

Optimization of extraction of crude polysaccharide from Cassia seed via ultrasonic assisted cellulase and study on its antioxidant activity

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Abstract. In order to improve the current low extraction rate of cassia polysaccharide and improve its extraction efficiency, the ultrasonic wave assisted cellulase extraction method of cassia polysaccharide is first proposed and optimized. Orthogonal test is conducted to analyze the influence of six factors related to cellulase and ultrasonic wave on the extraction of cassia polysaccharide, and at the same time to explore the antioxidant activity of the extracted polysaccharide in vitro. The results show that the main and secondary factors affecting the extraction rate of cassia polysaccharide are pH > ultrasonic power > enzyme dosage > enzymatic hydrolysis temperature > enzymatic hydrolysis time > ultrasonic time. The optimal conditions are: enzyme dosage: 1.6%, enzymatic hydrolysis time: 80 min, enzymatic hydrolysis temperature: 52 °C, enzymatic hydrolysis pH: 6.4, ultrasonic time: 30 min, ultrasonic power: 276 W, the polysaccharide yield under the optimal conditions is 12.29% ± 0.21%, which is 5.31% and 14.43% higher than the single enzyme extraction method and ultrasonic extraction method reported in the literature, respectively, indicating that the composite extraction method is superior to the single extraction method. The antioxidant activity analysis shows that the half inhibitory concentrations of cassia polysaccharide against DPPH·, ABTS·+, ·OH, ·O₂- free radicals are 0.894 mg/mL, 1.175 mg/mL, 1.908 mg/mL and 7.601 mg/mL, respectively, which shows good antioxidant activity in vitro. Therefore, ultrasonic assisted cellulase extraction of crude polysaccharide from cassia seed is a new efficient and rapid extraction method.

Keywords: Cassia seed; Polysaccharides; Cellulase; Ultrasonic assisted extraction; Anti-oxidation; Process optimization

1. Introduction

Cassia seed is a homology of medicine and food which is first recorded in Shennong Materia Medica Classic [1, 2]. It has various active functions such as clearing liver and improving eyesight, purging bowel, diuresis, lowering blood pressure, lowering blood lipids, anti-oxidation, anti-atherosclerosis and treating diabetes [3, 4, 5]. Among the many chemical components of cassia seed, polysaccharide has a considerable amount [6, 7], which plays a crucial role in the pharmacological and physiological effects of cassia seed, such as antioxidant, immune regulation, lipid-lowering, anti-tumor and other biological activities [6, 8], among which antioxidant activity is more significant [9]. However, with the deepening of the research on cassia polysaccharide, its low extraction rate, low purity [10] and easy structure destruction [15] have become the main problems in the current research on cassia polysaccharide.

Generally speaking, changing the extraction method will change the extraction rate of cassia polysaccharide [11, 12]. Currently, five extraction methods for cassia polysaccharide are water extraction alcohol extraction, enzyme extraction, high pressure assisted extraction, microwave assisted extraction and ultrasonic assisted extraction [2, 10, 13], among which water extraction alcohol extraction is the most widely used. The enzyme extraction method is first proposed and applied by Feng et al. [14] in 2018, and the yield of most polysaccharides is between 7% and 14%, with a maximum yield of 18.6% [2]. According to previous studies [2, 10, 13], there are many researches on the single extraction method of cassia seed polysaccharide, but few researches on the

combination of two or more extraction methods of cassia seed polysaccharide. Based on the research theory of Jiang & Kang [15, 17], this study for the first time combines enzyme extraction method and ultrasonic extraction method to extract polysaccharide from cassia seed. Orthogonal test is carried out to determine the optimum extraction conditions of polysaccharide from cassia seed, and its antioxidant activity in vitro is investigated. It will provide scientific basis and theoretical basis for the further development and application of cassia seed and its polysaccharide in the field of antioxidant in the future.

