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Study on the Optimization of Extraction Technology of Anemonin from *Pulsatilla chinensis* and Its Inhibitory Effect on *Alternaria panax*

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Abstract: Background: The main antibacterial component of *Pulsatilla* chinensis is anemonin, which has strong antibacterial effect, so it is studied. The *Pulsatilla chinensis* is one of the diseases that are difficult to solve in the growth of ginseng, but chemical control not only causes harm to the environment, but also has residues on ginseng itself. *Pulsatilla* is a good medicinal plant. It will have a certain effect on the prevention and treatment of ginseng disease, and it can do no residue, no harm to ginseng. Objective: This experiment can provide effective data support for the prevention and treatment of *Pulsatilla* chinensis. Method: In this experiment, Pulsatilla Chinensis was used as experimental materials to extract plant by reflux, ultrasonic extraction methods, the content of anemonin in each single factor experiment was determined by HPLC. According to the content, the response surface analysis method was used to find the best extraction process, and the mycelium growth rate method was further used to determine the antifungal effect of *Alternaria panax*. Results: The EC_{50} value of *P. Chinensis* extract to *A. panax* was methanol extract < acetone extract < ethanol extract in order from small to large; The optimum extraction conditions of anemonin, the effective component of *P. Chinensis* methanol extract, were as follows: methanol volume fraction 87%, extraction time 2 h, solid-liquid ratio 20 (V: m), the optimum extraction amount was 3.760 $\mu g \cdot g^{-1}$.

Keywords: High Performance Liquid Chromatography, Response Surface Analysis, Anemonin, *Alternaria panax*

1. Introduction

Pulsatilla chinensis (Bunge) Regel, ranunculaceae perennial herbs have the effect of clearing heat, detoxifying, cooling blood and stopping dysentery. Recent studies have shown that fresh juice, Decoction and ethanol extract of Pulsatilla chinensis have obvious antimicrobial effects [1]. Studies on pharmacology and pharmacochemistry of modern Chinese medicine have found that Pulsatilla chinensis mainly contains Ranunculin and its decomposition products, triterpenoid saponins, etc [2-3]. In which, under the action of enzymatic hydrolysis, Ranunculin is decomposed into proto-Pulsatillarin, which is rapidly polymerized into dimer Pulsatillarin in air; at the same time, the main antimicrobial components of Pulsatilla chinensis are protoanemonin and anemonin [4-6], which have strong antimicrobial activity [7]. It is mainly manifested in the control of various bacteria [9-11] such as corn leaf spot pathogen [8], Staphylococcus aureus, etc.

Some scholars have used Ranunculus [12], Huihui garlic [13], huohuocao [14], Maozhaocao [15], Clematis northeast [16] as raw materials to extract and study anemone. However, there are few studies on extracting anemonin from plant Pulsatilla chinensis.

In this study, Pulsatilla chinensis was used as raw material, and the extraction conditions of Pulsatilla chinensis crude powder were optimized by response surface methodology on the basis of single factor experiments. The bacteriostatic effect of Pulsatilla chinensis was determined in order to provide a new material source and theoretical basis for the extraction

and utilization of Pulsatilla chinensis crude powder.

2. Materials and Methods

2.1. Test Material

Strain tested: *Alternaria panax* Whetz provided by the Department of Crop Cultivation and Farming, College of Agriculture, Yanbian University.

Pulsatilla chinensis powder: Purchased in Shaanxi Senfu Natural Products Co., Ltd.

Test medium: Potato glucose agar medium (PDA), Beijing Luqiao Technology Co., Ltd.

2.2. Testing Instruments and Drugs

Test instrument: Hitachi high performance liquid chromatograph; chromatographic column: HITACHI LaChrom C_{18} (250 mm×4.6 mm, 5 μm); Ultrasonic cleaning machine; RE-2000B Rotary evaporator, DLSB-5/20 Low temperature cooling circulating pump; OSB-2100 Constant temperature water bath; Constant temperature light incubator.

Test drugs: Petroleum ether, methanol, chloroform, ethyl acetate, acetone, n-butanol, anhydrous ethanol (all analytical pure), glucose (Tianjin comio Chemical Reagent Co., Ltd.); resveratrol standard (Keli technology development company of Guangzhou analysis and test center); methanol (Chromatographic grade) (SEMER Fisher Technology (China) Co., Ltd.).

