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Original Research Article

Extraction and Purification of Flavonoids from *Radix Puerariae*

Pengyue Li, Yang Lu*, Shouying Du*, Jie Bai, Huimin Liu, Qingli Guo and Yiwang Guo
School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing 100102, China

For correspondence: **Email: pengyuelee@126.com, dushouying@263.net, landocean@163.com; **Tel:** 010-84738615, 010-84738615*

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Abstract

Purpose: To develop an efficient method for the purification of flavonoids from *Radix puerariae*
Methods: Optimal extraction technology was obtained using orthogonal test. Through adsorption and desorption tests, 8 resins with different polarity, diameter, and surface area were studied. Finally, a novel macroporous resin, HPD200A, was applied in the work. Desorption tests were performed in a glass column packed with HPD200A resin, and several parameters of purification were studied. The content of puerarin and flavonoids in the samples were tested by a HPLC and a UV-Vis Spectrophotometer, respectively.
Results: Optimum extraction technology was confirmed as follows: extraction time is 1.5 h; ratio of solvent to material 12; and repetition of extraction 3 times. Optimum purification technology was confirmed as follows: decoction is concentrated into 1.05 ~ 1.10 g/ml; 95 % ethanol is added to remove the impurities and the volume ratio is 1.5 : 1; the concentration of loading sample is 0.25 g/ml (based on the weight of the crude drug), the volume of loading sample is 2 BV (bed volume). The mobile phases of desorption are water (2BV) and 30 % ethanol (4BV) in succession. The contents of puerarin and flavonoids in the extract were improved greatly after the purification procedure, by up to 32 and 75 %, respectively.
Conclusion: This efficient method has potential for purification and preparation of flavonoids in the future.

Keywords: Puerarin, Flavonoids, Extraction, Purification, Macroporous resin.

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INTRODUCTION

Radix puerariae, a traditional Chinese medical herb, has been described by so many Chinese herbal books, Chinese Pharmacopoeia and Japanese Pharmacopoeia. Nowadays in China, many related beverages, healthcare products and preparations are now on sale. Preliminary studies have shown that the key components are flavonoids, they have a variety of biological activities [1-3] including improving of insulin resistance [4] and anti-oxidation [5].

Therefore, developing an efficient method for the production of high quality products has become

an issue of concern in food and pharmaceutical industries.

Thus, the purpose of this work was to optimize the technology of water extraction and develop an efficient method for the purification of flavonoids. The information of this study is significant for optimization of extraction, resin selection and purification procedures.

EXPERIMENTAL

Chemicals, materials, macroporous resins, and apparatus

The standards of puerarin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), HPLC-grade Methanol was purchased from Mreda technology Inc. (USA), HPLC-grade Water was purchased from Hangzhou Wahaha Group Co., Ltd (Zhejiang, China). Other reagents were all of analytical grade.

Radix puerariae material was obtained from Beijing Hua Miao Medicine Engineering Technology Development Center, Beijing, China.

Eight macroporous resins, including HPD600, ADS-7, HPD450, HPD750, HPD722, AB-8, HPD200A and D101 were obtained from Cang Zhou Bon Adsorber Technology Co., LTD. (Hebei, China). Physical and chemical parameters of the resins were provided by manufacturer (Table 1 of Supporting Information Section).

LC20AHPLC (Shimadzu Corporation, Japan) with SPD-20A UV detector (Shimadzu Corporation, Japan) and Kromasil C_{18} Column (150mm×4.6mm) (TIANHE, China), Analytical balance (Sartorius, Germany), 752PC UV-Vis Spectrophotometer (Shanghai Spectrum Instrument Co, Ltd, Shanghai, China) were used.

Extraction of *Radix puerariae*

The orthogonal test with three factors (ratio of solvent to material, extraction time and repetition of extraction) and three levels was designed to solve the optimal technology of the extraction (Table 2 of Supporting Information Section). And the L_9 $(3)^4$ table was designed to detect the effects of three factors on extracting efficiency.

