound Instrument Co., Jiangsu, China, 40 kHz), with different extraction parameters, including extraction temperature, extraction time, and water to raw material ratio. A water bath was used to monitor the temperature of extraction process (501-A, Shanghai Instrument Co., Ltd.). After treatment, the mixture extract was centrifuged (4000 r/min, 20 min). Insoluble residue was re-extracted to recover the residual water-soluble polysaccharides. The supernatants were collected, concentrated and then mixed with four-fold amount of absolute ethanol. The mixture was stirred vigorously and then kept overnight at 4 °C. The precipitate was collected by centrifugation (5000 r/min, 20 min) and freeze drying to get crude BCP.

Content of polysaccharide in crude BCP was measured by the phenol-sulfuric acid method as reported [7].

2.3. BBD for the extraction of BCP

Box-Behnken Deign (BBD) with three independent variables (extraction temperature, X_1; extraction time, X_2; ratio of water to raw material, X_3) at three levels was used to design the best combination of extraction variables. The 17 experimental points of the independent variables and their levels were listed in Table 1.

2.4. Purification of BCP

Crude BCP was purified by DEAE-52 cellulose ion exchange chromatography and Sephadex G-100 gel filtration chromatography [8]. First, the BCP solution (10 ml, 2 mg/ml) was added to a DEAE-52 cellulose column (2.6 × 30 cm), and then eluted with different concentrations of NaCl solutions at a flow rate of 60 ml/h. Each tube collected 10 ml elute. Polysaccharides in each tube were detected

by the phenol-sulfuric acid method. The same fractions were collected, dialyzed and further purified by Sephadex G-100. Solution was eluted with deionized water at a flow rate of 15 ml/h, each tube collected 10 ml elute. At last, the purified fractions were collected and freeze drying.

Yield of purified polysaccharide (%) was calculated as shown in the following equation:

$$\text{Yield}(\%) = m_p/m_c \times 100 \quad (1)$$

where m_p and m_c were the weight of purified and crude polysaccharide, respectively.

2.5. Characterization of BCP

2.5.1. Chemical analysis of BCP

The content of carbohydrates in purified BCP was determined by phenol-sulfuric acid method [7]. The content of sulfate radicals [9], uronic acids [10] and protein [11] were determined by the classical method.

2.5.2. Determination of monosaccharide compositions

Monosaccharide compositions of BCP were analyzed by GC according to the previous reports with modifications [12]. Briefly, the polysaccharides samples (5 mg) were hydrolyzed (2 M trifluoroacetic acid, 120 °C, 2 h) and evaporated to dry. Then hydrolyzate was added with hydroxylamine hydrochloride (10 mg), inositol (internal standard, 5 mg), and pyridine (0.6 ml). Reaction for 30 min (90 °C) and then adding acetic anhydride (1 ml), continue to react for 30 min. At the same time, the monosaccharide standards of rhamnose, xylose, arabinose, fucose, mannose, glucose and galactose were acetylated. Then, all the derivatives were analyzed by a 6890N GC (Agilent Technologies, USA) instrument equipped with flame ionization detector and an HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 μm). The operation conditions of GC were as following: flow rates of N_2, H_2 and air were 25, 30 and 400 ml/min, respectively; the oven temperature was maintained at 120 °C for 3 min, and then increased gradually to 210 °C (3 °C/min); the temperatures of oven, detector and inlet were 210, 280 and 250 °C, respectively.

2.5.3. Determination of molecular weights of BCP

The molecular weights of BCP samples were characterized by HPLC according to the reported method with slight modifications [13]. The purified polysaccharides (9.5 mg) were dissolved, then passed through a 0.45 μm filter, and applied to a gel-filtration chromatographic column of TSK-GEL G5000PW$_{xl}$ column with a TSK G-3000PWxl column (7.5 × 300 mm, Tosoh Corp., Japan). The columns were kept at 35 °C, eluted with 0.02 M KH_2PO_4 solution at a flow rate of 0.6 ml/min. Detector was the Waters 2414 RID. Preliminary calibration of the column was conducted by using dextrans with different molecular weights (5200, 11,600, 48,600, 148,000, 410,000, 668,000 and 1,482,000).

2.5.4. FT-IR analysis of BCP

The powder of BCP samples was mixed with proper amount of KBr powder, and then pressed into piece for Fourier transform infrared (FT-IR) measurement. The FT-IR spectrum of BCP was determined by the Nicolet 5700 spectrometer (Thermo Electron, Madison, WI, US) at the frequency range of 4000–400 cm^{-1}.

2.6. Antioxidant activities in vitro of BCP

2.6.1. Assay of superoxide radical scavenging activity

Superoxide radical scavenging activity of BCP and its purified fractions was determined in accordance with the reported method [12]. Briefly, 1 ml BCP solutions with different concentrations (0.04, 0.12, 0.24, 0.4, 1.0, 2.0 mg/ml) were mixed with NBT solution (146 μmol/L, 1.0 ml), PMS solution (61 μmol/L, 1.0 ml) and NADH solution (452 μmol/L, 1.0 ml). The mixture was shaken and kept at 25 °C for 5 min. The absorbance at 560 nm was measured. The blank was set as water and 0.1 M phosphate buffer instead of BCP sample and NBT solution, respectively. The scavenging activity on superoxide radical was calculated by the following formula:

$$\text{Superoxideradicalscavengingactivity}(\%) = (A_0 - A_1 + A_2)/A_0 \times 100 \quad (2)$$

where A_0 was the absorbance of controlled sample (water instead of BCP solution), A_1 was the absorbance of the tested sample, and A_2 was the interference test (phosphate buffer instead of NBT solution). Ascorbic acid (Vc) was used as positive control.