2.3. Test Method

2.3.1. Preparation of the Mother Liquor of Pulsatilla Chinensis Extract

Weighing 5.00 g pulsatilla chinensis powder crude powder into 150 mL conical bottle, adding 50 mL methanol, acetone and ethanol to extract three times under the action of ultrasound, then filtering to get the filtrate. The filtrate is then concentrated and evaporated in a rotary evaporator at about 50 C in vacuum to obtain viscous liquid. The obtained viscous liquid was fully mixed with methanol, acetone and ethanol, and the extract was obtained after dissolution. Preservation of the extract in low temperature and dark.

2.3.2. Optimization of the Extraction Method of the Effective Components in the Extract of Pulsatilla Chinensis Extract

Conditions for high performance liquid chromatography of anemonin:

Chromatographic column: HITACHI LaChrom C_{18} (250 mm×4.6 mm, 5 μm); The mobile phase is methanol: water (25:75 volume fraction); ultraviolet detector, The detection wavelength was 220 nm, the flow rate was 0.8 mL/min, the column temperature was 34 C, and the injection volume was 10 mL.

Preparation of sample solution:

The extracts under different single factor extraction conditions were used as the original solution of the test sample, and then 0.5 mL of the original solution of the test sample was precisely measured in a 10 mL volume bottle, diluted and volumed with methanol, which was used as the test solution of the test sample. The sample was filtered with 0.22 um filter before injection.

Preparation of reference solution:

Accurately weigh 1.8 mg of Anemonin reference substance and place it in a 100 mL capacity bottle. Dilute it with methanol and fix the volume. Prepare the original solution containing 18 UG of Anemonin per mL for reserve. The reference solution was diluted and volumed with methanol in a 10 mL volumetric bottle. Filtration with 0.22 um membrane before injection.

Investigation of linear relations:

Under this chromatographic condition, 10 L of each reference solution was precisely absorbed and injected into the liquid chromatography to determine the peak area. Drawing standard curve with mass concentration as abscissa X and peak area as ordinate Y.

Precision test:

Under this chromatographic condition, the same reference solution was weighed and sampled three times with a sample volume of 10 UL each time. RSD of the peak area of Anemonin was determined and calculated.

Repeatability test:

Under this chromatographic condition, the same sample solution was sampled for five repetitive tests, each sample volume was 10 L, the peak area was determined, and the content of Anemonin in the sample and the relative standard deviation (RSD) were calculated.

Single factor test:

(1) Effect of solvent on the extraction amount of anemonin

Weigh 5.00g of the crude powder of pulsatilla chinensis, add 50ml of petroleum ether, methanol, ethyl acetate, acetone, n-butanol, ethanol (the ratio of material to liquid is 1:10) respectively, and extract it by ultrasonic for 30min. According to the preparation method of the test solution in 2.3.2, treat the extract, prepare the test solution, inject the sample according to the chromatographic conditions, and record the peak area.

(2) Effect of methanol volume fraction on the extraction amount of anemonin

Weigh 5.00g of the crude powder of pulsatilla chinensis, add 50ml of methanol with different volume fractions: 50%, 60%, 70%, 80%, 90%, and extract it by ultrasonic for 30min. According to the preparation method of the test solution in 2.3.2, treat the extract, prepare the test solution, inject the sample according to the chromatographic conditions, and record the peak area.

(3) Effect of extraction times on the extraction amount of anemonin

Weigh 5.00g of the crude powder of pulsatilla chinensis, add 80% methanol, and extract it once, twice and three times respectively by ultrasonic. According to the preparation method of the test solution in 2.3.2, treat the extract, prepare the test solution, inject the sample according to the chromatographic conditions, and record the peak area.

(4) Effect of material liquid ratio on the extraction amount of anemonin

The crude powder of pulsatilla chinensis was weighed at

5.00 g, and the ratio of feed to liquid was 1:5, 1:10, 1:15, 1:20 and 1:25. 80% methanol was added to the powder, respectively. The ultrasonic extraction time was 30 minutes. According to the preparation method of the test solution in 2.3.2, treat the extract, prepare the test solution, inject the sample according to the chromatographic conditions, and record the peak area.

(5) Effect of time on the extraction amount of anemonin

Weigh 5.00 g of the crude powder of pulsatilla chinensis, add 50 ml of 80% methanol, and extract 0.25 h, 0.5 h, 1 h, 1.5 h and 2 h respectively under the condition of 60°C. According to the preparation method of the test solution in 2.3.2, treat the extract, prepare the test solution, inject the sample according to the chromatographic conditions, and record the peak area.

