Optimization of Extraction Process for Total Flavonoids from *Penthorum chinense* Pursh and Comparison of Their Contents from Different Parts

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Abstract [**Objectives**] This study was conducted to optimize the extraction process of total flavonoids from *Penthorum chinense* Pursh and compare their contents from different parts. [**Methods**] Single factor and orthogonal experiments were designed to optimize the extraction process of total flavonoids from *P. chinense* Pursh with the volume fraction of ethanol, the ratio of material to liquid, heating reflux extraction time and extraction times as factors, and the content of total flavonoids as the index. A verification test was carried out. The optimized extraction process was adopted to compare the contents of total flavonoids from different parts of *P. chinense* Pursh. [**Results**] The best extraction process was extracting the powder of *P. chinense* Pursh for 2.0 h with 20 times of 55% ethanol by reflux twice. Under this condition, the contents of total flavonoids were 3.63%, 8.90%, 11.28%, and 4.36% from stems, leaves, flowers and whole grass of *P. chinense* Pursh, respectively. [**Conclusions**] The process is reasonable, feasible and stable, and can effectively extract total flavonoids from *P. chinense* Pursh. The contents of total flavonoids from different parts of *P. chinense* Pursh were quite different, and the value was higher in the leaves and flowers, so the proportions of leaves and flowers should be paid attention to in the industrial processing of *P. chinense* Pursh.

Key words *Penthorum chinense* Pursh, Total flavonoids, Orthogonal experiments, Extraction process, Content determination

1 Introduction

Penthorum chinense Pursh, belonging to *Penthorum* of Saxifragaceae, is mainly produced in Sichuan, Guizhou, Hunan and other places, and used as a medicine in the folk with its whole grass. It was officially approved by the National Health Commission as a new raw-food material in June 2020. It is a typical medicinal and edible plant in Gulin, Luzhou, Sichuan. *P. chinense* Pursh is mild in nature, bitter and slightly pungent in taste, non-toxic, and attributive to the liver and kidney meridians, and has the effects of clearing away heat and toxic materials, reducing jaundice and resolving dampness, promoting blood circulation and removing stasis, and inducing diuresis to alleviate edema. *P. chinense* Pursh has a unique therapeutic effect on liver diseases and is widely used in modern clinical practice to treat various types of hepatitis, cholecystitis, fatty liver, *etc.*[1].

P. chinense Pursh contains chemical components such as flavonoids, lignans, coumarins, acetophenones, tannins, triterpenes, organic acids, esters, and volatile oils. At present, about 120 compounds have been isolated from *P. chinense* Pursh, including more than 40 flavonoids[2-6], which are the main components of *P. chinense* Pursh[6-7]. Modern research has shown that the total flavonoids of *P. chinense* Pursh have anti-inflammatory[8], antioxidant[9], anti-tumor[10-11], anti-liver injury[12-14], and anti-liver fibrosis[15] effects.

The content of total flavonoids is an important indicator for quality control of traditional Chinese medicine, and its most classic measurement method is UV spectrophotometry. UV spectrophotometry mainly includes colorimetry, differential spectrophotometry, and direct determination methods, among which colorimetry is the most common, and included in various editions of the *Pharmacopoeia of the People's Republic of China*[16]. There has been no report on the determination of total flavonoids in different parts of *P. chinense* Pursh currently. In this study, for the first time, a determination method for the content of total flavonoids in *P. chinense* Pursh was established by $AlCl_3$ colorimetry, with pinocembrin as the control. On this basis, the extraction process of total flavonoids from *P. chinense* Pursh was optimized with the content of total flavonoids as the index; and the total flavonoids in different parts (stems, leaves, flowers and whole grass) of *P. chinense* Pursh were extracted by the optimized process, and their contents were compared, aiming to provide basis for rational development and comprehensive utilization of *P. chinense* Pursh.

