Study on Ultrasonic Enzyme-Assisted Extraction Process and Antioxidant Activity of Flavonoids from Sedum spectabile boreau flowers

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Abstract—Using *Sedum spectabile* boreau flowers as raw material, single factor and Box-Behnken response surface test were used to optimize the extraction conditions of *Sedum spectabile* boreau flavone assisted by ultrasonic enzymatic method, and the antioxidant activity of *Sedum spectabile* boreau flavone was analyzed in vitro. The results showed that the optimal extraction conditions of total *Sedum spectabile* boreau flavonoids were ultrasonic action time of 60 min, ethanol concentration of 35%, liquid-solid ratio (ml /g) was 20:1, and the amount of enzyme (cellulase: pectinase =1:1) was 3.2g.The study on the antioxidant activity of total flavonoids of *Sedum spectabile* boreau flower showed that its antioxidant capacity was gradually enhanced with the increase of the mass concentration of the test, which had a certain dose-effect relationship.

1. Introduction

Sedum spectabile Boreau is a perennial herb belonging to Sedum family. It is also known as gorgeous Sedum, long medicine, macrophyllum Sedum, Polygonum japonica, and sedum[1].The whole Sedum can be used as medicine and can be harvested throughout the year. *Sedum spectabile* Bboreau nature is flat, bitter taste, slightly sour, with the effect of dispelling wind and dampness, promoting blood circulation and removing blood stasis, stopping pain and clearing heat and detoxification, internal treatment of laryngitis, hematemesis, urticaria disease, mastitis, external treatment of injuries, boils and abscess, hypertension, sore throat, toothache and other symptoms. The eight treasures of Setien are recorded in the Chinese medicine classics such as Haiyao Materia Medica, Kaibao Materia Medica and Compendium of Materia Medica. It is a kind of plant that is easy to cultivate and grows in a wide range[2].

In recent years, some experts and scholars at home and abroad have also begun to conduct a large number of studies on the plants of Sedum family, but most of the studies focus on the reports of Notoginseng sedum and Rhodiola rosea, and there are few reports on *Sedum spectabile* boreau, mainly on the introduction and domestication and breeding of *Sedum spectabile* boreau, cultivation technology, physiological characteristics, chemical composition and application in garden plant landscape[3].After in-depth study of *Sedum spectabile* boreau, we found that it and its extracts contain very complex, rich and diversified biological functional substances, including a variety of natural active alkaloids, steroids, tannins, flavonoids and their derivatives, condensed tannins and cyanogen compounds, etc.[4],making flavonoid compounds in the medical effect is very significant[5].

2. Materials and methods

2.1. Materials and reagents

The samples were collected from Jiangsu *Sedum spectabile* Horticultural Base and identified as genuine.

Rutin standard, pectinase, cellulase 50μ·mg^{-1} purchased from Shanghai Yuanye Biotechnology Co., LTD. Ascorbic acid (VC), Sinopharm Group Chemical Reagent Co., LTD. DPPH, Shanghai Maclin Biochemical Technology Co., LTD. Tris, Shanghai Qiyuan Biotechnology Co., LTD. Anhydrous ethanol g was purchased from Anhui Ante Food Co., LTD. All other reagents were analytically pure.

2.2. Instruments and equipment

Ultraviolet spectrophotometer UV2550: Shanghai Zhicheng Analytical Instrument Manufacturing Co., LTD; Centrifuge TGLW16: Shanghai Zhaodi Biotechnology Co., LTD; Electronic balance FA1104B: Shanghai Yue Ping Scientific Instrument Co., LTD; Electric thermostatic air drying oven DHG-9101 OSA: Shanghai Sanfa Scientific Instrument Co., LTD; Visible spectrophotometer 1101: Shanghai Yuan analysis Instrument Co., LTD; Chinese herbal medicine crusher XL-04B:

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Changzhou Guohua Electric Appliance Co., LTD; Vertical pressure steam sterilization pot GI100T: Shanghai Boxun Industrial Co., LTD; Electronic analytical Balance AUY20: Shimadzu Instrument Co., LTD; Biochemical incubator SHP-250: Shanghai Sanfa Scientific Instrument Co., LTD; Rotary evaporator RE52CS: Shanghai Yarong biochemical instrument factory.

2.3. Experimental method

2.3.1. Sample pretreatment

The *Sedum spectabile* boreau flowers were dried in an oven at 55℃ for 2 hours, crushed, screened through 80 mesh, and stored for later use.

