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ORIGINAL ARTICLE

Modeling and Optimum Extraction of Multiple Bioactive Exopolysaccharide from an Endophytic Fungus of *Crocus sativus* L

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ABSTRACT

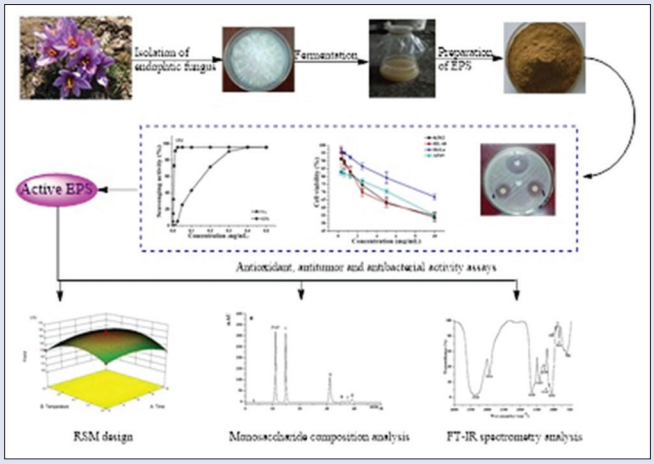
Background: *Crocus sativus* L. (saffron) is a scarce plant that has been used as food flavoring agent, coloring agent, and traditional herbal medicine. **Methods:** The bioactivity of exopolysaccharide (EPS) extracted from an endophytic fungus of *C. sativus* was examined for the first time by antioxidative, antitumor, and antibacterial assays. The extraction conditions for EPS were optimized by combining the response surface methodology with Box-Behnken design. **Results:** EPS exhibited excellent scavenging activities against 1,1-diphenyl-2-picrylhydrazyl, hydroxyl and superoxide anion radicals, and moderate cytotoxicities against K562, A549, HL-60, and HeLa cells. The optimum extraction conditions for EPS were as follows: precipitation time of 16 h, precipitation temperature of 3.7°C, pH 7.2, and ratio of ethanol to fermented broth of 5:1 (L/L). Under the optimized conditions, the yield of EPS reached 162 ± 6 μg/L which was close to the predicted one (165 μg/L). Moreover, high-performance liquid chromatography of monosaccharide composition showed that EPS comprised mannose, glucose, galactose xylose, and arabinose in a molar ratio of 25.6:16.5:1.0:3.8:5.4. **Conclusion:** EPS may be an eligible substitute for *C. sativus* and a potential bioactive source applicable to pharmaceutical and food industries.

Key words: Bioactivity, *Crocus sativus* L., endophytic fungus, exopolysaccharide, response surface methodology

SUMMARY

- Exopolysaccharide (EPS) from endophytic fungus of *Crocus sativus* was studied for the first time
- EPS extraction was optimized by combining response surface methodology with Box-Behnken design
- Monosaccharide composition and EPS structure were identified by high-performance liquid chromatography and Fourier-transform infrared spectroscopy.

Abbreviations used: EPS: Exopolysaccharide, RSM: Response surface methodology, BBD: Box-Behnken design, DPPH: 1,1-diphenyl-2-picrylhydrazyl, V_C: Ascorbic acid, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, LB: Luria Bertani, DMSO: dimethyl sulfoxide, PMP: 1-phenyl-3-methyl-5-pyrazolone, FT-IR: Fourier transform-infrared, HPLC: High-performance liquid chromatography, 3D: Three-dimensional, 2D: Two-Dimensional.



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INTRODUCTION

Exopolysaccharides (EPSs), also known as extracellular polysaccharides, have been employed as a crucial source of microbial polysaccharides. They are produced by the metabolic processes of fungi, bacteria, blue-green algae, and some other microorganisms.[1] Fungus-derived polysaccharides have diverse chemical structures as well as antioxidant, antitumor, immunomodulatory, and anti-inflammatory activities.[2] Compared with polysaccharides from mycelia and plants, it is easier to obtain EPSs from fermented broth with similar pharmacological and physiological functions.[3]

As a perennial stemless herb, *Crocus sativus* L. (saffron) is cultivated widely in Iran and slightly planted in China. Saffron is one of the most expensive spices in food and flavoring fields, also as a composition in commercially processed foodstuffs such as herbal teas, seasoning mixes, pasta, and rice.[4] Furthermore, saffron is a biologically active ingredient with antitumor, antioxidant, and anti-inflammatory properties.[5] However, this herb has an overlong growth cycle and unstable product quality during conventional cultivation.[6] Recently, Schulz *et al.* reported that the metabolites isolated from endophytic fungi exhibited higher bioactivities than those of the host plant.[7] Therefore, several biologically active compounds have been obtained from the cultures of endophytic

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fungi since then.[8] Nevertheless, EPS isolated from the endophytic fungi of *C. sativus* has never been reported hitherto.

We have previously obtained EPS through water extraction and ethanol precipitation.[9] In the extraction process, the yield of EPS is affected by precipitation time, precipitation temperature, and pH. The yield can be elevated by optimizing the extraction process, and EPS can be produced in a more compact space within a shorter time, also with lower contamination risk, convenient control, and facile downstream processing.[10] Response surface methodology (RSM) is an effective statistical and mathematical strategy for optimizing processes, even when complex interactions exist. Besides, it is less laborious and time-consuming than other methods by reducing the number of experimental trials that are required to assess interactions and multiple variables.[11,12] Box-Behnken design (BBD) is a statistical experimental design for RSM. As an independent quadratic design, it contains no fractional factorial or embedded factorial design. The treatment combinations in this design are in the center and at the midpoints of process space edges, needing three levels of each factor.[13] Above all, RSM-BBD can better arrange and interpret experiments than other studies on the extraction of EPS.