2 Materials

2.1 Raw materials and reagents The stems, leaves and flowers of *P. chinense* Pursh and its whole grass (including stems, leaves and flowers) were provided by Gulin Hongan Pharmaceutical Co., Ltd., and were identified as the stems, leaves, flowers and whole grass of *P. chinense* Pursh by Professor Shui Pixian of Southwest Medical University. Before use, they were dried in a 60 ℃ oven to constant weight, and crushed and sieved with a

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No. 4 sieve to obtain coarse powder of each part of *P. chinense* Pursh. The reagents used included rutin standard (batch number: 153-18-4, Guizhou Dida Biological Co., Ltd.), pinocembrin standard (batch number: 480-39-7, Chengdu Push Biotechnology Co., Ltd.), and $AlCl_3$, methanol, $NaNO_2$, $Al(NO_3)_3$ and NaOH, all of which were analytically pure, and the water used was ultrapure.

2.2 Main instruments SHIMADZU2450 ultraviolet-visible spectrophotometer, Shimadzu Company, Japan; HH-W three-purpose thermostatic water tank, Jintan Medical Instrument Factory, Jiangsu Province; 1 ∶ 100 000 analytical balance, Mettler-Toledo Instrument Co., Ltd.; UPH-1-20L ultrapure water system, Sichuan ULUPURE ultrapure Technology Co., Ltd.

3 Methods and results

3.1 Qualitative test on different parts of *P. chinense* Pursh

The stems, leaves and flowers were qualitatively analyzed by hydrochloric acid-magnesium powder reaction, lead acetate reaction and concentrated ammonia water test. The results showed that the stems, leaves and flowers of *P. chinense* Pursh were all positive for hydrochloric acid-magnesium powder, lead acetate and concentrated ammonia water, which indicated that the stems, leaves and flowers of *P. chinense* Pursh all contained flavonoids.

3.2 Study on extraction process of total flavonoids from *P. chinense* Pursh

3.2.1 Sample preparation. (i) Preparation of reference solution. First, 12.5 mg of pinocembrin was accurately weighed, and added with 55% ethanol to dissolve it. Subsequently, the obtained solution was transferred to a 25 mL volumetric flask, and diluted to constant volume to obtain a reference solution of pinocembrin with a concentration of 0.5 mg/mL.

(ii) Preparation of test solution. About 1 g of *P. chinense* Pursh sample was accurately weighed from the dried powder of stems, leaves and flowers of *P. chinense* Pursh and its whole grass (including stems, leaves and flowers), respectively. Each powder was added into a 50 mL round-bottomed flask, and then added with 20 mL of ethanol solution. Reflux extraction was performed with heating twice, 2.0 h each time. Each extraction system was filtered to obtain filtrates, which were merged and concentrated under reduced pressure to about 25 mL. Each concentrate was transferred to a 100 mL volumetric flask, and diluted to constant volume with 55% ethanol to obtain a test solution with a crude drug concentration of about 10 mg/mL.

3.2.2 Determination methods. Accurately, 1 mL of reference substance or test substance was transferred into a 10 mL volumetric flask, and added with 4 mL of 55% ethanol solution of 1% aluminum trichloride. The obtained solution was diluted to constant volume with 55% ethanol, and after standing at room temperature for 10 min, its absorbance was measured at 310 nm. The content of total flavonoids in the sample was calculated according to the standard curve.

3.2.3 Methodological investigation. (i) Investigation of linear relation. Accurately, 0, 0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 mL of pinocembrin reference solution were transferred into 10 mL volumetric flasks, respectively, and each solution was diluted to constant volume with 55% ethanol. The absorbance was determined according to Section **3.2.2**. A standard curve was drawn with concentration C as the abscissa and absorbance A as the ordinate, for linear regression analysis. The regression equation for the pinocembrin standard curve was $A = 8.588\,2C + 0.005$, with a correlation coefficient of $R^2 = 0.999\,9$, indicating a good linear relation of pinocembrin in the range of 0 – 0.1 mg/mL.

