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Study on the Extraction and Purification of Total Flavone in Etirfel Preparation

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The aim of the study was to optimize and discuss the extraction and purification of total flavone in etirfel preparation using macroporous absorption resin. Orthogonal test was adopted to optimize the purification of total flavone in etirfel preparation, with water as the solvent, using the content of total flavone in etirfel preparation as the evaluation indicator. The extraction temperature, extraction time and solvent consumption were chosen as the investigational factors to screen the optimal resin *via* the static experiments on the two types of macroporous absorption resin, namely using AB-8 and polyamide to investigate the adsorbtion and elution of total flavone in etirfel preparation by optimizing the extraction process parameters. The optimal extraction condition was obtained using a single 1 h extraction technology, at 50 ºC, with a extract-water ratio of 1:10. The optimal condition for purification of total flavone using 10 g polyamide resin are as follows. The adsorption was carried out at a flow rate of 0.25 mL/min, the loading solution volume was 25 mL, the diameter-height ratio was 1:3 and the eluent flow rate was 1.5 mL/min. The proprietary extraction process for the highest content of total flavone was obtained in this study through the optimization of the extraction and purification, providing a chemical basis for further pharmacological studies.

Key Words: Extraction, Total flavone, Etirfel.

INTRODUCTION

Etirfel preparation which belongs to a Chinese herbal compound preparation composed of both monarch drugs including fructus terminaliae immaturus, fructus chebulae and emblic leaf flower fruit and ministerial drugs is prepared by using traditional Uighur medicine manufacturing process[1]. Of 20 kinds etirfel preparation. The common characteristic effects of etirfel preparation are decrease of abnormal black bile, bile juice and aphlegm, killing of pathogens, astringent and xeransis, cleaning of blood, pain relief, clearing obstruction, tranquilizing mind, promoting digestion and improving eyesight. The therapeutic effects of etirfel preparation can be maintained for 5-12 h[2]. As an Uighur traditional medicine, etirfel preparation is mainly used in the treatment of atherosclerosis[3,4], hyperlipidemia[5,6], hypertension[7], leucoderma[8,9], eczema[10,11], atopic dermatitis[12,13], psoriasis[14], nervous system related diseases, urinary system diseases, ENT diseases and gynecological diseases[15]. Etirfel preparation has a confirmed therapeutic effect with widely clinical use and less side effects[16], however, there is no uniform standard of quality control for its preparation and the material basis for the therapeutic effect remain to be clarified. Therefore, we made a systematic study on the process of the extraction of total flavonoids and of the purification by using macroporous absorption resin and established the optimal proprietary extraction and purification process for total flavone. Macroporous resin is a material commonly used to separate flavonoids and due to the different adsorption mechanisms, different type of macroporous absorption resin have different advantages and disadvantages in separation and purification[17]. According to the characteristics of macroporous resin, we select AB-8 resin and polyamide resin to investigate proprietary extraction and purification process for total flavone in etirfel preparation.

EXPERIMENTAL

UV2100 ultraviolet and visible spectrophotometer (USA UNICO (Shanghai) Instrument Co. Ltd.); analytical balance FA2004 (Shanghai Balance Instrument Plant); rotary evaporator RE-52AA (Shanghai Yarong Biochemical Instrument Plant); high speed refrigerated centrifuge GL-21M (Changsha Xiangyi Centrifuge Instrument Co., Ltd); Glass resin column (Beijing Xinweier Glass Instrument Co. Ltd); Digital thermostatic water bath HH-8 (Guohua Electrical Appliance Co. Ltd.); high performance liquid chromatograph (Beijing Chuangxin Tongheng Science and Technology Co. Ltd.).

Macroporous absorption resin was purchased from Cangzhou Baoen Adsorbing Material Technology Co. Ltd. Sodium nitrate was obtained from Anshan Zhiao Chemical

Reagent Research Institute). Sodium hydroxide was a product of Tianjin Dalu Chemical Reagent Plant). Aluminium nitrate (Tianjin Damao Chemical Reagent Plant); Rutin (National Institute for the Control of Pharmaceutical and Biological Products); emblic leaf flower fruit (Weifang Shenzhou Tiancheng Chinese Herbal Decoction Co. Ltd.); Fructus terminaliae immaturus (Weifang Shenzhou Tiancheng Chinese Herbal Decoction Co. Ltd.); fructus chebulae (Weifang Shenzhou Tiancheng Chinese Herbal Decoction Co. Ltd.). All the traditional herbs were prepared in the form of powder.