In this study, EPS was isolated from an endophytic fungus *Penicillium citreonigrum* CSL-27 of *C. sativus* for the first time, and its bioactivity was evaluated by antioxidant, antitumor, and antibacterial assays. Moreover, the extraction conditions of EPS were optimized by RSM-BBD, and the actual values were close to the predicted ones.

MATERIALS AND METHODS

Materials and chemicals

C. sativus was purchased from Henan Agricultural University (Zhengzhou, China), and *P. citreonigrum* CSL-27 was isolated from *C. sativus* and stored at the China Center for Type Culture Collection (Wuhan, China). Bacterial strains: two Gram-positive bacterial strains (*Bacillus subtilis* and *Staphylococcus aureus*) and six Gram-negative bacterial strains (*Pseudomonas aeruginosa*, *Coli-aerogenes*, *Salmonella typhi*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Shigella dysenteriae*) were obtained from Department of Microbiology and Immunology of the Foundation Institute and the Microbiology Laboratory of the First Affiliated Hospital of Guangdong Pharmaceutical University (Guangzhou, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was bought from Wako Pure Chemical Industries (Tokyo, Japan). The assay kits for hydroxyl and superoxide anion radicals were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Ascorbic acid (V_C) was obtained from Shanghai Runjie Chemical Reagent Co., Ltd. (Shanghai, China). 3-(4,5-dimethylthiazole)-2,5-diphenyltetraazolium bromide (MTT) and dialysis tubing (molecular weight cutoff, 8000–14000 Da) were provided by Sigma Chemicals Co.(St. Louis, MO, USA). Human erythroleukemia cell line K562, human lung adenocarcinoma cell line A549, human promyelocytic leukemia cell line HL-60, and human cervical carcinoma cell line HeLa were bought from Guangzhou Jinan Biomedicine Research and Development Center Co., Ltd. (Guangzhou, China). Luria Bertani (LB) agar was obtained from Guangdong Huankai Microbial Sci. and Tech. Co., Ltd.(Guangzhou, China). The other reagents were all analytically pure.

Microorganism and culture conditions

P. citreonigrum CSL-27 isolated from the corm of *C. sativus* was conserved at 4°C in our laboratory. All operations were conducted in a sterile environment. The fungus was cultured at neutral pH in a 500 mL Erlenmeyer flask containing 250 mL of PYG medium (g/L): glucose 10, peptone 2, NaCl 2 and yeast extract 1, and then on a rotary shaker (TCYQ, Taicang Laboratory Equipment Factory; Jiangsu Province, China) constantly at 120 rpm and 28°C for 7 days.

Preparation of exopolysaccharide

Fermented broth was concentrated at 60°C in a water bath, and then, the insoluble residue was separated by a four-layer filter cloth. Fourfold of 95% ethanol (v/v) was added into the broth and maintained overnight at 4°C. EPS was collected by centrifugation at 3,000 rpm for 10 min. The resulting precipitate was washed with acetone and absolute ethanol successively, dialyzed against running water for 48 h, and finally lyophilized. Carbohydrates were determined by the phenol-sulfuric acid method[14] and the EPS yield was calculated according to the following equation:

$$Y\ (\mu g/L) = (v_1 \times c \times m)/(m_1 \times v)$$

Where Y is the yield of EPS; v_1 is the constant volume of EPS; c is the concentration of EPS; m is the total mass of EPS; m_1 is the mass of EPS for reaction; and v is the volume of EPS for precipitation.

Assay for scavenging activity of exopolysaccharide against 1,1-diphenyl-2-picrylhydrazyl radical

The scavenging activity of EPS against DPPH radical was assayed as previously described with minor modifications.[15] Briefly, 3.5 mL of EPS solution at 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.025 mg/mL was added to 3.5 mL of DPPH solution (0.1 mM) in 80% ethanol. Afterward, the mixture was vigorously shaken and left in dark at room temperature for 30 min. V_C was utilized as positive control. The effective concentration at which 50% of DPPH radicals were scavenged by EPS was defined as EC_{50} (mg/mL). Then, the absorbance was detected by a spectrophotometer (Shimadzu UV2550, Japan) at 517 nm. The scavenging rate of DPPH radical was calculated with the equation below:

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

Where A_0 is the absorbance of DPPH solution without tested sample, A_1 is that of tested sample (DPPH solution with either positive control or sample), and A_2 is that of sample without DPPH solution.

Assay for scavenging activity of exopolysaccharide against hydroxyl radical

The scavenging activity of EPS against hydroxyl radical was tested through the Fenton's reaction with minor modifications.[16] A reaction mixture containing 0.2 mL of ferrous sulfate and 0.2 mL of salicylic acid-ethanol was added in a test tube. Subsequently, 0.2 mL of EPS solution at 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, or 0.0125 mg/mL was added. The reaction was started by adding 1.0 mL of H_2O_2. After incubation at 37°C for 30 min, the Griess reagent was added to the resulting mixture. The solution absorbance was detected at 550 nm, using V_C as positive control. The effective concentration at which 50% of hydroxyl radicals were scavenged by EPS was defined as EC_{50} (mg/mL). The scavenging effect was assessed according to the following equation:

$$\text{Scavenging activity}\ (\%) = (A_0 - A)/A_0 \times 100\%$$

Where A_0 is the absorbance of control (distilled water instead of sample) and A is that of sample.