2. Materials and methods

2.1 Materials and instruments

Materials: Cassia seed; 95% anhydrous ethanol; Cellulase; Citric acid; Sodium citrate; Phenol; Concentrated sulfuric acid; Glucose standard; 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH); ABTS; Potassium persulfate; Potassium chloride; Sodium chloride; Disodium hydrogen phosphate; Potassium dihydrogen phosphate; Ferrous sulfate; Hydrogen peroxide; Phenanthroline; Tris; Hydrochloric acid; Na₂EDTA; Pyrogallol

Instruments: PL203/01 electronic analytical balance; KDM type temperature control heating sleeve; Electric thermostatic water bath; Ultrasonic cell shredder; RE-52AA rotary evaporator; SHZ-D (III) circulating water type multi-purpose vacuum pump; SC-3614 low speed centrifuge; FD-1A-50 vacuum freeze dryer; Multifunctional enzyme marker; TU-1810 UV-visible spectrophotometer; MP522 pH meter.

2.2 Extraction of crude polysaccharide from cassia seed

The dried cassia seed was crushed through a 90-mesh sieve, and the sieved powder was added into 80% (v/v) ethanol at a solid-liquid ratio of 1:10 (m/v) for refluxing and degreasing for 120 min [8]. After drying, 3.00 g dried defatted cassia seed powder was accurately weighed and added to cellulase aqueous solution at a certain pH at a ratio of 1 to 20 (m/v). After mixing, it was enzymolized at a specific temperature for a period of time. After that, the enzyme was inactivated by high-temperature water bath for 10 min, cooled to normal temperature, sonicated, centrifuged at 4500 r/min for 10 min, took the supernatant, concentrated by rotary evaporation at 75 °C and 90 r/min for 10 min, and the volume of concentrated liquid was recorded as V. At 3: 7 (v/v) volume ratio: added anhydrous ethanol to the concentrated liquid, mixed well and left it in the refrigerator at 4 °C for 12 h, centrifuged at 4500 r/min for 10 min and discarded the supernatant, added anhydrous ethanol at 3 V volume to the precipitation and stirred well, centrifuged at 4500 r/min for 15 min to retain the precipitation, and dissolved the precipitation with water. The crude polysaccharide of cassia seed was obtained by freeze-drying at -50 °C [15].

2.3 Determination of total sugar content of cassia seed

2.3.1 Drawing of glucose curve

Phenol-concentrated sulfuric acid method was used [16]. Absorbed 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 mL of glucose standard solution (0.1 mg/mL) into a centrifuge tube, added distilled water to 1 mL, shook well, then added 0.5 mL phenol solution of 50 mg/mL and 2.5 mL concentrated sulfuric acid in turn. After the mixture was mixed and reacted at normal temperature for 30 min, 200 µL was removed into 96-well plate in order to determine its absorbance at 490 nm, and no glucose solution was added as a blank control. Each group of tests was parallel three times, and the glucose standard curve was drawn, as shown in Fig. 1.

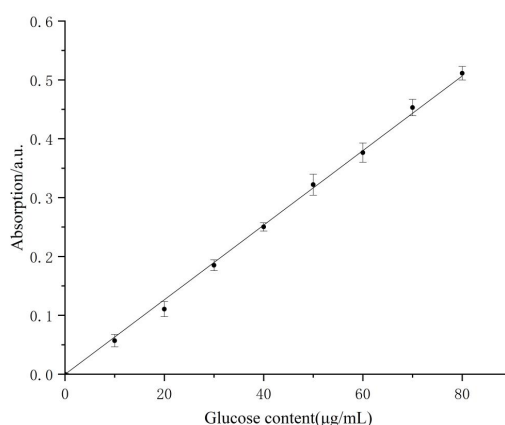


Fig. 1. Glucose standard curve

According to the glucose standard curve in Figure 1, regression equation $y=0.0065x-0.0084$, $R^2=0.9988$ was obtained.

