Process Optimization of Nuciferine with Cellulase Auxiliary Ultrasonic-Assisted Extraction from Leaves of *Nelumbo Nucifera* Using Response Surface Methodology

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Abstract. The cellulase auxiliary ultrasonic-assisted extraction process of nuciferine from *Nelumbo nucifera* leaves was optimized by response surface methodology. The extraction content of nuciferine was measured by HPLC. On the basis of single factor experiment, ethanol concentration, liquid-solid ratio, and extraction times were selected as independent variables, and the extraction content of nuciferine was selected as response value. Box-Behnken design was adopted and optimized extraction process in this experiment, the optimum extraction conditions were confirmed. The results showed that the optimum extraction conditions were 10% cellulase, 87% ethanol concentration, 1:26g/mL solid-liquid ratio, 3 times and 25 minutes. The content of nuciferine was 0.343mg/g. The extraction rate of nuciferine was 0.034% after cellulase was added, while the extraction rate of nuciferine without cellulase was 0.010%. The extraction process obtained was reasonable, this experiment can provide a more excellent and reliable method for extracting nuciferine from leaves of *Nelumbo nucifera*.

Keywords: Leaves of *Nelumbo Nucifera*, Nuciferine, Cellulose, Response Surface Methodology

1. Introduction

The lotus leaves are the leaves of *Nelumbo nucifera*, and the petiole is the lotus stem. The parts where the leaves meet the petiole are the lotus pedicle (also known as the lotus nose), all of which can be used as medicine [1]. Nuciferine is an aporphine type alkaloid in leaves of *N. nucifera*, which is the main lipid-lowering active component [2]. Modern medicine has proved that nuciferine has the therapeutic effect of reducing fat, reducing weight, anti-free radical, inhibiting hypercholesterolemia and arteriosclerosis. In addition, it also has the effect of anti-mitosis, and has strong antibacterial effect [3].

The application of these chemical components in the chemical, food and pharmaceutical industries proves that people are more and more interested in developing and optimizing the extraction process.
of natural sources, especially when the content of nuciferine is known, the extraction conditions have a significant impact on its type and yield.

In recent years, the development direction of extraction technology is to find efficient and innovative techniques to extract natural active ingredients, in order to improve extraction rate, shorten extraction time and reduce solvent consumption. Ultrasound-assisted extraction (UAE) [4] and microwave-assisted extraction (MAE) [5] usually conform to these requirements. Compared with other techniques, UAE is driven by the driving force of acoustic cavitation, which is conducive to the rupture of cell wall and the improvement of extraction process. Ultrasound has the advantages of simple operation and safety, because it is carried out under atmospheric pressure and ambient temperature, using a proper amount of solvent is repeatable, and requires relatively short processing time [6]. Response surface methodology (RSM) is a statistical method to find the optimal process parameters and solve the multivariable problem by using the reasonable experimental design method and getting certain data through experiments, using multiple quadratic regression equation to fit the functional relationship between factors and response values, and analyzing the regression equation [7].

The purposes of this paper were to investigate ultrasound-assisted extraction as a means applicable for the extraction of nuciferine with cellulase auxiliary from leaves of \textit{N. nucifera}, to evaluate the influence of main extraction parameters on extraction and comprehensive effect, and to optimize the operation parameters to maximize the content of nuciferine in RSM extraction solution.

2. Experimental

2.1. Materials
Leaves of \textit{N. nucifera} were collected from Botanical Garden of Jilin Agricultural Science and Technology University, Jinlin Province. Plant samples were air dried in the shade at ambient temperature and milled immediately before extraction. After 40 mesh sieves, it was dried to constant weight in a 60°C oven. The powder was stored in darkness in a dry, cool place until treatment. Herbarium voucher samples are deposited in the Laboratory of Pharmaceutical Chemistry, Jilin Agricultural Science and Technology University.

2.2. Experimental Design
A $3^4$ full factorial experimental designs were used to the process parameters for the yield of nuciferine (dependent parameter). The range of process parameters (independent variables) was investigated preliminarily: concentrations of ethanol (50%, 70%, 90%, $X_1$), solid - liquid ratio (1:20, 1:25, 1:30 g/mL, $X_2$) and extraction times (2, 3, 4, $X_3$). All the trial runs were performed in triplicate.

