Preparation of high purity apigenin from Adinandra nitida leaves by antisolvent recrystallization method

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Abstract

High purity apigenin(AP) was prepared by antisolvent recrystallization method from old Adinandra nitida leaves. The procedure was as follows: Adinandra nitida leaves were extracted by boiling water, and the extracting solution was hydrolyzed with sulfuric acid to obtain crude apigenin(58.9%); the purity of crude apigenin was further improved by antisolvent recrystallization method. A satisfactory purity(98.47%) and yield(86.65%) was obtained under the optimum purifying condition. Scanning electronic microscopy(SEM),differential scanning calorimetry(DSC), Fourier transform infrared spectroscopy(FTIR) and HPLC-MS/MS were investigated.

Title: Preparation of high purity apigenin from Adinandra nitida leaves using acid hydrolysis

in situ extraction followed by antisolvent recrystallization method

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Key words: Adinandra nitida, Antisolvent recrystallization, apigenin, purification, response surface method.

Statement of Novelty

Adinandra nitida is a plant growing in southern China. It is usually used to make tea. As we know, tea is usually made from the young leaves of plants, so the old leaves are discarded. The novelty of this study is to prepare high-purity apigenin from the discarded old leaves of Adinandra nitida, and the preparation method is antisolvent recrystallization, which has never appeared in the purification of apigenin.

1. Introduction

Adinandra nitida, known as Shiyacha by Chinese, is a plant growing in southern China. Its leaves have been used as tea and Chinese herbal medicine for hundreds of years by local people.[1]Shiyacha has the special flavor of returning to sweet after drinking. It not only refreshes and quenches thirst, expels greasiness and dullness, but also has the effects of anti-inflammatory, detoxification, blood pressure reduction, antipyretic and hemostasis.[2-4] It is commonly used by the people to prevent and treat various diseases such as inflammation, nameless swelling and pain, and trauma. It can not only receive satisfactory curative effect, but also has no side effects. Shiyacha has these effects because it is rich in flavonoids, and the total flavonoid content in Shiyacha was more than 20%.[5] Some published reports have proved that the Shiyacha are abundant in apigenin glucosides, such as camellianin A and camellianin B, especially camellianin A, which can be hydrolyzed to apigenin.[6]

Apigenin(Fig. 1), chemically known as 4', 5, 7-trihydroxyflavone, is a kind of yellow powder and belongs to flavones.[7-9] The molecular weight of apigenin is 270.24 g/mol.[10]Apigenin is extensively found in a variety of plants, such as parsley, celery, onions, oranges, chamomile, maize, rice, tea, wheat sprouts, and some grasses.[11,12] As a plant-derived flavonoid, apigenin is gradually gaining tremendous appreciation because of its amazing pharmacological properties.[10] This compound has a variety of biological activities, first of all, anti-inflammatory, anti-cancer, antioxidant and anti-allergic activities.[13-16] The antitumor effects of apigenin have been proved by inhibiting the activity of several cell proliferation regulating enzymes, nitric oxide inhibition, prostaglandin (PG) production and selective apoptotic effects on tumor cells.[9,17] Beyond that, recent studies have shown that as antiviral drugs, it has potential effects on hepatitis C virus (HCV), herpes simplex virus type I (HSV-1), influenza virus and human immunodeficiency virus type I (HIV-1).[18,19]

Although apigenin has great pharmacological potential, there are few reports about apigenin purification. Generally speaking, common purification methods, such as recrystallization and liquid-liquid extraction, are used to purify drugs. Liu et al.[6]purified the apigenin by ethanol recrystallization, increasing the purity of apigenin from 63.93% to 93.05%. These methods are cheap and easy to operate, but they are often inefficient, accompanied by the consumption of a large number of organic solvents. High speed countercurrent chromatography and preparative high performance liquid chromatography (HPLC) are often used to purify drugs, but these are time-consuming and laborious, and are not suitable for industrial production. Antisolvent recrystallization, which used to be used to change the crystal form of drugs or produce ultrafine particles, now is also used to purify drugs. This method has the advantages of simple preparation process, simple operation, low cost and high yield. It has a broad application prospect in the pharmaceutical industry. Previously, antisolvent recrystallization has been successfully used to purify ginkgo flavonoids and ellagic acid, the purity of Ginkgo flavonoids and ellagic acid reached 53.85% and 98.07% respectively.[20,21]

Normally, the tender leaves of Adinandra nitida was used to make tea, however, the old leaves was abandoned, which resulting in a waste of this resource. In view of the above background, the objective of this study was to obtain high purity AP by antisolvent recrystallization method from old Adinandra nitida leaves which can not be used to make tea. The effects of changing the recrystallization temperature, the drug concentration, the stirring time and the volume ratio of antisolvent to solvent on the purity and yield of AP were investigated using a factorial design, response surface methodology (RSM) with a central composite design(CCD). Under the optimal conditions provided by the RSM, high-purity AP was prepared with considerably simple process.

