Original Article

The Optimum Extraction Process for *Radix glycyrrhizae* and *Angelica dahurica* (Fisch.) Benth.et Hook with Orthogonal Design

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ABSTRACT

Background: To develop the optimal extractive technique of the *Radix glycyrrhizae* and *Angelica dahurica* (Fisch.) Benth.et Hook in the Fengshiding dropping pill. **Methods:** Single factor experiments were carried out on the effect of water-ethanol extraction by the multiple guidelines grading of the dried extract quantity, the contents of liquiritin and imperatorin. Furthermore, orthogonal experimental design was used for the optimization of cross-functional process. The dried extract quantity and the contents of liquiritin and imperatorin were used as targets. The interaction among solid-liquid ratio, ethanol concentration, extraction time and extraction times was investigated by the analysis of variance. Data processing was carried out with the multiple guidelines grading method for optimizing the extraction condition. **Results:** The optimal extracting condition was founded to be successively 3 decoctions with 12 times of 75% alcohol for 1 h. **Conclusion:** The optimal process is stable and feasible.

Key words: *Radix glycyrrhizae*, *Angelica dahurica* (Fisch.) Benth.et Hook, Liquiritin, Imperatorin, Orthogonal test.

INTRODUCTION

The prescription of the Fengshiding dropping pill comes from the Fengshiding tablet that exists in preparation of Chinese medicine in twelfth volumes from drug standard of ministry of public health (Standard No. WS3-B-2297-97). The Fengshiding tablet is a traditional Chinese preparation and made up by *Alangium chinense, Cynanchum paniculatum*, *Radix glycyrrhizae* and *Angelica dahurica* (Fisch.) Benth.et Hook.[1] It has good effect on promoting blood circulation and analgesia in the anti-rheumatic arthritis or anti-rheumatoid arthritis,[1] which has been for clinical practice for many years with definite therapeutic effects. However, *Angelica dahurica* powder can be directly used as medicine in the Fengshiding tablet, which leads to low content of digestion components and high daily doses *in vivo*. In order to improve patient compliance and the preparation quality, the formulation and preparation of the Fengshiding tablet need to be re-optimized.

Recent studies have shown that licorice extract and its bioactive component liquiritin can be applied for the treatment of inflammation-related disorders, such as oxidative liver damage and inflammation diseases;[2] *Angelica dahurica* extract[3] and its bioactive component imperatorin[4] can be applied for the anti-inflammatory effects and the antiallergic effects. Therefore, the representative extract of liquiritin and imperatorin lays an anti-inflammatory medicinal material foundation for development of the Fengshiding dropping pill. In addition, several methods, including organic solvent extraction, microwave-assisted extraction,[5] and ultrasonic-cassisted extraction (UAE),[6] have been widely employed for the extraction of single herb. However, few reports have focused on combination extraction process of *Radix glycyrrhizae*

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and *Angelica dahurica* (Fisch.) Benth.et Hook for liquiritin and imperatorin.

In the present study, based on the common chemical properties of the representative extract of liquiritin and imperatorin (structure shown in Figure 1), we chose ethanol–water combination extraction of liquiritin[2,7,8] and imperatorin[9,10,11] from *Radix glycyrrhizae* and *Angelica dahurica* (Fisch.) Benth. et Hook according to similarity-intermiscibility theory. In order to estimate and optimize the factors affecting extraction to achieve maximum recovery, we investigated the effect of water-ethanol extraction on the multiple guidelines grading of the dried extract quantity, the contents of liquiritin and imperatorin by single factor experiment. A second-order polynomial model was set up to predict the acquired the multiple guidelines grading using orthogonal test. The interaction among solid-liquid ratio, ethanol concentration, extraction time and extraction times was investigated by the analysis of variance. Finally, the optimal extraction condition was obtained and provided a basis for new drug development of the Fengshiding dropping pill.

MATERIALS AND METHODS

Materials

The medicinal materials of *Radix glycyrrhizae* and *Angelica dahurica* (Fisch.) Benth.et Hook were purchased from the Kangyuan Pharmaceutical Co. Ltd. in Anhui province, China. The Control products of liquiritin (batch code: 111610-200604) and imperatorin (batch code: 110826-200712) were purchased from China Materia Medica Biological Product Inspection Institute. Methanol was chromatographic grade and the other chemicals were of analytical grade.