2.2.1. Response surface methodology
The optimal combination of process parameters are determined with RSM at the tree level. The output of homoplantaginin (related parameter Y) was measured in three times, and the average value was used for regression analysis. For predict the optimum conditions, the design expert software is used to analyze the experimental data and fit it to the empirical second-order polynomial regression model:

$$Y = f(X_1, X_2, \cdots, X_i) + \varepsilon$$

Among them, Y is the observation value (the dry matter yield of homoplantaginin mg/g). Where $f(X_1, X_2, \cdots, X_i)$ is the function of $X_1, X_2, \cdots, X_i$ (the independent variable), $\varepsilon$ is the error term. In response surface analysis, the first step is to get the regression equation, and then through the reasonable value of the self catalaplasm, to show the optimal value, which is the purpose of response surface design test.

The experimental design, regression and graphical analysis were expressed by the Design Expert software package (Trial version 8.0.0, USA). Variance analysis is used to assess the significance of independent parameters and their interaction, the sufficiency of the model and the statistical significance of regression coefficient.
2.3. Ultrasound-assisted Extraction

Microwave extraction was carried out by a Microwave Synthesizer (“Xiangwu”, XH-100A, China). Ground plant material (1.00 g) adding 10 mL 10% cellulase (50u/mg, “Shanghai yuanye Bio-Technolony Co., Ltd.”) and pH 5.0 citric acid buffer solution, and different concentration (50%, 70%, 90%) of solid-liquid ratio (1:20, 1:25, 1:30 g/mL) were put in a series of Erlenmayer flasks (50 mL). The extraction was carried out for 25 min at various extraction times (2, 3, 4). After the extraction cycle, the liquid extracts were condensed with a rotary evaporator at 45 °C. The filtrations of the two extractions were combined and transferred into a 50 mL volumetric flask and adjusted to the volume with 60% ethanol. The solution was ultrasonicated for 10 min, filtered through a 0.45 μm filter for High Performance Liquid Chromatograph.

2.4. Determination of Nuciferine Content

2.4.1. Preparation of Control Solution

2.3 mg nuciferine was accurately weighed and placed a 10mL volume bottle, added methanol to scale and shaked well, then obtained 0.23mg/mL standard solution.

2.4.2. The HPLC Conditions

The chromatographic conditions were used by ZORBAX SB-C18 column (4.6 × 150 mm, 5 μ m), the mobile phase was consisted of 0.1% triethylamine and 0.2% acetic acid solution (A)-acetonitrile (B), 0~15 min 40%~80% B; 15~20 min 80% ~90% B; 30~35 min 40% B. The column temperature was 30 °C, the injection volume was 1 μL and the flow rate was 1 mL/min, the detection wavelength was 287 nm[8].

2.4.3. Determination of the Standard Curve

The above standard solution was drawn accurately and injected into 2, 4, 6, 8 and 10 μL respectively, and the chromatographic peak area was recorded according to the above chromatographic conditions "2.4.2". The standard curve was drawn for linear regression with mass concentration (X) as transverse coordinate and chromatographic peak area (Y) as longitudinal coordinate.

3. Results and Discussion

3.1. The Standard Curve

![Figure 1](image_url)

Figure 1. High-performance liquid chromatography of nuciferine

The regression equation of nuciferine was y = 940.56x - 0.84, R² = 0.9933.

3.2. RSM Modeling and Process Optimization

According to the combination design principle of Box-Behnken, 17 experiments were carried out in random order and made in three times to study affluence of different variable quantity on the yield of
nuciferine. Table 1 showed the factors and their levels that affect the production of nuciferine, and the experimental and predicted the results.

