Optimization Of Extraction Condition And Quantification Of Total Flavonoids In *Elaeagni Folium*

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ABSTRACT

Introduction: *Elaeagni Folium* has been used as a crude drug to cure lung deficiency cough, asthma and shortness of breath for a long time in China. The research aimed to optimize the extraction condition and measure the amount of total flavonoids in this medicinal material.

Methods: Reflux extraction was an efficient method, compared with sonication and soxhlet extraction, to extract total flavonoids. On the basis of single factor test, the $L_9(3^4)$ orthogonal test was used in the optimization of technological parameters by investigating the ethanol concentration, extraction time and the material /solvent ratios. Using 0.1 mol·L^{-1} aluminum chloride ($AlCl_3$) solution as chromogenic agent, the total flavonoids content was measured by ultraviolet and visible spectrophotometry.

Results: The optimal extraction parameters showed that: ethanol concentration was 85%, ratio of solvent to raw material 40:1 and extraction time 1.5 h. The amount of total flavonoids in the leaf of *Elaeagnus pungens* Thunb. collected in different harvest time and habitats was 20.8-33.2mg·g^{-1}.

Conclusions: It indicated that the amount of total flavonoids had significant difference in this crude drug from different habitats but at the same time, while the total flavonoids content was stable in the samples collected in different harvest time but at the same habitat.

Key words: *Elaeagni Folium.*; Total flavonoids; Extraction conditions; Orthogonal test

INTRODUCTION

Elaeagni Folium is the dried leaf of *Elaeagnus pungens* Thunb. subordinated to *Elaeagnaceae*. It is recorded by Compendium of Materia Medica that *Elaeagni Folium* has been used as a crude drug to cure lung deficiency cough, asthma and shortness of breath for a long time. The leaf is primary raw material for making "Hai Zhu Chuan Xi Ding Tablet", which treats bronchial asthma effectively [1-2]. Pharmacological experiments showed that the extract of *Elaeagni Folium* possessed obvious effects of preventing asthma, relieving cough and removing phlegm. It may conjecture that the effective fractions are flavonoids and alkaloids [3-4]. Researches also studied that the flavonoids isolated from the leaf such as kaempferol glucoside, kakkalide and 3'-O-methylquercetin significantly inhibited proliferation of human gastric carcinoma cell SGC -7901 and cervical cancer cells Hela in vitro[5] .Many studies also reported the chemical constituents of flavonoids were kaempferol, kaempferol glucoside, quercetin, 3'-O- methylquercetin and so on[6-7]. However, the extraction technique of total flavonoids in *Elaeagni Folium* has not been reported. Thus, the best condition of extraction was obtained by studying single-factor tests and orthogonal design test. With the yield of total flavonoids as an index, the amount of total flavonoids of *Elaeagni Folium* collected in different habitats and harvest time was measured. This study provided experimental foundation for quality control, exploitation and utilization of this crude drug.

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MATERIALS

Leaf of *E.pungens* Thunb. was collected in Hubei province of China and authenticated by Dr. Dingrong Wan, a professor in Pharmacy college of South-central University for Nationalities.

The standard rutin was purchased from National Institutes for Food and Drug Control of China (Batch

Table1: Comparation of flavonoids yield with different extraction method

method	Sample (g)	Volume of 70% ethanol (ml)	Extraction time (h)	Flavonoids yield (%)	Average (%)
Soxhlet extraction	4.0001	80	2	1.17	1.16
	4.0003	80	2	1.14	
Reflux extraction	3.9996	80	2	2.16	2.14
	4.0003	80	2	2.11	
Sonication	4.0001	80	2	1.92	1.95
	4.0002	80	2	1.97	

number: 100080-200707). Aluminum chloride crystal ($AlCl_3 \cdot 6H_2O$) and alcohol employed were analytical reagent.

METHODS AND RESULTS

Sample Preparation

The dried leaf of *E. pungens* Thunb. was pulverized. 1 g of this power was sonicated with 20 ml of 70% alcohol for 30 min .The testing solution was obtained by filtering the extraction.

Standard solution preparation

Weigh accurately 5.7mg rutin reference standard in 50 ml of volumetric flask, dissolve and make up to mark with 70% alcohol. A total of 0.114 mg·ml^{-1}of standard solution was prepared.

Detection wavelength selection

2 ml of rutin solution and 1 ml of the testing solution were accurately pipetted into a 25 ml volumetric flask respectively, with 3 ml of 0.1mol·ml^{-1} aluminium chloride($AlCl_3$) solution separately[8-10]. Then the mixture was diluted to volume with 70% alcohol and shaken up. After 30 min, the absorption spectra of testing solution and reference solution were gained by wavelength scanning at 300-600 nm, showing a maximum absorption wavelength at 410 nm.