2.3.2. Preparation of total flavonoids extract and determination of total flavonoids content

According to the method of Yun et al. (2023), the standard curve of rutin was drawn, and the regression equation y=11.798x+0.001, R^2=0.9997 was obtained, showing a good linear relationship. The *Sedum spectabile* boreau flowers powder was extracted under the conditions of 30% ethanol, solid-liquid ratio 1:20, ultrasonic time 60 min and ultrasonic power 175 W. After the end of ultrasound, the waste residue was extracted, concentrated by rotary evaporation to no alcohol taste, and the total flavonoid extract of *Sedum spectabile* boreau flowers was obtained by constant volume of pure water in a volumetric bottle to 250 mL. Dilution was 10 times, absorbance value A was measured at 510 nm wavelength by standard curve method, and the mean value was taken by parallel measurement for 3 times. Calculate the total flavonoid content of *Sedum spectabile* boreau flowers according to the following formula: total flavonoid content =C×n×V/W×100%, where: C is the mass fraction of flavonoids corresponding to the measured absorbance value, g/mL; n is the dilution factor of 10; V is the volume of the sample, mL; W is the sample mass, g[6].

2.3.3. Single factor experiment on extraction of total flavonoids

The total flavonoids in *Sedum spectabile* boreau flowers were extracted according to the extraction method of 2.3.2 total flavonoids. Four influencing factors, such as ethanol concentration, liquid-solid ratio, ultrasonic action time and enzyme dosage, were used as single factors for single factor experiment. How four different influencing factors affect the extraction of total flavonoids of *Sedum spectabile* boreau flowers was studied (Table 1) [7].

Table 1 Response surface test factors and levels

Factor	Levels		
	-1	0	1
Aliquid-solid ratio (ml/g)	15:1	20:1	25:1
Bethanol concentration (%)	30	35	40
Cultrasonic action time (min)	40	60	80
Denzyme dosage (g)	2.4	3.2	4.0

2.3.4. The extraction conditions of total flavonoids were optimized by response surface analysis

On the basis of single factor experiment, the liquid-solid ratio (A), ethanol concentration (B), ultrasonic action time (C) and enzyme dosage (D) were further investigated. Design-Expert.V8.0.6.1 software was used to take the extraction rate of total flavonoids of *Sedum spectabile* boreau compound as the response value, and response surface analysis with four factors and three levels was adopted.

3. Results and analysis

3.1 Results of single factor experiment

3.1.1 Effect of liquid-solid ratio on the extraction rate of total flavonoid compounds

At five different liquid/solid ratios of 10:1, 15:1, 20:1, 25:1 and 30:1, 8g *Sedum spectabile* boreau powder and different volumes of 30% ethanol were added respectively, ultrasounded at 50℃ for 40 minutes, 1.6g double enzyme (cellulase : pectinase=1:1) was added, pH was adjusted to 4.5, and enzymatic hydrolysis was performed at 50℃ for 2h. 0.25ml supernatant was centrifugated to measure the absorbance, and the effects of different liquid-material ratio on the extraction rate of total flavonoids *Sedum spectabile* boreau were obtained, as shown in Fig. 1.

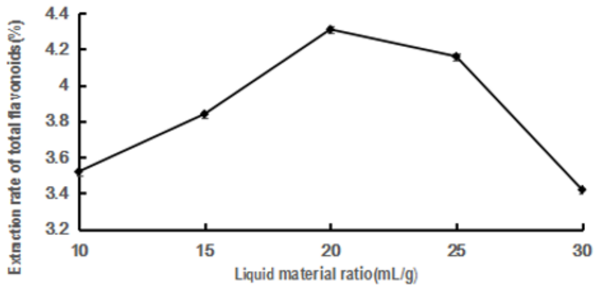


Fig.1 Effect of liquid-material ratio on the yield of total flavonoids

As shown in Fig. 1, when the ratio of liquid to solid was less than 20:1, the extraction rate varied with the ratio of liquid to solid. When the liquid/solid ratio was increased above 20:1, the extraction rate tended to be stable. This may be because the flavonoids were basically completely dissolved in the 20： 1 solvent, considering the extraction rate of the flavonoids, Solvent consumption, energy loss and other factors, the choice of liquid-material ratio of 20:1 is appropriate.

