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Research Article

Extractions and Purification of Nuciferine from Lotus Leaves

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Abstract: Four kinds of extraction methods such as Acid-Ethanol Extraction (AEE), Ultrasound-Assisted Extraction (UAE), Microwave-Assisted Extraction (MAE) and Enzyme-Assisted Extraction (EAE) were used to extract Nuciferine from lotus leaves. The crude Nuciferine was further purified by re-crystallization. Results showed that compared with AEE, MAE and EAE, UAE exhibited highest extraction ratio (97.05%) with the irradiation power of 400 W at 50°C for 30 min, which indicates UAE is an efficient, rapid and simple Nuciferine extraction technique. The mixed solvent of acetone, petroleum ether, methanol and acetonitrile rather than single solvent was shown to be the most effective for re-crystallization of Nuciferine while stirring (600 rpm) at a storage temperature of 10°C for 24 h, resulting in higher purity (96.85%) and yield (94.7%). The purity of Nuciferine was increased from an average of 53.19% of the crude product to an average of 96.85% of purified product that were detected by the HPLC analysis. So, UAF combined with re-crystallization can be applied to extraction and purification of Nuciferine to improve its industrial mass production and increase greatly economic additional value.

Keywords: Extractions, nuciferine, purification, recrystallization

INTRODUCTION

The lotus leaves have been used as a traditional Chinese herb for various medicinal purposes in oriental medicine, such as cleaning heat, losing weight, treating sweating, hematemesis, epistaxis, hemoptysis, hematuria, metrorrhagiam and anti-HIV activity, etc., (Agnihotri *et al*., 2008; Guinaudeau *et al*., 1975; Guinaudeau *et al*., 1983; Han *et al*., 1989; Kashiwada *et al*., 2005; Kang *et al*., 2005; Huang *et al*., 2007). Recently, the lotus leaves have also been prepared healthy tea and as food supplements, thus showing very promising market perspective (Li *et al*., 2000). Lotus leaves taste slightly bitter and contain alkaloids such as N-nornuciferine, O-nornuciferine, Nuciferine and Roemerine, while Nuciferine is the main alkaloid in the lotus leaves (Li *et al*., 2000). These active ingredients were isolated and the chemical structures were determined, especially Nuciferine (Fig. 1). Currently, Nuciferine has been widely used as a hygienical component for lowering blood pressure and lipid, as well as some new potential drugs. Recently, many pharmaceutical and healthcare services have produced the supplements containing different Nuciferine purities. The low Nuciferine purity (<20%) is applied for the weight-loss supplement and the high Nuciferine purity (>80%) is use to make some medicines for treating the pathological demand patients, such as obesity, hypotension, hypercholesterolemia,

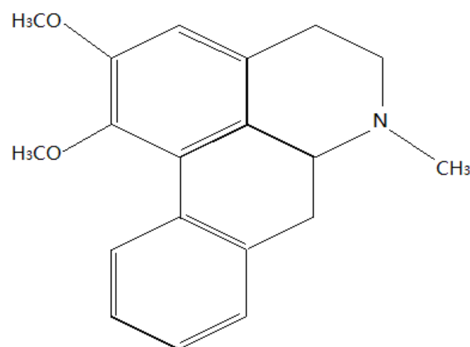

Fig. 1: The chemical formula of nuciferine

arteriosclerosis, etc. So, it 's necessary to develop a cost-effective and industrial process for preparing different purities of Nuciferine in order to meet different business requirements (Zheng *et al*., 2010; Chen *et al*., 2007; Luo *et al*., 2005).

Nuciferine is a kind of aporphine type alkaloid derived from benzylisoquinoline. The studies showed that Nuciferine is an alkalescent alkaloid, which leads to insolubility in water but solubility in acid aqueous solution (Kupchan *et al*., 1963; Wang, 2008). The mechanism is that Nuciferine can combine with acid to produce salt dissolving in water. Besides, Nuciferine is lipophilic substance dissolving in organic solvents such as ethanol, methanol, chloroform, etc. The traditional extraction method, Acid-Ethanol Extraction (AEE), is