Standard curve preparation

Each standard solution of rutin (0.00 ml, 1.00 ml, 2.00 ml, 4.00 ml, 6.00 ml, 8.00 ml) was spiked into 25 ml measuring flask and conducted by the method stated in "Detection wavelength selection". 30min later, the absorbance of the mixtures were measured at 410 nm using a spectrophotometer against an appropriate blank solution. Using the absorbability as ordinate and the concentration as abscissa, the specification curve was obtained. The calibration curve presented a linear response within the concentration range of 4.56-36.48 μg·ml^{-1}. The regression equation was $Y = 0.0233X + 0.0043$, $R^2 = 0.9997$.

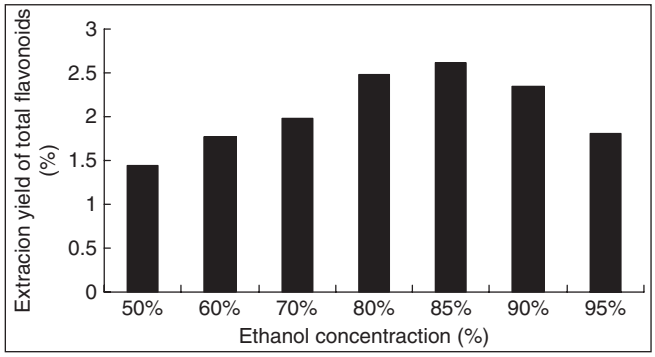

Figure1: Effect of ethanol concentration on the extraction yield of total flavonoids

Total flavonoids determination

1g of leaf was used in each extraction experiment with appropriate different concentration of alcohol.1 ml of each extract and 3 ml 0.1 mol·L^{-1} $AlCl_3$ were mixed in a 25 ml volumetric flask and the volume was made up with corresponding concentration of alcohol. The mixture was shaken and allowed to settle for 30 min at room temperature. The absorbance was measured at 410 nm. The extraction yield of flavonoids was calculated from the standard curve.

Optimization of extraction condition

Extract method selection To select efficient extract method, reflux, sonication and soxhlet extraction were compared. The results showed that reflux could provide the highest extraction yield (Table 1).

The single factor test

To determine the single factor experiment condition, the effect of three main factors including ethanol concentration, extraction time, material /solvent ratios were studied.

The extractions were performed with different concentrations of ethanol, namely 50%, 60%, 70%, 80%, 85%, 90%, 95% (v/v). Figure 1 showed that the total flavonoids amount increased with rise of concentration of ethanol from 50% to 85%. However, the flavonoids yield began to

decline, if the alcohol concentration continued to increase. Therefore, the optimal ethanol concentrations were 80%, 85% and 90%.

The effects of the material /solvent ratios from 1:10 to 1:40 on the extraction yield were investigated. Figure 2 manifested that the amount of flavonoids increased significantly with rise of the ratio (material/solvent) in a range of 1:10-1:30(w/v).Nevertheless, when the ratio was greater, the yield of extraction almost became steady. In the consideration of economy, the range of material / solvent ratios could be 1:20, 1:30, 1:40.

The effects of the extraction time from 1h to 2.5h on the extraction yield were measured. From figure 3, it could be found that the extraction yield improved with the increase of the extraction time from 1 to 2h, and didn't change significantly after 2h .Thus, the extraction time 1, 1.5, 2h were chosen for the following experiment.

Design for L9(34) orthogonal test

Considering the above experiment results, concentration of ethanol(A),material ratio(B) and extraction time (C) were selected as the experimental factors and a $L_9(3^4)$ orthogonal test was carried out. Factors and their levels were summarized in Table 2.