2.6.2. Assay of reductive potential

The reductive potential of BCP was determined according to the method of [14]. Phosphate buffer (0.2 M, 1.0 ml) and 1.0 ml 1% (w/v) $K_3Fe(CN)_6$ solution were mixed with 0.5 ml BCP samples (0.04, 0.12, 0.24, 0.4, 1.0 mg/ml), and then incubated at 50 °C for 20 min. After these, cool to room temperature. 1.0 ml trichloroacetic acid (10%, w/v) and 0.25 ml 0.1% (w/v) $FeCl_3$ were added to the mixture solution. The absorbances of mixed reaction solution were measured at 700 nm. Increased absorbance of the reaction mixture indicates an increase of reduction capability.

2.6.3. Ferrous metal ions chelating activity

The chelating of ferrous ions by BCP was estimated by the method of [15]. Briefly, 0.4 ml BCP solutions were mixed with 0.05 ml 2.0 mM $FeCl_2$. Then added ferrozine (5.0 mM, 0.2 ml), adjusted total volume to 4 ml by deionized water. The mixture was shaken vigorously and set aside for 10 min. At last, absorbance of the solution was measured at 562 nm against the blank (water instead of BCP sample and $FeCl_2$ solution, respectively). The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated according to the following formula:

$$\text{Metalcheiatingeffect}(\%) = (A_0 - A_1 + A_2)/A_0 \times 100 \quad (3)$$

where A_0 is the absorbance of control sample (water instead of BCP solution), A_1 is the absorbance of the tested sample, and A_2 is the absorbance of test sample solution (water instead of $FeCl_2$ solution).

2.7. 7 In vitro anti-inflammatory effects of BCP

2.7.1. Cell culture and cytotoxicity assay

RAW264.7 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 U/ml). RAW264.7 cells (5×10^4/well) were cultured in 96-well plates and incubated at 37 °C in a wet atmosphere containing 5% CO_2 for 12 h. Then treated with different concentrations of samples (0, 20, 40, 80 and 160 μg/ml). After 24 h, cell viability was evaluated by MTT assay [16].

2.7.2. Model establishment

RAW264.7 cells were divided into the following groups: Group I (blank control group, normal RAW264.7 cells without LPS or BCP treatment); Group II (model control group, added with 10 mg/l LPS); Group III–VI (experimental groups, added with 10 mg/l LPS and treated by BCP-1 with different concentration); Group VII–X (experimental groups, added with 10 mg/l LPS and treated by BCP-2 with different concentration).

2.7.3. Determination the level of nitric oxide (NO)

Indirect detection of NO production by Griess reaction [17]. RAW264.7 cell was pipetted into a 96-well plate (100 μl/well), each

Table 3
The chemical compositions and contents of carbohydrate, protein, uronic acid and sulfate of BCP-1 and BCP-2.

Sample	Carbohydrate (%)	Sulfuric radical (%)	Protein (%)	Uronic acid (%)	Sugar component (%)		
					Glucose	Arabinose	Galactose
BCP-1	89.71 ± 1.08	0.76 ± 0.26	0.23 ± 0.01	2.24 ± 0.08	42.57	32.14	25.29
BCP-2	92.56 ± 1.63	4.74 ± 0.80	0.26 ± 0.02	2.57 ± 0.07	28.65	35.22	36.13

Table 4
Anti-inflammation results of BCP.

Group	Treatment (Dose)	Effect of BCP on xylene-induced ear swelling		Effect of BCP on vascular permeability increase induced by intraperitoneal acetic acid	
		Ear swelling (mg)	Inhibition rate (%)	Evan's blue (mg/ml)	Inhibition rate (%)
I	Model group	16.4 ± 1.88	/	10.3 ± 0.57	/
II	Ibuprofen (200 mg/kg)	6.5 ± 0.93**	60.37	3.0 ± 1.22**	70.87
III	BCP-1(100 mg/kg)	13.9 ± 2.58*	15.24	9.3 ± 0.53**	9.71
IV	BCP-1 (200 mg/kg)	11.2 ± 2.06**	31.71	6.9 ± 1.13**	33.01
V	BCP −1 (400 mg/kg)	9.2 ± 1.72**	43.90	5.8 ± 1.24**	43.69
VI	BCP-2(100 mg/kg)	13.4 ± 2.43**	18.29	8.9 ± 0.88**	13.59
VII	BCP-2 (200 mg/kg)	11.5 ± 2.35**	29.88	6.5 ± 1.24**	36.89
VIII	BCP −2 (400 mg/kg)	8.8 ± 2.18**	46.34	5.3 ± 1.13**	48.54

* $P < 0.05$, compared with the model group.
** $P < 0.01$, compared with the model group.

group treatment with corresponding LPS or BCP for 24 h. 50 μl cell culture solution was taken from each well and tested with Griess reagent. The absorbance of each well was read at 540 nm using an ELISA plate reader. The level of NO generated in the medium was calculated according to the standard curve obtained by $NaNO_2$.

2.7.4. Determination the level of inflammatory cytokine

RAW264.7 cell was pipetted into a 24-well plate (100 μl/well), each group treatment with corresponding LPS or BCP for 24 h. The supernatant was measured, and TNF-α, IL-6 and IL-8 levels were determined in accordance with the specification of ELISA kits.