Assay for scavenging activity of exopolysaccharide against superoxide anion radical

The scavenging activity of EPS against superoxide anion radical was evaluated by a method previously described.[17] In brief, EPS was dissolved by distilled water into various concentrations (2.0, 1.0, 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/mL), and incubated with 50 mL of tris-HCl (pH 8.2) for 20 min at 30°C. Afterward, 0.1 mL of tris-HCl was added into each sample solution that was thereafter incubated with 0.1 mL of 1, 2, 3-phentriol for 40 min at 37°C. Finally, the reaction system was mixed with 2 mL of Griess reagent. Using distilled water solution of V_C

as positive control, the absorbance was detected at 550 nm. The effective concentration at which 50% of superoxide anion radicals were scavenged by EPS was defined as EC_{50} (mg/mL). The ability to scavenge superoxide anion radicals was evaluated by the following equation:

Scavenging activity (%) = $(A_0 - A)/A_0 \times 100\%$

Where A is the absorbance of test sample mixed with reaction solution and A_0 is that of control group in which distilled water was used instead of sample.

Cytotoxicity assay

The cytotoxicity assay was evaluated *in vitro* using the MTT assay according to a previous method.[18] K562, A549, HL-60, and HeLa cells were inoculated at the density of 8×10^3/well into 96-well microplates and incubated in a 37°C incubator (Thermo Model-3111, USA) with humidified atmosphere and 5% CO_2. After 24 h, they were treated with EPS solutions at various concentrations (0.31, 0.62, 1.25, 2.5, 5.0, and 10.0 mg/mL) and incubated at 37°C for another 24 h. Control wells were prepared by adding culture medium (100 μL), followed by further incubation for 48 h. After 20 μL of MTT solution (5 mg/mL) was added, they were incubated for another 4 h, from which the medium was then carefully removed. Finally, the reaction was terminated by adding 100 μL of DMSO. The absorbance of each well was measured at 570 nm in a microplate spectrophotometer (Bio-Rad Model-680, USA) to count viable cells. The viability was calculated by the equation below:

Viability (%) = $(A_1/A_0) \times 100\%$

Where A_1 is the absorbance of test sample and A_0 is that of control.

Antibacterial assay

The antibacterial activity of EPS was determined by the agar disc diffusion method with slight modifications.[19] The test pathogenic bacteria were cultured for 24 h at 37°C and adjusted into 0.5×10^8 CFU/mL with sterile saline. Bacterial suspensions (0.2 mL) were evenly swab-inoculated on the surface of LB agar. Sterile filter paper discs (diameter: 6 mm) were impregnated with 100 μL of EPS solutions (concentrations: 150 and 200 mg/mL) and placed on the LB plate surfaces. Then, the plates were incubated for 24 h at 37°C in a self-regulating thermostat (SPX-250C, Shanghai Boxun Industry and Commerce Co., Ltd; Shanghai, China). The antibacterial activity was tested through measuring the diameter (mm) of clear inhibition zone around each disc. Streptomycin sulfate and 0.9% saline solution were used as positive control and negative control, respectively. All tests were performed in triplicate.

Single-factor experimental design

The effects of complex precipitation time, precipitation temperature, pH, and ethanol/fermented broth ratio on the yield of EPS were studied by single-factor experiments. During optimization, one factor was altered while keeping the others constant in every experiment.[20] All experiments were performed in triplicate.

Response surface methodology design

The extraction of EPS was optimized by BBD using three independent variables. Experiments were constructed on the basis of BBD with four factors at three levels, with each independent variable coded at −1, 0, and +1. Precipitation time (h), precipitation temperature (°C), pH, and ratio of ethanol to fermented broth (L/L) were used as independent variables, and EPS yield (Y, μg/L) was employed as response-dependent variable. All the trials were conducted in triplicate. Table 1 summarizes the noncoded and coded values of independent variables. The variables of *X* were coded as *x* by the following equation:

$xi = (Xi - X0)/\Delta X \quad i = 1, 2, 3, 4$

Table 1: Independent variables and levels in Box-Behnken design

Variables	Factor level		
	−1	0	1
Time A (X_1)	12	16	20
Temperature B (X_2)	3	4	5
pH C (X_3)	6.5	7	7.5
Ratio D (X_4)	3	4	5

Where x_i is the coded value of variable; X_i is the actual value of variable; X_0 is the actual value of *X*i in the center; and Δ*X* is the step change.

To predict the optimum conditions, the results were analyzed using Design-Expert software (Version 8.0.6, USA) and fitted by a second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_i X_i^2 + \sum_{i=1}^{4} \sum_{j=1}^{4} \beta_{ij} X_i X_j$$

Where *Y* represents the response variable, β_0 is the constant coefficient, *β*i, *β*ii, and *β*ij are the regression coefficients of linear, quadratic, and interaction terms, respectively, and *X*i and *X*j are different independent variables (i ≠ j).

The experimental design data were designed and the predicted responses were calculated by Design Expert. Analysis of variance (ANOVA) was utilized. The fitness of the polynomial equation was expressed by adjusted-R^2 ($R2$ adj) and the coefficient of determination R^2. By using the *F*-test, statistical significance was determined at the probability (*P*) of 0.05, 0.01 or 0.0001,[21] and the significances of regression coefficients were determined as well. Statistical calculations were thereafter performed using the regression coefficients to generate dimensional and contour maps based on the regression models.