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often applied to extract Nuciferine. However, extraction ratio is very low by using this traditional method. So, based on this method, some assisted methods are investigated, such as Ultrasound-Assisted Extraction (UAE), Microwave-Assisted Extraction (MAE), Enzyme-Assisted Extraction (EAE), etc. These methods are relatively simple to perform. They can shorten extraction times and decrease the release of toxic pollutants through reducing organic solvent consumption (Khoddami *et al*., 2013; Lin *et al*., 2008; Ma *et al*., 2010; Zhong *et al*., 2007). However, the comparison between different extraction methods has still not been studied systematically. Besides, High-speed countercurrent chromatography, membrane separation technique and column chromatography have been used to obtain high purity Nuciferine (Chen *et al*., 2007; Zheng *et al*., 2010). Many studies indicate that the extraction and purification technologies of Nuciferine are extremely complex, especially purification technology. Some purification methods, such as high-speed countercurrent chromatography, membrane separation technique and column chromatography can obtain high purity Nuciferine, but the yield is very low and the cost of production is very high, which leads these purification technologies not to be used for mass production.

Re-crystallization is a simple, energy-efficient and environmentally friendly process that is widely applicable and has a low investment cost. This technology maximizes the quality of the final product and also results in a higher value-added product. The degree of super-saturation during purification via solution re-crystallization is an important parameter that has an influence on the purity of the crystal. Reducing solubility by the addition of an organic solvent leads to super-saturation. The type and amount of organic solvent in the solution re-crystallization are important parameters (Liu *et al*., 2006; Khoddami *et al*., 2013). Highly-purified Nuciferine is crystalline powder whose color is from light yellow to white according to its purity level. The higher the purity of Nuciferine is, the lighter the color of Nuciferine is. Therefore, the purpose of this study was to determine the optimal extraction and re-crystallization purification of Nuciferine. We attempted to develop an extraction and re-crystallization method capable of more conveniently and efficiently purifying Nuciferine in high purity and yield and systematically optimizing the main extraction and re-crystallization parameters by using High Performance Liquid Chromatography (HPLC). The results of this study will become a good foundation for industrial mass production of Nuciferine to improve greatly economic additional value of lotus leaves.

METHODOLOGY

Chemical: Dried lotus leaves were purchased from Chinese medicinal herbs store (Hefei, China). The content of Nuciferine was about 13%. Standard sample of Nuciferine was purchased from National Institutes for Food and Drug Control. Cellulase (100,000 U/g) was purchased from Sigma. Ethanol, Hydrochloric acid (HCl), sodium hydroxide, chloroform, ethyl acetate, acetone and petroleum ether were purchased from Nankai University Chemical Co., (Tianjin, China). Acetonitrile, methyl alcohol, triethylamine and acetic acid used for HPLC analysis were purchased from Tedia Company, Inc. (Fairfield, Ohio, USA) and chromatographic grade. All other reagents and solvents were of analytical grade.

The extraction of crude nuciferine:

Acid-Ethanol Extraction (AEE): (50.0 g) dried and powdered lotus leaves (20 meshes) were soaked and extracted 3 times by 500 mL 70% ethanol containing 0.15% hydrochloric acid stirring at 60°C for 6 h using a magnetic stirrer. The extract solution was filtered and combined. Then, sodium hydroxide was added to the filtrate to reach neutralization and concentrated. Ethyl acetate was added to the concentrated solution to extract Nuciferine. 40% sodium hydroxide was added to the concentrated extract to obtain flocculation precipitation. The precipitation was dried by rotary vaporization under reduced pressure at 30°C. Crude Nuciferine was obtained which was used for further isolation and separation. Nuciferine purity (%) was analyzed and determined by using HPLC. Extraction ratio (%) was calculated. Every value was measured three times and then averaged.

Ultrasound-Assisted Extraction (UAE): (50.0 g) dried and powdered lotus leaves (20 meshes) were extracted three times by 500 mL 70% ethanol containing 0.15% hydrochloric acid using an ultrasound bath (30 KHz, 400 W) at 50°C for 30 min. The extract solution was filtered and combined. Then, sodium hydroxide was added to the filtrate to reach neutralization and concentrated. Ethyl acetate was added to the concentrated solution to extract Nuciferine. 40% sodium hydroxide was added to the concentrated extract to obtain flocculation precipitation. The precipitation was dried by rotary vaporization under reduced pressure at 30°C. Crude Nuciferine was obtained which was used for further isolation and separation. Nuciferine purity (%) was analyzed and determined by using HPLC. Extraction ratio (%) was calculated. Every value was measured 3 times and then averaged.