2.3.3. Inhibition of the Extract of Pulsatilla Chinensis on the Mycelial Growth of the Black Spot of Ginseng

The mycelial growth rate method was used to determine the bacteriostatic effect of different extracts on Ginseng black spot pathogen. After compounding each extract with different concentration, the potato agar-glucose medium sterilized by high temperature and high pressure was added into the super-clean workbench, and the extracts were added into the plate with the concentration of 2.08, 4.17, 8.33, 16.67, 33.33, 66.67 and 133.33μg·mL^{-1} respectively, three times per treatment. Repeat. The cake was made on the edge of the colony with a sterile perforator with an inner diameter of 7 mm and placed in the center of the plate containing the extract. The cake was cultured in a constant temperature incubator at 25°C without the extract as a control. After 6 days of culture, the colony diameter was measured, and the inhibition rate of mycelium growth was calculated according to the following formula.

Diameter of colony growth = mean diameter for 3 times-7 mm;

$$\text{Inhibition rate of mycelium growth}(\%) = \frac{(\text{Control colony growth diameter} - \text{Treat colony growth diameter})}{\text{Control colony growth diameter}} \times 100$$

Using the logarithm of the treatment concentration as the abscissa, the inhibition growth rate under different concentrations is converted into the probability value as the ordinate to draw the virulence curve, and the linear regression equation, correlation coefficient and the value of inhibition medium concentration (EC50) of single agent are obtained.

2.4. Data Processing

Using Excel and SPSS statistical software to deal with variance, multiple comparison, standard curve drawing, etc.

3. Results and Analysis

3.1. Optimized Extraction Method of Effective Components from Pulsatilla Chinensis Extract Chinensis

3.1.1. Linear Relationship of Standard Curve

The regression equation of the standard concentration of anemonin to the peak area was y=57262x-99.234 (r = 0.9999, n = 5). Meet the requirements of analysis.

3.1.2. Precision Test

Take 18 μg·mL^{-1} standard mother liquor of anemonin and inject it for 5 times continuously. The average peak area of anemonin is 788435, and its RSD is 0.05%, less than 2%. It shows that the precision is good and meets the requirements of analysis.

3.1.3. Repeatability Test

Weigh accurately the crude powder of Pulsatilla chinensis, prepare the sample solution according to the single factor test of Pulsatilla chinensis, and determine it under the chromatographic conditions of 2.3.2, meeting the requirements of analysis. See Table 1 for the results.

Table 1. *The content of anemonin under different treatments.*

Different treatment	Anemonin content (μg·g^{-1})	RSD %
petroleum ether	-	-
methanol	0.084	0.719
ethyl acetate	-	-
acetone	-	-
N-butanol	-	-
absolute ethanol	-	-
1 time	1.595	0.026
2 time	2.873	0.149
3 time	3.625	0.007
50%	0.103	0.697
60%	1.165	1.466
70%	1.063	0.652
80%	1.549	1.549
90%	2.460	1.338
100%	0.084	0.719
1:5	4.313	1.262
1:10	1.578	1.094
1:15	0.169	1.228
1:20	1.113	1.943
1:25	4.351	1.617
0.25 h	0.847	1.668
0.5 h	1.578	1.094
1 h	0.783	1.781
1.5 h	0.937	1.386
2 h	4.841	0.952

3.1.4. Single Factor Test Results

In fact, the extraction amount of anemonin is affected by extraction temperature, extraction time, material liquid ratio, ethanol volume fraction and solvent. In order to determine the best extraction conditions, the following single factor experiment was designed, with extraction amount of anemonin as the final evaluation index, and multiple comparisons were carried out. The results are as follows

(1) Effect of solvent on the extraction amount of anemonin

Figure 1 shows that different solvents have influence on the

extraction amount of anemonin, and methanol is the largest, 0.084 μg·g^{-1}. Through the analysis of variance of the average value of 5 times of extraction amount of anemonin, the results showed that under the significant level of 0.05, P<0.05, different solvents had significant differences in the extraction amount of anemonin.