2.3.2 Determination of total sugar content

The method of determining the total sugar content involved phenol-concentrated sulfuric acid [16]. 0.5 mL crude polysaccharide solution of cassia seed was placed in a centrifuge tube, distilled water was added to 1 mL, and then shook well, 0.5 mL phenol solution of 50 mg/mL and 2.5 mL concentrated sulfuric acid were added successively. After mixing, reacted at normal temperature for 30 min, 200 μ L was removed by pipetting gun and placed in a 96-well plate to determine its absorbance at 490 nm. The yield of polysaccharide (Y_p) from cassia seed was calculated as follows:

$$Y_p = \frac{CV_1m_2n}{m_3m_1} \times 100\% \quad (1)$$

Where: C is the calculated polysaccharide concentration, g/mL; V_1 is the constant volume of crude polysaccharide solution, mL; m_1 is the mass of defatted cassia powder weighed, g; m_2 is the quality of extracted crude polysaccharide, g; m_3 is the mass of weighed crude polysaccharide, g; n is the dilution ratio of crude polysaccharide solution.

2.4 Optimization of extraction technology by orthogonal experiment

With reference to the extraction factor design method proposed by Jiang & Kang et al. [15, 17] and modified, the orthogonal experiment was designed with cellulase dosage (A), enzymolysis time (B), enzymolysis temperature (C), enzymolysis pH (D), ultrasonic auxiliary time (E) and ultrasonic power (F) as independent variables and polysaccharide yield as index, as shown in Table 1. Each group of experiments was conducted in parallel for 3 times. The results were expressed as the mean \pm standard deviation.

Table 1 Orthogonal test factor level table

Level	A/%	B/min	C/ $^{\circ}$ C	D	E/min	F/W
1	0.8	40	44	4	15	168
2	1.2	60	48	4.6	20	195
3	1.6	80	52	5.2	25	222
4	2	100	56	5.8	30	249
5	2.4	120	60	6.4	35	276

2.5 Analysis of antioxidant activity

2.5.1 Detection of DPPH free radical scavenging ability of cassia crude polysaccharide

Referred to the method of Chen et al. [18] and made adjustments. The crude polysaccharides were prepared into sample solutions with different concentrations of 0.2, 0.4, 0.8, 1.2, 1.6, 2.0 and 2.4 mg/mL. The 3.0 mL sample solution and 1.0 mL DPPH ethanol solution (0.1 mmol/mL) were added into the centrifuge tube successively and mixed, and after standing for 30 min of light avoidance at normal temperature. 200 μ L was removed successively into the 96-well plate, and the absorbance A1 at 517 nm [19] was detected. The absorbance A2 and A0 of ethanol sample solution and DPPH ethanol solution plus distilled water were determined by the same method. The scavenging activity (SA1) was determined by the formula (2).

$$SA_1(\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\% \quad (2)$$

2.5.2 Detection of ABTS cationic free radical scavenging ability of cassia crude polysaccharide

Referred to the method of Han et al. [20]. ABTS cationic free radical mother liquor was obtained by mixing 7.0 mmol/L ABTS solution with 2.45 mmol/L potassium persulfate solution 1:1 and standing at normal temperature for 12-14 h in the dark. Diluted the mother solution with a pH 7.4 phosphate buffer so that it had an absorbance of 0.700 ± 0.02 at 734 nm. The crude polysaccharide was prepared into sample solutions with different concentrations of 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 3.0, 3.5 and 4.0 mg/mL, and 1 mL of each was taken into a centrifuge tube, followed by 4 mL of ABTS cationic free radical diluent. After shaking for 30 s, it was left for 10 min at normal temperature away from light. The absorbance value A_i was detected at 734 nm [21]. The absorbance value A0 of distilled water instead of sample solution was determined by the same method. The scavenging activity (SA2) was determined by the formula (3).

$$SA_2(\%) = \frac{A_0 - A_i}{A_0} \times 100\% \quad (3)$$

2.5.3 Detection of hydroxyl radical scavenging ability of cassia crude polysaccharide

The Phenanthroline-Fe²⁺ method was used [22]. The crude polysaccharide was prepared into sample solutions with different concentrations of 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 3.0, 3.5 mg/mL, and 0.5 mL Phenanthroline solution (1 mmol/L), 1 mL phosphate buffer (pH 7.4), and 1.5 mL sample solution were successively added into the centrifugation tube. Shook well, added 0.5 mL FeSO₄ solution (1 mmol/L), mixed well, added 0.5 mL hydrogen peroxide solution (3.0 mmol/L), and reacted for 60 min at 37 °C in a water bath, then removed 200 μ L successively into the 96-well plate to detect the absorbance value A_S at 536 nm. The same method was used to determine the absorbance value (A_P) of the sample solution replaced by distilled water and the absorbance value (A_B) of distilled water instead of hydrogen peroxide solution. The percentage of scavenging activity (SA3) was calculated according to the formula (4).