Microwave-Assisted Extraction (MAE): (50.0 g) dried and powdered lotus leaves (20 meshes) were extracted three times by 500 mL 70% ethanol containing 0.15% hydrochloric acid using an microwave heating (2000 MHz, 700 W) at 50°C for 20 min. The extract solution was filtered and combined. Then, sodium hydroxide was added to the filtrate to reach neutralization and concentrated. Ethyl acetate was added to the concentrated solution to extract Nuciferine. 40% sodium hydroxide was added to the concentrated

extract to obtain flocculation precipitation. The precipitation was dried by rotary vaporization under reduced pressure at 30°C. Crude Nuciferine was obtained which was used for further isolation and separation. Nuciferine purity (%) was analyzed and determined by using HPLC. Extraction ratio (%) was calculated. Every value was measured 3 times and then averaged.

Enzyme-Assisted Extraction (EAE): (50.0 g) dried and powdered lotus leaves (20 meshes) were soaked completely in 500 mL pH 4.5 citric acid buffer solution and 0.15% cellulase was added, stirring for 2 h at 50°C using a magnetic stirrer. Then, 1000 mL 0.5% hydrochloric acid solution were added to enzyme hydrolysis solution and extracted for 24 h, then concentrated and adjusted pH value to 3. The concentrated solution was centrifuged (5000 rpm, 15 min) to obtain the supernatant and precipitation. Thereafter, chloroform was added to the supernatant to extract Nuciferine. The extracts were combined and evaporated to dryness by rotary vaporization under reduced pressure at 30°C. Crude Nuciferine was obtained which was used for further isolation and separation. Nuciferine purity (%) was analyzed and determined by using HPLC. Extraction ratio (%) was calculated.

Re-crystallization purification of nuciferine: The obtained crude Nuciferine was diluted to 50 mL with 95% ethanol and filtrated through a 0.45 μm filter to remove macromolecular substances such as protein, polysaccharide, etc. To efficiently induce crystal formation by a reduction in solubility, the Nuciferine solution was slowly added drop wise in several kinds of organic solvents (acetone, petroleum ether, methanol and acetonitrile) while stirring (600 rpm). Then, acicular crystal was produced at a storage temperature of 10°C for 24 h. The Nuciferine crystal was washed with the organic solvents used for the re-crystallization process in order to obtain a clear crystal product. Finally, the crystal product was dried under vacuum at 35°C for 24 h and analyzed by HPLC.

HPLC qualitative and quantitative analysis:
Chromatographic conditions: A Waters 1500 liquid chromatography (Waters, USA) consists of a 1515 and 1525 binary gradient pump, an injector with a 200 μL injection loop (Rheodyne®, IDEX Corporation, USA) and Waters 2489 UV/Vis detection. A guard column, SB-G (6×50 mm, 10 μm) and a Welch Materials C18 (4.6×250 mm, 5 μm) were connected in tandem. The mobile phase used for HPLC was acetonitrile and 0.1% triethylamine aqueous solution filtered through 0.45 μm Micro PES membrane filter (Membrane Co., Germany). A linear gradient elution of solvent A (acetonitrile) and solvent B (0.1% triethylamine aqueous solution) was used. The gradient elution was programmed as follows: 0-10 min, 30-60% A; 10-25 min, 60-80% A; 25-30 min, 80-100% A. The injector and columns were maintained at 30°C. The HPLC system was performed at a flow rate of 1.0 mL/min at 30°C. The wavelength was set at 270 nm and the injection volume was 20.0 μL. The Nuciferine sample and standard solution was accurately filtered through 0.45 μm filters before injecting into the chromatographic system.

Linearity: Nuciferine standard was prepared in methyl alcohol in range of 1.0-80.0 μg/mL. The least squares linear regression analysis was used to determine the slope and intercept. Every value was measured at least three times and then averaged.

Statistical analysis: Results are expressed as mean±standard deviations of triplicate analyses for each sample unless otherwise stated. Statistical significance was assessed with one-way Analysis of Variance (ANOVA) using the ORIGIN 7.5 (OriginLab Inc. USA) for windows programme. Treatment means were considered significantly different at $p<0.05$.

The precision and accuracy of the analytical method was determined by assaying the Quality Control (QC) samples with low, medium and high concentrations. Each sample was triplicate. The accuracy was determined by comparing the calculated concentration which was obtained from the calibration curves to the theoretical concentration. The extraction recoveries were measured at three different QC concentrations by comparing peak areas extracted with those of the same concentration of mixed standard solution ($n = 3$).