Monosaccharide composition analysis

After acid hydrolysis and reaction of 1-phenyl-3-methyl-5-pyrazolone (PMP), the monosaccharide composition of EPS was detected with high-performance liquid chromatography (HPLC) as reported before.[22] Briefly, sample (5 mg) was hydrolyzed by 2 mL of trifluoroacetic acid (2 M) for 4 h at 120°C. Subsequently, the hydrolysate was dried under vacuum and redissolved by 1 mL of water. The resulting solution (100 μL) was mixed by 200 μL methanol solution of PMP (0.5 M) and 200 μL of NaOH solution (0.3 M) and reacted for 30 min at 70°C. The reaction was terminated through neutralization with 450 μL of HCl solution (0.3 M), and the product was then partitioned three times with chloroform. Afterward, the collected aqueous layer was filtered through a 0.45 μM filter membrane and subjected to HPLC that was conducted by a Phenomenex GEMINI-NX C_{18} column (250 nm × 4.6 nm, 5 μm) on an Agilent 1200 instrument at 30°C. Potassium phosphate-buffered saline solutions (0.1 M, pH 6.7) containing 83% acetonitrile (solvent A) and 17% acetonitrile (solvent B) were used as mobile phases, and the wavelength of UV detection was set at 250 nm. The monosaccharide components were finally identified through comparing their retention times with those of standard saccharides.

Fourier transform-infrared spectroscopy

Fourier transform-infrared (FT-IR) spectroscopy, which has been widely employed to study molecular vibrations and polar bonds between different atoms, can reveal the functional groups and glycosidic bonds in polysaccharides. IR spectroscopy of EPS was carried out by an FT-IR spectrometer (Perkin Elmer Spectrometer 100, Wellesley, USA). Dried EPS powders were mixed with potassium bromide and compressed into a 1 mm pellet for FT IR spectroscopy from 4000 to 400 cm^{-1}.[23]

RESULTS AND DISCUSSION

1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity

The free radical-scavenging activity of EPS has commonly been evaluated by the DPPH radical scavenging model. As to the mechanism, DPPH radical is scavenged by accepting hydrogen, thereby being converted into the nonradical form (DPPH-H). Therefore, the antioxidant activity of EPS originated from the hydrogen-donating ability.[24] As presented in Figure 1a, DPPH radicals are scavenged by EPS in a concentration-dependent manner, with EC_{50} of 0.14 mg/mL. When the EPS concentration increased to 0.4 mg/mL, the DPPH radical scavenging activity reached 95.50% which was close to that of V_C (95.93%).

Hydroxyl radical scavenging activity

Of all reactive oxygen species, hydroxyl radical is highly oxidative, being able to react with most biomacromolecules in living cells and to severely damage adjacent ones.[25] As shown in Figure 1b, hydroxyl radicals are scavenged by EPS in a concentration-dependent manner, with EC_{50} of 0.16 mg/mL. At 0.2 mg/mL, EPS managed to scavenge 94.42% of hydroxyl radicals, similar to V_C did (98.43%).

Superoxide anion radical scavenging activity

Compared to other radicals generated by photochemical and biological reactions, superoxide anion radical has a much longer lifetime and is less reactive. This radical causes tissue damage and diseases by generating secondary radicals such as hydroxyl radicals and H_2O_2 through dismutation and some other reactions. Thus, the antioxidant activity of EPS against superoxide anion radical can be detected by assessing its scavenging effect.[26] As shown in Figure 1c, superoxide anion radicals are scavenged by EPS in a dose-dependent manner, with EC_{50} of 0.60 mg/mL. Since 1.0 mg/mL EPS scavenged 74.54% of superoxide anion radicals, it had a higher scavenging activity.

Cytotoxicity assay

Dou *et al.* reported that the extracts of *C. sativus* exhibited high cytotoxicities against K562, A549, HL-60, and HeLa cells,[27] so these tumor cells were chosen to investigate the cytotoxicity of EPS. As shown in Figure 2, EPS evidently inhibits the proliferation of these cells in a dose-dependent manner. In the presence of 10 mg/mL EPS, the proliferation of K562, A549, HL-60, and HeLa cells was inhibited by 46.16, 44.97, 44.95, and 33.10%, respectively. Therefore, EPS displayed potential cytotoxicities against K562, A549, and HL-60 cells

Antibacterial activity

The disc diffusion method was employed to detect the antibacterial activity of EPS. The growths of tested microorganisms are inhibited differently. Notably, EPS showed a moderate activity against *S. aureus* in a concentration-dependent manner. The inhibitory effect of 200 mg/mL EPS was close to that of streptomycin sulfate. In addition, EPS exerted weak inhibitory effects on *B. subtilis*, *E. coli*, *P. aeruginosa*, *C. aerogenes*, *S. typhi*, *S. dysenteriae*, and *K. pneumoniae*.

Effect of precipitation time on exopolysaccharide yield

The effect of precipitation time on EPS yield was studied at 4°C, pH 7.0, and ethanol/fermented broth ratio of 4:1 (L/L). The precipitation time was set at