3.1.2 Effect of ultrasonic time on total flavonoid content

Add 160ml of 30% ethanol to 8g Sedum spectabile boreau powder, ultrasonic for 20, 40, 60, 80 and 100min at 50℃, add 1.6g double enzyme (cellulase: pectinase=1:1), adjust the pH to 4.5, enzymatic hydrolysis at 50℃ for 2h, centrifuge 0.25ml supernatant solution to measure absorbance. The effect of ultrasonic action time on the extraction rate of total flavonoids Sedum spectabile boreau was obtained, and the results were shown in Figure 2.3.

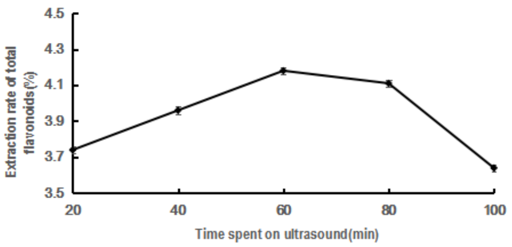


Fig.2 Effect of ultrasonic extraction time on the yield of total flavonoids

As can be seen in Figure 2, the extraction rate of flavonoids was lower at 20 minutes of ultrasound.

At 60min, the extraction rate increased significantly and then the ultrasonic time was extended and the extraction rate was slightly higher. Possibly due to the long ultrasonic treatment, the temperature of the solution gradually increases, which affects the stability of the flavonoids, resulting in a decrease in the extraction rate. From the point of view of saving time, 60 minutes is reasonable.

3.1.3 Effect of ethanol concentration on the extraction rate of total flavonoids

At the end of 8g *Sedum spectabile* boreau, 160ml of 25%, 30%, 35%, 40%, 45% ethanol was added, respectively, under the condition of 50℃ for 60min, 1.6g double enzyme (cellulase: pectinase=1:1) was added, the pH was adjusted to 4.5, the enzyme hydrolysis was conducted in a water bath at 50℃ for 2h, and 0.25ml supernatant was centrifugated to measure the absorbance. The effect of ethanol concentration on the extraction rate of total flavonoid *Sedum spectabile* boreau was obtained, and the results were shown in Fig.3.

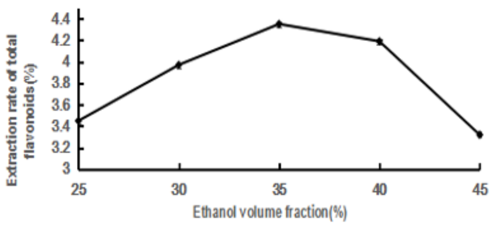


Fig.3 Effect of ethanol concentration on total flavonoid extraction

As can be seen from Fig.3, the extraction rate of total flavone compounds increases significantly when the concentration of ethanol is less than 35%; when the concentration of ethanol is greater than 35%, the extraction rate of total flavone compounds gradually decreases. This may be because the properties of total flavone compounds change with increasing ethanol concentration. Therefore, from the perspective of extraction rate, the ethanol concentration of 35% was selected as the ethanol concentration for the extraction of total flavonoids from Sedum *spectabile* boreau.

3.1.4 Effect of the amount of enzyme on the extraction rate of total flavonoids

Add 160ml of 30% ethanol to 8g *Sedum spectabile* boreau powder and ultrasonic for 60min at 50℃. Then add 1.6g, 2.4g, 3.2g, 4.0g, 4.8gdouble enzymes ((cellulase: pectinase=1:1)) respectively, adjust the pH to 4.5, and enzymolize at 50℃ for 2h. 0.25ml supernatant was centrifugally taken to measure the absorbance, and the effect of the amount of enzyme on the extraction rate of total flavonoids *Sedum spectabile* boreau was obtained, as shown in Fig.4.

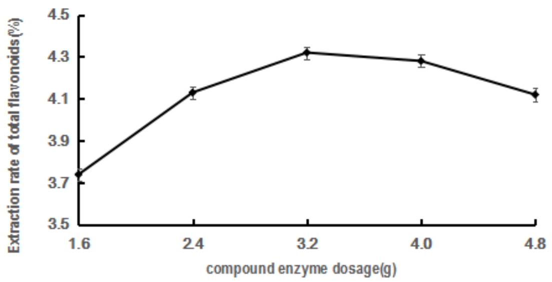


Fig.4 Effect of enzyme dosage on total flavonoid extraction rate

As can be seen from Fig.4, when the amount of double enzyme (cellulase + pectinase) is less than 3.2g, the extraction rate of total flavonoids increases with the increase in the amount of double enzyme; when the amount of double enzyme is greater than 3.2g, the extraction rate of total flavonoids gradually decreases with the amount of double enzyme. From the point of view of extraction rate, reagent consumption and other factors, 3.2 g of double enzyme was selected as the amount of enzyme for the extraction of total flavonoid compounds.