2.8. Methods for testing acute inflammation of BCP in vivo

2.8.1. Experimental animals

In this study, the male NIH mice (8-week-old) were provided by Experimental Animal Center of Guangzhou University of Chinese Medicine (the qualified certificate No. was SCXK 2013-0020). Male NIH mice of grade of specific pathogen free with body weight (BW) of 20.0 ± 2.0 g were used for the evaluation of anti-inflammatory activities of BCP. The mice were kept in the conditions of room temperature (25 ± 2 °C), humidity (55 ± 10)%, 12 h light and dark cycle. They were allowed free access to drinking water and standard pellet diet. All procedures were conducted in strict accordance with the Chinese legislation on the use and care of laboratory animals during the entire experimental period.

2.8.2. Determine the dose of BCP

Dosage of ibuprofen in positive control mice was obtained according to an equivalent conversion for adult clinical conventional dosage. Compared to the positive control of ibuprofen, the concentration of BCP in medium-dose group was defaulted as the equals of ibuprofen dosage. For the center with concentration of BCP in medium-dose group, its concentration in the low-dose group and high-dose group was respectively chose based on the experimental results of dose-effect relationship and side effects. As results, concentration of BCP in the low-dose, medium-dose and high-dose group were determined as 100, 200 and 400 mg/kg of body weight (BW), respectively.

2.8.3. Xylene induced ear oedema

After a 7-day acclimation, mice were randomly divided into five different groups with 10 mice in each group. Mice in model group (Group I) was given the same dose of deionized water. Mice in positive control group (Group II) were treated with ibuprofen (200 mg/kg BW per day). Mice in Group III ~ V treated by BCP-1 with the dosage of 100, 200 and 400 mg/kg BW per day, respectively. Mice in Group VI ~ VIII treated by BCP-2 with the dosage of 100, 200 and 400 mg/kg BW per day, respectively. All treatments were conducted by gastric gavage for 7 consecutive days. Afterwards, all mice were fast but can still drink water freely for 8 h before the experiment. All mice were applied with 20 μl xylene evenly on the anterior and posterior surface of right ear 0.5 h after the last administration. 30 min after xylene application, mice were sacrificed and both ears were removed. The same parts of left and right ears were removed by a 5 mm cork borer, and the removed ear pieces were weighed and recorded. The weight difference between right and left ear was calculated as inflammatory swelling index. The inhibitory rate of ear swelling was calculated according to the formula below:

$$\text{Inhibitoryrateofearswelling}(\%) = (w_1 - w_2)/w_1 \times 100 \quad (4)$$

where w_1 is the weight of right ear piece, w_2 is the weight of left ear piece.

2.8.4. Vascular permeability increased by acetic acid in mice

The dosage of ibuprofen and animal management were the same with Section 2.8.3. Afterwards, feeding was stopped but water was given without restriction for 8 h before the experiment. Intravenous injection of 0.5% Evans blue solution (0.1 ml/10 g) was given in each mouse's tail, followed by intraperitoneal injection of 0.8% acetic acid solution (0.2 ml/10 g). Thirty minutes after i.p. administration of acetic acid, mice were sacrificed. Their abdomens were opened to expose the entrails, and washed with normal saline (5 ml), which was then collected and centrifuged for 5 min (1500 rpm). The absorbance of the supernatant was detected at 590 nm. The inhibition of the increase of abdominal capillary permeability was calculated according to the formula below:

$$\text{Inhibitoryrateofpermeabilityincrease}(\%) = (c_1 - c_2)/c_1 \times 100 \quad (5)$$

where c_1 was the concentration of Evans blue solution of the model group, c_2 was the concentration of Evans blue solution of administration group.

2.9. Statistical analysis

All statistical analyses were carried out by SPSS for Windows, Version 18.0 (SPSS, Chicago, IL). Experimental values are expressed as mean ± standard deviation (SD) and evaluated by one-way analysis of variance (ANOVA) followed by the Duncan's multiple-range tests.

3. Results and discussion

3.1. Model fitting and optimization for extraction of BCP

3.1.1. Model fitting

Using multiple regression analysis on experimental data by the Design Expert version 8.0.0, the correlation between response variables and the extraction yield of BCP was associated with the following second-order polynomial equation:

$$Y = -16.28050 - 0.31210X_1 + 0.36565X_2 + 1.07600X_3 - 0.009X_1X_2 - 0.0096X_1X_3 - 0.0022X_2X_3 + 0.021160{X_1}^2 - 0.00021{X_2}^2 - 0.012440{X_3}^2$$

The variance of the quadratic regression model showed that the determination coefficient (R^2) was 0.9248. Meanwhile, coefficient of the variation was 0.66, which indicated the high level of precision and a large number of experimental data were very reliable. The values of regression coefficient in second-order polynomial equation were listed in Table 2. The smaller the *P*-value is, the more significant the corresponding coefficient is. Accordingly, X_1, X_1X_2, ${X_1}^2$ and ${X_3}^2$ were significant ($P < 0.05$).

3.1.2. Optimization for extraction of BCP

Process variables and corresponding experimental data were shown in Table 1. And the response surface plots and contour plots as shown in Fig. 1. Through the calculation of Design-Expert, the optimal extraction parameters the extraction of BCP were extraction temperature 60.0 °C, extract 35.0 min and ratio of water to raw material 24.5 ml/g. Under these conditions, the maximum predicted yield of BCP was 8.43%, which was well consistent with the actual extraction yield (8.37 ± 0.36%, n = 3).