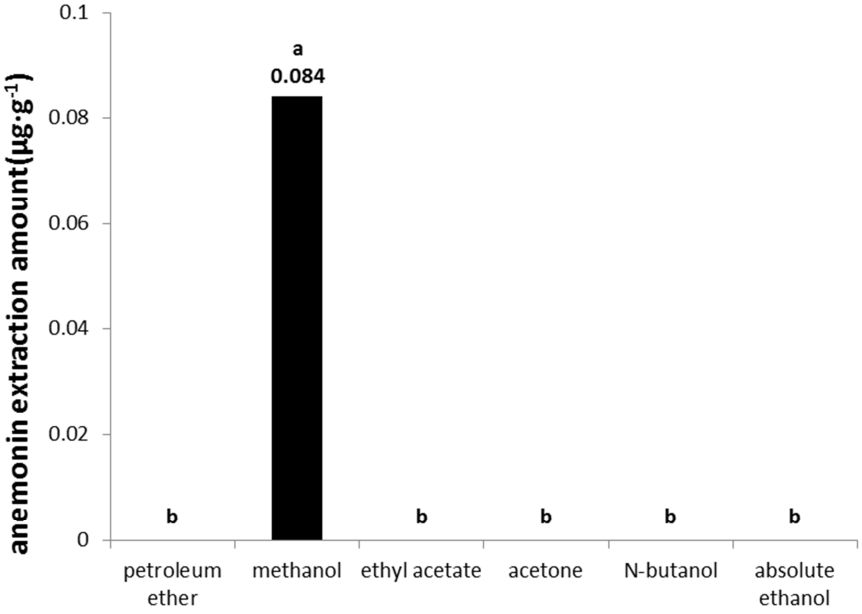


Figure 1. *The extraction amount of anemonin under different solvents.*

(2) Effect of methanol volume fraction on the extraction amount of anemonin

It can be seen from Figure 2 that when the volume fraction of methanol is 90%, the extraction amount of anemonin in the crude powder of pulsatilla chinensis (content of anemonin per gram of pulsatilla chinensis) is the highest, which is 2.460 μg·g^{-1}, followed by the volume fraction of 80%, and the extraction amount of anemonin is 1.549 μg·g^{-1}. At the same time, the influence of different volume fractions on the extraction amount of anemonin is also different. Through the analysis of variance of the average value of 5 times of anemonin extraction amount, the results show that at the significant level of 0.05, P value is less than 0.05, and different methanol volume fractions have significant difference on the extraction amount of anemonin.

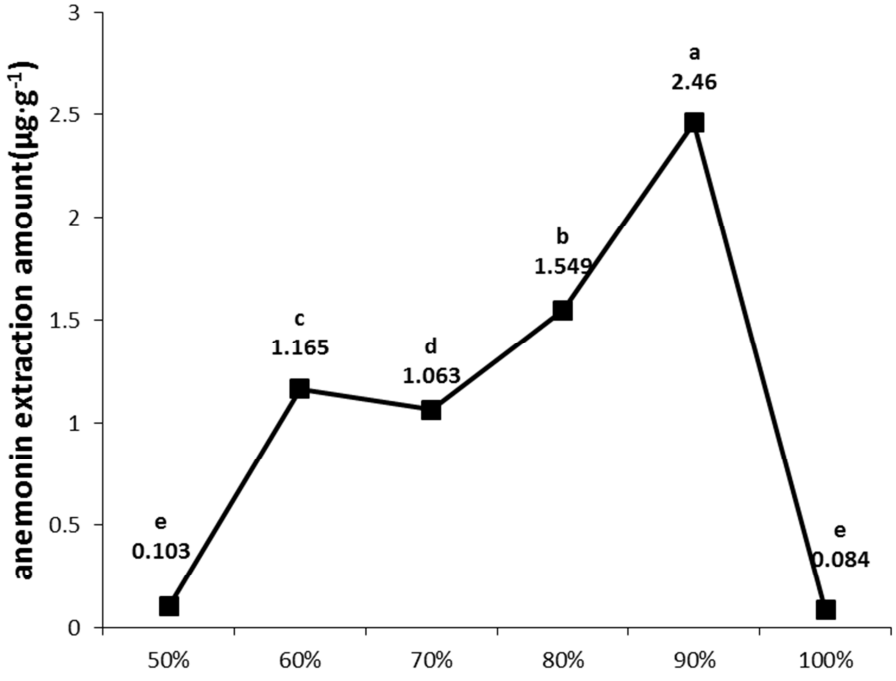


Figure 2. *The extraction amount of anemonin under different methanol volume fractions.*

(3) Effect of extraction time on the extraction amount of anemonin

It can be seen from Figure 3 that the extraction amount of anemonin is affected by the extraction time. At 2 hours, the extraction amount reaches the maximum value of 4.841 $\mu g \cdot g^{-1,}$ and there is little difference in other extraction time.