3.2 Response surface analysis for extraction of total flavonoids from compounds

As shown in Table 2, the quadratic multinomial regression equation obtained by regression analysis is: $Y=4.77+0.15A+0.17B+0.17C+0.16D+0.088AB+0.066AC+0.062AD+0.13BC+0.063BD+0.067CD-0.37A^2-0.39B^2-0.39C^2-0.35D^2$

Table 2 Response surface analysis experimental design and results

Experiment number	A	B	C	D	Total flavonoids extraction rate Y (%)
1	1	0	1	0	4.36
2	-1	0	0	1	3.95
3	0	0	1	-1	3.965
4	0	-1	-1	0	3.736
5	0	0	-1	-1	3.788
6	0	1	-1	0	3.826
7	-1	0	0	-1	3.78
8	-1	1	0	0	3.978
9	0	0	0	0	4.709
10	1	1	0	0	4.48
11	0	1	1	0	4.438
12	1	0	0	-1	3.958
13	0	0	0	0	4.802
14	1	0	0	1	4.375
15	0	0	-1	1	3.999
16	0	-1	0	1	3.967
17	0	0	0	0	4.807
18	-1	0	1	0	3.969
19	0	-1	0	-1	3.767
20	0	-1	1	0	3.836
21	0	0	1	1	4.446
22	1	-1	0	0	3.916
23	0	0	0	0	4.806
24	0	1	0	-1	3.945
25	-1	-1	0	0	3.766
26	0	0	0	0	4.708
27	-1	0	-1	0	3.769
28	0	1	0	1	4.396
29	1	0	-1	0	3.898

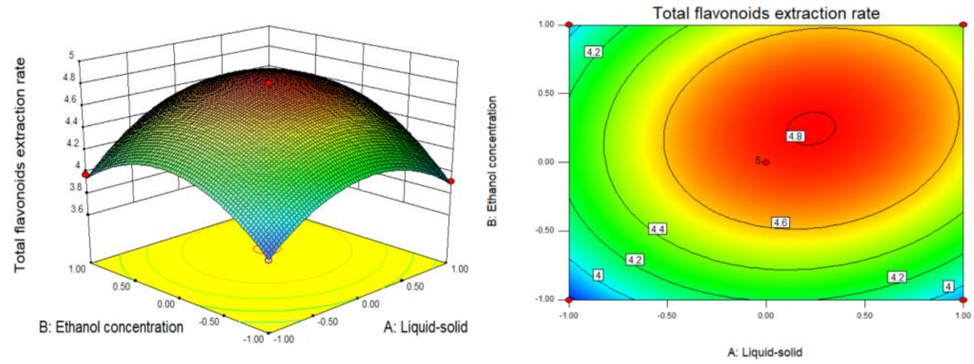
To test the validity of the regression equation and to further determine the degree of influence of various factors on the extraction rate of total flavonoids, analysis of variance and response surface analysis were performed on the regression model and the results are shown in Table 3.