Determination of total flavone extraction technology

Establishment of standard curve[18]: Rutin reference was added into an oven at 120 ºC and the drying procedure was not finished till the constant weight was obtained. Methanol was added to 10.6 mg rutin reference to a final volume of 25 mL. Accurate volume of 0, 1.0, 2.0, 3.0, 4.0 and 5.0 mL Rutin reference solution was add into six 25 mL volumetric flasks respectively, 1.0 mL 5 % sodium nitrite solution was then added and shaken evenly and kept for 6 min, 1.0 mL 10 % aluminum nitrate solution was then added, shaken evenly and kept for 6 min again, 5.0 mL of 4 % sodium hydroxide solution was added and methanol was added to reach a final volume of 25 mL; the flasks were shaken evenly and stood in shade for 15 min and then measured the absorbance A at 510 nm. The regression equation of rutin content (mg/mL) and absorbance is shown in Fig. 1.

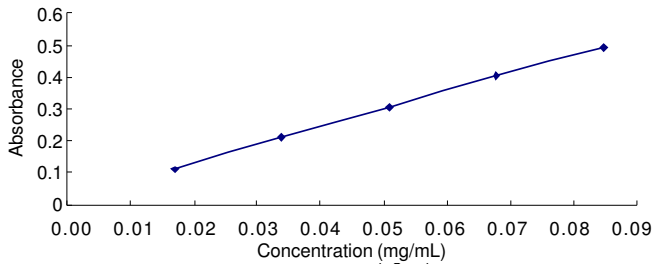

Fig. 1. Standard curve of rutin (UV)

Ultraviolet spectrophotometer is adopted to obtain the regression equation of rutin content (mg/mL) and absorbance at 510 nm: Y = 5.6073X + 0.0195, r = 0.9995 and the linear range is 0.01696-0.08480 mg/mL.

Extraction of total flavone: The orthogonal design of the assay is as follows. 10 g fructus terminaliae, 10 g immaturus, 10 g fructus chebulae and 10 g emblic leafflower fruit were added into 500 mL flasks, with water as the solvent to investigate extraction temperature (50, 80 and 100 ºC), extraction time (1, 1.5 and 2 h), extraction times (once, twice and three times) and extraction solvent consumption (5×, 10× and 15×). The extracted samples were dried in vacuum at 80 ºC untill the constant weight was obtained by using a three-time measurement within 24 h (Table-1).

Measurement of samples[19,20]: After moisture was removed, the dried rutin extract was dissolved in distilled water, shaken evenly, filtered and to reach a final volume of 1000 mL with distilled water and then 1 mL of the prepared solution was added to a 25 mL volumetric flask for use in next step. The measurement of the content of total flavone was described in above section.

TABLE-1
ORTHOGONAL DESIGN FOR TOTAL FLAVONE EXTRACTION

Level	Factor			
	Extraction temperature (ºC)	Extraction time (h)	Extraction times (times)	Solvent capacity (multiple)
1	50	1	1	5
2	80	1.5	2	10
3	100	2	3	15

Determination of total flavone purification[21-24]

Pretreatment of resin: Polyamide resin was prepared for use after soaked in distilled water for 24 h.

Determination of static adsorption capacity: 3 g treated dry resin was accurately weighed and added into 50 mL conical flask and then 10 mL total flavone sample solution which was extracted with the optimal proprietary purification process, was added and treated by using ultrasound for 20 s in every 10 min interval for 2 h and left for 24 h to make it reach saturated adsorption and then 1 mL upper solution was obtained and added into a 25 mL volumetric flask to measure the content of total flavone according to aforementioned procedures and to calculate its concentration. The saturated adsorption capacities was calculated using the following formula: saturated adsorption capacity = [(initial concentration - concentration after adsorption) × adsorption solution volume]/resin amount (mg/g).