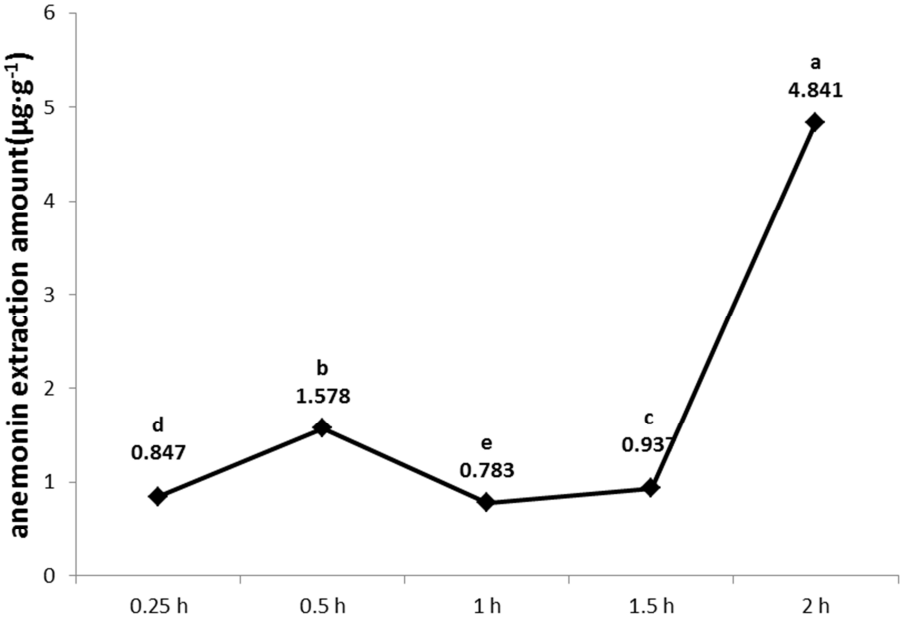
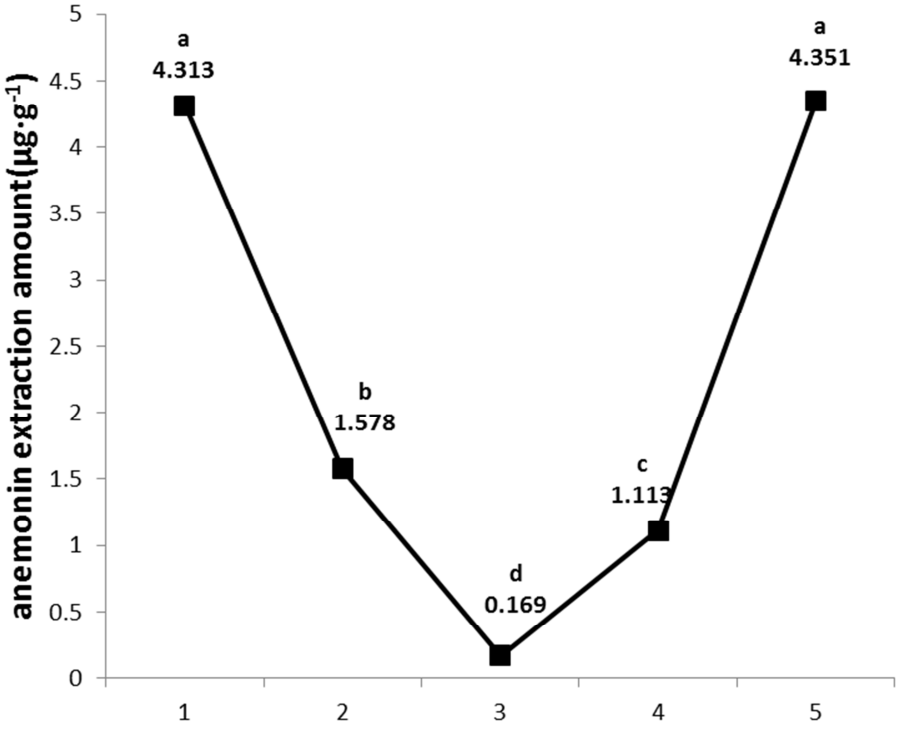


Figure 3. *The extraction amount of anemonin under different extraction time.*

(4) Effect of the ratio of material to liquid on the extraction amount of anemonin

It can be seen from Figure 4 that when the solid-liquid ratio (the ratio of the mass of the crude powder of pulsatilla chinensis to the volume of the extraction solvent) is 1:25 and 1:5, the extraction amount of anemonin is larger, which is 4.351 and 4.313$\mu g \cdot g^{-1}$, respectively, and the extraction amount is significantly higher than that the extraction amount of anemonin when the ratio of material to liquid is 1:10, 1:15 and 1:20.



