Optimization of extraction process and investigation of antioxidant effect of polysaccharides from the root of *Limonium sinense* Kuntze

Xinhui Tang[1], Lifang Yan[1,2], Jing Gao[2], Haiyan Ge[2], Haidong Yang[2], Na Lin[2]

[1]Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection, Yancheng Teachers University, Yancheng - 224002, [2]School of Pharmacy, Jiangsu University, Zhenjiang - 212013, People's Republic of China

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ABSTRACT

Objective: To optimize the extraction technology for polysaccharides from the root of *Limonium sinense* (Girard) Kuntze, Plumbaginaceae and evaluate the antioxidant capacity of polysaccharides from *L. sinense* (LSEP) **Materials and Methods**: One-singer factor and response surface methodology(RSM) were established to extract the polysaccharides from *L. sinense*. Then, the 1,1-diphenyl-2-picrylhydrazyl free radical, hydroxyl radical(.OH), and 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt free radical assays were established to measure the antioxidant capacity of the LSEP *in vitro*. **Results:** According to analysis, extraction temperature significantly affected extraction yield. The optimum extraction conditions for LSEP were as follows: extraction temperature, 95°C; ultrasonic time 50 minutes; and dosage liquor ratio, 1: 12. Under these conditions, the experimental yield of crude LSEP was 12.80±0.19% which was well matched with the predicted models. The antioxidant capacity data suggested that LSEP has strong antioxidant activity. **Conclusion:** One-singer factor and RSM were used to extract of LSEP are simple and feasible and LSEP could be developed as a nutraceutical agent for itsstrong antioxidant activity.

Key words: Antioxidant activities, optimization extraction, polysaccharide, response surface methodology

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INTRODUCTION

Limonium sinense (Girard) Kuntze, Plumbaginaceae, is a kind of herb mainly distributed along seashores and salts marshes in southern China, Ryukyus (Japan), and western Taiwan of China, and has been used traditionally for treating bleeding, piles, fever, hepatitis, diarrhea, bronchitis, and other disorders.[1] The hepatoprotective effect of *L. sinense* against carbon tetrachloride and D-galactosamine intoxication in rats was reported[2] and the mechanism underlying its hepatoprotective effects was found to be related to the mitochondrial protection.[3-5] Recently, obvious antitumor activity and immune modulation effect of *L. sinense* were also found.[6] And, the polysaccharides, which are known as compound to possess numerous biological activities, are one of the most abundant components in *L. sinense*. However, there was no report about the optimization of extraction conditions for polysaccharides from *L. sinense* (LSEP).

Response surface methodology (RSM) is an effective tool for optimizing the process, especially when many factors and interactions affect desired response. It has been successfully used for optimizing complex process, extraction technology, conditions of enzyme reaction, and so on.[7-9] The advantage of RSM is that it can reduce the number of experimental trials and evaluate the interactions between multiple parameters, which are less laborious and time-consuming than other optimizing process, and more effective and precise than many approaches.

In this paper, we aim to optimize the production process of LSEP from the *L. sinense* Kuntze root by RSM, employing a the Box-Behnken design (BBD) (3 factors and 3 levels) to study the effects of extraction temperature, ultrasonic time, and ratio of material to water on the crude extract of LSEP

Address for correspondence:
Prof. Jing Gao, School of Pharmacy, Jiangsu University, Zhenjiang, 212013, People's Republic of China.
E-mail: jinggao@ujs.edu.cn

from the root of *L. sinense* Kuntze. And then, the antioxidant activities were assayed by testing the scavenging abilities on hydroxyl radicals, 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), and 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radicals.

MATERIAL AND METHODS

Materials

The whole plant of *L. sinense* (Girard) Kuntze, Plumbaginaceae, was collected from the Yancheng Sea Beach in Jiangsu, P. R. China, and identified by Mr. Yu Yanqiu (Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection, Yancheng Teachers University) in December 2008. The root of *L. sinense* was washed and oven dried at 60°C until the moisture level was constant. D-glucose was from Amresco Inc. All other reagents were of analytical grade.

Extraction and determination of crude polysaccharides from *L. sinense*

The root of *L. sinense* were dried, crushed, and extracted under the ratio of it to water (1 : 8-1 : 16), ultrasonic time (20-80 minutes), extracting time (1-5 hours), temperature (60-100°C), and extraction times (1-4 times). The combined extraction were centrifuged at 5 000 r/minutes for 15 minutes, precipitated with 95% ethanol,[8] and then precipitated by the addition of dehydrated alcohol to a final concentration of 80 % (v/v). The precipitates (LSEP) collected by centrifugation (2 000 g for 10 minutes, at 20°C) were washed by dehydrated alcohol for three times and dried under reduced pressure. Sugar content was measured by phenol–sulfuric method using D-glucose as a standard. The purity of LSEP is calculated as the sugar content of extraction/dried crude polysaccharide weight.