Extraction process of liquiritin and imperatorin

Ten g of mixture medicinal material (prescription proportion: *Radix glycyrrhizae*: *Angelica dahurica*=2.5:1, w/w) was blended with complex solvent of ethanol and water. The values of solid-liquid ratio, ethanol concentration, extraction time, and extraction times were set according to the requirement of experiment. The extract was filtered using a sand core funnel, and then fixed to 250 mL with the same extracting solution. The physic liquor 10 μL was precisely sampled to determination of the contents of liquiritin and imperatorin by HPLC (LC-20AT, Shimadzu Corporation, Japan) and 25 mL was precisely sampled to determination of the dried extract quantity. The dried extract quantity was calculated using the following equation:

The dried extract quantity = weight of dried 25mL physic liquor×250 / 25 / 10×100%

Determination method of liquiritin and imperatorin

The determination was carried out over a Shim-pack VP-ODS C_{18} column (250×4.6, 5 μm) at the temperature of 30°C. The mobile phase[1] consisted of methanol (A) – water (B) with the gradient elution (0-3 min, 45% A; 3-6 min, 55% A; 6-8 min, 65% A; 8-50 min, 65% A). The flow rate: 0.6 mL/min, and detection wavelength was set at 276 nm (detecting liquiritin) and 300 nm (detecting imperatorin). Figure 2 shows HPLC chromatograms of determination.

Preparation of reference substance: precisely sampled the standard liquiritin 2.67 mg and imperatorin 3.66 mg into 25 mL capacity bottle, ultrasonic dissolving in appropriate methanol, added methanol to scale, and shook up.

The establishment of standard curve: precisely transfered 1, 2, 3 4, 5 mL the solution to 10 mL capacity bottle, added methanol to scale, shook up, and determined it at 276 nm and 300 nm wavelength at the same time. The regression equations were obtained as $Y=25094X-6154.2$ ($r=0.9997$, $n=5$, liquiritin) and $Y=86702X+29706$ ($r=0.9996$, $n=5$, imperatorin). The linear ranges were 10.68-53.40 μg/mL for liquiritin and 14.64-73.20 and g/mL for imperatorin. This method was simple, and had good linear relationship.

Experimental design

Single factor experiments[12]

Active components were extracted using complex solvent of ethanol and water in boiling status. The

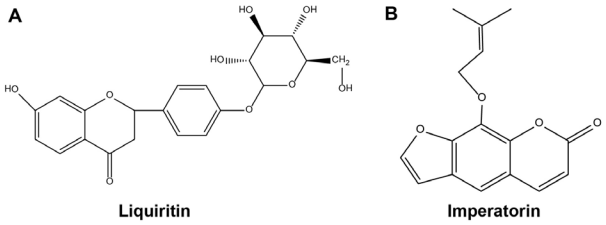

Figure 1: Chemical structures. (A) Chemical structure of liquiritin (B) Chemical structure of imperatorin.

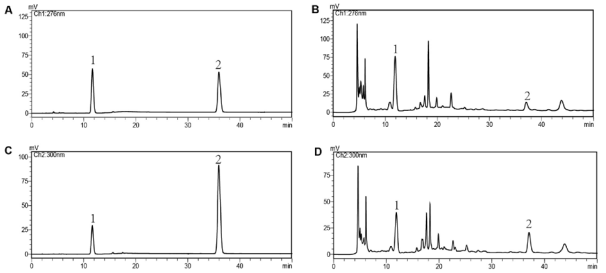

Figure 2: HPLC chromatograms of reference substance (A) and sample (B) at 276 nm, and of reference substance(C) and sample (D) at 300 nm 1-liquiritin 2-imperatorin.

proportion of ethanol in the solvent varied in the range of 55–95% (v/v). The best solvent concentration was selected according to the value of overall desirability (OD). The value of OD was calculated using the following equation: OD= (liquiritin content / the maximum of liquiritin content)×0.4 +(imperatorin content / the maximum of imperatorin content)×0.4+(extract rate / the maximum of extract rate)×0.2.

Active components were extracted using the best solvent selected in the previous step. The solid-liquid ratio varied from 1:8 to 1:16 while fixing the extraction time constant at 1h.

Using the best ethanol concentration selected in first step, active components were extracted during various extraction time course ranges from 0.5 to 4 h at the optimum solid-liquid ratio determined in the second step.

Using the best ethanol concentration selected in first step and the optimum solid-liquid ratio determined in the second step, active components were extracted during various extraction times from 1 to 5 at the optimum extraction time determined in the third step.

The ranges of three factors were determined for L_9 (3^4) orthogonal test according to the results of experiments.

Experiment of L_9 (3^4) orthogonal test[13]

Optimization of extracting condition was carried out using L_9 (3^4) orthogonal test, which was consisted of 9 experimental runs and used as visual analysis and variance analysis for optimization.