$$SA_3(\%) = \frac{A_S - A_P}{A_B - A_P} \times 100\% \quad (4)$$

2.5.4 Detection of scavenging ability of crude polysaccharide of cassia seed

Pyrogallol autooxidation method was used [23]. The crude polysaccharide was prepared into sample solutions with different concentrations of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10.0 mg/mL. 1 mL sample solution, 2.5 mL Tris-HCl buffer solution (containing 1 mmol/L Na₂EDTA, pH 7.4, 0.05 mol/L) and 0.1 mL pyrogallol solution (60 mmol/L, dissolved in 1 mmol/L hydrochloric acid) were added into the quartz colorimetric plate successively. After mixing, Started the timer, checked the absorbance value at 325 nm every 30 s to 300 s, recorded A₀, 30 S and A₀, 300 s, the difference between the two was ΔA_S ; The absorbance value of 3.5 mL Tris-HCl buffer and 0.1 mL pyrocatechol solution as blank controls was determined by the same method, and A₀, 30 S and A₀, 300 s were recorded, and the difference between the two was ΔA_0 . The percentage of scavenging activity (SA4) was calculated by using the formula (7).

$$\Delta A_S = A_{S, 300s} - A_{S, 30s} \quad (5)$$

$$\Delta A_0 = A_{0, 300s} - A_{0, 30s} \#(6)$$

$$SA_4(\%) = \frac{\Delta A_0 - \Delta A_5}{\Delta A_0} \times 100\% \#(7)$$

2.6 Data processing

Microsoft Excel software was used for orthogonal experiment design and data processing, and SPSS 26.0 was used to analyze the difference significance of factors. The glucose standard curve and antioxidant correlation curve were plotted using Origin 2018.

3. Results and discussion

3.1 Orthogonal test

According to the study of Jiang & Kang et al. [15, 17] and slightly adjusted, the yield of cassia polysaccharide was taken as the evaluation index, and the effects of cellulase dosage, enzymatic hydrolysis time, enzymatic hydrolysis temperature, enzymatic hydrolysis pH, ultrasonic time and ultrasonic power on the yield of cassia polysaccharide were investigated by L25 (56) orthogonal test to determine the optimal process. The results of orthogonal test and range analysis were shown in Table 2, and the analysis of variance was shown in Table 3.

Table 2 Results and analysis of orthogonal experiment

Test	A/%	B/min	C/°C	D	E/min	F/W	Yp/%
1	1	1	1	1	1	1	4.23 ± 0.09
2	1	2	2	2	2	2	6.32 ± 0.32
3	1	3	3	3	3	3	7.98 ± 0.57
4	1	4	4	4	4	4	8.36 ± 0.19
5	1	5	5	5	5	5	10.10 ± 0.54
6	2	1	2	3	4	5	8.41 ± 0.00
7	2	2	3	4	5	1	9.70 ± 0.20
8	2	3	4	5	1	2	9.83 ± 0.16
9	2	4	5	1	2	3	5.03 ± 0.13
10	2	5	1	2	3	4	6.55 ± 0.06
11	3	1	3	5	2	4	10.34 ± 0.29
12	3	2	4	1	3	5	7.13 ± 0.39
13	3	3	5	2	4	1	6.94 ± 0.02
14	3	4	1	3	5	2	8.08 ± 0.36
15	3	5	2	4	1	3	9.76 ± 0.48
16	4	1	4	2	5	3	6.66 ± 0.17
17	4	2	5	3	1	4	6.96 ± 0.08
18	4	3	1	4	2	5	10.67 ± 0.32
19	4	4	2	5	3	1	8.60 ± 0.43
20	4	5	3	1	4	2	6.71 ± 0.07
21	5	1	5	4	3	2	8.28 ± 0.17
22	5	2	1	5	4	3	9.36 ± 0.22
23	5	3	2	1	5	4	4.75 ± 0.11
24	5	4	3	2	1	5	7.47 ± 0.13
25	5	5	4	3	2	1	7.01 ± 0.11
k1	7.40	7.58	7.78	5.57	7.65	7.30	
k2	7.90	7.90	7.57	6.79	7.88	7.84	
k3	8.45	8.03	8.44	7.69	7.71	7.76	
k4	7.92	7.51	7.80	9.36	7.95	7.39	
k5	7.37	8.02	7.46	9.65	7.86	8.76	
R	10.77	5.28	9.77	40.78	3.05	14.59	