Static adsorption-elution performance experiment: Filter it after static saturation adsorption till there is no moisture on resin surface, accurately add 30 mL distilled water, make ultrasound treatment for 20 s every 10 min for 2 h, measure eluent concentration and calculate the elution rate. Elution rate = [eluent concentration × eluent volume]/saturated adsorption capacity × 100 %.

Determination of adsorption flow rate: Make the total flavone sample solution with the mass concentration of 7.0890 mg/mL flow through resin column, make the dynamic adsorption with three resin columns, respectively at the flow rate of 0.25, 0.5 and 1.0 mL/min, check the effluent with 1 % $FeCl_3$ solution, when it presents positive reaction, record the loading volume and calculate the adsorption capacity of total flavone. Elute the three resin columns with water and then 70 % ethanol, collect the ethanol eluent, weigh the weight, measure the amount of total flavone and calculate the recovery rate of total flavone (recovery rate of total flavone = total flavone amount in eluent/total flavone adsorption capacity by resin).

Investigation of dynamic adsorption: Accurately weigh 11.9 mg rutin, settle in 25 mL volumetric flask with methanol and obtain 0.476 mg/mL rutin solution for future use. Withdraw the 0.01, 0.02, 0.04, 0.08, 0.16 and 0.32 mL from the above rutin solution and settle in 25 mL volumetric flasks for future use. Take the rutin solutions of the above 6 concentrations, with the chromatographic conditions as follows: chromatographic column: Kromasil C_{18} column (150 mm × 4.6 mm, 5 μm), mobile phase: methanol - 0.5 % phosphoric acid (38:62), flow rate 1 mL/min, detection wavelength 254 nm, column temperature 30 ºC, sampling volume 20 μL and measure the peak area.

Make the total flavone sample solution of 7.0890 mg/mL flow through polyamide resin column, make the dynamic adsorption at the flow rate of 0.25 mL/min, check the effluent with 1 % $FeCl_3$ solution, when it presents positive reaction and collect 1 share per 5 mL, totally 13 shares. HPLC is adopted to measure rutin content in each share according to the above chromatographic conditions and rutin leakage curve is adopted to investigate the dynamic adsorption capacity of polyamide to total flavone in etirfel preparation.

Determination of eluent concentration: According to the above determined conditions, take the sample solution to flow through polyamide resin column and make the pre-adsorption for 0.5 h. Elute the resin column with water till the eluent is colourless, check the effluent with 1 % $FeCl_3$ solution till it presents negative and then elute from low concentration to high concentration with ethanol of different concentrations till the effluent presents negative, checked with 1 % $FeCl_3$ solution. Dry the ethanol eluent of different concentrations by distillation, measure the extract weight, calculate the extract rate (dry extract rate = eluted dry extract amount/total extract amount corresponding to sampling volume × 100 %), dissolve the dry extract with ethanol of corresponding concentration, measure total flavone content and calculate total flavone content percentage (total flavone content percentage = total flavone amount/dry extract × 100 %).

Orthogonal design investigation on the levels of the factors influencing resin elution: The main factors influencing the elution performance of polyamide resin are resin diameter-height ratio and eluent flow rate and three levels for each factor can be selected, to optimize the elution conditions (Table-2).

TABLE-2
LEVELS OF THE FACTORS INFLUENCING POLYAMIDE RESIN ELUTION

Level	Factor	
	A Diameter-height ratio	B Elution flow rate (mL/min)
1	1:03	0.5
2	1:06	1.0
3	1:09	1.5

According to the above factor level table, L_9 (3^4) orthogonal test table is selected to investigate the influence of the three levels of the above two factors on the results. Accurately weigh 9 shares of 10 g polyamide resin, add 25 mL upper column solution, pre-adsorb for 0.5 h, adopt the corresponding flow rate, add water, 30 % ethanol, 50 % ethanol and 70 % ethanol in turn for elution, collect 70 % ethanol eluent, measure the total flavone content, score with flavone content percentage.

RESULTS AND DISCUSSION

Determination of total flavone extraction technology: The optimal process for total flavone extraction is shown in Tables 3 and 4.

Intuitive analysis shows that the sequence of the factors influencing total flavone extraction is B > D > C > A, *i.e.*, extraction time has the greatest influence; the second is the solvent consumption and then extraction times and extraction temperature. It can be seen from the analysis of variance that factor B (extraction time) and factor D (solvent consumption) have significant difference, therefore, we can obtain from the two analyses that the optimal extraction technology is 50 ºC, one extraction for 1 h and water volume is 10 times.