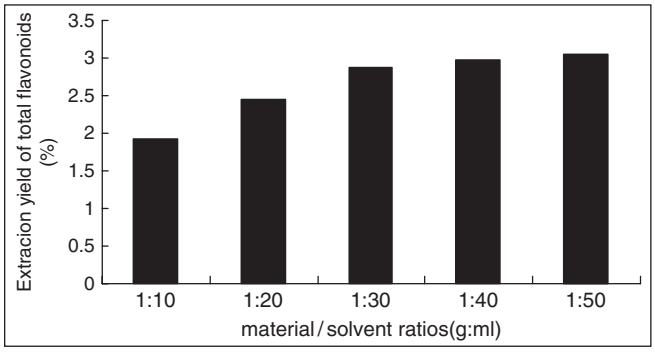

Figure 2: Effect of material ratios on the extraction yield of total flavonoids

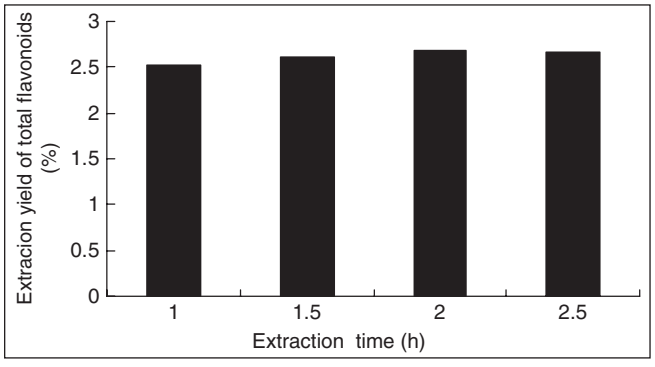

Figure 3: Effect of extraction time on the extraction yield of total flavonoids

The results of $L_9(3^4)$ orthogonal test were showed in Table 3, 4. Both results of intuitionistic analysis and variance analysis showed that the importance of three factors to affect extraction yield was B>A>C, with statistical significance. The optimal extraction condition was $B_3A_2C_2$, i.e., material/solvent ratios 1:40, concentration of ethanol 85% and extraction time 1.5h. Under the optimal conditions, the total flavonoids yield was 2.95%, which was higher than the orthogonal experimental result. This showed that orthogonal experiment was feasible.

Validation of the UV method

The repeatability was estimated by measuring the six samples under the optimal conditions in 1 day. The yields of total flavonoids were respectively 3.10%, 3.13%, 3.18%, 3.22%, 3.29% and 3.26%. The intermediate precision was evaluated by assaying the samples in duplicate on three consecutive days. The extraction yields were 3.15%, 3.11 % (day 1), 3.23%, 3.17% (day 2), 3.20%, 3.23% (day 3). Thus, the relative standard deviation (RSD) of repeatability and intermediate precision were 2.25% and 1.49% respectively. Recoveries ranged from 97% to 103 % with RSD2.25%, indicating the good accuracy of the proposed method.

Table 2: Factors and levels for orthogonal test

Level	Factors		
	A concentration of ethanol(%)	B ratios of liquid to solid	C extraction time(h)
1	80	20	1
2	85	30	1.5
3	90	40	2

Table3 : The results of $L_9(3^4)$ orthogonal experiment and range analysis

NO.	Factors				Flavonoids yield (%)
	A	B	C	D(Blank)	
1	1	1	1	1	2.20
2	1	2	2	2	2.70
3	1	3	3	3	2.54
4	2	1	2	3	2.45
5	2	2	3	1	2.56
6	2	3	1	2	2.87
7	3	1	3	2	1.67
8	3	2	1	3	2.44
9	3	3	2	1	2.63
k1	7.44	6.32	7.51	7.39	
k2	7.88	7.70	7.78	7.24	
k3	6.74	8.04	6.77	7.43	
k1'	2.48	2.11	2.50	2.46	
k2'	2.63	2.57	2.60	2.41	
k3'	2.25	2.68	2.26	2.48	
R	0.38	0.57	0.34	0.07	

Table 4: Variance analysis of the results

Source of error	Sum of Squares (SS)	Degree of freedom (f)	Mean square (S)	F value	P value	Significance
A	0.220	2	0.110	31.429	0.031	*(P<0.05)
B	0.553	2	0.276	79.000	0.012	*(P<0.05)
C	0.182	2	0.091	26.000	0.037	*(P<0.05)
D	0.007	2	0.004	1.000		

Table 5: Total flavonoids amount of samples collected in different harvest time and habitats

Harvest time	Habitat	Flavonoids amount (mg·g^{-1})	Average (mg·g^{-1})	RSD (%)
May 1, 2011	Huangmei, China	20.4		
		21.2	20.8	1.92
		20.8		
May 10, 2010	Wuhan, China	30.1		
		30.4	30.0	1.53
		29,5		
June 15, 2010	Wuhan, China	26.9		
		27.9	27.2	2.02
		27.0		
July 4, 2010	Wuhan, China	31.7		
		32.0	32.2	1.77
		32.8		
October 5, 2010	Wuhan, China	33.3		
		33.6	33.2	1.55
		32.6		

Sample determination

The samples collected in different harvest time and habitats were extracted by the optimal extraction method .According to the method illustrated in the "Total flavonoids determination", the total flavonoids content of each sample was also measured and the results were showed in Table 5.

DISSCUSION

To extract total flavonoids from *Elaeagni Folium*, the optimum condition obtained by using single-factor tests and orthogonal test was as follow: the ratio of solvent to raw material was 40:1, ethanol concentration 85% and extraction time 1.5 h. This result provided the reliable experimental basis for industrial production to extract the total flavonoids in this crude drug. Using rutin as standard substance and 0.1 mol·L^{-1} aluminum chloride ($AlCl_3$) solution as chromogenic agent, the total flavonoids content was measured by ultraviolet and visible spectrophotometry. The confirmation test indicated the optimal method was reproducible, accurate and feasible. Under this condition, the results represented that the amount of total flavonoids in the samples were 27.2-33.2mg·g^{-1} collected at Wuhan, Hubei Province but in the different harvest time from early summer to autumn. However, the total flavonoids content in this drug was 20.8mg·g^{-1} harvested in May at Huangmei, Hubei Province, which was tremendously different from the amount of the former. With total flavonoids as active ingredient, it indicated that the amount of total flavonoids was stable in this crude drug collected in different harvest time but at the same habitat, which provided basis for the further development and utilization.

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