(ii) Precision test. Accurately, 1 mL of pinocembrin reference solution was transferred into a 10 mL volumetric flask, and its absorbance was measured according to Section **3.2.2** continuously for 6 times on the same day, and twice a day for 3 d in total, so as to investigate the intra-day and inter-day precision. The intra-day and inter-day *RSD* values were 0.15% and 0.23%, respectively, indicating that the precision of the instrument was good.

(iii) Stability test. Accurately, 1 mL of the sample solution from stems of *P. chinense* Pursh was pipetted into a 10 mL volumetric flask, and its absorbance was measured according to Section **3.2.2** at 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h. The *RSD* was 0.38%, and the results showed that the test solution was stable within 3.0 h.

(iv) Repeatability test. Six portions of *P. chinense* Pursh stems were weighed, about 1 g for each part. Next, each portion was accurately weighed and prepared into a test solution according to Section **3.2.1**. Next, the absorbance values were measured according to Section **3.2.2**. The *RSD* was 1.23%, indicating that the method has good repeatability.

(v) Recovery test. About 0.5 g of the coarse powder from *P. chinense* Pursh stems with a known content (the content of total flavonoids was 3.61%) was weighed accurately, and a total of 9 portions were obtained. Next, pinocembrin standard was added according to 80%, 100% and 120% of the known total flavonoid content, respectively, and test solutions were prepared according to Section **3.2.1**. Absorbance values were determined according to Section **3.2.2**, and total flavonoid contents were calculated according to the standard curve. The differences between measured values and total flavonoid contents in test samples were divided by the amounts of reference substance added to calculate recoveries. The average recovery was 100.4% and the relative standard deviation *RSD* was 2.45%. The results are shown in Table 1.

Table 1 Recovery test of total flavonoids

Sample amount//g	Content mg	Addition amount mg	Measured amount mg	Recovery %	Average recovery %	*RSD* %
0.499 7	18.04	14.43	31.89	95.98	100.4	2.45
0.500 2	18.06	14.45	32.18	97.72		
0.499 9	18.05	14.44	32.95	103.20		
0.499 5	18.03	18.03	36.50	102.40		
0.499 8	18.04	18.04	36.96	104.90		
0.500 8	18.08	18.08	36.09	99.61		
0.500 0	18.05	21.66	39.51	99.07		
0.499 5	18.03	21.64	39.70	100.10		
0.500 3	18.06	21.67	39.93	100.90		

3.2.4 Single-factor experiment investigation of extraction process. (i) Investigation of ethanol concentration. Accurately, 5 portions of *P. chinense* Pursh stems were weighed, 1.0 g each, and 15%, 35%, 55%, 75% and 95% ethanol were added according to the material-to-liquid ratio of 1 ∶ 20, respectively. Next, reflux extraction was performed with heating once in a slightly boiling state for 1.5 h, and after cooling, weight compensation, filtration and merging of filtrates, the obtained filtrate was concentrated under reduced pressure to about 10 mL. Each concentrate was then transferred to 100 mL of volumetric flask, and diluted with 55% ethanol to constant weight. Absorbance values were measured according to the section of Section **3.2.2** to calculate the contents of total flavonoids in *P. chinense* Pursh. The results are shown in Fig. 1A. With the increase of ethanol concentration, the content of total flavonoids increased, and when the ethanol concentration was 55%, the content of total flavonoids reached its maximum. When the ethanol concentration exceeded 55%, the content of total flavonoids decreased with the increase of ethanol concentration. It might be because that the polarity of total flavonoids from *P. chinense* Pursh was similar to that of 55% ethanol. According to the principle that like dissolves like, the total flavonoids of *P. chinense* Pursh had the highest solubility in 55% ethanol solution, so its content was the highest.