Verification of the optimal conditions

Optimal conditions for active components extraction were obtained using the variance analysis model of orthogonal test. The practical acquired ratio was obtained under the optimal conditions. The acquired OD of experimental and predicted was compared in order to determine the validity of the model.

RESULTS

Results of single factor experiment

As can be seen from Figure 3A, the acquired OD as a function of ethanol concentration followed a parabola shape. The acquired OD increased with increasing of the ethanol proportion in the extraction medium up to 85% and then began to decline with the further increase of ethanol proportion in the extraction medium. As it was shown in Figure 3B, the acquired OD was increased gradually with increasing of the solid-liquid ratio and the maximum acquired OD of 99.14% was obtained when the solid-liquid ratio was 1:14. As observed in Figure 3C, the acquired OD as a function of extraction time followed a parabola shape. The acquired OD increased with increasing of the extraction time in the extraction medium up to 2 h and then began to decline with the further increase of extraction time in the extraction medium. As shown in Figure 3D, the acquired OD was increased slightly with increasing of the extraction times. This observation suggested that the extraction times had no significant effect on the acquired value of OD and fixed it on 3 times.

Table 1: Factors and levels.

Level	Factors		
	A ethanol concentration (%)	B solid-liquid ratio (times)	C extraction time (h)
1	65	12	1.0
2	75	14	2.0
3	85	16	3.0

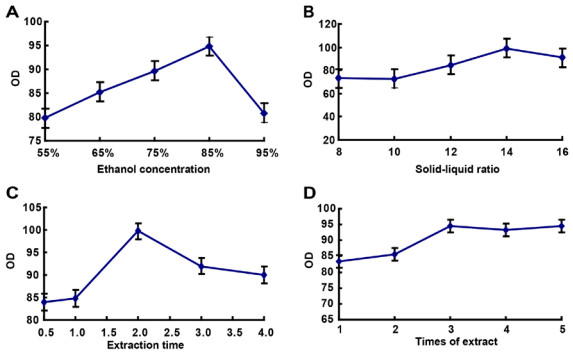

Figure 3: Results of single-factor experiments (A) ethanol concentration (B) solid-liquid ratio (C) extraction time (D) extraction times.

Results of orthogonal test

Based on the single-factor experiments, the following values influencing the acquired OD were selected as the ranges of independent variables. The levels of factors were shown in Table 1. The design arrangement and the experimental results were shown in Table 2. Analysis of variance was summarized in Table 3. As visual analysis shown in Table 2, the main influence factors on OD from big to small in proper order are A, B, and C. The *F*-test and *p* value in Table 3 indicated that ethanol concentration (*A*) had the largest effect on OD ($P < 0.05$), and the others had no significance on OD. Considering cost reduction and energy saving, the optimal extracting condition was founded to be successively 3 decoctions with 12 times of 75% alcohol for 1 h.

Table 2: Result of $L_9(3^4)$ orthogonal test.								
No.	A	B	C	D	extract rate/%	liquiritin content/mg/g	imperatorin content/mg/g	OD
1	1	1	1	1	22.73	1.6719	0.2620	90.74
2	1	2	2	2	20.11	1.7740	0.2959	95.12
3	1	3	3	3	20.86	1.6609	0.3032	94.30
4	2	1	2	3	18.57	1.7093	0.2863	91.09
5	2	2	3	1	21.88	1.8485	0.2978	98.54
6	2	3	1	2	18.00	1.7147	0.2968	92.09
7	3	1	3	2	18.63	1.5415	0.2487	82.56
8	3	2	1	3	20.24	1.5722	0.2788	88.61
9	3	3	2	1	18.11	1.5865	0.2554	83.96
K_1	93.387	88.130	90.480	91.080				
K_2	93.907	94.090	90.057	89.923				
K_3	85.043	90.117	91.800	91.333				
R	8.864	5.960	1.743	1.410				

Table 3: Analysis of variance.					
factor	sum of square	freedom	*F* value	*F* critical value	significance
A	148.440	2	43.788	19.000	$P<0.05$
B	55.256	2	16.300	19.000	
C	4.961	2	1.463	19.000	
error	3.39	2			

$F_{0.1}(2,2)=9.00$ $F_{0.05}(2,2)=19.00$ $F_{0.01}(2,2)=99.00$

Verification of the results

The suitability of OD was tested using the recommended optimal conditions. The experimental values (96.62, 97.51, 95.00, $n=3$) were found to be close to the predicted one.