(Note: 1-5 means that the solid-liquid ratio is 1:5, 1:10, 1:15, 1:20 and 1:25 respectively.)

Figure 4. *The extraction amount of anemonin under different solid-liquid ratio.*

(5) Effect of extraction times on the extraction amount of anemonin

It can be seen from Figure 5 that the extraction amount of anemonin increases with the increase of extraction times, among which the extraction amount of anemonin for three times is the most, which is 3.625 μg·g^{-1}.

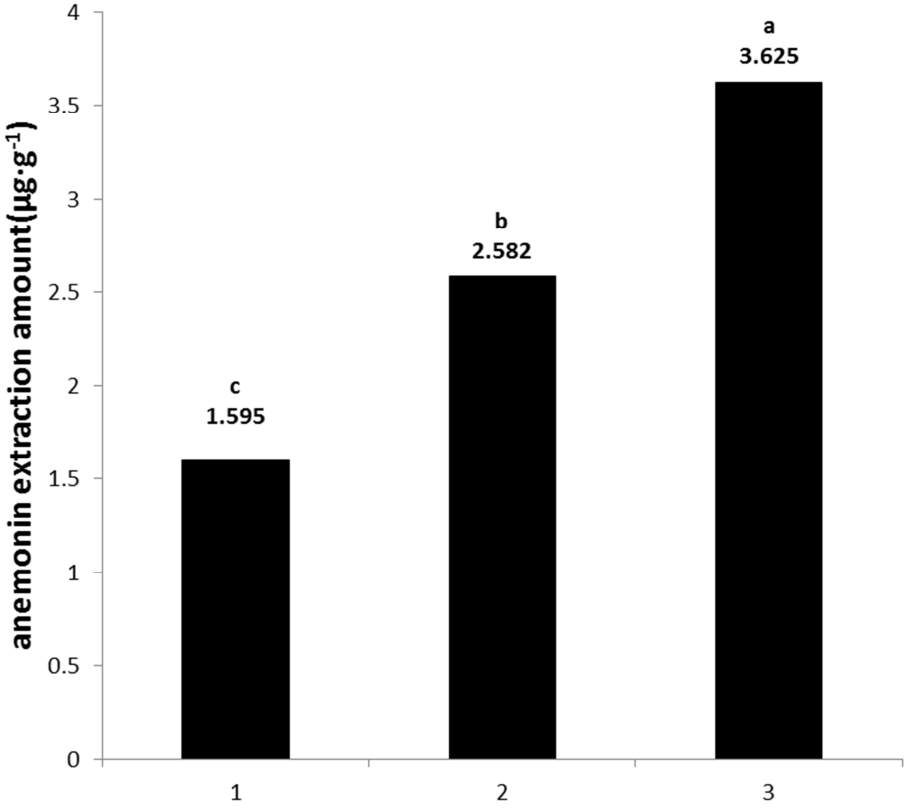


Figure 5. *The extraction amount of anemonin under different extraction times.*

3.1.5. Optimization of the Extraction Amount of Anemonin by Response Surface Experiments

Through the regression analysis of Design-expert software, the quadratic multiple regression equation of the extraction amount of anemonin to the coded independent variable methanol volume fraction X_1, extraction time X_2 and material liquid ratio X_3 is:

$$Y=2.82-0.X_1+0.52X_2+0.02X_3+0.51X_1X_2-0.47X_1X_3+0.32X_2X_3-0.96X_1^2+0.29X_2^2-0.46X_3^2$$

Table 2. *Results of response surface experiments.*

No.	X_1/%	X_2/h	X_3	Concentration of anemonin Y (μg·g^{-1})
1	80	0.5	15	2.82
2	100	0.5	15	0.09
3	80	2	15	3.20
4	100	2	15	2.51
5	80	1.25	5	1.76
6	100	1.25	5	1.20
7	80	1.25	25	2.54
8	100	1.25	25	0.10
9	90	0.5	5	2.52
10	90	2	5	2.55
11	90	0.5	25	2.12
12	90	2	25	3.43
13	90	1.25	15	2.78
14	90	1.25	15	2.46
15	90	1.25	15	2.67
16	90	1.25	15	2.87
17	90	1.25	15	3.32

The regression analysis results are shown in Table 3, and the response surface map and isoline are made, as shown in Figures 6-8. It can be seen from table 3 that according to the software analysis, the regression equation is significant ($P< 0.01$), and the correlation coefficient $R^2=0.9502$, indicating that the method is reliable and can better describe the test results. The equation can be used to replace the real test points for analysis, and predict the extraction amount of anemonin under different extraction conditions.