Static adsorption and desorption Tests

To optimize a resin, the adsorption capacity and desorption rate of 8 resins were studied by static adsorption tests It was carried out as follows, resin (5 ml) and decoction (100 ml) were put into a triangular flask for 24h at 25 ^{o}C. Then the resin was filtered out and the filtrate was collected in a 250 ml volumetric flask. The adsorption capacity of the resin was calculated according to Eq 1.

$$Q_e = (C_0V_1-C_eV_2)/V \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where Q_e is the adsorption capacity at adsorption equilibrium (mg/ml resin), C_0 and C_e are drug concentrations of initial stage and equilibrium stage (mg/ml), V_1 is the volume of the initial solution (ml), V_2 is the volume of volumetric flask (250ml) and V is the volume of the tested resins (ml).

During static desorption tests, the resin(filtered out in the adsorption test) was desorbed with 95 % ethanol (100ml). Desorption rate was calculated as in Eq 2.

$$E_0 = C_3V_3/Q_e \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where E_0 is the desorption ratio and C_3 is the drug concentration in the desorption solution (mg/ml) and V_3 is the volume of desorption solution.

Preliminary purification of the sample for dynamic adsorption and desorption tests

The water extract contains a lot of protein and starch which may be adverse to desorption. It is necessary to remove these impurities, so, the ethanol precipitation was made. The decoction was concentrated into different density, and then precipitated with 95 % ethanol. The precipitate was removed by filtration and the filtrate was collected. Take the retention rates of puerarin and flavonoids as indexes, optimize the proper density. Thereafter, the effect of 95 % ethanol volume was studied, using the same indices.

Through static adsorption and desorption tests, a suitable resin was chosen for dynamic tests. Dynamic adsorption and desorption tests were carried out in glass columns (12 mm ×30 mm) wet-packed with the selected resin (10 ml). Several items, like dynamic leakage curve and mobile phase, were studied.

Concentration of loading sample

After the ethanol-precipitation procedure, the filtrate was collected and ethanol was reclaimed by Rotary Evaporators. Loading samples (20 ml) of different concentrations (g/ml, based on the weight of crude drug) were prepared and passed through the glass column at the flow rate of 1 mL/min. The adsorption ratios of puerarin and

flavonoids were used as index to optimize the proper loading sample concentration.

Dynamic leakage curves of loading and water elution

The turning point on the loading curve was due to the fact that the resin in the column approached to its saturation. Considering the water-elution operation in the next step may also cause leakage. So the loading volume was defined according to the loss of target compounds in the whole process of loading and water elution. Each volume of the eluate was collected, tested by HPLC and UV-Vis Spectrophotometer, dried and weighed. The leakage curves and water-elution curves were drawn, and the loading volume was fixed accordingly.

Optimum mobile phase

After loaded and eluted by water, the resin columns were eluted by 4 times of bed volume (BV) of mobile phase (30, 40, 50, 70 and 95 % ethanol). The collected eluent was tested by HPLC and UV-Vis Spectrophotometer, dried and weighed. So the purity of the compounds can be obtained.

Dynamic desorption curve on resin

According to the parameters above, after the resin was loaded and eluted by water, it was washed with 8 BV of selected mobile phase at the speed of 0.5 ml/min. Each volume of the eluent was collected, tested by HPLC and UV-Vis Spectrophotometer, dried and weighed. From the desorption curve, the optimum volume of mobile phase was obtained according to desorption rate and purity of the target compounds.

Statistical analysis

Analysis of the data was performed by SAS 8.2 software based on Student's *t*-test or ANOVA tests. Differences were considered significant at $p < 0.05$.

RESULTS

Extraction technology of radix puerariae

The content of puerarin in radix puerariae was tested by HPLC according to Chinese Pharmacopoeia [7]. The content was 3.77 %.

A UV-Vis Spectrophotometer was used to determine the content of flavonoids in radix puerariae at 250 nm. The flavonoid content was calculated using linear equation (Eq 3) based on the calibration curve prepared in the puerarin concentration range of 1.04 to 10.40 µg/ml. The content is 9.62 %.

$$A = 0.0736C + 0.0231 \quad \text{..... (3)}$$

where A is the absorbance, C is the concentration of flavonoid.