Table 3 Analysis of variance of orthogonal test

Factor	SS	df	MS	F	P	Sig.
A	7.897	4	1.974	25.972	<0.0001	**
B	2.472	4	0.618	8.132	<0.001	**
C	5.773	4	1.443	18.989	<0.0001	**
D	118.471	4	29.618	389.653	<0.0001	**
E	0.638	4	0.159	2.097	0.11126	
F	13.376	4	3.344	43.994	<0.0001	**
error	1.900	25	0.076			

Where: “**” indicates that the difference is statistically significant ($P < 0.01$)

According to Table 2, the main order of influence on polysaccharide extraction rate was pH > ultrasonic power > enzyme dosage > enzymatic hydrolysis temperature > enzymatic hydrolysis time > ultrasonic time. The optimal combination was A3B3C3D5E4F5, that was, the amount of cellulase was 1.6%, the enzymatic hydrolysis time was 80 min, the enzymatic hydrolysis temperature was 52 °C, the enzymatic hydrolysis pH was 6.4, the ultrasonic time was 30 min, and the ultrasonic power was 276 W. As shown by variance analysis in Table 3, except that ultrasound time had no significant effect on polysaccharide yield ($P > 0.05$), other factors had significant effects on polysaccharide yield ($P < 0.01$). The scheme was verified and three parallel tests were carried out under the above optimal extraction process conditions. The average yield of the three extracts was $12.29 \pm 0.21\%$, which was generally higher than the yield of some common extraction methods reported previously [2], and the extraction yield of cassia polysaccharide under this condition was higher than the highest value $10.67 \pm 0.32\%$ in the orthogonal test scheme, indicating that the optimization scheme was feasible. Meanwhile, compared with 11.67% of enzyme extraction method proposed by Jiang [15] and 10.74% of ultrasonic extraction method proposed by Kang et al. [17], the results were increased by 5.31% and 14.43%, respectively, indicating that the composite extraction method was more beneficial to the extraction of cassia polysaccharide than the single extraction method.

3.2 Antioxidant activity

2.2.1 Scavenging effect of crude polysaccharide from *cassia* seed on DPPH·

As shown in Fig. 2, in the concentration range of 0.2-1.2 mg/mL, the scavenging effect of crude polysaccharide of cassia seed on DPPH· was positively correlated with the mass concentration of crude polysaccharide. When the concentration of crude polysaccharide was 1.2 mg/mL, the DPPH· clearance rate was $60.24\% \pm 0.98\%$, reaching a peak value, and then becoming stable with the increase of concentration. The polynomial fitting was carried out for the crude polysaccharide within the concentration of 0.2-1.2 mg/mL, and the fitting equation was $y = 41.81x^3 - 87.845x^2 + 78.931x + 19.772$, $R^2 = 0.9999$. The median inhibitory concentration of crude polysaccharide of cassia seed on DPPH· was calculated by the fitting equation as 0.894 mg/mL.

