Optimization of Extraction Process of Qingdu Jianpi Mixture by Orthogonal Experimental Design

Jiangcun WEI[1,2△], Bing QING[1△], Jinqing PENG[2], Xiaofen FENG[1], Weiwei CHEN[2], Wen ZHONG[1*], Zujie QIN[1,2*]

1. International Zhuang Medical Hospital Affiliated to Guangxi University of Chinese Medicine, Nanning 530201, China; 2. Guangxi University of Chinese Medicine, Nanning 530200, China

Abstract [**Objectives**] To optimize the optimal extraction process of Qingdu Jianpi Mixture. [**Methods**] Taking water addition ratio, extraction time and extraction times as process investigation factors, psoralen content, astilbin content and dry extract yield as evaluation indicators, the main influencing factors and level range of the extraction process of Qingdu Jianpi Mixture were determined on the basis of single factor test method, and the optimal weight coefficient was screened by AHP-entropy method mixed with weighting method. Combined with $L_9(3^4)$ orthogonal experiment, the best extraction process was obtained. At the same time, thin-layer chromatographic identification was used to identify *Ficus simplicissima* Lour. and *Smilax glabra* Roxb. in the medicinal liquid. [**Results**] The best extraction process: add 1∶12 water to the prescription decoction pieces, extract under reflux for 2 times, 1.5 h per time, and combine the filtrate to 250 mL. Thin layer chromatography analysis showed that the spots of *Ficus simplicissima* Lour. and *Smilax glabra* Roxb. in the medicinal solution were the same as those of reference substances at the corresponding positions, and the negative control had no interference. [**Conclusions**] The experimental method is reasonable and feasible, and the process is reliable, which can provide experimental reference for the subsequent application of in-hospital preparations and research and development of Qingdu Jianpi Mixture.

Key words Qingdu Jianpi Mixture, AHP-entropy method, Orthogonal design, Extraction process

1 Introduction

The original Zhuang prescription of Qingdu Jianpi Mixture comes from the clinical experience prescription of Chief Physician Feng Xiaofen, Department of Gynecology, International Zhuang Medical Hospital affiliated to Guangxi University of Chinese Medicine. It is used clinically as a decoction, and consists of *Ficus simplicissima* Lour., *Smilax glabra* Roxb., stir-baked *Atractylodes macrocephala* with bran, yam and other traditional Chinese medicines. It has the effects of invigorating spleen and replenishing qi, removing dampness and turbidity, detoxifying and clearing away heat, and it can be mainly used to treat disorders for women such as abdominal aversion to cold caused by spleen deficiency, internal stagnation or downward flow of damp-heat, that is, gynecological diseases such as irregular menstruation and dysmenorrhea as called by modern medicine. In the prescription, *F. simplicissima* Lour. and *S. glabra* Roxb. are the main ones, which have the effects of invigorating spleen and promoting qi, removing dampness and detoxifying; stir-baked *Atractylodes macrocephala* with bran, yam, and stir-baked *Atractylodes lancea* (Thunb.) DC. with bran have the effects of invigorating the spleen and replenishing qi[1-3], helping *F. simplicissima* Lour. to strengthen the spleen and promote qi, and *Microcos paniculata* L., *Rhizoma Dioscoreae Septemlobae*[4], and holly bark help *S. glabra* Roxb. to dispel dampness and give play to detoxification effect; the medicine *Bupleurum* soothes the liver and promotes yang, white peony root nourishes blood and softens the liver and spleen, *etc.* It is combined with licorice, plantain, dried tangerine peel and schizonepeta spike to regulate kidney, bladder and intestine channels, so as to improve transportation and transformation process of dampness and turbidity in the body.

In this study, according to the preparation requirements of medical institutions, the clinical experience prescription was developed into a mixture. On the basis of a single factor, the best weighting coefficient screened by the mixed weighting method was combined with the $L_9(3^4)$ orthogonal test method to obtain the best extraction process[4], and the identification of *F. simplicissima* Lour. and *S. glabra* Roxb. in the liquid medicine was studied by thin-layer chromatographic identification.

2 Materials

2.1 Instruments LC-2030 PDA high performance liquid chromatograph, TGL-16G high-speed desktop centrifuge (Shanghai Anting Scientific Instrument Factory), UPH-IV-20TN ultrapure water machine (Sichuan ULUPURE Technology Co., Ltd.).