3.2. Separation and purification of BCP

Crude BCP was extracted by the optimal extraction conditions, and then it was separated through DEAE-52 cellulose. As shown in Fig. 2, two different elution peaks (F_1 and F_2) were appeared and collected, then these two fractions were filtered by Sephadex G-100, respectively. One single elution peak means F_1 and F_2 were homogeneous component. These two purified products called BCP-1 and BCP-2, and then collected. The yields of BCP-1 and BCP-2 were 13.2% and 9.7%, respectively.

3.3. Characterization of BCP

3.3.1. Contents of carbohydrate, sulfate, protein and uronic acid in BCP

The contents of carbohydrate, sulfate, protein and uronic acid in BCP-1 and BCP-2 were shown in Table 3. The content of carbohydrate in BCP-1 and BCP-2 were 89.71% and 92.56%, respectively, while BCP-2 contained the higher amount of sulfate, protein and uronic acid.

3.3.2. Monosaccharide composition of BCP

As shown in Table 3, Fig. 3A and B, BCP-1 and BCP-2 was mainly composed of glucose, arabinose and galactose. In addition, the content of galactose in BCP-2 was relatively higher than that in BCP-1.

3.3.3. Molecular weights of BCP

As shown in Fig. 3C, the standard regression curve equation was log Mw = −0.2017t + 9.8758, r = 0.9949 (t was the retention time, min). By the calculation of regression curve, the average molecular weights of BCP-1 and BCP-2 were 11.6 and 26.7 KDa, respectively.

3.3.4. FT-IR spectrum of BCP

As shown in Fig. 3D, the broad and strong peak around 3415 cm^{-1} in infrared spectrum was hydroxyl groups' stretching vibration [18]. The weak peaks around 2930 cm^{-1} and 1320 cm^{-1} were the C—H asymmetric stretching vibration [19]. The peak around 1612 cm^{-1} was assigned to the C=O asymmetric stretching vibration absorption peak [20]. The strong and extensive absorption peak around 1000–1200 cm^{-1} were the stretching vibrations of C—O—C glycosidic and C—OH side groups band vibrations [21]. The above absorption peaks were characteristic absorptions of polysaccharides. Furthermore, peak around 1071 cm^{-1} was absorption of furan glycosides [22]. The absorption peak at 854 cm^{-1} was indicating that α-type glycosidic linkages existing in BCP.

3.4. Antioxidant activity in vitro of BCP

3.4.1. Superoxide radical scavenging activity of BCP

Polymorphonuclear neutrophils and macrophage would produce reactive oxygen species in phagocytosis or by other factors after stimulation. Reactive oxygen species could promote the chemotactic migration of white blood cells, lead to lysosomal rupture and increase vascular permeability, and then produce an inflammatory response [16]. Therefore, inhibiting the formation of active oxygen is one of the mechanisms of anti-inflammatory drugs [23]. Generally, the model of evaluation antioxidant activity includes scavenge activity of superoxide radical, reductive potential, ferrous ion chelating ability, *etc* [24].

As showed in Fig. 4A, BCP-1 and BCP-2 all showed ideal ability of scavenging superoxide radical. And their antioxidant activities were positively correlated with the concentration (0.04–2.0 mg/ml). Among these, BCP-2 showed strongest scavenging ability of superoxide radical at each concentration. After the concentration of 500 μg/ml, scavenging ability of BCP-1 and BCP-2 would not significantly increase and trend to be similar.

3.4.2. Reductive potential of BCP

As shown in Fig. 4B, at concentration of 40- 240 μg/ml, the reducing power of BCP-1 and BCP-2 were enhanced. When the concentrations were higher than 120 μg/ml, the reductive activity of Vc was much stronger than that of BCP-1 and BCP-2. Beside these, the reductive activity of BCP-2 was higher than that of BCP-1.

3.4.3. Ferrous ion chelating ability of BCP

Fig. 4C showed ferrous ion chelating activities of BCP. The ferrous ion chelating activity of BCP-1 and BCP-2 were correlated with the concentration from 0.04 to 0.4 mg/ml. When the concentrations were higher then 0.4 mg/ml, the ferrous ion chelating activities of BCP-1 and BCP-2 were not increased.