2. Materials and methods

2.1 Materials

The leaves of Adinandra nitida was obtained from Guangdong. Reference standard of apigenin was purchased

from Shanghai Aladdin Biological Technology Co. Ltd. Methanol (HPLC grade), acetonitrile (HPLC grade) and ethanol (analytically pure) were supplied by Shandong Yuwang Industrial Co. Ltd. Chemical Branch (Shandong, China). N-Methyl pyrrolidone (NMP) and sulfuric acid were of analytical grade and obtained from Tianjin Zhiyuan Chemical Reagent Co.Ltd. Deionized water was prepared with Hitech-K Flow Water Purification System (Hitech Instruments Co. Ltd., Shanghai, China).

2.2 Purification of apigenin by antisolvent recrystallization

2.2.1 Method for preparing apigenin from the leaves of Adinandra nitida

Apigenin was prepared according to the previous study[5], slightly modified as follows: 50 g of dried leaves of Adinandra nitida were ground into powder using a miller and then suspended in 500 mL water. The mixture was boiled for 1 h and then filtered. Under this condition, the filter residue was extracted again. And then the aqueous extract was combined. After cooling, the aqueous extract was mixed with sulphuric acid in the ratio of 1:50 (by volume). Then the mixture was heated for 20 min in a 60 °C water bath and then filtered to collect the precipitate. The precipitate was washed with water on the filter until neutral pH was obtained and then dried in an oven at 50 °C for 24 h. Finally, 2.04 g crude AP with a purity of 58.9% were obtained. The overall yield of AP per gram leaves was 2.4%.

2.2.2 Purification process

The antisolvent recrystallization method was applied to purify apigenin. 1 g of crude product obtained was added to 7 mL NMP and placed in a 60 °C water bath under stirring for about 5 min. Then the mixture was centrifuged at 6000× g for 5 min to obtained the supernatant which was apigenin solution, and then the apigenin solution was dropped into a certain volume of deionized water at a certain recrystallization temperature under stirring with certain speed intensity. After the apigenin solution was completely dropped into deionized water, the stirring time was measured. After recrystallization, the suspension was instantly centrifuged at 6000× g for 10 min, and the precipitate obtained was washed twice with deionized water then dried in an oven at 50 °C for 12 h.

2.3 Optimization of theantisolvent recrystallization process

Single factor test were used to investigate the effect of the four factors (the recrystallization temperature, the drug concentration, the stirring time and the volume ratio of antisolvent to solvent) on the purity of apigenin. The recrystallization temperature was between 30-80 °C, the drug concentration was between 60-140 mg/mL, the stirring time was between 5-30 min, and the volume ratio of antisolvent to solvent was between 5:1-30:1.

On the basis of single-factor experiments, in which the ultimate range of four variables including the recrystallization temperature, the drug concentration, the stirring time and the volume ratio of antisolvent to solvent were determined, central composite design (CCD, with α= 2.000) under the response surface methodology (RSM) was employed to further optimize the conditions. 30 experiments were carried out for four factors at five levels. The recrystallization temperature (X_1), the drug concentration (X_2), the stirring time (X_3) and the volume ratio of antisolvent to solvent (X_4) as the independent variables (Table 1). Two responses (purity (Y_1) and yield (Y_2)) were selected in order to be optimized. The experimental design protocols and corresponding results were presented in Table 2.

2.4 High-performance liquid chromatography analysis and quantification

Standard stock solutions of standard AP (1 mg/mL) and purified AP (1 mg/mL) were prepared by dissolving the samples in NMP and diluted with methanol. The solutions were stored at 4 °C in the dark before analysis by HPLC.

The chromatographic system (Waters Corporation, Milford, MA, USA) consisted of a 1525 binary HPLC pump, a 2489 UV/Visible detector and a Diamonsil C18 reverse-phase column (250 mm×4.6 mm, 5 μm, China). The mobile phase for separation of the target analyses was water:methanol:phosphoric acid

(30:70:0.2, v/v/v). The other analysis conditions were: a mobile phase flow rate of 1.0 mL/min; injection volume of 20 μL. The wavelength used for detection of apigenin was 353 nm. Good linearity of the calibration curve for apigenin between 0.78125 and 1000 μg/mL were obtained. The linear equation for apigenin were Y = 82190X - 34308 (R^2 = 0.9999).