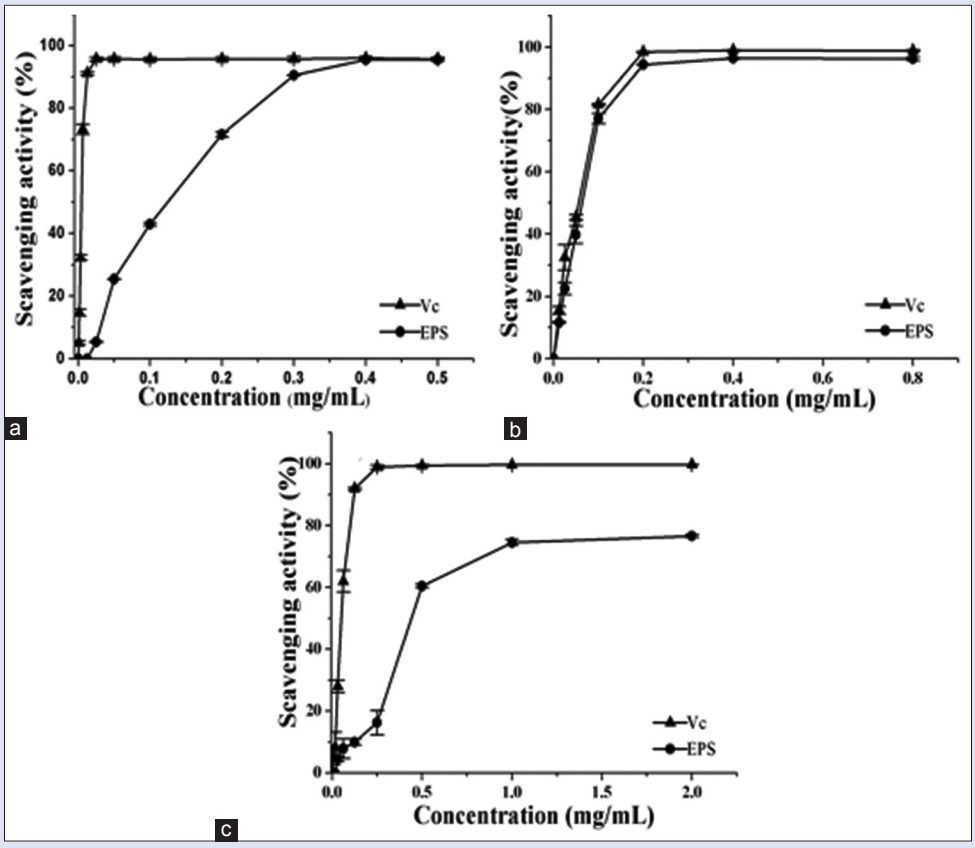

Figure 1: *In vitro* antioxidant activities of exopolysaccharide. (a) 1,1-Diphenyl-2-picrylhydrazyl radical; (b) hydroxyl radical; (c) superoxide anion radical. The values shown are means ± standard deviation of triplicate measurements

4, 10, 16, 22, and 28 h. The yield of EPS rose with increasing precipitation time from 4 to 16 h. When the precipitation time exceeded 16 h, the yield was hardly affected. Thus, the optimum precipitation time was 16 h.

Effect of precipitation temperature on exopolysaccharide yield

The effect of precipitation temperature (2°C, 3°C, 4°C, 5°C, and 6°C) on EPS yield was evaluated, with the other factors (time, pH, ratio) fixed (20 h, pH 7.0 and 4:1 L/L). The yield increased to maximum (164 μg/L) as the precipitation temperature ranged from 2 to 4°C. As the temperature further rose from 4°C to 6°C, the yield dropped. Thus, 4°C was selected as the optimum precipitation temperature in subsequent experiments.

Effect of pH on exopolysaccharide yield

The effects of pH values from 6.0 to 8.0 on EPS yield were tested, with all the other conditions kept constant (20 h, 4°C and 4:1 L/L). The yield increased with rising pH, reaching a critical value (163 μg/L) at pH 7.0. Accordingly, pH 7.0 was selected thereafter.

Effect of ratio of ethanol to fermented broth on exopolysaccharide yield

The effect of ethanol/fermented broth ratio on EPS yield was studied for 20 h at 4°C and pH 7.0. The yield increased rapidly when this ratio ranged from 2:1 to 4:1 (L/L). However, this yield slowly increased when water/raw material ratio varied from 4:1 to 6:1 (L/L). Therefore, 4:1 (L/L) was selected as the optimum ethanol/fermented broth ratio.

Statistical analysis and model fitting

Extraction conditions can be efficiently optimized by methods such as BBD and factorial design using experiments with reduced number. According to the results of single-factor experiment herein, a dependent variable as well as four independent factors (i.e., precipitation temperature, precipitation time, pH and ethanol/fermented broth ratio) were studied by RSM-BBD. Table 2 lists the experimental conditions and outcomes of 29 runs using BBD design. The process stability was tested by conducting 5 center point runs.

With multiple regression analysis, the relationship between response variable and test variables can be revealed by the second-order polynomial equation generated using Design-Expert. The equation in terms of coded factors is shown below:

$$Y\ (\mu g/L) = -1446.96+2.24X_1 - 10.06X_2+434.59X_3+20.44X_4+0.88X_1X_2 +1.63X_1X_3 - 0.06X_1X_4+24.01X_2X_3 - 14.03X_2X_4+18.52X_3X_4 - 0.52X_1^2 - 14.42X_2^2 - 44.66X_3^2 - 10.17X_4^2$$

Where Y is the EPS yield, and X_1, X_2, X_3, and X_4 are the coded variables for precipitation time, precipitation temperature, pH, and ethanol/fermented broth ratio, respectively.

Table 3 summarizes the ANOVA results of this model. The effects of lower than 0.05 and 0.01 are considered significant and highly significant, respectively. Given a very high *F*-value (63.03) and a very low *P* value (0.0001), the model was indeed highly significant. In addition, the determination coefficient (R^2) was 0.9844, so the fitted model could explain 98.44% of variations. *R*2 adj is required to study the effects of independent variables, which should be close to *R*2 for a robust statistical model. *R*2 adj was 0.9688 herein, suggesting that the model could predict most variations (>96%) of the extraction yield. Data in the experimental domain at points excluded from the regression were represented by the lack of fit measuring the model's failure. As indicated by *F*-value of 1.58 and *P* value of 0.3489 for yield, the lack of fit was insignificantly related to noise-induced pure error. The experimental values were reliable because the coefficient of variation was 1.61%. The signal-to-noise ratio of this model was measured as 25.520 by adequate precision, suggesting an adequate signal to navigate the design space.