Table 1. Design and Results of Box-Behnken Test

| Run | Coded Factors | Uncoded Factors | NY (mg/g) |
|-----|---------------|----------------|----------|
| X1  | X2  | X3  | Ethanol Concentrations (X1, %) | Solid-liquid, (X2, g/mL) | Extraction Times | EV | PV |
| 1   | 0   | 0   | 90 | 1:25 | 4 | 0.22 | 0.218 |
| 2   | -1  | -1  | 70 | 1:20 | 2 | 0.11 | 0.051 |
| 3   | -1  | 1   | 50 | 1:30 | 3 | 0.09 | 0.064 |
| 4   | 1   | -1  | 90 | 1:20 | 3 | 0.25 | 0.276 |
| 5   | -1  | 0   | 50 | 1:25 | 2 | 0.03 | 0.053 |
| 6   | 1   | 0   | 90 | 1:25 | 2 | 0.26 | 0.292 |
| 7   | 1   | 1   | 90 | 1:30 | 3 | 0.31 | 0.260 |
| 8   | 0   | 0   | 70 | 1:25 | 3 | 0.30 | 0.354 |
| 9   | 0   | 0   | 70 | 1:25 | 3 | 0.30 | 0.304 |
| 10  | -1  | 0   | 50 | 1:25 | 4 | 0.09 | 0.062 |
| 11  | 0   | 1   | 70 | 1:30 | 2 | 0.17 | 0.183 |
| 12  | 0   | 0   | 70 | 1:25 | 3 | 0.30 | 0.273 |
| 13  | 0   | 1   | 70 | 1:30 | 4 | 0.16 | 0.222 |
| 14  | -1  | -1  | 50 | 1:20 | 3 | 0.10 | 0.152 |
| 15  | 0   | -1  | 70 | 1:20 | 4 | 0.17 | 0.153 |
| 16  | 0   | 0   | 70 | 1:25 | 3 | 0.30 | 0.318 |
| 17  | 0   | 0   | 70 | 1:25 | 3 | 0.30 | 0.268 |

NY – nuciferine yield, EV – experimental values, PV – predicted values.

Based on the experimental data, a second-order polynomial model was established to predict the yield of nuciferine from leaves of N. nucifera: Y = -1.76116 + 0.027076 X1 - 3.62500 × 10^-3 X2 + 0.63082 X3 + 9.0 × 10^-4 X1 X2 - 1.0375 × 10^-3 X1 X3 - 0.015750 X2 X3 - 1.39250 × 10^-4 X1^2 - 0.059700 X2^2 - 0.09145 X3^2

Table 2 was given the summary results of ANOVA of quadratic model, indicating the importance and sufficiency of the selected model.

The F-value (4.28) and P-value (0.0250 < 0.05) demonstrated that the established model had statistically significant effects. R^2 and Adj. R^2 were 0.8611 and 0.6825 respectively, suggesting that the model was in good fit of the experimental data. The low coefficient of variation (4.92%) also confirmed the good reproducibility of the model. The lack of fit further verified the correctness of the model (0.0832 > 0.05). Through the significance (P < 0.05) coefficient of the second-order polynomial model, the effects of three independent variables (ethanol concentration, solid-liquid ratio and extraction times) on nuciferine yield were reported. The lower values of the variables F and P, the greater the impact on the response. It can be seen from Table 2 that the extraction concentration (X1) had a significant impact on the reaction (P < 0.05); the solid-liquid ratio (X2) and extraction times (X3) had no significant impact on the response (P > 0.05). In addition, there was significant difference in X3^2 (P < 0.05).

The three-dimensional (3D) response surfaces showed the significant interaction of independent variables on nuciferine yield (P < 0.05). Figure 2 illustrated the interaction of two process factors at the same time, while the third factor was fixed at its intermediate level.
Table 2. Analysis of Variance (ANOVA) of Box-Behnken Test