RESULTS AND DISCUSSION

Optimization chromatographic separation conditions: We investigated the separation of aqueous acetonitrile, aqueous acetonitrile containing 0.1% ammonia and aqueous acetonitrile containing 0.1% triethylamine as mobile phase on C18 column. The peaks were very broad and overlapped in these solvent systems except for aqueous acetonitrile containing 0.1% triethylamine. So, a linear gradient elution of solvent A (acetonitrile) and solvent B (0.1% triethylamine aqueous solution) was adopted. The gradient elution was programmed as follows: 0-10 min, 30-60% acetonitrile; 10-25 min, 60-80% acetonitrile; 25-30 min, 80-100% acetonitrile.

Through the HPLC analysis of standard Nuciferine sample, the curve was obtained between sample concentration and peak area. The result showed that linear relationship was observed in the calibration curves in the range of 1.0-80.0 μg/mL. The regression equation was $y = 246000x-12700$. In the equation, y and x represent the peak area and the concentration of

Table 1: Recovery, RSD and LOD studies of nuciferine in the range of different concentration

Concentration (μg/mL)	Concentration determined (μg/mL)	Recovery (%)	RSD% (n = 3)	LOD (μg/mL)
1	1.037	99.64	0.01	0.23
20	20.348	102.42	0.07	0.17
40	40.153	99.78	0.04	0.28
60	60.189	101.64	0.05	0.36
80	81.043	104.32	0.02	0.14

the Nuciferine samples, respectively. According to the regression equation, the extraction ratio can be calculated exactly. Some parameters such as recovery, Relative Standard Deviation (RSD) and Limits of Detection (LOD) were determined under the above optimized conditions. The results were summarized in Table 1. Calibration graphs plot in the form of peak area versus standard concentration of Nuciferine was run for all analyses. Good linearity was observed with the regression coefficients (R^2 = 0.9999). The LODs of the analyses were ranged from 0.14 to 0.36 μg/mL. The reproducibility study was carried out on three repeated measurements. From Table 1, the method had good Reproducibility (RSD) obtained was between 0.01 and 0.07. The recoveries were in the range of 99.64 and 104.32%. It can be concluded that the HPLC method performed has high separation efficiency, good resolution and higher sensitivity.