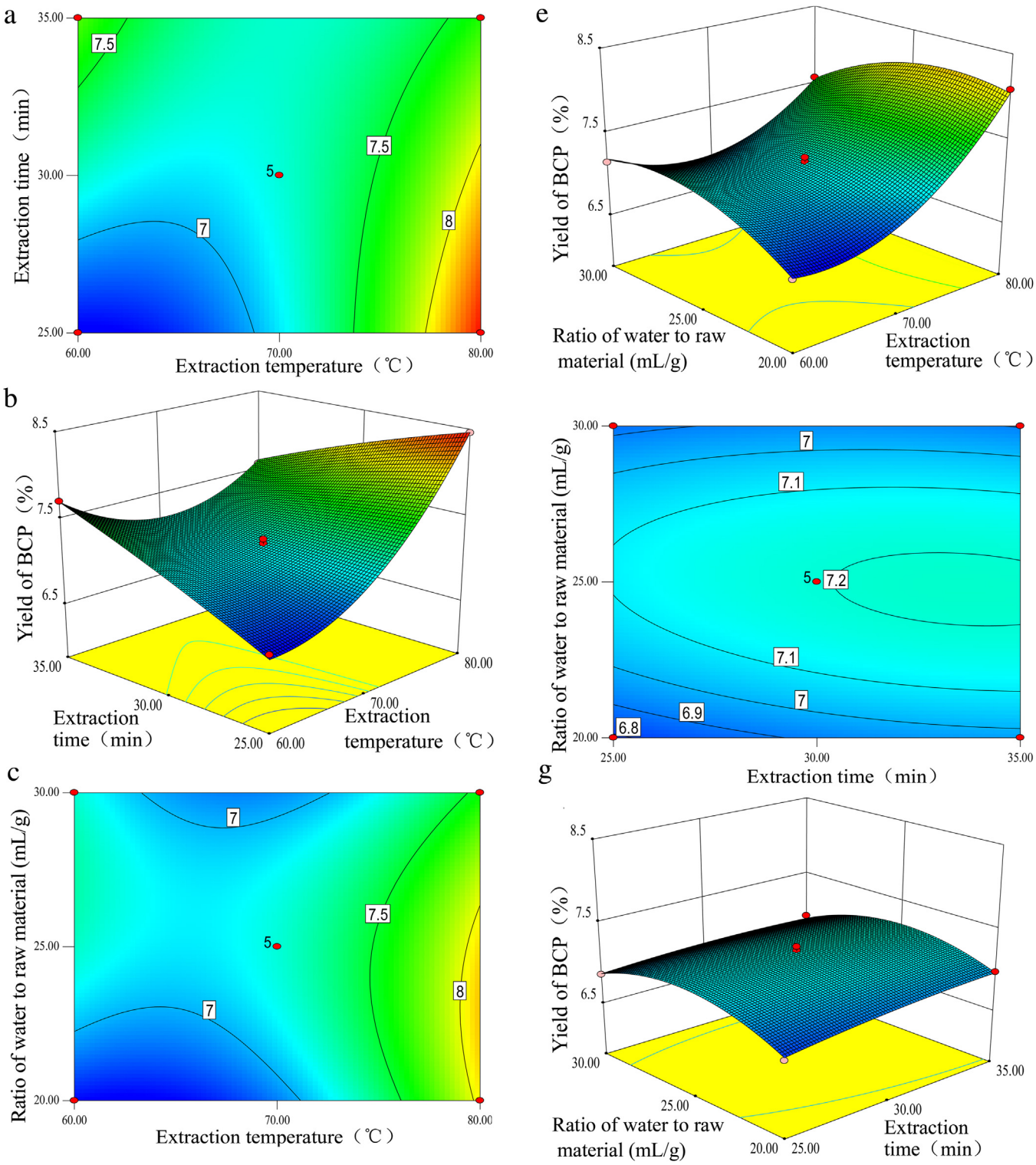

Fig. 1. Response surface plots (a, c and e) and contour plots (b, d and f) showing the effects of extraction temperature, extraction time and ratio of water to raw material their mutual effects on the extraction yield of BCP.

3.5. *Anti-inflammatory experiment of BCP* in vitro

3.5.1. *Cytotoxicity of BCP-1 and BCP-2*

Cytotoxicity of BCP-1 and BCP-2 on RAW264.7 cells were tested before to evaluate the anti-inflammatory activity. As shown in Fig. 5A, both BCP-1 and BCP-2 showed no obvious cytotoxicity on RAW264.7 cells at concentrations 20–160 μg/ml. Therefore, BCP-1 and BCP-2 at the indicated concentrations of 20, 40, 80 and 160 μg/ml were selected to conduct assay of anti-inflammatory activity.

3.5.2. *Effect of BCP on the release of NO from LPS stimulated RAW264.7 cells*

The role of nitric oxide in the body has dual nature, which is both has ability to protect and damage [25]. NO produce by induced