2.5 Morphology analysis

The raw apigenin and the purified apigenin were observed by SEM. They were fixed on the silicon wafer and sputtered with gold to a thickness of about 100 nm. The shape and the surface characters tested under high vacuum conditions at an accelerating voltage of 15.0 kV were observed and recorded on the scanning electron microscope (Quanta 200, FEI; The Netherlands).

2.6 Fourier-transform infrared spectroscopy (FTIR)

To investigate the chemical structures of the purified apigenin, FTIR was utilized in the wavenumber range of 400-4000 cm^{-1}at a resolution of 4 cm^{-1} with KBr disks method. Each sample was accurately weighed 0.5 mg, then mixed with KBr of 200 mg respectively, and grinded into fine powders, then pressed into transparent slices for analysis.

2.7 Differential scanning calorimetry (DSC)

DSC measurements of the standard AP and purified AP were obtained in the temperature range of 40-500°C using STA 449 F5 Jupiter® instrument under dynamic nitrogen atmosphere (50 mL/min) and a heating rate of 10 °C/min in hermetically sealed alumina crucibles with approximately 5 mg of the samples.

2.8 High performance liquid chromatography-mass spectrometry (HPLC-MS)

The LS–MS spectrum was monitored using a triple quadrupole mass spectrometer (API 3000, MDS Sciex, Sunnyvale, USA) equipped with an APCI interface. The purified AP and standard AP were dissolved in methanol of chromatographic grade, preparing the solution of 100 μg/mL. According to the testing conditions, the solution of 100 μg/ml could be diluted. The mass spectrometer was operated in electrospray ionization (ESI) positive ion mode with the ion spray voltage 5500 V. The other parameters were as follows: collision gas was at 6 L/min, curtain gas was at 10 L/min, and nebulizing gas was at 12 L/min. The m/z ranged from 100 amu to 1000 amu.

3 Result and discussion

3.1. Investigation of single factor

3.1.1. Effect of recrystallization temperature

To determine the effect of recrystallization temperature, the experiments were performed under various temperatures of 30, 40, 50, 60, 70 and 80 °C, and the other parameters were kept at 60 mg/mL, 10 min and 10:1, for drug concentration, stirring time, and the volume ratio of antisolvent to solvent, respectively. As can be seen from Fig. 2a, the purity of AP increased dramatically when the recrystallization temperature increased from 20 degC to 80 degC, and when the recrystallization temperature was higher than 80 degC, the purity of AP changed slightly. This indicated that the supersaturation of the impurities decreased when the recrystallization temperature increased, which leading to the reduction of impurity's precipitation and the increasing of the purity of AP. The highest purity of AP was obtained at 80 degC, so the temperature range of 50–80 degC was selected for the following experiments.

3.1.2. Effect of drug concentration

The drug concentration was optimized in the range from 60 to 160 mg/mL, and the investigation was conducted at the condition of 50 degC for recrystallization temperature, 10 min for stirring time and 10:1 for the volume ratio of antisolvent to solvent. From Fig. 2b, we can conclude that the purity of AP increased dramatically when the drug concentration increased from 60 to 160 mg/mL. The reason was that the increasing of drug concentration leaded to the increasing of drug's supersaturation, therefore, the

precipitation of drug increased which caused the increasing of the purity of drug. Considering the solubility of AP in solvent was high, the range of 80-160 mg/mL was selected for further optimization.

3.1.3. Effect of stirring time

Different stirring time (5, 10, 15, 20, 25 and 30 min) were selected and compared to determine the optimum range for purity of AP. The other experimental conditions applied were recrystallization temperature of 50 degC, drug concentration of 60 mg/mL and volume ratio of antisolvent to solvent of 10:1, respectively. Fig. 2c shows that the purity of AP significantly increased as the stirring time increased from 5 to 20 min and then decreased with further increase in time from 20 to 30 min. This may be because, with the extension of time (0-20 min), the drug gradually separated out and the purity of AP increased. However, when the time continued to extend (20-30 min), the impurities precipitated more and the purity of AP reduced slightly. So a stirring time range of 15-30 min was selected for the following experiments.