(ii) Investigation on material-to-liquid ratio. Accurately, 5 portions of *P. chinense* Pursh stems were weighed, 1.0 g each, and 55% ethanol was added according to the material-to-liquid ratios of 1 ∶ 5, 1 ∶ 10, 1 ∶ 20, 1 ∶ 30 and 1 ∶ 40, respectively. Next, heating reflux extraction was performed once in a slightly boiling state for 1.5 h, and after cooling, weight compensation, filtration and merging of filtrates, the obtained filtrate was concentrated under reduced pressure to a certain volume. Next, each concentrate was then transferred to 100 mL of volumetric flask, and diluted with 55% ethanol to constant weight. Absorbance values were measured according to the section of Section **3.2.2** to calculate the contents of total flavonoids in *P. chinense* Pursh. The results are shown in Fig. 1B. With the material-to-liquid ratio increasing, the content of total flavonoids in *P. chinense* Pursh increased, and the content of total flavonoids in *P. chinense* Pursh was the highest when the material-to-liquid ratio was 1 ∶ 20. It was because that with the material-to-liquid ratio increasing, the contact surface between the medicinal powder and solvent molecules is increasing, and the dissolution rate of total flavonoids in *P. chinense* Pursh is increasing, thus increasing the yield of total flavonoids in *P. chinense* Pursh. When the ratio of material to liquid exceeded 1 ∶ 20, the yield of total flavonoids in *P. chinense* Pursh decreased slightly.

(iii) Investigation on extraction time. Accurately, 5 portions of *P. chinense* Pursh stems were weighed, 1.0 g each, and 20% of 55% ethanol was added, respectively. Next, heating reflux extraction was performed once in a slightly boiling state for 1.0, 1.5, 2.0, 2.5 and 3.0 h, respectively, and the results are shown in Fig. 1C. With the extraction time increasing, the content of total flavonoids in *P. chinense* Pursh increased, and the content of total flavonoids was the highest when the extraction time was 2.0 h. However, the content of total flavonoids decreased after the extraction time exceeded 2.0 h. It might be because that a too-short extraction time led to incomplete extraction of total flavonoids, and the longer the extraction time, the more complete the extraction of total flavonoids, but the structure of flavonoid chemical components might be destroyed at high temperature for a long time, resulting in a decline in the extraction rate of total flavonoids.

(iv) Investigation on extraction times. Accurately, 3 portions of *P. chinense* Pursh stems were weighed, 1.0 g each, and 20 mL of 55% ethanol was added, respectively. Next, heating reflux extraction was performed in a slightly boiling state for 2 h, once, twice and thrice, respectively, and the results are shown in Fig. 1D. The content of total flavonoids extracted once was obviously lower than those extracted twice and thrice, which was due to that too-few extraction times led to incomplete extraction of total flavonoids. With the extraction times increasing, the content of total flavonoids increased and was finally stabilized.

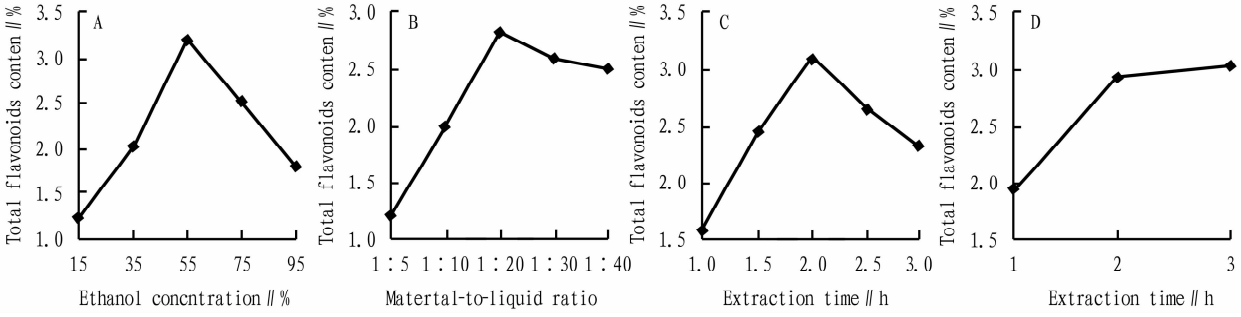

Fig. 1 Effects of different factors on the extraction of total flavonoids from *Penthorum chinense* Pursh