DISCUSSION

The Fengshiding tablet, a traditional Chinese preparation, is composed of *Alangium chinense, Cynanchum paniculatum, Radix Glycyrrhizae* and *Angelica Dahurica* (Fisch.) Benth.et Hook.[1] However, the Fengshiding tablet has many defects such as the indistinctness of its effective substances, low content of digestion component, high daily doses, poor stability and quality control. In order to improve patient compliance and the preparation quality, the formulation and preparation of the Fengshiding tablet need to be re-optimized. Following the original preparation functions, we must fully consider the characteristics and the roles of each medicinal material in the prescription, when the preparation is improved. On the basis of the latest achievements of modern pharmacology research, we made clear its effective components and extracts. The combination extract taken liquiritin and imperatorin as representatives from *Radix glycyrrhizae* and *Angelica dahurica* (Fisch.) Benth.et Hook, which showed significant activity of anti-inflammatory and analgesic,[14,15,16] played an important role in the Fengshiding dropping pill. Furthermore, recent studies have indicated that the extraction of salicin[17,18,19] from *Alangium chinense* has the antinociceptive and anti-inflammatory activity, and steam distillation of paeonol[20,21,22] from *Cynanchum paniculatum* has suppressed swelling, inflammatory reaction and cell impairment. The new extraction procedure for *Alangium chinense* or *Cynanchum paniculatum* in the Fengshiding dropping pill is definitely worth study in the future.

In the present study, we focused on the optimal extractive technique of the *Radix glycyrrhizae* and *Angelica dahurica* (Fisch.) Benth.et Hook in the Fengshiding dropping pill. The dried extract quantity and the contents of liquiritin and imperatorin used as targets, we employed single factor experiments combined with orthogonal experimental design to improve the preparation process. Our results demonstrated that the optimal process for *radix glycyrrhizae* and *angelica dahurica* (Fisch.) Benth.et Hook was stable and feasible, which could be applied in

the future drug development of the Fengshiding dropping pill.

Of note, to find an easy way to analyse the speciments, we had tried a gradient solvent system (water, methanol and acetonitrile).[1] The final choice of mobile phase was methanol-water, which can effectively separate the two markers (liquiritin and imperatorin) simultaneously. This mobile phase was relatively simple to operate and less harmful to the chromatographic column and instrument. In order to further shorten the analytical time and improve the sensitiv ity of analysis results, gradient elution was carried out with adopting double wavelength for determination of two kinds of ingredients simultaneously. It had been proved that the experimental establishment of the chromatographic conditions had got a good segregative result.

CONCLUSION

The optimum extraction process for *Radix glycyrrhizae* and *Angelica dahurica* (Fisch.) Benth.et Hook in Fengshiding dropping pill was as follow: successively 3 decoctions with 12 times of 75% alcohol for 1 h. Our verification of the results demonstrated that the optimal process for *Radix glycyrrhizae* and *Angelica dahurica* (Fisch.) Benth.et Hook was stable and feasible. The precise combination extract of *Radix glycyrrhizae* and *Angelica dahurica* (Fisch.) Benth.et Hook laid a solid medicinal material foundation for new drug development of the Fengshiding dropping pill.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ABBREVIATION USED

UAE: Ultrasonicassisted extraction; **OD:** Overall desirability; **HPLC:** High performance liquid chromatography.

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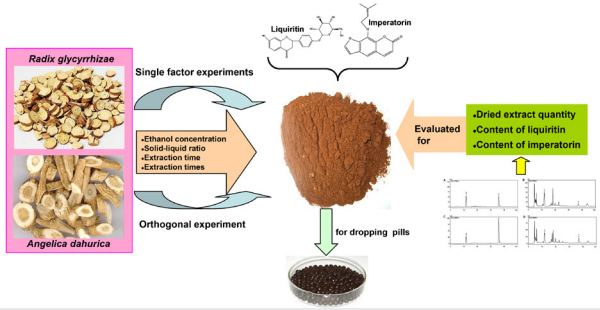
PICTORIAL ABSTRACT



SUMMARY

- A validated method for estimation of liquiritin and imperatorin in the ethanol–water combination extraction from *Radix glycyrrhizae* and *Angelica dahurica* (Fisch.) Benth.et Hook through RP-HPLC.
- Data processing was carried out with the multiple guidelines grading method for optimizing the extraction condition.
- Single factor experiments and orthogonal test were carried out to develop the optimal extractive technique of the *Radix glycyrrhizae* and *Angelica dahurica* (Fisch.) Benth.et Hook in the Fengshiding dropping pill.
- The optimal extracting condition was founded to be successively three decoctions with 12 times of 75% alcohol for 1 h. This extracting process was stable and feasible and provided a basis for new drug development of the Fengshiding dropping pill.

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