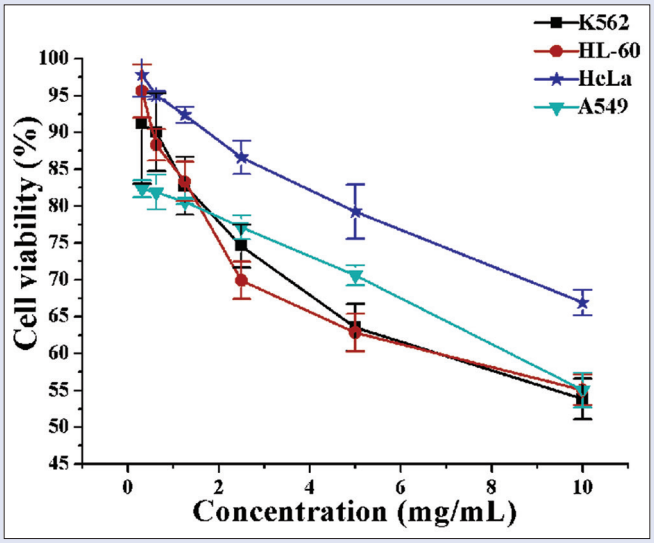

Figure 2: *In vitro* cytotoxicities of exopolysaccharide against K562, A549, HL-60, and HeLa cells. The values shown are means ± standard deviation of triplicate measurements

Table 2: Coded levels of independent variables and responses

n[a]	Coded variable level[b]				Yield (μg/L)[c]	
	X_1	X_2	X_3	X_4	Observed	Predicted
1	12	3.0	7.0	4.0	138±3	138
2	20	3.0	7.0	4.0	131±4	133
3	12	5.0	7.0	4.0	133±13	132
4	20	5.0	7.0	4.0	140±16	141
5	16	4.0	6.5	3.0	130±3	132
6	16	4.0	7.5	3.0	120±8	119
7	16	4.0	7.0	3.0	139±4	137
8	16	4.0	7.5	5.0	161±9	161
9	12	4.0	7.0	3.0	127±3	127
10	20	4.0	7.0	3.0	131±16	130
11	12	4.0	7.0	5.0	151±2	150
12	20	4.0	7.0	5.0	154.±2	153
13	16	3.0	6.5	4.0	145±1	142
14	16	5.0	6.5	4.0	121±7	119
15	16	3.0	7.5	4.0	122±7	123
16	16	5.0	7.5	4.0	149±9	148
17	12	4.0	6.5	4.0	138±10	139
18	20	4.0	6.5	4.0	135±11	134
19	12	4.0	7.5	4.0	137±3	138
20	20	4.0	7.5	4.0	147±3	146
21	16	3.0	6.5	5.0	145±2	148
22	16	5.0	7.0	3.0	136±4	137
23	16	3.0	7.0	5.0	162±0	159
24	16	5.0	7.0	5.0	131±18	133
25	16	4.0	7.0	4.0	161±10	159
26	16	4.0	7.0	4.0	157±8	159
27	16	4.0	7.0	4.0	159±8	158
28	16	4.0	7.0	4.0	158±16	158
29	16	4.0	7.0	4.0	156±20	158

[a]Experiments were conducted in a random order. [b]X_1: precipitation time (h), X_2: precipitation temperature (°C), X_3: pH, X_4: ratio of ethanol to fermented broth (L/L). [c]The yield represented the amount of EPS was extracted from per liter of the fermentation broth. Observed: The data were collected from three replicate extractions. Each value the mean ± standard deviation (*n*=3). Predicted: The calculation of predicted responses were carried out using Design Expert software

Table 3: Analysis of variance of the fitted quadratic polynomial model for optimization

Source	Sum of squares	Df	Mean	*F*	*P*	Significant
Model	4624.09	14	330.29	60.03	<0.0001	Significant
A-Time	16.33	1	16.33	3.12	0.0993	
B-Temperature	3.51	1	3.51	0.67	0.4267	
C-pH	86.57	1	86.57	16.52	0.0012	
D-Ratio	1436.54	1	1436.54	274.15	<0.0001	Significant
AB	49.00	1	49.00	9.35	0.0085	
AC	42.25	1	42.25	8.06	0.0131	
AD	0.25	1	0.25	0.048	0.8302	
BC	629.01	1	629.01	120.04	<0.0001	
BD	556.64	1	556.64	106.23	<0.0001	
CD	297.97	1	297.97	56.86	<0.0001	
A^2	443.24	1	443.24	84.59	<0.0001	
B^2	1182.13	1	1182.13	225.60	<0.0001	
C^2	817.10	1	817.10	155.94	<0.0001	
D^2	622.81	1	622.81	118.86	<0.0001	
Residual	73.36	14	5.24			
Lack of Fit	58.56	10	5.86.	1.58	0.3489	
Pure Error	14.80	4	3.70			
Cor Total	4697.45	28				
C.V. %	1.61					
R^2	0.9844					
Adj R^2	0.9688					

CV: Coefficient of variation

The significance of every coefficient and the interaction strength between variables was examined by *P* value. Table 2 presents that linear coefficient (X_4), interaction coefficients (X2 1, X2 2, X2 3and X2 4), and quadratic term coefficients (X2 1, X2 2, X2 3and X2 4) are significant, with low *P* values ($P < 0.001$). In contrast, the other term coefficients were insignificant.