Table 3. *Regression analysis results.*

Source	Sum of squares	df	Mean square	F value	p-value
X_1	5.160	1	5.160	46.510	< 0.010
X_2	2.150	1	2.150	19.350	< 0.010
X_3	0.003	1	0.003	0.029	0.870
X_1X_2	1.040	1	1.040	9.410	0.018
X_1X_3	0.880	1	0.880	7.970	0.026
X_2X_3	0.410	1	0.410	3.690	0.096
X_1X_1	3.880	1	3.880	35.030	< 0.010
X_2X_2	0.370	1	0.370	3.300	0.112
X_3X_3	0.890	1	0.890	8.020	0.025
regression	14.820	9	1.650	14.850	< 0.010
residual	0.780	7	0.110		
Missing item	0.370	3	0.120	1.210	0.413
Pure error	0.410	4	0.100		
Total deviation	15.600	16			

The results of variance analysis showed that methanol volume fraction and extraction time had significant effect on the extraction of pulsatin ($P < 0.01$). The order of factors influencing the extraction amount of anemonin is methanol volume fraction > extraction time > solid-liquid ratio.

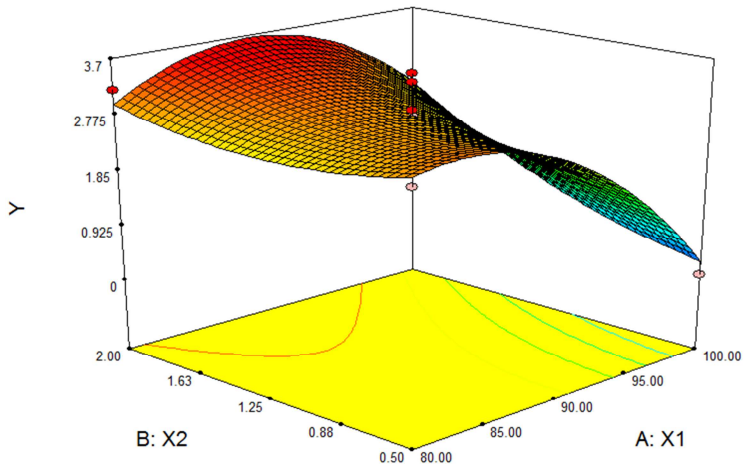


Figure 6. *Response surface and contour map of Y=f (X_1, X_2).*

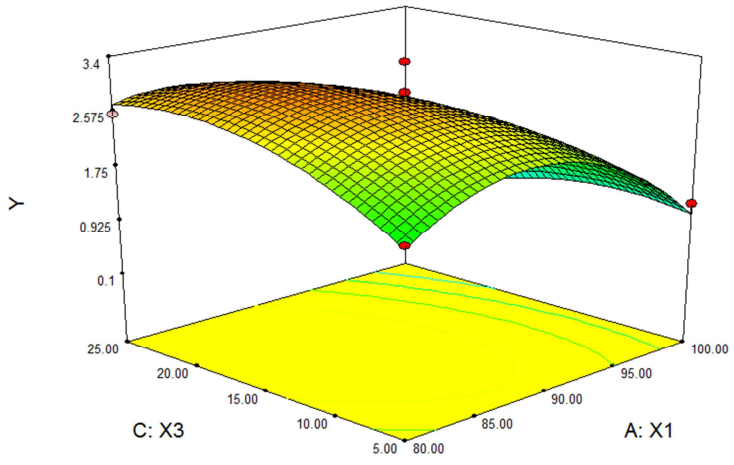


Figure 7. *Response surface and contour map of Y=f (X1, X3).*

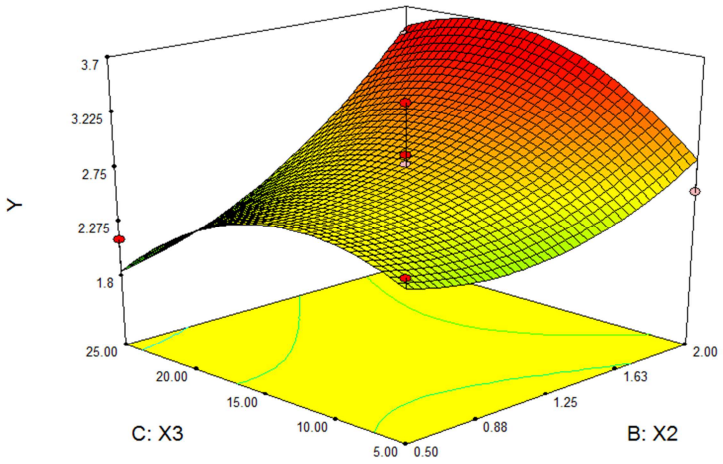


Figure 8. *Response surface and contour map of Y=f (X2, X3).*

According to the regression equation to draw the analysis chart, examine the shape of the fitted response surface, Figures 6-8 intuitively gives the response surface 3D map and isoline map of the interaction of various factors. From the isoline and the highest point of the response surface, it can be seen that the highest point of the response surface in the selected range is the extreme value. From the response surface optimization diagram, it can be seen that the most important factor affecting the extraction amount of anemonin is the methanol volume fraction, followed by the extraction time. Selecting the appropriate methanol volume fraction and time can obtain a higher extraction amount of anemonin.

Based on the partial integration of the bivariate multiple equation, the coding values of X_1, X_2, X_3 are X_1=87.24%, X_2=2 h, X_3=20 when the maximum response value (y) is analyzed by Design-expert software. The corresponding extraction conditions were 87% methanol, 2 hours extraction time, 20 (V: m) material liquid ratio and 3.760 μg·g^{-1} theoretical optimum.

In order to verify the reliability of the above results, the above extraction process was adopted: ultrasonic extraction was carried out for 2 hours with 87% methanol as solvent and 20 (V: m) as the ratio of material to liquid, and the extraction amount of pulsatin measured by parallel test was 3.755 μg·g^{-1}, which was close to the theoretical value, indicating that the model can better reflect the extraction conditions of anemonin.

3.2. Inhibitory Effect of the Extraction Amount of Pulsatilla Chinensis Against a. Panax

See Table 4 for the bacteriostatic test results of different extracts of pulsatilla chinensis by mycelial growth rate method. The EC_{50} values of the extracts of pulsatilla chinensis to the pathogen were methanol extract(21.58 μg·mL^{-1})<acetone extract(28.84 μg·mL^{-1})<ethanol extract(35.71 μg·mL^{-1}).