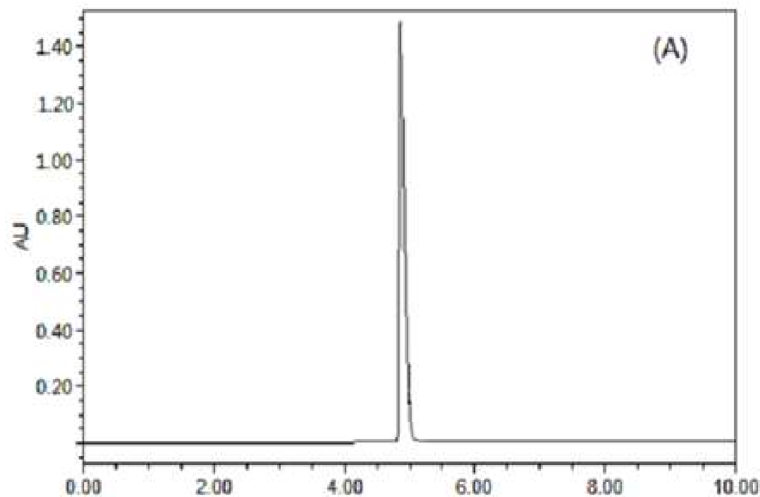
The optimal conditions of four extraction methods: The univariate method was used in all extraction methods of Nuciferine for optimization of the different parameters including extraction time, extraction temperature, concentration of extraction solvent, power, etc. Besides, the orthogonal experiment indicates that each single factor has notability influence on the extraction ratio of four methods for their F value <0.05 and 0.01 (in Table 2). The results are shown in Fig. 2. It can be seen in Fig. 2A that for AEE, the extraction ratio increased when hydrochloric acid concentration was changed from 0.01 to 0.20%. No significant increase in efficiencies was observed when it was increased to 0.15%. Similarly, the extraction ratio increased when ethanol concentration varied from 10 to 90%. It was obvious that the optimum extraction time and temperature should be set at 6 h and 60°C, respectively. From Table 2, we can see that the extraction ratio could reach the maximum value (58.78%) under the optimal conditions such as 70% ethanol concentration, 0.15% hydrochloric acid concentration, 60°C and 6 h. Nuciferine purity determined by HPLC was 23.57%. For EAE (Fig. 2B), the extraction ratio of Nuciferine increased when enzyme concentration was changed from 0.05 to 0.25%. No significant increase in efficiencies was observed when it was increased to 0.15%. The extraction ratio of Nuciferine was sensitive to changes in temperature and pH. It was obvious that the extraction ratio of Nuciferine increased with pH increasing when pH was less than 4.5. While it decreased with pH increasing when pH was more than 4.5. So, the optimum pH should be 4.5. The optimum extraction time and temperature should be set at 2 h and 60°C, respectively. From Table 2, we can see that the extraction ratio could reach the maximum value (82.20%) under the optimal conditions such as 0.15% cellulose, pH 4.5, 2 h and 50°C. Nuciferine purity determined by HPLC was 32.29%. In Fig. 2C, for UAE, the extraction ratio of Nuciferine was increased with ultrasound power increasing. No significant increase in efficiencies was observed when it was increased to 400 W. It was obvious that the optimum extraction time and temperature should be set at 30 min and 50°C, respectively. From Table 2, we can see that the extraction ratio could reach the maximum value (97.05%) with the irradiation power of 400 W at 50°C for 30 min. Nuciferine purity determined by HPLC was 53.19%. For MAE, as shown in Fig. 2D, the extraction ratio of Nuciferine was increased with microwave power increasing. No significant increase in efficiencies was observed when it was increased to 700 W. It was obvious that the optimum extraction time and temperature should be set at 20 min and 50°C, respectively. From Table 2, we can see that the extraction ratio could reach the maximum value (83.79%) with the irradiation power of 700 W at 50°C for 20 min. Nuciferine purity determined by HPLC was 39.05%. So, among the main extraction methods, it was concluded that UAF is the optimum extraction method of Nuciferine based on the extraction ratio and purity.

Table 2: Analyses of orthogonal experiment of four extraction methods

Method	Factors	M.S.	F-value	Pr>F	Extraction ration (%)	Purity (%)
AEE	a (6 h)	30.45630	46.22	<0.0001		
	b (60°C)	3.81500	5.79	0.0242	58.78	23.57
	c (0.15%)	16.01520	24.30	0.0002		
	d (70%)	6.22980	9.45	0.0061		
EAE	a (2 h)	28.30430	98.54	<0.0001		
	b (50°C)	2.94170	10.24	0.0048	82.20	32.29
	e (0.15%)	20.82760	72.51	<0.0001		
	f (4.5)	5.43300	18.91	0.0006		
UAE	a (30 min)	6.33450	122.60	0.0004		
	b (50°C)	2.56440	10.01	0.0341	97.05	53.19
	g (400 W)	6.72450	29.41	0.0056		
MAE	a (20 min)	3.45630	98.35	0.0003	83.79	39.05
	b (50°C)	4.39287	100.05	0.0043		
	h (700 W)	5.77650	35.68	0.0018		

a: Time; b: Temperature; c: HCl concentration; d: Ethanol concentration; e: Enzyme concentration; f: pH; g: Ultrasound power; h: Microwave power; M.S.: Mean square

Fig. 2: The optimation of different extraction methods; (A): AEE; (B): EAE; (C): UAE; (D): MAE