Response surface plot and contour plot analyses

Two-dimensional (2D) contour plots and three-dimensional (3D) response surface were graphically represented to visualize the regression equation and to better understand the relationship between each variable and response together with the interactions between two random variables. A circular contour plot suggests a negligible interaction between corresponding variables, and an elliptical one indicates that the interaction between corresponding independent variables is ideal and crucial.[28] In this study, the relationships between EPS yield and any two independent variables were explored by 2D contour plots and 3D response surfaces, with the other one kept constant at zero level.

In Figure 3a, the effects of precipitation time, precipitation temperature, and their interactions on EPS yield are illustrated. The yield first increased to maximum with rising precipitation temperature or precipitation time and dropped thereafter. Moreover, the full elliptic contour suggests positive, significant synergistic effects of precipitation time and temperature on yield. Likewise, the yield of EPS first increased evidently with extended precipitation time, and then leveled off [Figure 3b]. However, pH alone affected the yield negatively within the experimental range. As exhibited in Figure 3c, the yield rises evidently with increasing precipitation time and ethanol/fermented broth ratio in the beginning and thereafter reduces. As shown in Figure 3d, precipitation temperature affects yield more markedly than pH does. As presented in Figure 3e, ethanol/fermented broth ratio has a significantly stronger effect on yield than precipitation temperature. In addition, yield was significantly affected by ethanol/fermented broth ratio but not pH [Figure 3f].

Validation of prediction model

To validate whether this model equation was suitable for predicting the optimum response values, four independent experiments were carried out under the following optimum conditions: precipitation time of 16 h, precipitation temperature of 3.7°C, pH 7.2, and ethanol/fermented broth ratio of 4.9:1 (L/L). Since it was difficult to control the optimum ratio during actual extraction, it was adjusted to 5:1 (L/L). Under the optimized conditions, the experimental yield of EPS was 162 ± 6 μg/L, basically being consistent with the predicted one (165 μg/L). Therefore, the model was indeed suitable for optimizing the extraction process

Monosaccharide composition

Monosaccharides were identified according to the retention times of standard saccharides, with the contents calculated by corresponding peak areas. As shown in Figure 4, EPS consists of mannose, glucose, galactose xylose, and arabinose in a molar ratio of 25.6:16.5:1.0:3.8:5.4.

Fourier transform-infrared spectroscopy

The fingerprint regions and functional groups of EPS were characterized by FT-IR spectroscopy. The FT-IR spectrum of EPS shows characteristic absorption bands at 3368, 2934, 1650, 1415, 1249, 1130, 1052, 916, 814, and 582 cm^{-1} [Figure 5]. There are stretching vibration peaks of O-H and C-H in a methylene group (-CH_2-) at 3368 cm^{-1} and 2934 cm^{-1}, respectively. Furthermore, a weak symmetrical stretching peak at approximately 1415 cm^{-1} and an asymmetrical one at 1650 cm^{-1} can be assigned to deprotonated carboxylic group (COO-), indicating that EPS was an acidic polysaccharide. The signal at 1249 cm^{-1} represents the stretching vibration of C=O groups. The bands in the range of 1200–1000 cm^{-1} and 350–600 cm^{-1} suggest that the monosaccharide of EPS has pyranose rings. Given a weak peak at 916 cm^{-1} and a peak at 814 cm^{-1}, EPS mainly contained α-glycosidic bonds. The absorption bands of EPS are consistent with those reported before.[29]

Up to now, this is the first study on EPS isolated from the endophytic fungus of *C. sativus*. Taken together, EPS is a potentially eligible substitute for *C. sativus* and a feasible new bioactive source in the food industry

CONCLUSION

We herein detected the antioxidant, antitumor, and antibacterial activities of EPS. Besides effectively scavenging DPPH, hydroxyl, and superoxide