2.2 Reagents and medicinal materials Psoralen (batch No.: 230129, content≥98%) and astilbin (batch No.: 230212, content≥98%) reference substances were purchased from Sichuan

Received: November 13, 2024 Accepted: May 21, 2025

Supported by Huang Ruisong's National Famous Old Traditional Chinese Medicine Expert Inheritance Studio Construction Project [GuoZhongYiYaoRenJiaoHan(2022)75]; Hospital Pharmacy Research Project of Guangxi Pharmaceutical Association (GXYXH-202404); 2024 Youth Science Fund Project of International Zhuang Medical Hospital (2024GZYJKT005); High-level Traditional Chinese Medicine Key Discipline Construction Project of National Administration of Traditional Chinese Medicine (ZYYZDXK-2023165); National Old Pharmaceutical Workers Inheritance Studio Construction Project of National Administration of Traditional Chinese Medicine [GuoZhongYiYaoRenJiaoHan(2024)255]; Talent Cultivation Project-"Young Crop Project" of International Zhuang Medical Hospital Affiliated to Guangxi University of Chinese Medicine (2022001); Guangxi Traditional Chinese Medicine Multidisciplinary Innovation Team Project (GZKJ2309).

△These authors contributed equally to this study.

Jiangcun WEI, master, assistant researcher; Bing QING, bachelor, engineer. *Corresponding author. Zujie QIN, master, professor; Wen ZHONG, bachelor, chief pharmacist.

Zhibiao Biotechnology Co., Ltd.; acetonitrile (chromatographically pure, Fisher, USA), methanol (chromatographically pure, Fisher, USA), analytical methanol, analytical acetic acid, analytical phosphoric acid.

3 Methods and results

3.1 Determination of psoralen and astilbin

3.1.1 Chromatographic conditions. A reverse chromatographic column Agilent ZORBAX Eclipse Plus C_{18} (250 mm × 4.6 mm, 5 μm) was used, psoralen (244 nm, column temperature 30 ℃) was used with acetonitrile-0.1% phosphoric acid as the mobile phase, astilbin (293 nm, column temperature 35 ℃) was used with methanol-0.15% acetic acid as the mobile phase; the injection volume of 10 μL and flow rate of 1.0 mL/min were used as the conditions for testing. The mobile phase elution program is shown in Table 1.

Table 1 Elution program

Psoralen			Astilbin		
Time//min	Acetonitrile//%	0.1% phosphoric acid//%	Time//min	Acetonitrile//%	0.1% phosphoric acid//%
0 – 20	27	73	0 – 15	28	72
20 – 50	27 – 32	73 – 68	15 – 35	28 – 30	72 – 70
50 – 60	32 – 35	68 – 65	35 – 41	30	70

3.1.2 Preparation of standard stock solution. 0.58 and 18.32 mg of psoralen and astilbin control samples were accurately weighed respectively, and 50% and 60% methanol were added respectively to volume of 25 mL, making stock solutions with concentrations of 22.74 and 17.718 1 mg/mL.

3.1.3 Preparation of sample solution. The prescription medicinal materials were weighed, mixed with 1∶10 distilled water, soaked for 1.0 h, and extracted under reflux twice, 1.0 h per time. The two filtrates obtained by filtration with a 250-mesh filter cloth were combined, concentrated and filled to volume of 250 mL, as a sample solution.

3.1.4 Preparation of test solution. 1 mL of the sample solution under Section **3.1.3** was pipetted, evaporated in a water bath, and mixed with 50% (psoralen) and 60% methanol (astilbin) respectively to make the volume to 2 mL. The supernatant after centrifugation was taken and filtered with a 0.22 μm microporous membrane to serve as the test solution.

3.1.5 Preparation of negative control solution. The negative prescriptions except *F. simplicissima* Lour. and *S. glabra* Roxb. were weighed separately, to prepare the respective single negative control solutions according to the methods specified in Section **3.1.3** and **3.1.4** (lack of *F. simplicissima* Lour., lack of *S. glabra* Roxb.)

3.1.6 Investigation of linear relationship. The reference stock solutions under Section **3.1.2** were accurately pipetted and prepared into a series of solutions with a maximum concentration ratio of 1∶20. The peak area of the above reference solutions was measured according to the chromatographic conditions under Section **3.1.1** to get the regression equation: $Y_{psoralen} = 6.799\,8 \times 10^7 X_{psoralen} + 1.475\,7 \times 10^3$, correlation coefficient $r = 0.999\,9$ ($n = 6$); $Y_{astilbin} = 2.213\,0 \times 10^7 X_{astilbin} + 5.626\,4 \times 10^4$, correlation coefficient $r = 0.999\,9$ ($n = 6$).