Experimental design and statistical analysis

After determining the preliminary range of the extraction variables through a single-factor test, a central composite design (CCD) with four independent variables (X1, extraction temperature; X2, ultrasonic time; X3, ratio of material to distilled water) at three levels was performed for statistical calculation, and the variables were coded according to

$$\psi_i = (X_i - X_0)/\geq X_i \quad .....(1)$$

Where, ψ_i is a coded value of the variable; X_i the actual value of variable; X_0 the actual value of the X_i on the center point; and $\geq X_i$ the step change value. The range of independent variables and their levels are presented in Table 1, which was based on the results of preliminary experiments. The extraction yield of LSEP was the dependent variables. As seen from Table 2, the complete design consisted of 15 experimental points, and the experiment was carried out in a random order.

Table 1: Independent variables and their levels used in the response surface design

Independent various	Factor levels		
	-1	0	1
Extraction temperature (°C)	80	90	100
Ultrasonic time (min)	20	40	60
Ratio of material to water	1 : 10	1 : 12	1 : 14

Table 2: Response surface central composite design (uncoded) and results for extraction yield of LSEP

X_1 (Extraction temperature (°C)	X_2 (Ultrasonic time (min))	X_3 (Ratio of material to water)	Extraction yield (%)
-1 (80)	-1 (20)	0 (1 : 12)	7.728
-1 (80)	0 (40)	-1 (1 : 10)	6.082
-1 (80)	0 (40)	1 (1 : 14)	6.326
-1 (80)	1 (60)	0 (1 : 12)	6.926
0 (90)	-1 (20)	-1 (1 : 10)	10.976
0 (90)	-1 (20)	1 (1 : 14)	8.972
0 (90)	1 (60)	1 (1 : 14)	9.936
1 (100)	-1 (20)	0 (1 : 12)	9.552
1 (100)	0 (40)	-1 (1 : 10)	9.24
1 (100)	0 (40)	1 (1 : 14)	11.082
1 (100)	1 (60)	0 (1 : 12)	13.11
0 (90)	1 (60)	-1 (1 : 10)	9.852
0 (90)	0 (40)	0 (1 : 12)	12.96
0 (90)	0 (40)	0 (1 : 12)	12.88
0 (90)	0 (40)	0 (1 : 12)	12.94

Data from the CCD were analyzed by multiple regressions to fit the following quadratic polynomial model.

$$Y = \beta_{k0+} \sum_{i=1}^{3} \beta_{ki} X_i + \sum_{i=1}^{3} \beta_{kii} X_i^2 + \sum_{i<j=2}^{3} \beta_{kij} X_i X_j \quad .....(2)$$

Y represents the response function. β_{k0} is an intercept. Where, β_{ki}, β_{kii}, and β_{kij} are the coefficients of the linear, quadratic, and interactive terms, respectively. And, accordingly, xi and x_j represent the coded independent variables, respectively. The fitted polynomial equation is expressed as surface and contour plots in order to visualize the relationship between the response and experimental levels of each factor and to deduce the optimum conditions.[10] The analysis of variance tables were generated and the effect and regression coefficients of individual linear, quadratic, and interaction terms were determined. The regression coefficients were then used to make statistical calculation to generate dimensional and contour maps from the regression models. SAS (Version 8.0, USA) software package was used to analyze the experimental data. *P* values of less than 0.05 were considered to be statistically significant.

Assays for antioxidant activities

1, 1-diphenyl-2-picryl-hydrazyl radical scavenging assay

The free radical scavenging activity of the polysaccharides was measured by DPPH test according to the method of Shimada with some modifications.[11] Solution of DPPH in methanol (0.2 mmol/l) was prepared daily before Ultraviolet (UV) measurements. 0.1 ml of the polysaccharides of different addition quantities (10-100 μg/ml) in methanol was thoroughly mixed with 3.9 ml of freshly prepared DPPH. The mixture was shaken well, allowed to stand for 30 minutes in the dark, and the absorbance was then measured at 517 nm against a blank. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity, which was analyzed from the graph plotted of inhibition percentage against compound concentration. V_C was used as positive control. The experiment was carried out in triplicate. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Scavenging rate (\%)} = [1 - (A_i - A_j) / A_0] \times 100$$

Where, A_0 is the absorbance of DPPH solution without sample; A_i is the absorbance of the test sample mixed with DPPH solution; and A_j is the absorbance of the sample without DPPH solution.