| Source | Sum of | df | Mean squares | F value | P value | P value |
|--------|--------|----|--------------|---------|---------|---------|
| Model  | 0.14   | 9  | 0.016        | 4.28    | 0.0250  | Significant |
| X_1    | 0.064  | 1  | 0.064        | 19.80   | 0.0030  |
| X_2    | 1.176×10^3 | 1 | 1.176×10^3  | 0.36    | 0.5651  |
| X_3    | 7.220×10^4 | 1 | 7.220×10^4  | 0.22    | 0.6506  |
| X_1X_2 | 1.296×10^3 | 1 | 1.296×10^3  | 0.40    | 0.5464  |
| X_1X_3 | 1.722×10^3 | 1 | 1.722×10^3  | 0.53    | 0.4888  |
| X_2X_3 | 9.923×10^4 | 1 | 9.923×10^4  | 0.31    | 0.5965  |
| X_1^2  | 0.013  | 1  | 0.013        | 4.05    | 0.0841  |
| X_2^2  | 0.015  | 1  | 0.015        | 4.65    | 0.0680  |
| X_3^2  | 0.035  | 1  | 0.035        | 10.91   | 0.0131  |
| Residual| 0.023  | 7  | 3.227×10^3   |         |         |
| Lack of fit | 0.018 | 3  | 5.879×10^3   | 4.75    | 0.0832  | Not significant |
| Error  | 4.951×10^3 | 4 | 1.238×10^3   |         |         |
| Total  | 0.16   | 16 |              |         |         |

CV = 27.57%  \quad R^2=0.8611  \quad \text{Adj}R^2=0.6825

*p<0.05 – significant, **p<0.01 – very significant.

It can be seen from Figure 2 that with the increase of concentration of ethanol from 50% to 90%, the yield of nuciferine added, but with the further increase of solid-liquid ratio, the yield of nuciferine decreased. With the increase of extraction times (from 2 times to 4 times), the yield of nuciferine increased slightly, but with the increase of solid-liquid ratio, the yield of nuciferine increased significantly. Compared with other analysis factors, the extraction concentration was the greatest and the extraction times had the least effect on the yield of nuciferine.

Figure 2. 3D response surface for yield of nuciferine

The optimum extraction process of nuciferine with cellulase auxiliary ultrasonic-assisted extraction with Design-Expert8.0.6 software was as follows: 87% ethanol concentration, 1:26g/mL solid-liquid ratio, 2 times. The extraction content was 0.343 mg/g by three parallel confirmatory tests. The difference between the model and the predicted value was 0.025%, which was not much different from the predicted value of the model, which fully verified the reliability of the model.

3.3. Confirmatory Test

3.3.1. Test without cellulose
1.00 g leaves of *N. nucifera* was placed in Erlenmayer flasks, 10 mL PH 5.0 citric acid buffer solution was added and placed in 55 °C water bathing for 30 min, then adding 26 mL 87% ethanol solution. The samples were extracted under conditions of 60 °C, 100W, 25min, 3 times with Ultrasonic Cleaner. The filtrate was combined and concentrated, the solution was fixed in a 10 mL volumetric flask with 87% ethanol solution, shaken well. The content of nuciferine was determined by "2.4.2". The extraction rate was 0.010%.
3.3.2. Test with cellulose.

1.00 g leaves of *N. nucifera* was placed in Erlenmayer flasks, added 10% cellulase and 10 mL of citric acid buffer solution with pH 5.0, and placed in 55 °C water bath for 30 min, then adding 26 mL 87% ethanol solution. The extraction conditions are the same as above “3.3.1”, the content of nuciferine was determined by “2.4.2”. The extraction rate was 0.031%.

The results showed that the extraction rate of nuciferine with cellulase was higher than that without cellulase.

4. Conclusion

In this study, based on the single factor experiment of ultrasonic-assisted extraction of nuciferine from *N. nucifera*, RSM was used to optimize cellulase auxiliary ultrasonic-assisted extraction process to extract nuciferine from *N. nucifera*. Through three factors, the optimum ultrasonic extraction process of three levels of Box-Behnken was 87% ethanol concentration, the ratio of solid to liquid was 1:26 g/mL, the extraction times was 3 times, the results showed that the average extraction content of nuciferine was 0.343 mg/g. And the extraction rate of nuciferine with cellulase was higher than that without cellulase. The results of response surface method were accurate, effective and reliable, and the model of interaction of different factors had a good fit within the experimental conditions. It can save reagents and raw materials, shorten the extraction time, improve efficiency, improve the bioavailability of nuciferine from *N. nucifera*, and provide a more effective method for the extraction of nuciferine from *N. nucifera*.

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