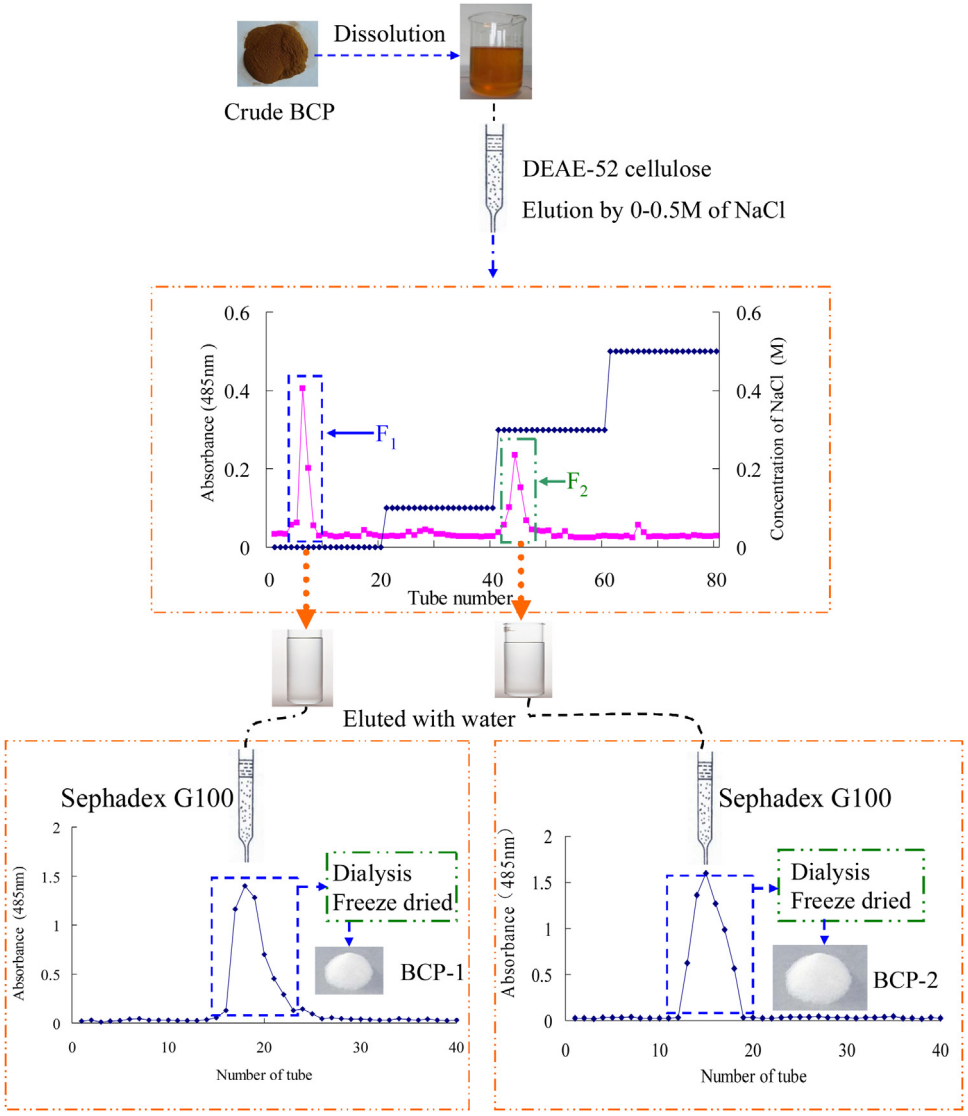

Fig. 2. Separation flow diagram of BCP.

enzyme distributed in the macrophages and inflammatory neutrophils mainly to play a toxic role. Therefore, it is important to regulate the synthesis and release of NO, reduce the cytotoxicity in the treatment of inflammatory diseases.

As shown in Fig. 5 B, compared with the blank control group (Group I), the model group released a large amount of NO ($P < 0.05$). After treatment by BCP, the NO secretion was decreased. And with the increase of BCP dose, the amount of NO secretion decreased. NO secretion was significantly reduced ($P < 0.05$) after BCP concentrations exceeded 40 μg/ml. This may be related to the inhibition of the activation of macrophages and the release of inflammatory cytokines associated with iNOS expression.

3.5.3. *Effect of BCP on the secretion of inflammatory cytokines in LPS stimulated RAW264.7 cells*

TNF-α is a major proinflammatory cytokine produced by macrophages in the inflammatory response [26]. It can regulate cell growth, differentiation and proliferation. In addition, it also can regulate the immune response and participate in a variety of inflammatory responses. IL-6 has outstanding contributions in both innate and acquired immunity. It can regulate the differentiation and activation of T lymphocytes, induce the activation of helper T cells, maintain the balance between regulatory T cells and helper T cells, and play an important role in chronic inflammation. IL-8 is also a common proinflammatory cytokine. Generally, the inflammatory response induced by TNF, IL-1, and IL-6 is mainly because they induce the production of IL-8 [27].

As shown in Fig. 5C–E, BCP-1 and BCP-2 had a significant effect on the secretion of inflammatory cytokines stimulated by LPS in RAW264.7 cells. Compared with model control group, BCP-1 and BCP-2 could significantly inhibit LPS stimulated RAW264.7 cell secretion of TNF-α, IL-6 and IL-8 when the concentration in range from 20 to 160 μg/ml. These results indicated that, the anti-inflammatory effects of BCP-1 and BCP-2 were related to the inhibition of the expression of proinflammatory cytokines.

3.6. *Anti-inflammatory experiment of BCP* in vivo

3.6.1. *Effect on xylene induced ear oedema*

The results present in Table 4 showed that six doses of BCP-1 and BCP-2 all exerted anti-inflammatory effect in this model. Their inhibition rate was significantly increased with the increase of dose. There was a significant dose effect relationship between the dose and the inhibition rate. At the dose of 400 mg/kg, the inhibition rate of BCP-1 and BCP-2 was as high as 43.90% and 46.34, respectively.