Table 3 Variance analysis of quadratic model of response surface test

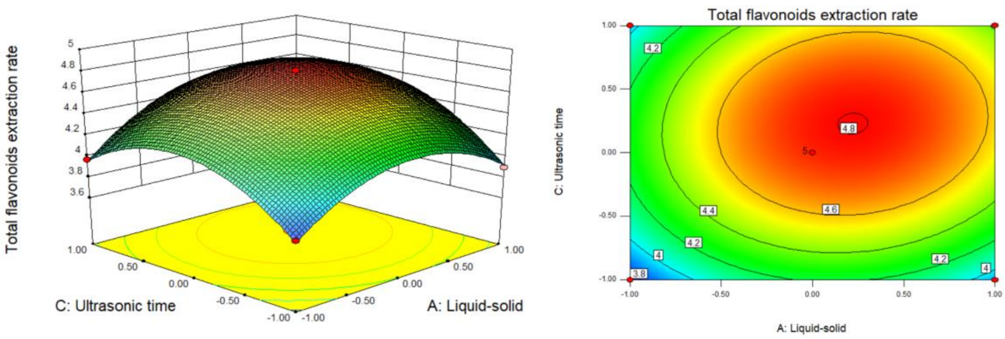
Source of variance	Sum of squares	Degree of freedom	Mean square	F-number	P	Significance
Model	3.78	14	0.27	119.80	<0.0001	***
A	0.26	1	0.26	116.35	<0.0001	***
B	0.36	1	0.36	159.00	<0.0001	***
C	0.33	1	0.33	147.42	<0.0001	***
D	0.31	1	0.31	137.55	<0.0001	***
AB	0.031	1	0.031	13.73	0.0024	**
AC	0.017	1	0.017	7.60	0.0154	*
AD	0.015	1	0.015	6.76	0.0210	*
BC	0.066	1	0.066	29.04	<0.0001	***
BD	0.016	1	0.016	6.98	0.0193	*
CD	0.018	1	0.018	8.08	0.0131	*
A2	0.89	1	0.89	395.98	<0.0001	***
B2	0.98	1	0.98	436.63	<0.0001	***
C2	1.00	1	1.00	442.33	<0.0001	***
D2	0.81	1	0.81	360.08	<0.0001	***
Residual error	0.032	14	2.257×10-3			
Missing fit	0.020	10	2.040×10-3	0.73	0.6887	
Pure error	0.011	4	2.797×10-3			
Total deviation	3.82	28				
		R^2	0.9917			
		R^2_{Adj}	0.9834			

Note: *** Difference is highly significant P<0.0001; ** Difference was highly significant P<0.01; * Difference was significant P<0.05.

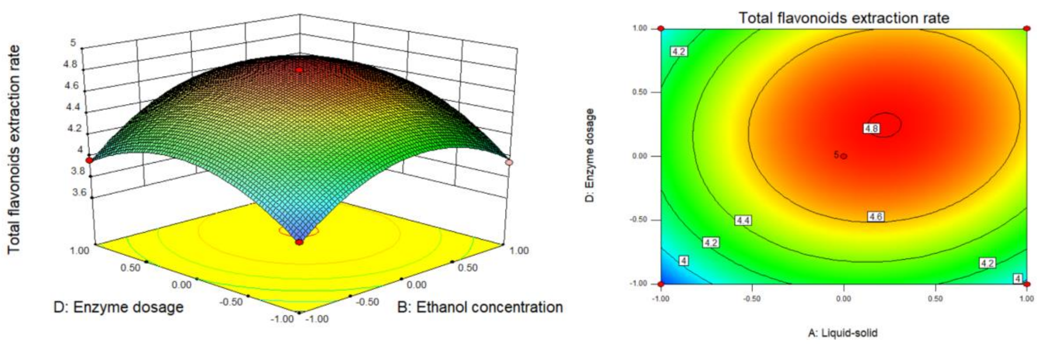
It can be seen from Table 3 that the P-values of A, B, C and D are all less than 0.0001, indicating that their influence on the extraction rate of total flavonoids of Sedum *spectabile* boreau is extremely significant. The quadratic terms A2, B2, C2 and D2 had significant effects on the extraction rate of total flavonoids[8].The above results showed that the factors had an interactive effect on the extraction rate of total flavonoids, rather than a simple linear relationship. The response surface diagram can well describe the interaction between different factors, and the response surface diagram is shown below.



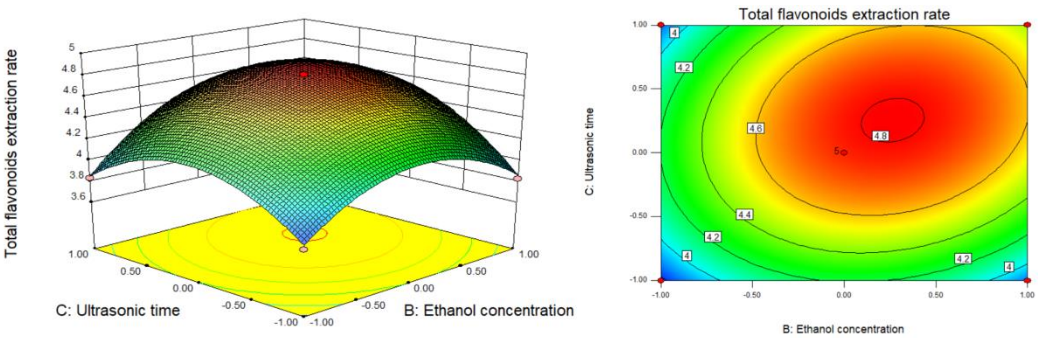
A. Liquid-solid ratio and ethanol concentration