2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical scavenging assay

For ABTS assay, the procedure followed the method of Du *et al*.[12] with some modifications. The stock solutions included 7 mmol/l ABTS+ solution and 2.45 mmol/l potassium per sulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 hours at room temperature in the dark. The solution diluted with methanol to obtain an absorbance at 734 nm was 0.70 ± 0.02 using the spectrophotometer. Fresh ABTS+ solution was prepared for each assay. LSEP (0.1 ml) were allowed to react with 3.9 ml of the ABTS+ solution for 6 minutes in a dark condition. Then, the absorbance was taken at 734 nm using the spectrophotometer.

$$\text{Scavenging rate (\%)} = [1 - (A_i - A_j) / A_0] \times 100$$

Where, A_0 is the absorbance of ABTS solution without sample; A_i is the absorbance of the test sample mixed with ABTS solution; and A_j is the absorbance of the sample without ABTS solution.

Hydroxyl radical scavenging assay

The assay was measured by the method of Rosales-Castro M[13] with a minor modification. Polysaccharides were dissolved in deionized water at the concentration of 0.1 to 1 mg/ml. The sample solution (2 ml) was mixed with 6 mmol/l of FeSO4 solution (2 ml) and 6 mmol/l H_2O_2 (2 ml) was then added to the reaction solution. The reaction solution was incubated for 10 minutes in room and then added 6 mmol/l of salicylic acid (2 ml) and incubated for 30 minutes. The absorbance of the mixture was measured at 510 nm against blank. The capability to scavenge hydroxyl radical was calculated using the following equation:

$$\text{Scavenging rate (\%)} = [1-(A_i-A_j)/A_0]\times 100$$

Where, A_0 is the absorbance of mixture solution without sample; A_i is the absorbance of the test sample mixed with reaction solution; and A_j is the absorbance of the test sample without salicylic acid.

Statistical analysis

All the data were shown in $\overline{X} \pm$ SD. Statistical significance was analyzed by one-way analysis of variance (ANOVA). *P* value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Optimization of extraction conditions

Effect of ratio of material to water on the extraction yield of polysaccharides from L. sinense

The yield of LSEP effected by different ratio of material to water from 1 : 8 to 1 : 16 was shown in Figure 1a, while other extraction variables were set as follows: ultrasonic time, 20 minutes; extraction time, 2 hours; extraction temperature, 100°C; and number of extraction, 2 times. The extraction yields of the polysaccharides increased with the ratio increasing from 1 : 8 to 1 : 16, and then it tended to go steady. The more water was used, the more ethanol was exploited to precipitate LSEP. Therefore, extraction ratio 1 : 10 to 1 : 14 was favorable for polysaccharides production.

Effect of ultrasonic time on the yield of polysaccharides from L. sinense

The yield of LSEP affected by different ultrasonic time (20, 40, 60, and 80 minutes) was shown in Figure 1b, when other extraction variables were set as follows: ratio of material to water, 1 : 12; extraction time, 2 hours; extraction temperature, 100°C; and number of extraction, 2 times. The yield of LSEP reached a maximum when the ultrasonic time was 60 minutes. After this point, the yield of LSEP started to a descending with increasing the ultrasonic time. This situation maybe due to the polysaccharide hydrolyses under some temperature with long ultrasonic time. Therefore, ultrasonic time range of 40 to 80 minutes was adopted in this experiment.