Table 4. *Inhibitory effect of different extraction of Pulsatilla Chinensis against A. panax.*

Test extract	Regression equation	EC_{50} value (μg·mL^{-1})	Correlation coefficient (r)
Methanol extract	y=2.4402+1.9188x	21.58	0.9153
Acetone extract	y=2.5688+1.6652x	28.84	0.9010
Ethanol extract	y=3.1510+1.1908x	35.71	0.8231

4. Discussion

According to the related literature, the main antibacterial component of pulsatilla chinensis is anemonin. Therefore, ultrasonic assisted extraction method is used to extract anemonin in this experiment. Single factor analysis and response surface analysis are carried out for the extraction process of solvent, solvent volume fraction, extraction time, material liquid ratio, extraction times, etc. the content is determined by HPLC, and the best extraction process is fitted. Art. This method has the advantages of simplicity and high sensitivity, and through the verification test of the best extraction process, it is found that it is close to the theoretical extraction value, which indicates that the extraction amount can be predicted by response surface analysis, thus proving the stability and rationality of the process, and providing the theoretical basis for the process conditions of extracting anemonin from pulsatilla chinensis. But in this experiment, only ultrasonic assisted extraction method is used, and other extraction methods are not studied, which needs further study.

The methods of inhibition circle and inhibition medium concentration are usually used to judge the bacteriostatic effect on plant pathogens. In this study, the crude powder of pulsatilla chinensis was used as the extraction material. The ultrasonic assisted extraction method and different solvents were used for extraction, and the indoor mycelial growth rate method was used for bacteriostatic study of the pathogen. The results showed that the bacteriostatic effect of different solvent extracts on the pathogen was different, among which the methanol extract of Pulsatilla chinensis had the best bacteriostatic effect, and the EC_{50} value was 21.58μg·g^{-1}. The results show that the methanol extract has a good antibacterial

effect, which is consistent with the study on the antibacterial effect of shenxiaohui et al [3] on the extract of pulsatilla chinensis. Due to the limited time, this experiment only carried out the mycelial growth rate test in the laboratory, but failed to carry out the field test. There was a certain difference in the bacteriostatic effect between this experiment and the field, so we should further study the field test.

5. Conclusion

The results showed that the EC_{50} value of the extract of pulsatilla chinensis to the pathogen of black spot of ginseng was methanol extract < acetone extract < ethanol extract, and the best extraction condition of pulsatilla chinensis was 87% methanol volume fraction, 2 hours, 20 (V: m), 3.760μg·g^{-1}.

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