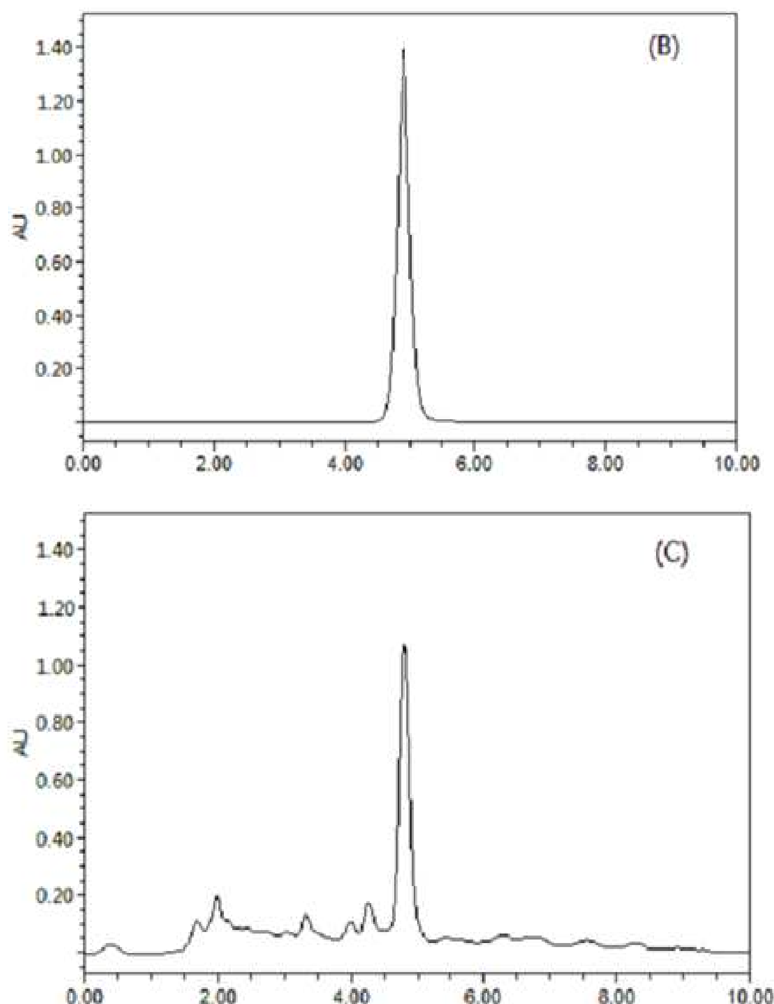


Fig. 3: HPLC chromatograms of; (A): Standard nuciferine; (B): Purified nuciferine; (C): Crude nuciferine

Re-crystallization purification of nuciferine: We tried re-crystallization from various kinds of solvents such as acetone, petroleum ether, methanol and acetonitrile and combination of solvents. We were not able to obtain crystals by re-crystallization from above single solvent, even if the crystals were added as seed crystals among re-crystallization. While the light yellow needle crystals were formed when the Nuciferine solution was slowly added drop wise in several kinds of organic solvents (acetone, petroleum ether, methanol and acetonitrile) while stirring (600 rpm) at a storage temperature of 10°C for 24 h. In Fig. 3, compared with the crude Nuciferine before re-crystallization, a higher purity (96.85%) and yield (94.7%) of the purified Nuciferine after re-crystallization were obtained by using HPLC analysis.

An efficient method including UAF and re-crystallization has been developed for extraction and purification of Nuciferine from lotus leaves. The optimum UAF and re-crystallization conditions were determined. Compared with AEE, MAE and EAE methods, UAF obtained highest extraction ratio (97.05%) and purity (53.19%) and consumed short extraction time (30 min), which demonstrates UAF is a simple, rapid, effective extraction method. A re-crystallization process was optimized to efficiently purify Nuciferine in high purity and yield. In this study, the mixed solvent of acetone, petroleum ether, methanol and acetonitrile rather than single solvent was shown to be the most effective for re-crystallization of Nuciferine, resulting in higher purity (96.85%) and yield (94.7%). Crystals were produced at a storage temperature of 10°C for 24 h. So, UAF combined with re-crystallization can be applied to extraction and purification of Nuciferine to improve its industrial mass production and increase greatly economic additional value.

CONCLUSION

An efficient method including UAF and re-crystallization has been developed for extraction and purification of Nuciferine from lotus leaves. The optimum UAF and re-crystallization conditions were

determined. Compared with AEE, MAE and EAE methods, UAF obtained highest extraction ratio (97.05%) and purity (53.19%) and consumed short extraction time (30 min), which demonstrates UAF is a simple, rapid, effective extraction method. A re-crystallization process was optimized to efficiently purify Nuciferine in high purity and yield. In this study, the mixed solvent of acetone, petroleum ether, methanol and acetonitrile rather than single solvent was shown to be the most effective for re-crystallization of Nuciferine, resulting in higher purity (96.85%) and yield (94.7%). Crystals were produced at a storage temperature of 10°C for 24 h. So, UAF combined with re-crystallization can be applied to extraction and purification of Nuciferine to improve its industrial mass production and increase greatly economic additional value.

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