The effects of three factors on the extracting efficiency of puerarin and flavonoids are shown in Tables 1 and 2.

As shown in the value of range of R in Table 1, factor C exerted the most significant effect on the extraction efficiency of puerarin. The order of importance that influenced the extraction efficiency of puerarin was found to be C > A > B. The optimal parameters of the technology were $A_2B_3C_3$.

Table 1: Orthogonal test results of extraction efficiency of puerarin

No.	A (Time, h)	B (Ratio of solvent to material)	C (Repetition of extraction)	Extracting efficiency of puerarin (%)
1	1	6	1	43.26
2	1	9	2	79.42
3	1	12	3	89.96
4	1.5	6	2	88.90
5	1.5	9	3	83.69
6	1.5	12	1	61.73
7	2	6	3	78.52
8	2	9	1	52.70
9	2	12	2	78.31
Ⅰi	212.64	210.68	157.69	
Ⅱi	234.32	215.81	246.63	
Ⅲi	209.53	230	252.17	
R	24.79	19.32	94.48	

Table 2: Orthogonal test results of extraction efficiency of flavonoids

No.	A (Time, h)	B (Ratio of solvent to material)	C (Repetition of extraction)	Extracting efficiency of total flavonoids (%)
1	1	6	1	44.63
2	1	9	2	88.04
3	1	12	3	96.74
4	1.5	6	2	87.00
5	1.5	9	3	94.07
6	1.5	12	1	65.22
7	2	6	3	83.07
8	2	9	1	56.75
9	2	12	2	82.00
Ⅰj	229.41	214.70	166.6	
Ⅱj	246.29	238.86	257.04	
Ⅲj	221.82	243.96	273.88	
R	24.47	29.26	107.28	

Factor C exerted the most significant effect on the extraction efficiency of flavonoids. The order of importance that influenced the extraction efficiency of flavonoids was found to be C > B > A. The optimal parameters of the technology were $A_2B_3C_3$ (Table 2). These optimal parameters had been proved reasonable and feasible through verification test, with the extraction efficiency of puerarin and total flavonoids being 92.63 and 94.78 %, respectively.

Adsorption capacities and desorption ratio

As was shown in Fig. 1, the adsorption capacities of puerarin and flavonoids on HPD200A resin were the highest among all these resins. The desorption ratio of HPD200A was also higher than the other resins.

Purification of decoction

The resins may be covered with water – soluble impurities, which led to a low desorption ratio in desorption experiments. So, it is necessary to purify the decoction before loading. So an ethanol-precipitation procedure was introduced in to improve the desorption ratio. The density of decoction and the volumes of ethanol were studied. The results are shown in Tables 3 and 4.

According to the retention rate of puerarin and flavonoids the density of decoction was set at 1.05~1.10 g/ml (Group C is significantly different from group A and B at $p< 0.05$). Puerarin and