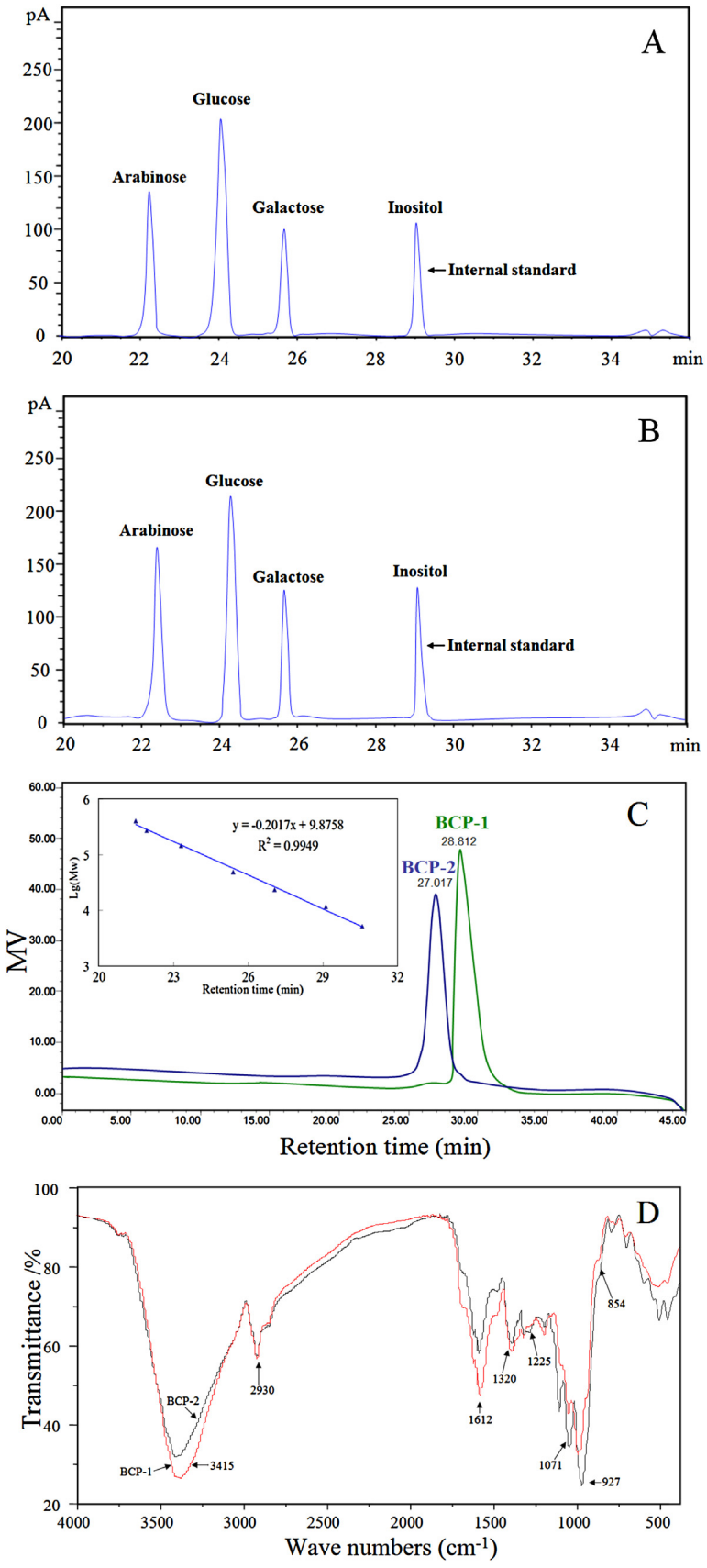

Fig. 3. GC chromatogram of monosaccharide of BCP-1 (A) and BCP-2 (B), HPLC chromatogram (C) and FT-IR spectra of BCP (D).

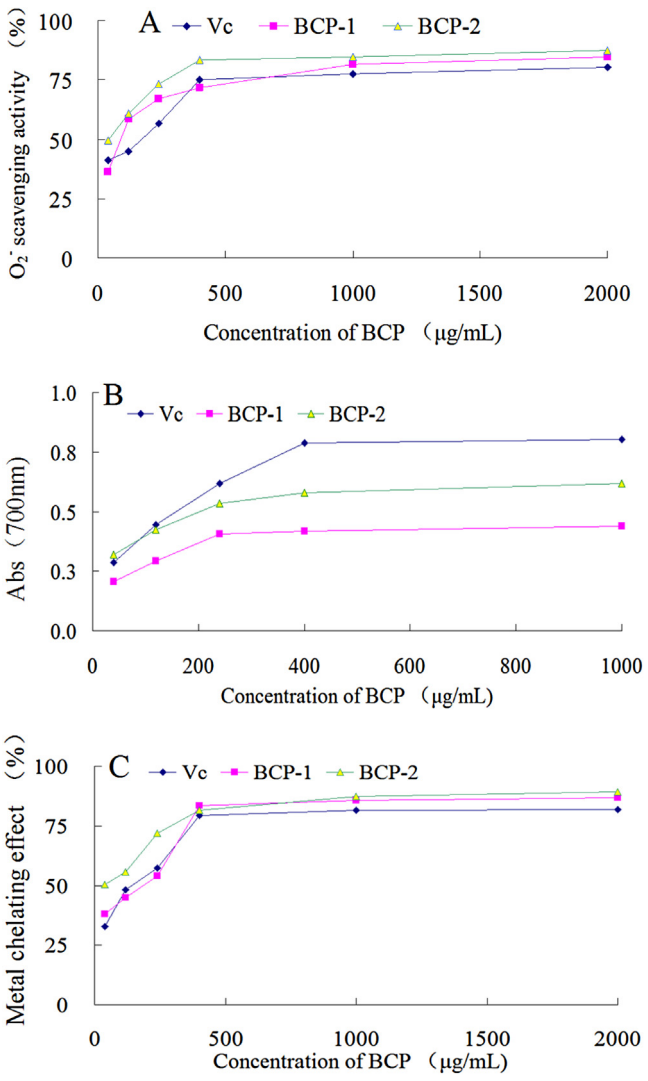

Fig. 4. Scavenging effects onsuperoxide radical (A), reductive potential (B) and Ferrous metal ions chelating activity (C) of BCP-1 and BCP-2.

However, their inhibitory activity was lower than that of ibuprofen at the same dose.

3.6.2. *Effect on vascular permeability increase*

Effect of BCP-1, BCP-2 and ibuprofen on vascular permeability induced by acetic acid were presented in Table 4. Compared with the model group, the vascular permeability was significantly inhibited in the experimental groups ($P < 0.01$). The inhibitory effect was correlated with the dose, in which high dose group (400 mg/kg) presented significantly higher inhibitory ability ($P < 0.01$) than medium dose (200 mg/kg) and low dose group (100 mg/kg). Besides, the inhibitory effect of BCP-2 was better than BCP-1, but there was no significant difference ($P > 0.05$).