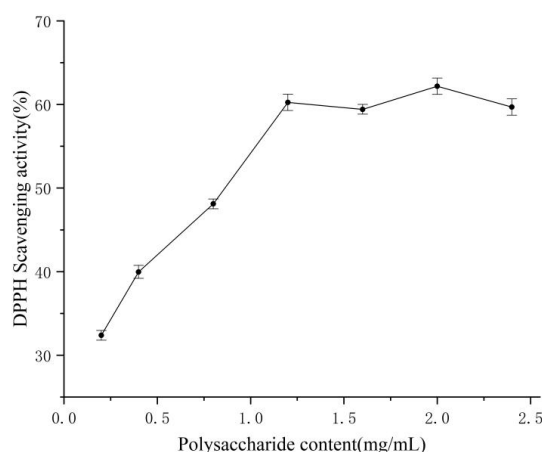


Fig. 2. Clearance of DPPH· by different concentrations of cassia polysaccharide

2.2.2 Scavenging effect of crude polysaccharide from *cassia* seed on ABTS·⁺

As shown in Fig. 3, the scavenging effect of cassia crude polysaccharide on ABTS·⁺ was positively correlated with the mass concentration of crude polysaccharide. When the mass concentration reached 3.0 mg/mL, the scavenging rate of ABTS·⁺ was $85.20\% \pm 0.26\%$, which reached the maximum value, and then tended to be stable with the increase of the concentration. The polynomial fitting of ABTS·⁺ was performed within the concentration of 0.2-2.4 mg/mL, and the fitting equation was $y = -7.2351x^2 + 49.806x + 1.4506$, $R^2 = 0.9998$. The half-inhibitory concentration of cassia crude polysaccharide on ABTS·⁺ was calculated by the fitting equation as 1.175 mg/mL.

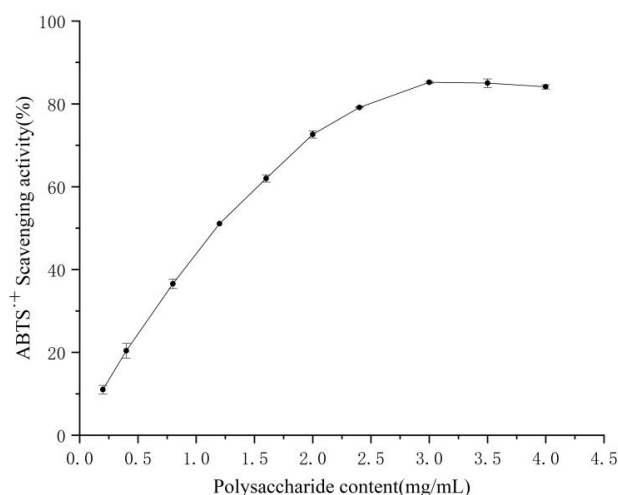


Fig. 3. The clearance rate of cassia polysaccharide to ABTS·⁺ at different concentrations

2.2.3 Scavenging effect of crude polysaccharide from *cassia* seed on ·OH

As shown in Fig. 4, the scavenging effect of cassia crude polysaccharide on ·OH showed a significantly positive correlation with the mass concentration of crude polysaccharide. When the concentration of crude polysaccharide reached 3.5 mg/mL, the scavenging rate of ·OH was $99.06\% \pm 1.25\%$, and the scavenging effect tended to be complete. ·OH was fitted by polynomial, and the fitting equation was $y = -1.8641x^4 + 14.113x^3 - 32.819x^2 + 48.447x + 3.7136$, $R^2 = 0.9972$. By the fitting equation, the median inhibitory concentration of cassia polysaccharide on ·OH was calculated to be 1.908 mg/mL.

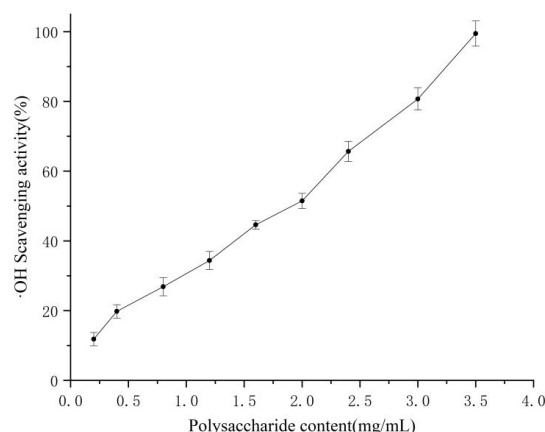


Fig. 4. The clearance rate of polysaccharide from cassia seed with different concentration to $\cdot\text{OH}$
 2.2.4 Scavenging effect of crude polysaccharide from *cassia* seed on $\cdot\text{O}_2^-$