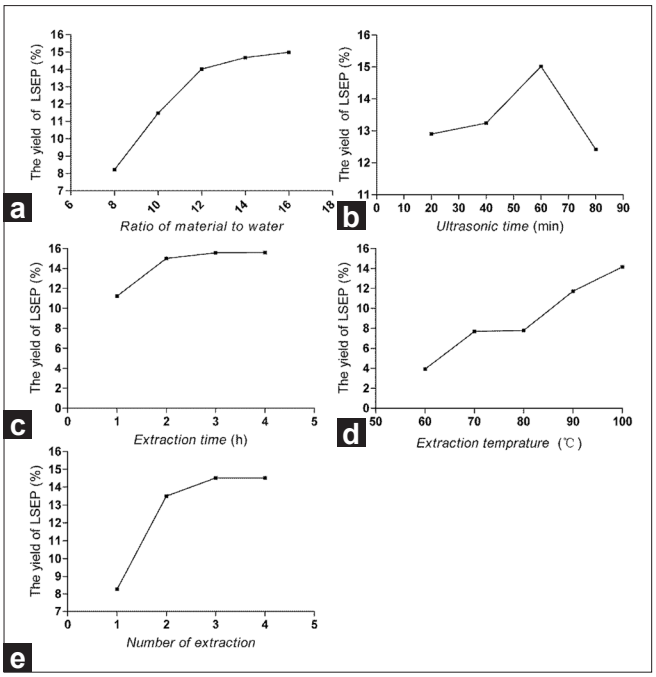

Figure 1: Effect of different extraction parameters on the yield of LSEP (ratio of material to water; ultrasonic time; extraction time; extraction temperature, and number of extraction)

Effect of extraction time on the yield of polysaccharides from L. sinense

The yield of LSEP affected by different extraction time (1, 2, 3, and 4 hours) was shown in Figure 1c, while other experimental conditions were as follows: ratio of material to water, 1 : 12; ultrasonic time, 20 minutes; extraction temperature, 100°C; and number of extraction, 2 times. The extraction yields of the polysaccharides increased with the increasing extraction time, and then it tended to go steady; however, considering the time and the economic benefits, the extraction time of 2 hours was selected as the optimal in the present experiment.

Effect of temperature on the yield of polysaccharides from L. sinense

As shown in Figure 1d, the effect of temperature on extraction yield was investigated. Different extraction temperature was set at 60, 70, 80, 90, and 100°C, while the other factors were set as follows: ratio of material to water, 1 : 12; ultrasonic time, 20 minutes; extraction time, 2 hours; and number of extraction, 2 times. The yield of LSEP increased with the increasing temperature and reached the peak value at 100°C, while the color is deepened from the extracted solution when the temperature was above 100°C. It is speculate that high temperature may affect the polysaccharide structure and activity, i.e., and the temperature (more than 100°C) easily decomposed polysaccharides to monosaccharide,which can impact the extraction rate. Therefore, 90-100°C was considered to be optimal extraction temperature in this experiment.

Effect of extraction number on the yield of polysaccharides from L. sinense

The yield of LSEP affected by different number of extraction (1-4 times) was shown in Figure 1e, while other experimental conditions were as follows: ratio of material to water, 1 : 12; ultrasonic time, 20 minutes; extraction time, 2 hours; extraction temperature, 100°C. The yield of LSEP unexpectedly gets the point when number of extraction is from 1 to 3 times; however, the yield of LSEP no longer obviously changed with the increasing number of extraction. However, considering the economic benefits and the time, 2 times was selected as the optimal extraction number in the present experiment.

According to the single-parameter study, we adopted extraction temperature of 80 to 100°C; ultrasonic time, 20 to 60 minutes; and ratio of material to water, 1 : 10 to 1 : 14 for RSM experiments.

Predicted model and statistical analysis

The design material and the corresponding results of RSM experiments to determine the effects of the three independent variables including extraction temperature (X_1); ultrasonic time (X_2); and ratio of material to water (X_3) are shown in Table 3. By employing multiple regression analysis on the experiment data, the predicted model was obtained by the following second-order polynomial function:

$$Y = 12.96 + 1.99X_1 + 0.35X_2 - 1.75E3X_3 + 1.09X_1X_2 + 0.40X_1X_3 + 0.57X_2X_3 - 2.67X_1^2 - 0.96X_2^2 - 2.11X_3^2$$

The fit statistics of extraction yield (Y) for the selected quadratic predictive model is shown in Table 3. The dependent variable and the independent variables were highly significant ($R^2 = 0.9624$), which indicated this experimental method is reliable. It suggests the regression equation can instead of the true test to analyze the experimental results. The ANOVA analysis is also shown in Table 3. *P* value is 0.0046 and the *P* values were used as a tool to check the significances of each coefficient. The smaller the *P* value was, the more significant the corresponding coefficient was.[14] It can be seen that the variable with the largest effect was the interaction effects of extraction temperature to ultrasonic time ($X_1 \times X_2$), followed by the linear terms of extraction time (X_1, $X_1 \times X_1$, and $X_3 \times X_3$). The other term coefficients (X_2, X_3, $X_1 \times X_3$, $X_2 \times X_3$, and $X_2 \times X_2$) were not influential ($P>0.05$).