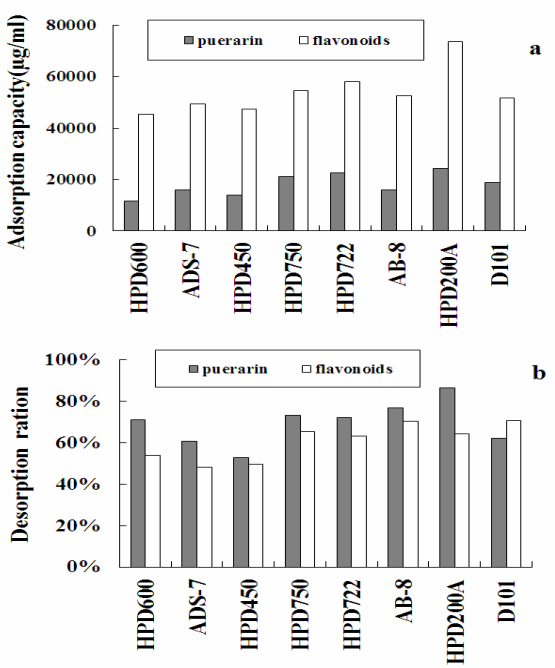

Fig 1: Adsorption capacity and desorption ratio of puerarin and flavonoids

Table 3: Effect of density on ethanol precipitation

Group	Density (g/ml)	Retention of puerarin (%)	Retention of flavonoids (%)
A	1.01~1.04	98.58	100.92
B	1.05~1.10	98.74	100.54
C	1.15~l.20	91.35	96.47

Table 4: Effect of ethanol volume on ethanol precipitation

Group	Ratio of concentrates to 95% ethanol	Retention of puerarin (%)	Retention of flavonoids (%)
A	1:1	92.09	92.21
B	1:1.5	93.39	96.53
C	1:2.5	95.69	97.91

Table 5: Effect of loading sample concentration on adsorption ratio

Concentration (g/ml of crude drug)	Adsorption ratio of puerarin (%)	Adsorption ratio of flavonoids (%)
0.1	95.31	93.37
0.25	95.90	91.27
0.5	94.16	90.80

flavonoids might be deposited when the density increased. According to the retention rate of puerarin and flavonoids, the ratio of concentrate to 95 % ethanol was set at 1:1.5 (Group A is significantly different from group B and C at $p < 0.05$). When the ratio was 1:1, the sediments were too loose, which was adverse to filtration. A ratio of 1:2.5 will cost a lot of ethanol. Hence, a moderate ratio, 1:1.5, was chosen.

After ethanol-precipitation, the desorption ratios of both puerarin and flavonoids were improved greatly. The ratios were 98.34 and 96.15 %, respectively.

Loading sample concentration

The adsorption ratios of puerarin and flavonoids were studied. There were no significant differences among these groups ($p > 0.05$), as shown in Table 5. However, the column was blocked up easily when the concentration was 0.5 g/ml of crude drugs. So the loading concentration was set at 0.25 g/ml of crude drugs.

Dynamic leakage curves of loading and water elution

The leakage curves of resin are shown in Fig 2. According to the curves of Fig 2a, flavonoids leaked when the loading volume was 2 times of bed volume (2BV), though puerarin was not released until the volume was 3.5BV. The water-elution curves of Fig 2b also showed that, water-elution caused great loss when the loading volume was 3.5BV. So, it is reasonable to set the loading volume at 2 BV. And water of 2 BV removed most of impurities, so the water volume was set at 2 BV, as was shown in Fig. 2c.

Mobile phase of desorption

Fig. 3a shows that the desorption ratios of puerarin and flavonoids in 30% group were lower than that in other groups. But the purity of puerarin and flavonoids, as was shown in Fig 3b, was higher. Ethanol with higher concentration would decrease the selectivity of target compounds. Thus, 30 % ethanol was chosen as the mobile phase for desorption.

The whole process of enrichment is shown in Fig 4 and Table 6.

Table 6: Whole process of enrichment

	Content in Radix Puerariae	Purity of target compound in intermediate product		
		After water extraction	*After ethanol precipitation*	*After Purification via resins*
Puerarin	3.77	10.00	12.84	32.40
Flavonoids	9.62	26.29	33.31	75.52