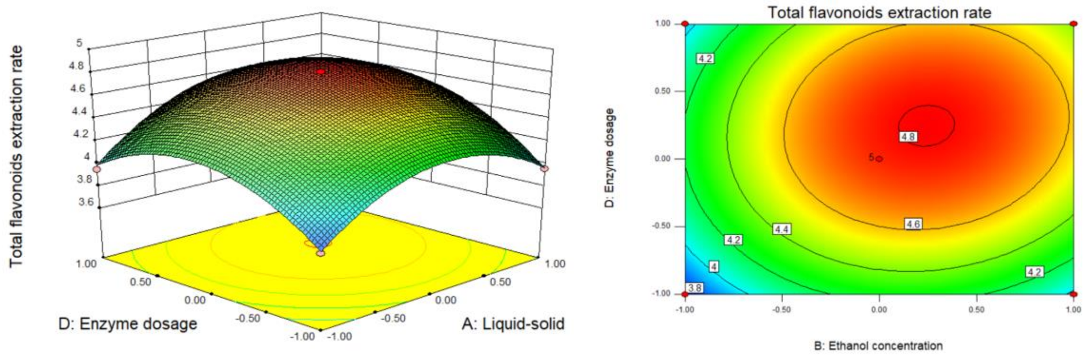
B. Liquid to material ratio and ultrasonic time



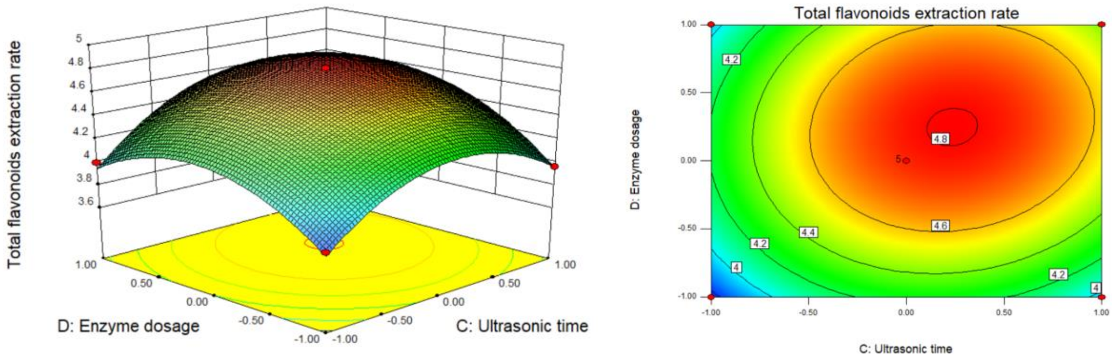
C. Liquid to material ratio and enzyme dosage



D. Ultrasound time and ethanol concentration



E. The amount of enzyme and the concentration of ethanol



F. Enzyme dosage and ultrasound time

Fig.5 Response surface and contour map of the influence of pairwise interaction on the yield of flavonoids

As can be seen from Fig.5, the response surface plot of the interaction between ethanol concentration and liquid/solid ratio is the steepest and the contours are dense, indicating that this interaction has the most significant effect on the extraction rate of flavonoids from *Sedum spectabile* boreau, while the other interaction terms are relatively insignificant.

Through the analysis and optimisation of the response surface, and the optimum process conditions for the extraction of total flavonoid compounds by double enzyme-assisted ultrasonography were obtained as follows: The liquid-solid ratio was 20.77:1ml/g, the ethanol concentration was 35%, the ultrasonic time was 60.51min, and the total amount of double enzyme was 3.209g. Under the above conditions, the extraction rate of total flavonoids was 4.9081%. Due to the limitations of the actual operation, the optimal extraction conditions were revised as follows: liquid-solid ratio 20:1ml/g, ethanol concentration 35%, ultrasonic action time 60min, and total dosage of double enzymes 3.2g. Three parallel experiments were carried out according to the modified conditions. The results showed that the extraction rate of total flavonoids was 4.90%, which was similar to the predicted value of the model. The experimental results were in good agreement with the predicted values of the model, and the parameters of the dual-enzyme-assisted ultrasonic extraction process optimised by the response surface analysis method are accurate and reliable and can be implemented.