The results showed that the linear relationship between psoralen (1.136 4 – 22.736 0 μg/mL) and astilbin (0.035 9 – 0.718 1 mg/mL) was good.

3.1.7 Precision test. The standard solution with known concentrations of psoralen (9.094 4 μg/mL) and astilbin (0.287 3 mg/mL) was injected six times consecutively according to the chromatographic conditions under Section **3.1.1**. The results showed that the *RSD* values of peak area for the two were 0.48% and 0.07% ($n = 6$), and the precision of the instrument was good.

3.1.8 Repeatability test. Six test solutions were prepared in parallel according to the method under Section **3.1.4**, and then injected respectively according to the chromatographic conditions under Section **3.1.1** to determine the content of psoralen and astilbin. The results showed that the *RSD* values of content determination values for the two were 0.79% and 1.62% ($n = 6$), respectively, indicating that the established method had good repeatability.

3.1.9 Stability testing. The test solution prepared under Section **3.1.4** was taken, and the content of psoralen and astilbin was determined at different time points of 0, 4, 8, 12, 16, and 24 h according to the chromatographic conditions under Section **3.1.1**. The results showed that the *RSD* of content for the two was 0.65% and 0.56% ($n = 6$), and the test solution had good stability within 24 h.

3.1.10 Sample recovery test. 0.5 mL of sample solution with known content of each component was pipetted, and 6 samples of the test solution prepared in parallel according to the method under Section **3.1.4** were added and recovered at a content ratio of 1∶1. Samples were injected separately according to the chromatographic conditions under Section **3.1.1**, the peak area of psoralen and astilbin was determined, and the recovery rate was calculated. The results showed that the average recovery rates were 101.80% and 103.35%, and the *RSD* values were 1.70% and 1.87%, respectively, all of which met their respective recovery limits.

3.2 Calculation of dry extract yield The liquid medicine under Section **3.1.3** was accurately pipetted into an evaporation dish (with constant weight), evaporated to dryness in a water bath, baked in an oven at 105 ℃ for 3 h, cooled in a drier for 30 min, quickly and accurately weighed, and the dry extract yield was determined.

Dry extract yield (%) = (Dry extract weight/Sampling vol-

ume) × (Liquid medicine volume/Medicinal material weight) × 100%.

3.3 Investigation of water absorption rate Multiple prescription medicinal materials were weighed, mixed with 1∶10 distilled water and soaked. The weight of the wet medicinal materials was recorded every 30 min and the water absorption rate was calculated. It can be seen from Fig. 1 that the water absorption weight of medicinal materials no longer increased significantly after 60 minutes of immersion, and the water absorption rate of medicinal materials was 93.36%. Therefore, it was determined that 0.93 times more water should be added during the first extraction.

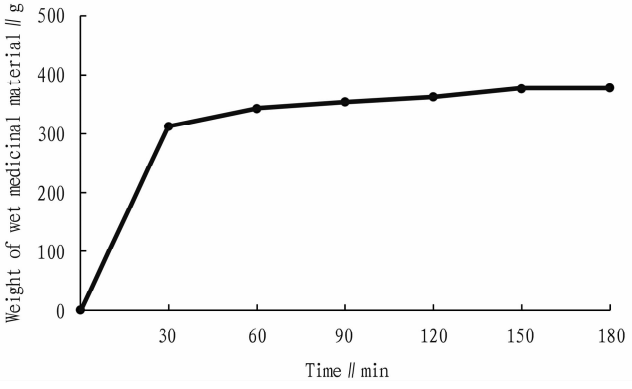

Fig. 1 Investigation results of water absorption rate of Qingdu Jianpi Mixture

3.4 Single factor test The prescription medicinal materials were accurately weighed, the extraction time was fixed at 1.0 h, and the reflux extraction was carried out twice. The water addition ratios (1∶8, 1∶10, 1∶12, 1∶14, and 1∶16) were investigated, and the test was conducted in parallel three times; the water addition ratio was fixed at 1∶10, the extraction time was 0.5, 1.0, 1.5, 2.0 and 2.5 h, and the test was performed three times in parallel; taking psoralen content, astilbin content and dry extract yield as evaluation indexes, the obtained data were imported into the SPSSPRO analysis platform, and the calculated investigation results are shown in Fig. 2 and Fig. 3.