As shown in Fig. 5, the scavenging effect of crude polysaccharide of cassia seed on $\cdot\text{O}_2^-$ showed a significantly positive correlation with the mass concentration of crude polysaccharide. When the concentration of crude polysaccharide reached 10 mg/mL, the clearance rate of $\cdot\text{O}_2^-$ was $58.68\% \pm 1.08\%$. $\cdot\text{O}_2^-$ was polynomial fitted in the concentration range of 1.0-8.0 mg/mL, and the fitting equation was

$y = -0.008x^6 + 0.2362x^5 - 2.6692x^4 + 14.616x^3 - 39.739x^2 + 53.556x - 20.067$, $R^2 = 0.9993$, By the fitting equation, the median inhibitory concentration of cassia polysaccharide on $\cdot\text{O}_2^-$ was 7.601 mg/mL.

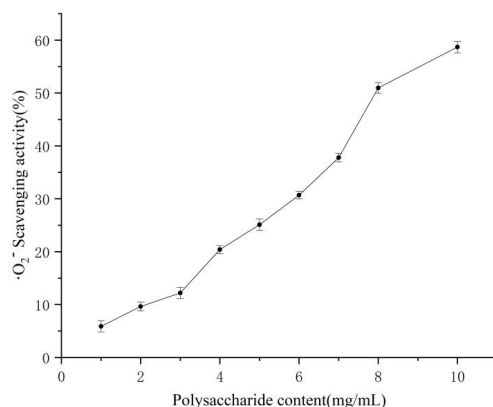


Fig. 5. The clearance of $\cdot\text{O}_2^-$ by different concentration of cassia polysaccharide

4. Conclusion

In this study, the ultrasonic assisted cellulase method is used to extract crude polysaccharide from cassia seed. Based on the research of Jiang & Kang et al. [15, 17], the factor level of orthogonal test is determined, and the optimal extraction process of crude polysaccharide from cassia seed is obtained through the range and variance analysis. The enzyme dosage is 1.6%, the enzymolysis time is 80 min, the enzymolysis temperature is 52 °C, the enzymolysis pH is 6.4, the ultrasonic time is 30 min, and the ultrasonic power is 276 W. Among the 6 factors, the ultrasonic time has no significant influence ($P > 0.05$), all the others have significant effects ($P < 0.01$), and the polysaccharide yield of cassia seed is $12.29\% \pm 0.21\%$, which is increased by 5.31% and 14.43% compared with the single enzyme extraction method proposed by Jiang et al. [15] and the single ultrasonic extraction method proposed by Kang et al. [17], further proving that the composite

extraction method is superior to the single extraction method. The scavenging effect of crude polysaccharide from cassia seed on DPPH·, ABTS·+, ·OH, ·O₂- has a certain dose effect within a specific concentration range [24,25]. At the same time, the half inhibitory concentration IC₅₀ of polysaccharides against these four kinds of free radicals are 0.894 mg/mL, 1.175 mg/mL, 1.908 mg/mL and 7.601 mg/mL, respectively. The maximum clearance rate of DPPH· and ABTS·+ at 1.2 mg/mL and 3.0 mg/mL, respectively, is 60.24% ± 0.98% and 85.20% ± 0.26% and tends to be stable, that is, it has a certain antioxidant saturation effect [26]. This study combines two extraction methods of crude polysaccharide from cassia seed, which provides the research basis and direction for the further development of polysaccharide extraction methods from cassia seed, and has certain guiding significance. However, the research on the separation and purification, molecular weight distribution, structural analysis, other physiological activities and other composite extraction methods of crude polysaccharide from cassia seed need to be further explored.

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