Response surface plot and contour plot

3D response surface and 2D contour plots were the graphical representations of regression function. They showed the type of interactions between two tested variables and the relationship between responses and

Table 3: Regression analysis analyzed by one-way analysis of variance for responses surface quadratic model analysis of variance table (Practical sum of squares-Type)

Source	Sum of squares	df	Mean square	F value	*P* value Prob.>F	
Model	80.55	9	8.95	14.23	0.0046	Significant
A - Extraction temperature	31.69	1	31.69	50.38	0.0009	
B - Ultrasonic time	0.96	1	0.96	1.53	0.2708	
C - Ratio of material to water	2.45E-05	1	2.45E-05	3.90E-05	9.95E-01	
AB	4.75	1	4.75	7.56	0.0404	
AC	0.64	1	0.64	1.02	0.3599	
BC	1.29	1	1.29	2.04	0.2121	
A^2	26.31	1	26.31	41.83	0.0013	
B^2	3.42	1	3.42	5.44	0.067	
C^2	16.43	1	16.43	26.12	0.0037	
Residual	3.14	5	0.63			
Lack of fit	3.13	3	1.04	118.38	0.0084	Significant
Pure error	0.018	2	8.81E-03			
Cor. Total	83.69	14				

experiment levels of each variable. Different shapes of the contour plots indicated different interactions between the variables. Circular contour plot indicated that the interactions between the corresponding variables were negligible, while elliptical contour plot indicated otherwise. In the present study, the response surface and contour plots were obtained by using SAS version 8.0 and are presented in Figure 2. As shown in Figure 2a, when extraction temperature (X_1) was fixed, ultrasonic time (X_2) and ratio of material to water(X_3) demonstrated quadratic effects on the extraction yields. The elliptical contour plot shown in Figure 2d indicated that the mutual interactions between ultrasonic time and ratio of material to water were significant. Figure 2b and Figure 2e show that when ultrasonic time (X_2) was fixed, the variations of yields were negligible with the extraction time increase. Figure 2c shows that when ratio of material to water (X_3) was fixed and ultrasonic time kept at lower level, the yield increased at first and then decreased with the increase of temperature (X_1). From Figure 2f, it indicated that the mutual interactions between temperature and ultrasonic time were much significant. By analyzing the plots, the predicted values (13.5%) of the tested variables for polysaccharides lied in the following condition: extraction temperature, 95°C; ultrasonic time, 50 minutes; and ratio of material to water, 1 : 12. In the optimal conditions, the experiment yield of crude LSEP was 13.0 %, which agreed with the predicted value. Therefore, the results indicated suitability of the model employed and the success of RSM in optimizing the extraction conditions.

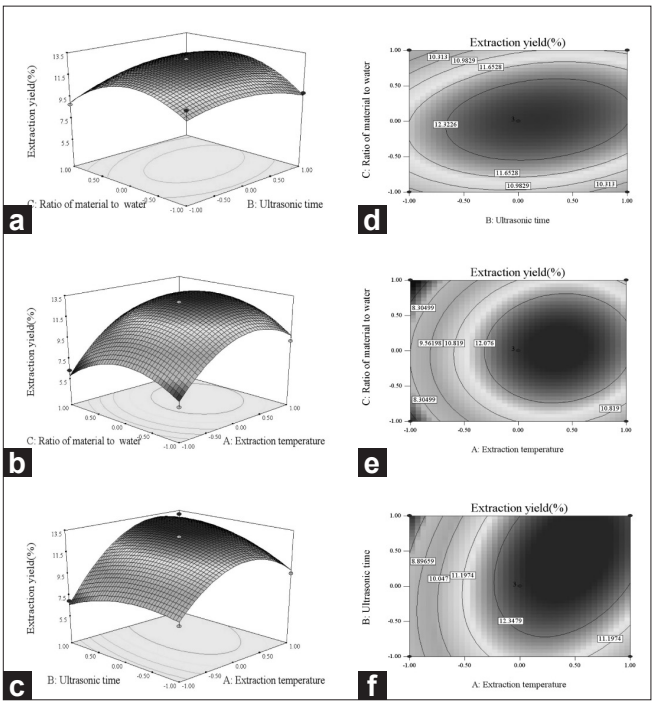

Figure 2: (a-c) Response surface plots for the effects of (a) ultrasonic time and ratio of material to water; (b) extraction temperature and ratio of material to water; (c) extraction temperature and ultrasonic time on the yield of polysaccharide; (d-f) Contour plots for the effects of (d) ultrasonic time and ratio of material to water; (e) extraction temperature and ratio of material to water; (f) extraction temperature and ultrasonic time on the yield of polysaccharide