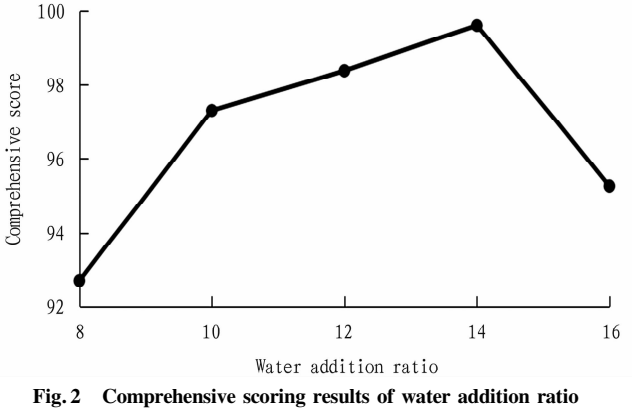

Fig. 2 Comprehensive scoring results of water addition ratio

Therefore, after overall consideration, the water addition ratio was selected to be 1∶8 – 1∶12; the extraction time of 1.0, 1.5 and 2.0 h was taken as the orthogonal investigation level range.

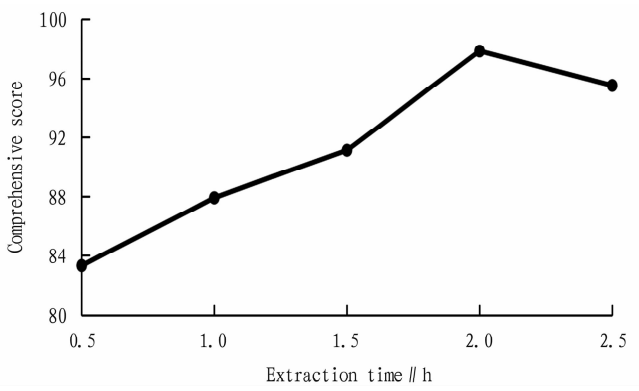

Fig. 3 Comprehensive scoring results of extraction time

3.5 Design of orthogonal test method On the basis of single-factor test, the water addition ratio (A) of 1∶8 – 1∶12, the extraction time (B) of 1.0 – 2.0 h, and the extraction times (C) of 1 – 3 were selected as the investigation factors, and the $L_9(3^4)$ orthogonal test was designed with the content of psoralen and astilbin and the dry extract yield as the evaluation indicators. The factor level table is shown in Table 2.

Table 2 Orthogonal factor level of extraction process

Level	Factor		
	Water addition ratio	Extraction time // h	Extraction times
	A	*B*	*C*
1	8	1.0	1
2	10	1.5	2
3	12	2.0	3

3.6 Orthogonal test results and analysis In this experiment, the content of psoralen, astilbin and dry extract yield were analyzed at the same time, and the weight coefficient of psoralen, astilbin and dry extract yield was calculated to be 28.5%, 32.9% and 38.6% by comprehensive weighted scoring method[5] (Table 3 – 4). The best index under the three component items was set as 100 points, and the other experimental items were scored according to the following formula: Comprehensive score = $Y_1/18.560\,5 \times 0.285 \times 100 + Y_2/0.753\,9 \times 0.329 \times 100 + Y_3/30.93 \times 0.386 \times 100$.

3.7 Verification test The three process parameter conditions obtained from the orthogonal test results were verified in three parallel batches, and the results are shown in Table 5. The results showed that the *RSD* of the content of the three batches of index components among groups was less than 3%, and there was no significant difference among the groups, indicating that the process was stable, reliable and repeatable.

In accordance with the demand of large-scale industrial production, the perspective of cost reduction, time and energy saving, and the principle of matching the value of the obtained product with the input cost and time, the best extraction process was finally obtained as $A_3B_2C_2$, that is, water addition ratio of 1∶12, heating and refluxing extraction twice, and 1.5 h per time was the best extraction process.

Table 3 Analysis of orthogonal test results

Experiment No.	*A*	*B*	*C*	Blank *D*	Psoralen content μg/mL	Astilbin content mg/mL	Dry extract yield//%	Comprehensive score
1	1	1	1	1	8.323 3	0.347 9	18.72	51.33
2	1	2	2	2	14.588 1	0.512 5	27.80	79.46
3	1	3	3	3	17.066 5	0.698 2	30.93	95.28
4	2	1	2	3	14.612 0	0.519 9	26.97	78.78
5	2	2	3	1	18.560 5	0.672 5	29.88	95.14
6	2	3	1	2	14.881 3	0.552 2	22.67	75.24
7	3	1	3	2	16.642 2	0.613 8	29.62	89.31
8	3	2	1	3	13.238 9	0.593 3	22.20	73.93
9	3	3	2	1	17.168 7	0.753 9	30.22	96.98
k_1	75.35	73.14	66.83	81.15				
k_2	83.05	82.84	85.07	81.34				
k_3	86.74	89.16	93.24	82.66				
R	11.38	16.03	119.65	1.51				