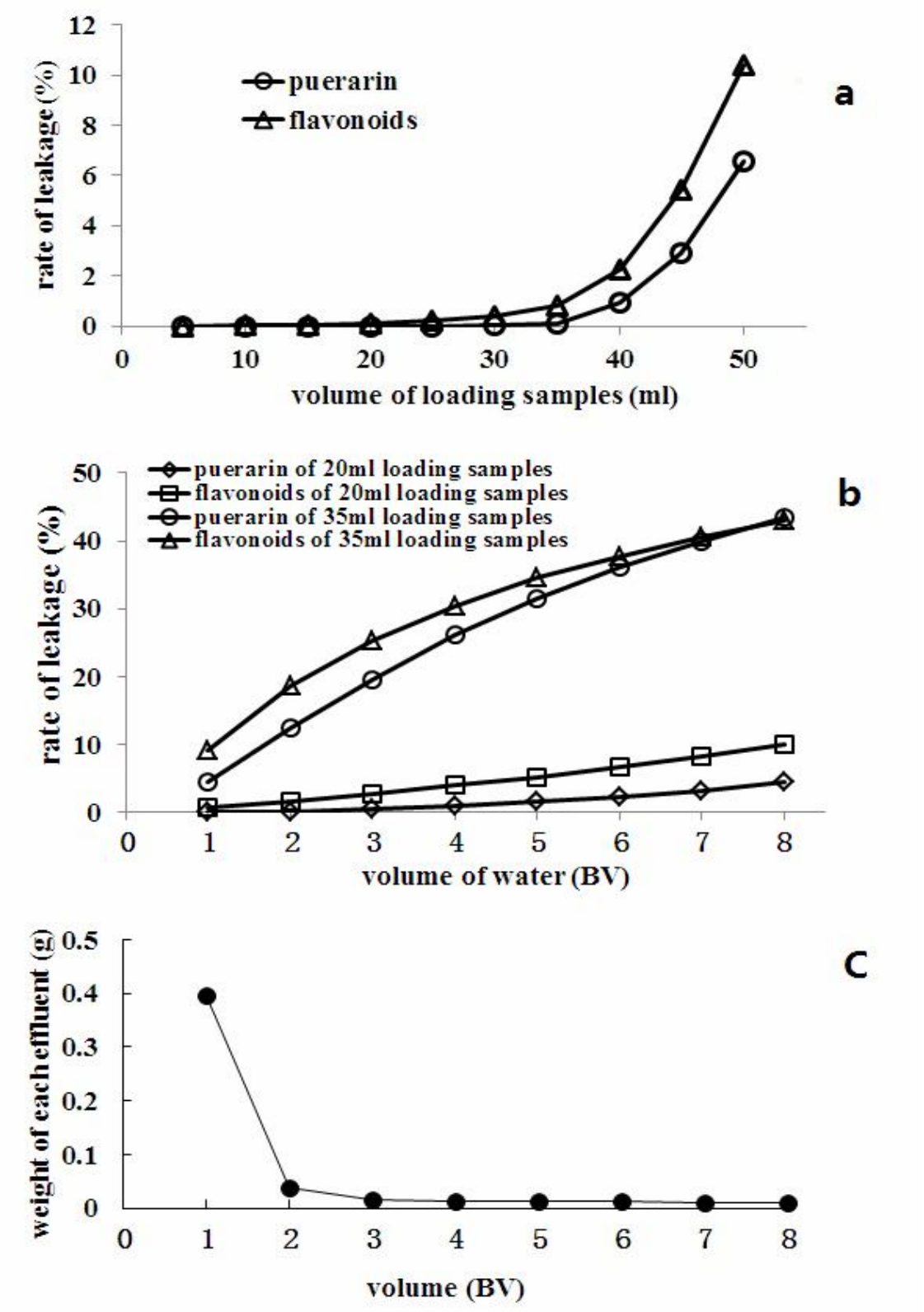

Fig. 2: Loading samples and volumes for water-elution a. Leakage curves, b. Leakage of different groups in water-elution procedure, c. Water-elution curves.