2.2.5 Optimization of extraction process parameters of total flavonoids from *P. chinense* Pursh. On the basis of the previous single-factor experiments, four factors, namely ethanol concentration, solid-liquid ratio, extraction time and extraction times, were selected as the investigation factors, and three levels were selected for each factor, to invest their effects on the content of total flavonoids in *P. chinense* Pursh serving as the index. The experiment was carried out according to the $L_9(3^4)$ orthogonal table. According to the results of intuitive analysis and variance analysis, the optimum extraction process parameters of total flavonoids from

P. chinense Pursh were optimized. The results are shown in Table 2 and Table 3.

Table 2 Analysis of orthogonal experiment on extraction process of total flavonoids

No.	Factor level				Total flavonoid content %
	Ethanol concentration (A)	Material-to-liquid ratio (B)	Extraction time (C)	Times (D)	
1	35% (A1)	1∶10(B1)	1.5 h(C1)	1 (D1)	1.53
2	35% (A1)	1∶20 (B2)	2.0 h(C2)	2(D2)	2.86
3	35% (A1)	1∶30 (B3)	2.5 h(C3)	3(D3)	2.56
4	55% (A2)	1∶10(B1)	2.0 h(C2)	3(D3)	3.56
5	55% (A2)	1∶20 (B2)	2.5 h(C3)	1(D1)	3.49
6	55% (A2)	1∶30 (B3)	1.5 h(C1)	2(D2)	3.31
7	75% (A3)	1∶10 (B1)	2.5 h(C3)	2(D2)	2.06
8	75% (A3)	1∶20 (B2)	1.5 h(C1)	3(D3)	2.41
9	75% (A3)	1∶30 (B3)	2.0 h(C2)	1(D1)	1.56
K_1	6.95	7.15	7.25	6.58	
K_2	10.4	8.76	7.98	8.23	
K_3	6.03	7.43	8.11	8.53	
k_1	2.32	2.38	2.42	2.19	
k_2	3.45	2.92	2.66	2.74	
k_3	2.01	2.48	2.70	2.84	
R	1.44	0.54	0.28	0.65	
Primary and secondary sequence	A > D > B > C				
Optimal level	A_2	B_2	C_3	D_3	

Table 3 Analysis of variance results

Source of variation	*SS*	*df*	*SM*	*F*	*P*
A	3.469	2	1.735	24.22	<0.05
B	0.493	2	0.247	3.443	>0.05
C (error term)	0.143	2	0.072	1.000	>0.05
D	0.735	2	0.367	5.130	<0.05

NOTE Factor C has a relatively small impact on the experimental results, so it is used as an error term; $F_{0.05}(2,2) = 19.00$.

It can be seen from Table 2 that the order of the effects of various factors on the extraction process of total flavonoids was A > D > B > C, and the optimal level combination obtained by intuitive analysis was $A_2B_2C_3D_3$. The results of variance analysis in Table 3 showed that after taking factor C with the smallest variance as the error term, factor A had a significant effect, while B, C and D had no significant effects. As for factors C and D, although the k value of C_3 was greater than that of C_2 and the k value of D_3 was greater than that of D_2, but the differences were both small (1.48% and 3.52%, respectively), and factors C and D had no significant effects on the content of total flavonoids. Consequently, C_2 and D_2 were selected from the perspective of industrial production. Based on the results of range analysis and variance analysis, the optimum extraction conditions of *P. chinense* Pursh were determined as $A_2B_2C_2D_2$, that is, extracting with reflux using 55% ethanol at the ratio of material to liquid of 1∶20 (g/mL) twice, 2.0 h each time.