3.3 Results of Sedum spectabile boreau flavonoid antioxidant activity

DPPH free radical is a kind of nitrogen-dominated free radical with very strong stability, which is widely used to determine the antioxidant activity of biological samples or biological extracts in vitro. When we want to evaluate the antioxidant activity of total flavonoids *Sedum spectabile* boreau, it is mainly based on whether total flavonoids *Sedum spectabile* boreau can freely and effectively remove DPPH. In addition, the ability to remove superoxide ions and hydroxyl radicals in vitro can also be used as evaluation indicators. Its antioxidant capacity is relatively strong, so it can play a very effective role in alleviating ageing[9].

According to the related studies of Li et al. mouse macrophages were used as the experimental objects, so that the antioxidant activity ability of total flavonoids of eight treasure sedum was effectively confirmed. It was found that it can significantly inhibit hydrogen peroxide in the body and inhibit the occurrence of oxidation reaction. Therefore, it can better protect the cells in the body. It is concluded that the higher the extraction concentration of total flavonoids, the more significant the inhibitory effect on the oxidation of hydrogen peroxide in vivo. This effect greatly improves the ageing of cells in the body and can inhibit the activity of oxidases such as catalase [10].

As shown in Fig.6, the total flavonoids of Setien have a certain antioxidant effect. As the concentration of total flavonoids increases, the scavenging ability becomes better. When the total flavonoids reach a certain concentration, the scavenging ability of DPPH free radicals, hydroxyl free radicals and superoxide ions gradually stabilises. The experimental results show that the eight treasures have antioxidant pharmacological effects, which can effectively alleviate the ageing of cells in the body.

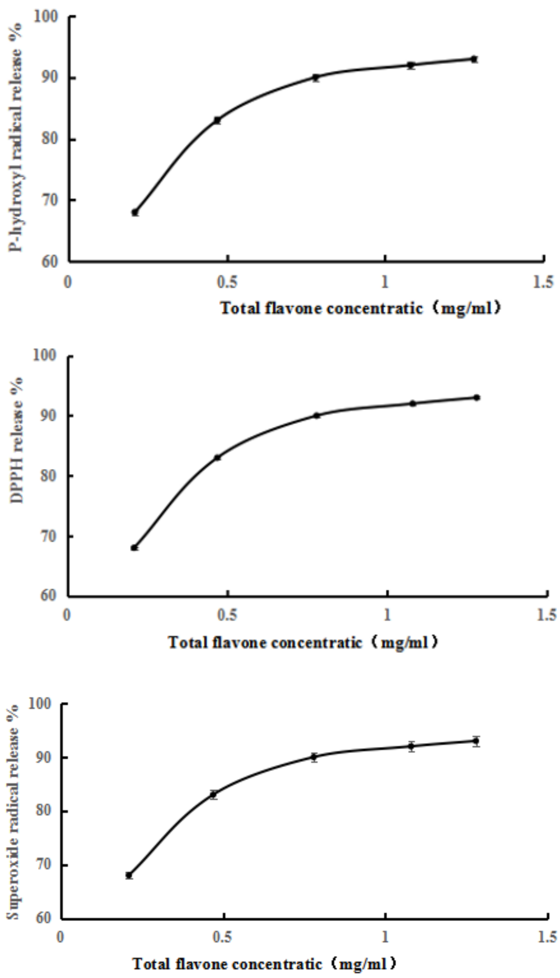


Fig.6 Total flavonoid scavenging of DPPH radicals, p-hydroxyl radicals and superoxide ions

4. Conclusion

Based In this experiment, ultrasonic-assisted enzymatic method was used to extract total flavonoids of *Sedum spectabile* boreau, and its pharmacological effect was studied and analysed. Ultrasonic action time, ethanol concentration, liquid-solid ratio and enzyme dosage were selected as response surface conditions to optimise the extraction process, and the optimal extraction conditions were obtained: ultrasonic action time was 60min, ethanol concentration was 35%, liquid-solid ratio (ml/g) was 20:1, and enzyme dosage (cellulase: pectinase =1:1) was 3.2g. The results show that the extraction rate is high and stable. As *Sedum spectabile* boreau itself contains more flavonoid compounds, it has good medicinal value and prospects for development and use. Studies on the pharmacological activities of flavonoids are relatively mature, and total flavonoids *Sedum spectabile* boreau have many pharmacological effects.

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