A large number of literatures reveal that bioactivities of polysaccharide are related to their own properties, such as main-chain configuration, monosaccharide compositions, relative molecular weight, types of glycosidic bond, and spatial configuration [28]. In terms of anti-inflammatory activity, polysaccharides from the same parts of the same plant, although their molecular weight and the composition of the monosaccharide were different, their anti-inflammatory activity had no significantly difference [29,30]. In addition, the presence of sulfate in the polysaccharides has a certain effect on the anti-inflammatory activity. For instance, sulfated

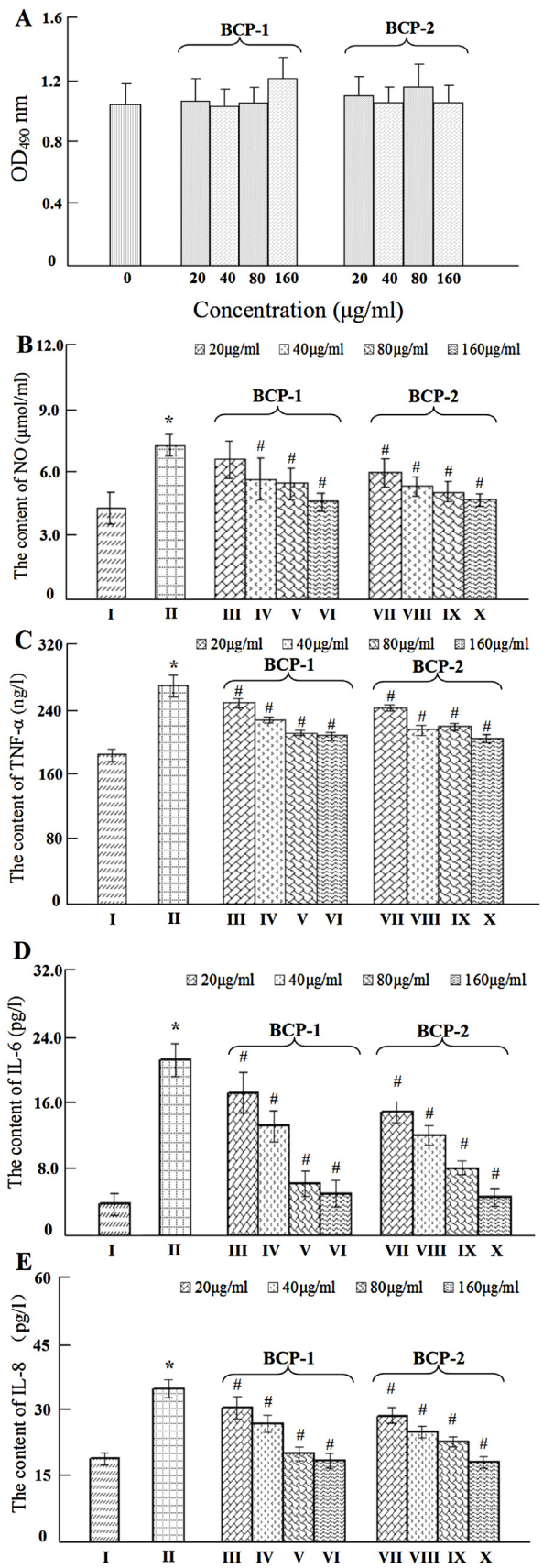

Fig. 5. (A) Effects of BCP-1 and BCP-2 on proliferation of RAW264.7 cells; (B) Effect of BCP on the secretion of NO in LPS induced RAW264.7 cells. Effects of BCP on the secretion of TNF-α (C), IL-6 (D) and IL-8 (E) in LPS induced RAW264.7 cells. Values are presented as means ± SD (n = 3). Superscript mark designate as a significant differences. * $P < 0.05$ compared with the blank control group. # $P < 0.05$ compared with the model control group.

modified polysaccharide of *Pleurotus eryngii*, its anti-inflammatory activity was significantly better than that of the unmodified one [31]. Our research also found that, BCP-2 had relatively more sulfuric acid, and its anti-inflammatory activity was better than BCP-1.

4. Conclusion

In the present study, two purified polysaccharides (BCP-1 and BCP-2) were extracted, separated and purified from Baphicacanthis Cusiae Rhizoma et Radix. Then, BCP-1 and BCP-2 were characterized by gas chromatography, high performance liquid chromatography and FT-IR. The results showed that BCP-2 had the relatively higher contents of sulfuric radical and uronic acid. In addition, the average molecular weights of BCP-1 and BCP-2 were 11.6 and 26.7 KDa, with the mainly composed of glucose, arabinose and galactose. Furthermore, BCP-1 and BCP-2 exhibited strong superoxide radical scavenging activity, reductive potential and ferrous ion chelating activity. For anti-inflammatory activity *in vitro*, BCP-1 and BCP-2 could exert their anti-inflammatory effect by inhibiting the secretion of NO and the expression of inflammatory cytokines in LPS-stimulated macrophages. For anti-inflammatory activity *in vivo*, BCP-1 and BCP-2 could significantly inhibit mice ear edema induced by xylene, and vascular permeability increase induced by intraperitoneal injection of acetic acid. These results suggest that BCP-1 and BCP-2 might be the active site that plays the role of anti-inflammatory, and they could be a new source of natural anti-inflammatory with potential value in supplements and medicine.

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