3.6 Results of verification test Accurately, 5 portions of coarse powder of *P. chinense* Pursh stems (sieved with a No. 4 sieve) were weighed, about 1 g each. Samples were prepared according to Section **3.2.1** and determined for the content of total flavonoids according to Section **3.2.2**. The average content of total flavonoids in the five repeated tests was 3.62%, and the relative standard deviation *RSD* was 1.02%, which was better than that in the orthogonal experiment, suggesting that the extraction process was reasonable and feasible.

3.7 Determination of total flavonoids in different parts of *P. chinense* Pursh Accurately, 3 portions of coarse powder of *P. chinense* Pursh stems, leaves and flowers and its whole grass were weighed, respectively, about 1 g each. Test solutions were prepared according to the method under Section **3.2.1**, and the content of total flavonoids was determined according to Section **3.2.2**. The results are shown in Table 4. Table 4 shows that the content of total flavonoids was quite different in different parts of *P. chinense* Pursh, showing the highest value in the flowers of *P. chinense* Pursh, followed by leaves and whole grass, and the lowest value was found in stems.

Table 4 Content of total flavonoids in different parts of *Penthorum chinense* Pursh ($n = 3$, %)

Part	Total flavonoid content	*RSD*
Stems	3.63	0.89
Leaves	8.90	0.57
Flowers	11.28	1.00
Whole grass	4.36	0.62

3 Discussion

In this study, pinocembrin was used as the reference substance, and measured by the $AlCl_3$ colorimetric method, the absorption spectrum of pinocembrin was basically similar to that of the total flavonoids of *P. chinense* Pursh, and both of them had the maximum absorption at 310 nm. Therefore, pinocembrin was chosen as the reference substance, and the total flavonoids of *P. chinense* Pursh was determined by $AlCl_3$ colorimetry. The method could truly reflect the content of total flavonoids in *P. chinense* Pursh.

There have been comparisons between heating reflux extraction and ultrasonic extraction methods for the selection of extraction methods. *P. chinense* Pursh was extracted once with 95% ethanol in a material-to-liquid ratio of 1∶20 (g/mL) for an extraction time of 1.0 h, and the total flavonoid contents in the stems, leaves, flowers and whole grass of *P. chinense* Pursh were 1.40%, 3.52%, 4.25%, and 1.81%, respectively, by heating reflux extraction; and the total flavonoid contents in the stems, leaves, flowers and whole grass of *P. chinense* Pursh extracted by ultrasound were 1.16%, 3.03%, 3.80%, and 1.51%, respectively. The total flavonoid contents in the stems, leaves, flowers and whole grass of *P. chinense* Pursh by heating reflux extraction were higher than those by ultrasonic extraction. Therefore, the heating reflux extraction method was chosen to explore the extraction process of total flavonoids in *P. chinense* Pursh and compare the total flavonoid contents in different parts of *P. chinense* Pursh.

On the basis of single factor experiments, four factors, name-

ly ethanol concentration (35%, 55%, 75%), material-to-liquid ratio (1∶10, 1∶20, 1∶30), extraction time (1.5, 2.0, 2.5 h) and extraction times (once, twice and thrice), were selected to carried out an orthogonal experiment. The optimum extraction conditions of total flavonoids from *P. chinense* Pursh obtained through the orthogonal experiment were as follows: 55% ethanol, material-to-liquid ratio 1∶20 (g/mL), twice, and 2.0 h each time. With the optimum conditions, the contents of total flavonoids in the stems, leaves, flowers and whole grass of *P. chinense* Pursh were 3.63%, 8.90%, 11.28% and 4.36%, respectively. The content of total flavonoids was quite different in different parts of *P. chinense* Pursh, showing the highest content in flowers of *P. chinense* Pursh, followed by leaves and whole grass, and the lowest value was found in stems. Therefore, it is suggested that the proportions of leaves and flowers should be paid attention to in the industrial processing of *P. chinense* Pursh. Therefore, it is suggested that the ratio of leaves to flowers should be paid attention to in the industrial processing of *P. chinense* Pursh.

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