TABLE-3
RESULTS OF ORTHOGONAL DESIGN ON TOTAL FLAVONE EXTRACTION TECHNOLOGY

Level		Factor						
		Extraction temperature (ºC)	Extraction time (h)	Extraction times (times)	Solvent capacity (multiple)	Dry paste rate (%) y_1	Total flavone content (%) y_2	Holistic Marking z
1		1	1	1	1	36.5	3.09905	85.522
2		1	2	2	2	47.7	3.233775	92.6938
3		1	3	3	3	35.9333	2.8613	79.7635
4		2	1	2	3	39.5	3.217925	89.3773
5		2	2	3	1	47.1667	2.8205	81.9477
6		2	3	1	2	35.8667	3.027725	83.628
7		3	1	3	2	56.6667	3.423975	100
8		3	2	1	3	34.9	3.027725	83.2807
9		3	3	2	1	49.9667	2.599775	78.6949
Intuitive analysis	K_1	86.003	91.643	84.154	82.065	–	–	–
	K_2	84.984	85.974	86.922	92.107	–	–	–
	K_3	87.325	80.695	87.237	84.141	–	–	–
	R	2.341	10.948	3.083	10.042	–	–	–

TABLE-4
ANALYSIS OF VARIANCE

Factor	Sum of squares of deviation from mean	Degree of freedom	F value	F marginal value	Statistical significance
Extraction temperature	8.272	2	1	19	
Extraction time	179.52	2	21.702	19	*
Extraction times	17.389	2	2.102	19	
Solvent capacity	168.87	2	20.415	19	*
Error	8.27	2			

Footnote: z = 0.1*y1 + 0.9y2.

Determination of total flavone purification: The results of measurement of static adsorption capacity and elution are given in Tables 5 and 6.

TABLE-5
RESULT OF THE MEASUREMENT OF STATIC RESIN SATURATION ADSORPTION CAPACITY

Resin model	Initial concentration of samples (mg/mL)	After absorption concentration (mg/mL)	After saturated the adsorption quantity (mg/g)(dry resin)
AB-8	7.0890	3.8794	10.6987
Polyamides	7.0890	2.5552	15.1127

TABLE-6
RESULT OF THE MEASUREMENT OF STATIC RESIN ELUTION (n = 3)

Resin model	After saturated the adsorption quantity (mg/g)(dry resin)	Elution quantity (mg/g) (dry resin)	Elution rate (%)
AB-8	10.6987	4.8792	45.6055
Polyamides	15.1127	5.1199	33.8781

It can be seen from Tables 5 and 6 that polyamide resin is easier to adsorb total flavone than AB-8 resin and at water elution, the elution rate is lower than the latter, so polyamide resin is selected.

According to Table-7, considering total flavone adsorption capacity and recovery rate, we determine that the optimal adsorption flow rate is 0.25 mL/min.

TABLE-7
DETERMINATION OF ADSORPTION FLOW RATE

Adsorption velocity (mL/min)	Total flavonoid dsorption quantity (mg/mL)	Flavonoids recovery rate (%)
0.25	5.3974	88.6542
0.50	5.2431	82.3275
1.00	4.9827	77.4863

The regression equation of rutin content (mg/mL) and peak area is shown in Fig. 2.

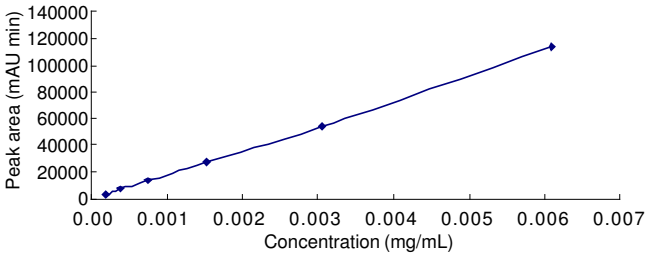

Fig. 2. Standard curve of rutin (HPLC)

HPLC is adopted to obtain the regression equation of rutin content (mg/mL) and peak area at 254 nm: $Y = 1.8697 \times 10^{-7} \times -656.62$ r = 0.9992 and the linear concentration range is 0.0001904-0.0060928 mg/mL.