3.1.4. Effect of volume ratio of antisolvent to solvent

A series of experiments was performed with various volume ratios of antisolvent to solvent (5, 10, 15, 20, 25, 30), and the consistent conditions were recrystallization temperature of 50 degC, drug concentration of 60 mg/mL, stirring time of 10 min. The results of which are shown in Fig. 2d. the purity of AP increased distinctly with increasing antisolvent volume for ratios up to 20. Thereafter, the purity of AP decreased slowly. The increase of the volume of antisolvent is helpful to the drug precipitation. It can be concluded that when the volume ratio reached 20, the drug had completely crystallized. However, above 20, the impurities had not completely crystallized and continued to crystallize, which resulting in the reduction of the purity of AP. Therefore, to minimize solvent usage and operating costs, a volume ratio range of antisolvent to solvent of 10-25 was considered optimal for the purification process.

3.2. Optimization of purification process using CCD

Based on the results of the single-factor experiments, the interactions between the main factors—recrystallization temperature, drug concentration, stirring time and volume ratio of antisolvent to solvent—were further studied by RSM. The results are shown in Table 3. The model F -values of 10.52 and 12.14 implied that the two models were all significant. Moreover, there were only 0.01% and 0.01% chances that a "Model F -Value" this large could occur due to noise for quadratic models, respectively. The "Lack of Fit F-value" of 4.14 and 3.63 implied the Lack of Fit was not significant relative to the pure error. There were 6.17% and 7.87% chances that a "Lack of Fit F-value" this large could occur due to noise. So the non-significant lack of fit implied that the model was suitable for data analysis. Values of "Prob > F" less than 0.0500 indicate model terms are significant. From Table 3, X_1 ,X_2 , X_4 ,$X_2{}^2$,$X_3{}^2$ are significant model terms, implied that these terms had significant effect on the purity of AP. And, the terms X_2 , X_4 ,$X_2{}^2$ and$X_4{}^2$ had significant effect on the yield of AP.The final purity(Y_1) and yield(Y_2) of AP were given by the following equations:

$$Y_1 = +97.33 + 0.37X_1 + 0.74X_2 + 0.77X_4 - 0.38X_2X_3 - 0.52X_2{}^2 - 0.52X_3{}^2 - 0.3X_4{}^2 \quad (1)$$

$$Y_2 = +84.73 + 2.38X_2 + 1.64X_4 - 1.18X_2{}^2 - 0.90X_4{}^2 \quad (2)$$

The response surfaces are shown in Fig. 3. Fig. 3a shows that the purity of AP was significantly influenced by drug concentration, increasing dramatically and then remaining constant with increasing drug concentration. The purity of AP increased when the recrystallization temperature increased. Fig. 3b depicts the interaction of recrystallization temperature and volume ratio of antisolvent to solvent on the purity. The purity of AP increased firstly and then changed slightly as the volume ratio of antisolvent to solvent increased. The purity of AP increased when the recrystallization temperature increased. Fig. 3c shows that the purity of AP increasing increased strongly and then remained almost constant when the drug concentration increased; however, the influence of stirring time first increased and then decreased. The three-dimensional response surface for the interaction of stirring time and volume ratio of antisolvent to solvent is shown in Fig. 3d. The purity of AP increased firstly and then decreased as the stirring time increased. However, the purity of AP increased as the volume ratio of antisolvent to solvent increased.

Fig. 4a depicts the interaction of drug concentration and stirring time on the yield of AP. The yield increased strongly and then remained almost constant as the drug concentration increased. The effect of stirring time on the yield was not obvious. From Fig. 4b,the yield increased strongly and then remained almost constant as the volume ratio of antisolvent to solvent increased. However, the influence of recrystallization temperature on the yield of AP was not significant. The three-dimensional response surface for the interaction of drug concentration and the volume ratio of antisolvent to solvent is shown in Fig. 4c. The effect of drug concentration on the yield of AP was larger than that of the volume ratio. With increasing drug concentration, the yield of AP increased strongly and then remained almost constant; and the volume ratio was the same.

Regression model prediction analysis showed that, the optimal experimental conditions were as follows: recrystallization temperature 62.5 degC, drug concentration 109.2 mg/mL,stirring time 19.15 min,volume ratio of antisolvent to solvent 17.5; the purity and yield of AP could reach 98.47% and 86.65% under this condition. The parallel verifying test was carried out for 3 times under the optimal condition, the result was that the purity and yield of AP reached 98.43% and 86.66% respectively. Therefore, these results showed that the model was reliable. After that, all of the following experiments were carried out using the purified AP produced under this optimal condition.