Table 4 Analysis of variance

	Sum of squares of deviations	Degree of freedom	Variance	*F* value	Significance
Water addition ratio (*A*)	202.42	2	101.21	49.50	<0.05
Extraction time (*B*)	390.96	2	195.48	95.61	<0.05
Extraction times (*C*)	1 096.95	2	548.47	268.27	<0.01
Error (*D*)	4.09	2	2.045		

Table 5 Analysis of verification results

No.		Psoralen			Astilbin			Dry extract yield		
		Content μg/mL	Mean value μg/mL	*RSD*//%	Content mg/mL	Mean value mg/mL	*RSD*//%	Content %	Mean value %	*RSD*//%
$A_3B_3C_3$	1	17.509 8	17.698 4	0.87	0.807 2	0.808 8	1.52	30.71	30.75	1.47
	2	17.798 8			0.804 3			31.24		
	3	17.786 7			0.815 0			30.31		
$A_3B_3C_2$	1	15.110 1	15.375 6	1.50	0.762 4	0.752 3	1.33	28.60	28.74	0.62
	2	15.525 0			0.751 9			28.68		
	3	15.491 8			0.742 5			28.94		
$A_3B_2C_2$	1	15.654 6	15.812 9	1.52	0.674 3	0.675 9	0.56	28.26	28.41	1.43
	2	16.089 8			0.680 2			28.75		
	3	15.694 2			0.673 1			28.23		

4 Discussion

4.1 Selection of evaluation indexes and evaluation methods

In the theory of traditional Chinese medicine, leukorrheal disease is dampness syndrome, and the treatment is mainly to eliminate dampness; to cure dampness, it lies in the spleen. The main medicine of the prescription, *F. simplicissima* Lour., is a traditional Chinese medicinal material that can be used as medicine and food in Lingnan area. It is included in the *Quality Standard of Yao Medicinal Materials in Guangxi Autonomous Region*. It can strengthen the spleen and lung, promote qi and remove dampness by diuresis, and also has various pharmacological effects such as relieving cough, resolving phlegm and relieving asthma, anti-inflammatory and analgesic, antibacterial and antioxidant effects[6]. Modern studies have shown that flavonoids in its various active ingredients are the material basis for the effect of invigorating spleen and replenishing qi, in which psoralen is often used as the basis for the evaluation and identification of the intrinsic quality of *F. simplicissima* Lour.[7-9]. *S. glabra* Roxb. has the effects of removing dampness and detoxifying, strengthening spleen and stomach, *etc*. *S. glabra* Roxb. can be added to daily soup drinks in hot-and-damp climates. There are a variety of active ingredients in *S. glabra* Roxb., including flavonoids (astilbin, isoastilbin), sterols and phenylpropanoids[10]. Modern research has found that astilbin, a flavonoid in *S. glabra* Roxb., plays a major role in anti-inflammation, dehumidification, easing joint movement and uric acid lowering[11-13]. Moreover, psoralen and astilbin are used as indexes for content determination in local standards[6] and national standards[14] of corresponding medicinal materials; the dry extract yield is an important reference item in the extraction process of traditional Chinese medicine compound preparations. Therefore, the main medicine psoralen in *F. simplicissima* Lour., astilbin in *S. glabra* Roxb. and the dry extract yield were used as the evalua-

tion indexes of extraction process.

4.2 Pre-experimental investigation There are many Chinese herbal medicines in compound preparations of traditional Chinese medicine, which also leads to many impurities, and the separation of impurities in the process of liquid chromatography identification or thin layer identification is particularly important[15]. In the pre-experiment, the content determination of methanol-water, acetonitrile-water, acetonitrile-0.1% phosphoric acid and methanol-0.15% glacial acetic acid in the fluidity system was investigated. It was found that during the content determination methods of psoralen and astilbin, it was difficult to separate impurities when using the same mobile phase and determination method. In order to save time and mobile phase, separate determination methods were established. Psoralen had good resolution, good peak shape and strong specificity under acetonitrile-0.1% phosphoric acid system; astilbin had good resolution and peak shape in methanol-0.15% glacial acetic acid. In the preliminary experiment, the thin-layer chromatographic identification of *F. simplicissima* Lour. and *S. glabra* Roxb. in the extract was preliminarily studied, and the suitable developing agent was selected, and the effective ingredients and impurities were effectively separated, and there was no interference from negative samples, which provided reference for the establishment of subsequent quality standards of the mixture.

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