It can be seen from Fig. 3 that when the sampling volume is 25 mL, rutin starts to leak, so when 7.0890 mg/mL total flavone sample undergoes dynamic adsorption at 0.25 mL/min, the adsorption capacity of 10 g resin is 25 mL.

According to Table-8, considering dry extract rate and total flavone content percentage, we determine that 70 % ethanol is the optimal eluent.

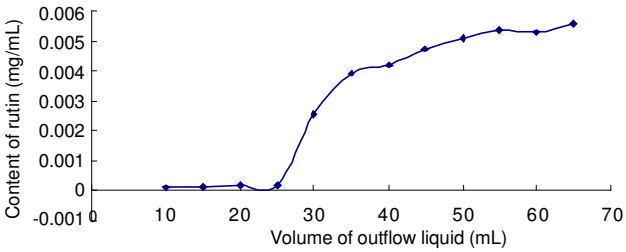

Fig. 3. Leakage curve of rutin on polyamide resin column (HPLC)

TABLE-8
ELUENT SELECTION

Eluent concentration	Dry paste rate (%)	Total flavone content (%)
30 % ethanol	13.76	6.59
50 % ethanol	8.11	29.81
70 % ethanol	1.76	48.68
95 % ethanol	0.71	10.33

It can be seen from Table-9 that the factors influencing resin elution have no significant difference, so the resin column diameter-height ratio of 1:3 and elution flow rate of 1.5 mL/min are the optimal elution conditions.

With water as the solvent and the extraction temperature, extraction time, extraction times and solvent consumption as the investigational factors, orthogonal test is adopted to optimize the technology for the extraction of total flavone in etirfel preparation and the optimal technology is 50 ºC, one extraction for 1 h and water volume is 10 times. Through the investigation on static adsorption and elution of total flavone in etirfel preparation on AB-8 resin and polyamide resin, we determine that total flavone in etirfel preparation has better adsorption and elution on polyamide resin. Through the investigations on adsorption flow rate, dynamic adsorption, eluent concentration and the orthogonal design on the levels of the factors influencing resin elution, we finally determine that the optimal technology for the purification by 10 g polyamide resin is that the adsorption flow rate is 0.25 mL/min, the loading solution volume is 25 mL, the diameter-height ratio is 1:3 and the eluent flow rate is 1.5 mL/min. Through the purification by the optimal technology of polyamide resin, the content of total flavone in etirfel preparation increases from *ca.* 3-48 %, indicating that polyamide resin has extremely high efficiency in purifying total flavone in etirfel preparation.

According to literature reports, total flavone has the functions of reducing blood sugar, reducing blood fat, protecting cardiac muscle, reducing blood pressure, antiarrhythmia, protecting liver and kidney, anticoagulation, relieving pain, antiinflammation, antioxidation, slowing down aging, antitumor, preventing osteoporosis, *etc.*[25]. Therefore, total flavone in etirfel preparation may be the material basis for etirfel preparation to treat arteriosclerosis, hyperlipidaemia, hypertension, *etc.*; further pharmacological studies and verifications should be made.

ACKNOWLEDGEMENTS

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TABLE-9
LEVELS OF POLYAMIDE RESIN ELUTION FACTORS

Level	Factor				
	A Diameter height ratio	B Elution flow rate (mL/min)	Blank	Blank	Total flavone content (%)
1	1	1	1	1	46.52
2	1	2	2	2	42.31
3	1	3	3	3	39.25
4	2	1	2	3	51.67
5	2	2	3	1	49.88
6	2	3	1	2	44.76
7	3	1	3	2	46.36
8	3	2	1	3	47.95
9	3	3	2	1	45.18
Intuitive analysis	K_1	42.693	48.183	46.410	47.193
	K_2	48.770	46.713	46.387	44.477
	K_3	46.497	43.063	45.163	46.290
	R	6.077	5.120	1.247	2.716
Variance analysis	Sum of squares of deviation from mean	56.559	41.698	–	–
	Degree of freedom	2	2	–	–
	F value	1.151	0.849	–	–
	F critical value	6.940	6.940	–	–

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