The HPLC chromatograms of extract of Adinandra nitida leaves, acidified extract of Adinandra nitida leaves, crude AP and purified AP were shown in Fig.5. From Fig.5a, the retention time of camellianin A was 6 min. Compared with the chromatograms of extract of Adinandra nitida leaves, the chromatographic peak of camellianin A of acidified extract of Adinandra nitida leaves was almost disappeared, demonstrating that most of camellianin A had been hydrolyzed into AP. From Fig.5b, the retention time of AP was 20 min and there were some peaks of impurities in the chromatogram of crude AP, however, no impurity peaks were found in the chromatogram of purified AP, demonstrating that the impurities had been separated.

3.3. Morphology Analysis

Scanning electron microscopy was applied to evaluate the morphological characteristics of crude apigenin and high purity apigenin lyophilized powder, and the results were shown in Fig. 6. From Fig. 6a we can see that the particles of the raw apigenin presented relatively large sizes and irregular shapes. The morphology image of the purified apigenin lyophilized powder was illustrated in Fig. 6b. The power were regular squares, with uniform size. In conclusion, the antisolvent recrystallization method not only can improve the purity of drugs, but also can change the crystal structure of drugs, which may be helpful to improve the other properties like bioavailability, stability etc.

3.4. FTIR Analysis

The FTIR spectra of standard AP and purified AP are shown in Fig. 7. The main spectrum peaks of the standard AP were at 3276, 2616, 1653, 1607, 1500, 1356, 1244, 1179, 1030, 904, 825, 737, 630, 574 and 500 cm^{-1}, respectively. The broad absorption band at 3276 cm^{-1} was the stretching vibration of –OH. The intensive band at 1653 cm^{-1} proved the existence of C=O group in the structure of the standard AP. However, the purified AP have the absorption bands at 3276, 2616, 1653, 1607, 1500, 1356, 1244, 1179, 1030, 904, 825, 737, 630, 574 and 500 cm^{-1}, respectively. Thus, it can be preliminarily determined that compared to standard AP the chemical structure of purified AP remained unchanged.

3.5. DSC Analysis

The DSC curves of standard AP(a) and purified AP(b) were shown in Fig. 8. Compared to purified AP, standard AP showed a sharper endothermic peak. The endothermic peak area ratio of purified AP to standard AP was 50.4%, demonstrating that 49.6% of purified AP has become amorphous and the water solubility of purified AP may possibly improved.

3.6. LC-MS/MS Analysis

The LC-MS/MS chromatograms of purified AP and standard AP were shown in Fig. 9. The chromatograms

illustrated that in positive mode the m/z of the molecular ion peak of purified AP and standard AP was 271.10. Therefore, the molecular weight of purified AP and standard AP was 270.10, which was coincident with 270.1 provided by the relative reference[6]. Therefore, combined with the results of FTIR and LC-MS/MS, we can draw a conclusion that the chemical structure of AP didn't change in the purification process.

4. Conclusions

A simple and efficient antisolvent recrystallization approach was developed and applied in the purification of AP. With NMP as solvent and water as antisolvent, the purity of obtained AP was extremely high. Optimized by single-factor experiments and RSM, the optimal conditions were determined as: recrystallization temperature 62.5 degC, drug concentration 109.2 mg/mL, stirring time 19.15 min, volume ratio of antisolvent to solvent 17.5. Under the optimal conditions, the purity of AP was 98.43% and the yield of purified AP was 86.69%, which was in close agreement with the predicted values by the model of 98.47% and 86.65%, repectively. An amount of 2.08 g AP with 98.43% purity was obtained from 100 g Adinandra nitida leaves.

In addition, SEM analysis showed that the particle morphology of purified AP was significantly different from that of crude AP, and its particle size was much smaller than that of crude AP. It can be obtained that the peaks of FTIR and HPLC–MS of the purified AP coincided well with that of the standard AP. DSC analysis demonstrated that purified AP has partly become amorphous, which will lead to higher water solubility. Therefore, the purified AP obtained by this method has certain reference value for further drug application. On the other hand, this study was help to the comprehensive utilization of Adinandra nitida resource.

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Author Contributions

Li Wang and Hongda Cai performed the experiments; Xiuhua Zhao and Fengjian Yang wrote the manuscript; Tongtong Feng and Xiaohu Liu conceived and designed the research, Li Wang, Xiaohu Liu and Fengjian Yang analyzed the data and interpreted the results.

Consent for publication

We agreed with the journal policy and provided our consent for the publication.

Competing interests

The authors declare no conflict of interest.

Availability of data and materials

All data generated and analysed during this study are included in this article.

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