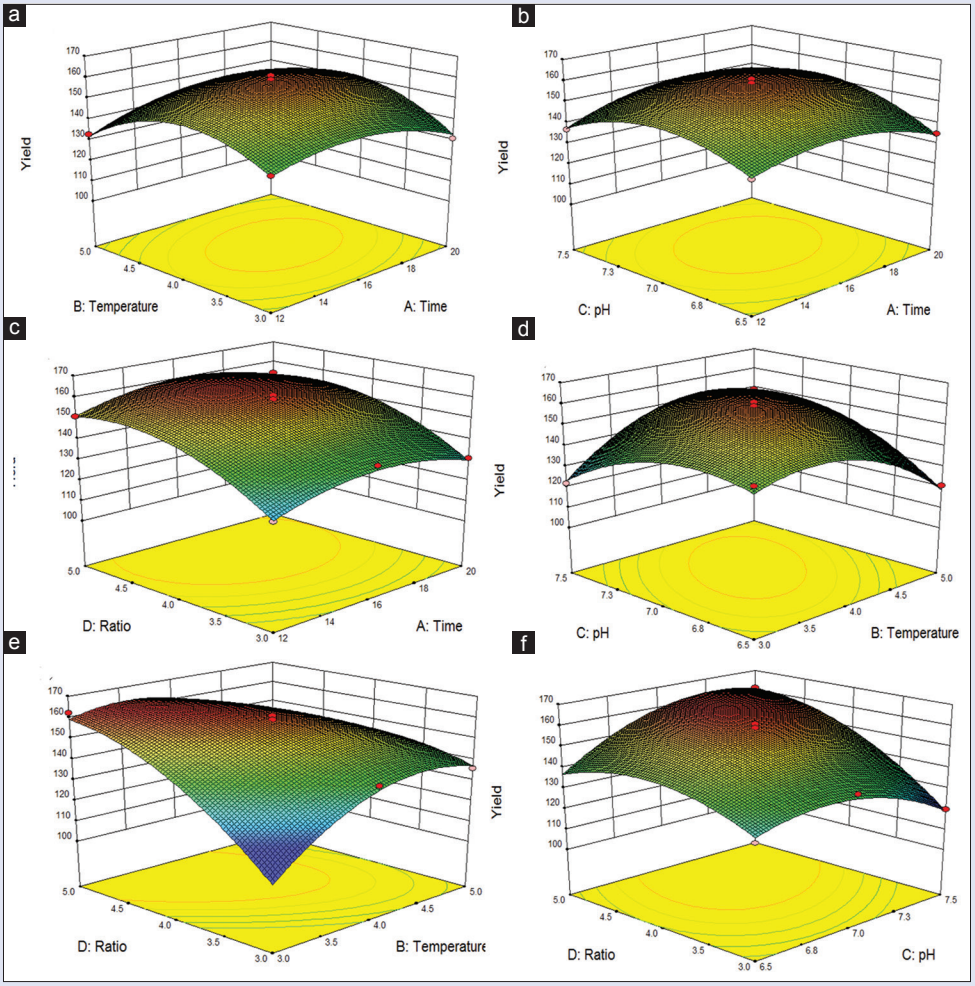

Figure 3: Response surfaces and contour plots for effects of variables (X_1: Precipitation time; X_2: Precipitation temperature; X_3: pH; X_4: Ratio of ethanol to fermented broth) on exopolysaccharide yield

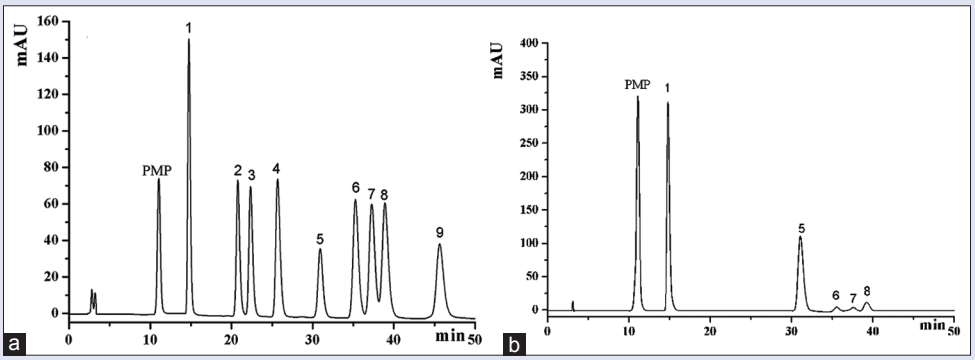

Figure 4: high-performance liquid chromatography analysis of 1-phenyl-3-methyl-5-pyrazolone derivatives of (a) standard monosaccharide samples (1 - mannose, 2 - rhamnose, 3 - glucuronic acid, 4 - galacturonic acid, 5 - glucose, 6 - galactose, 7 - xylose, 8 - arabinose, 9 - fucose) and (b) hydrolysate of exopolysaccharide

anion radicals, it also exhibited remarkable cytotoxicities against K562, A549, HL-60, and HeLa cells. Then, the extraction conditions for EPS, including precipitation time (h), precipitation temperature (°C), pH, and ratio of ethanol to fermented broth (L/L), were optimized by RSM-BBD. Under optimum conditions (time of 16 h, temperature of 3.7°C, pH 7.2 and ratio of 5:1 (L/L)), the experimental yield of EPS was 162 ± 6 μg/L which matched the predicted one (165 μg/L). The results indicated that the established model was adequate and precise. EPS comprised mannose, glucose, galactose xylose, and arabinose in a molar ratio of 25.6:16.5:1.0:3.8:5.4. In addition, the FT-IR spectrum of EPS

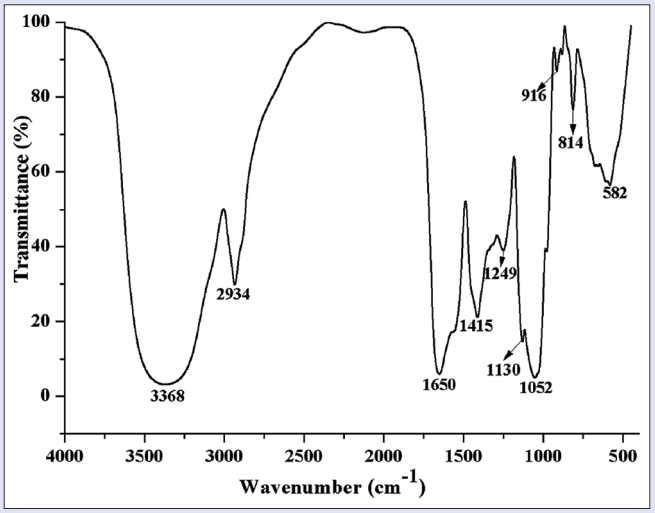

Figure 5: Fourier transform-infrared spectrum of exopolysaccharide in the range of 4000–400 cm^{-1}

showed a characteristic pyranose structure and that it mainly contained α-glycosidic bonds. To the best of our knowledge, EPS isolated from the endophytic fungus of *C. sativus* was studied for the first time. In summary, EPS is a promising substitute for *C. sativus* and also a potential novel bioactive source in pharmaceutical and food industries.

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Conflicts of interest

There are no conflicts of interest.

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