Verification of predictive model

The suitability of the model equations for predicting optimum response values was tested under the following conditions: extraction temperature, 95°C; ultrasonic time, 50 minutes; and ratio of material to water, 1 : 12. This set of conditions was determined to be optimum by the RSM optimization approach and was also used to validate experimentally and predict the values of the responses using the model equation. A mean value of 12.80 ± 0.19

(%) (n = 3), obtained from real experiments, demonstrated the validation of the RSM model, indicating that the model was adequate for the extraction process.

Antioxidant activity analysis

Oxidation is essential to many organisms for the production of energy in biological processes.[15] Reactive oxygen species,[13] in the forms of superoxide anion ($.O_2^-$), hydroxyl radical (.OH), and hydrogen peroxide (H_2O_2), are generated by normal metabolic processes or from exogenous factors and agents that can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides.[16] Polysaccharides have exhibited strong antioxidant properties and can be developed as novel potential antioxidants.[17] Antioxidant activities of LSEP were assayed by using the models of scavenging DPPH, ABTS, and hydroxyl radicals.

Scavenging activity of 1, 1-diphenyl-2-picryl-hydrazyl radicals

In the DPPH test, the antioxidants are able to reduce the stable DPPH radical to the yellow-colored diphenylpicrylhydrazine. The scavenging ability of the samples is shown in Figure 3. Figure 3a shows that the scavenging rate of LSEP on the DPPH radical increased dose-dependently and up to 97% at a dose of 80 μg/ml, which is similar to 100 μg/ml V_C.

Scavenging activity of 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radicals

The scavenging ability of the samples on ABTS radicals is shown in Figure 3. Figure 3b shows that the scavenging effects of LSEP on the ABTS radicals increased in a concentration-dependent manner. The scavenging effects of 600 μg/ml LSEP (97 %) was higher than those of 100 g/ml V_C (60%), which suggested that LSEP had a strong antioxidant activity.

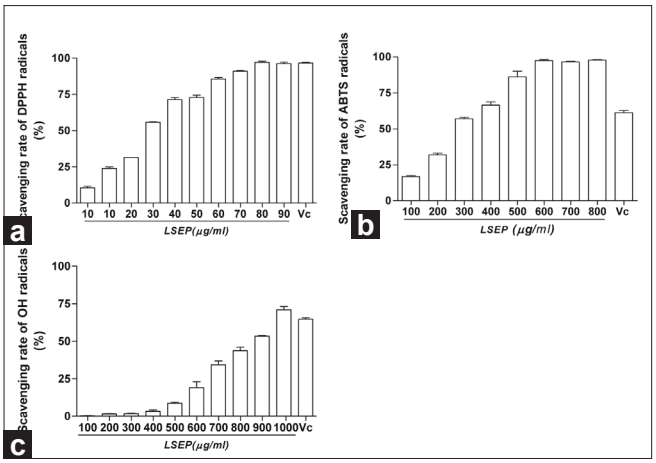

Figure 3: Antioxidant effect of LSEP against different kind of free radicals. (a) scavenging activity of 1, 1-diphenyl-2-picryl-hydrazyl radicals; (b) scavenging activity of 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radicals; (c) scavenging activity of hydroxyl radicals; data are presented as mean values (n = 3)

Scavenging activity of hydroxyl radicals

The scavenging effect of LSEP on hydroxyl radicals is shown in Figure 3c. The scavenging effect of LSEP increased with increasing concentration. The scavenging rate of 1 mg/ml LSEP was 70%, which is similar to the effect of V_C (1 mg/ml).

CONCLUSIONS

The single-factor experiments and BBD along with RSM were applied for optimizing extraction LSEP in this study. The optimal conditions for the production of polysaccharide were as follows: extraction temperature, 95°C; ultrasonic time, 50 minutes; and ratio of material to water, 1 : 12. In the optimal conditions, the experiment yield of LSEP was 12.80 ± 0.19 (%), which was agreed with the predicted value. The experimental conditions allow a fast and cost-saving process in extraction of polysaccharide from the root of *L. sinense* Kuntze. And, at the same time, LSEP showed obvious scavenging activities on DPPH, ABTS, and hydroxyl radicals, which indicated that LSEP had antioxidant activity against various reactive oxygen species (ROS) and could be developed in the near future.

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