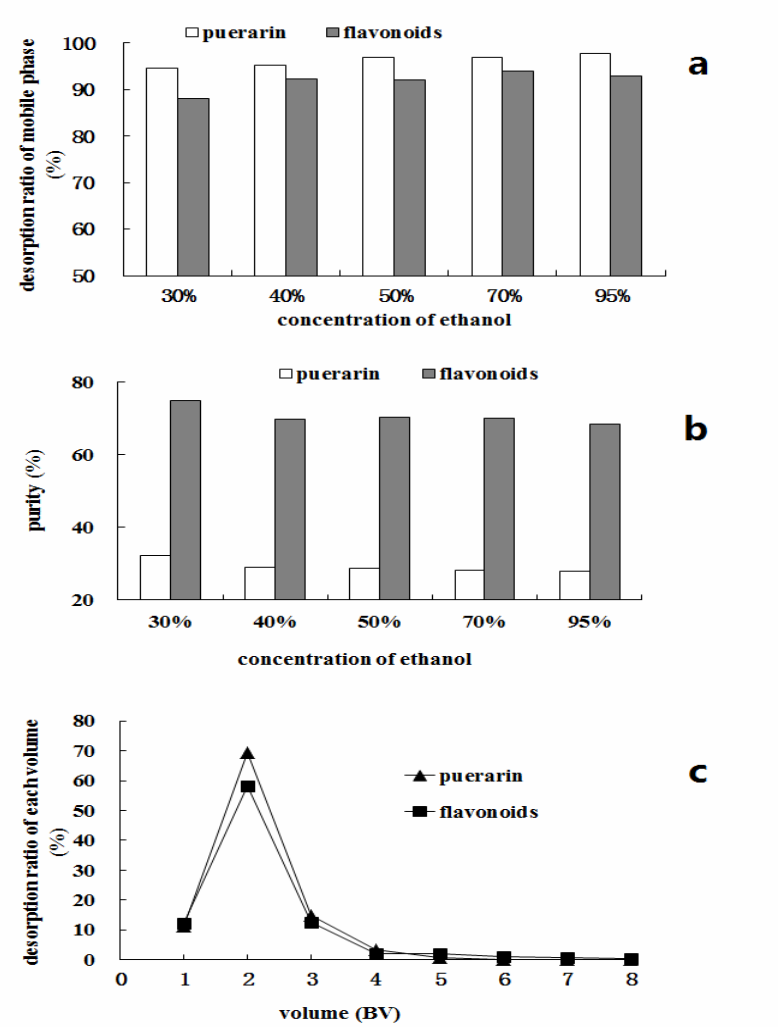

Fig 3: Mobile phase and the dynamic desorption curves. **Note:** (a) Desorption ratios of different groups, (b) Purity of different groups, (c) Desorption curves

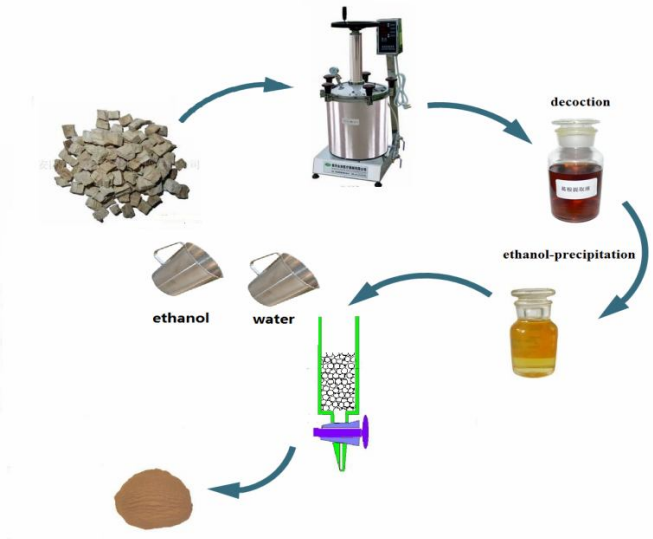

Fig 4: Whole process of enrichment

The dynamic desorption curves were obtained based on the volume of mobile phase and the desorption ratio of each volume. As can be seen in Fig 3c, puerarin and flavonoids could be desorbed by approximately 4 BV of mobile phase. The eluate solution was dried and weighted to calculate the contents of puerarin and flavonoids. The contents of puerarin and flavonoids reached 32 and 75 %, respectively. The contents were improved greatly after the purification procedure.

DISCUSSION

Now different kinds of new technologies were studied to extract flavonoids from Radix Puerariae, including microwave-assisted extraction [8], ultrasonic extraction and pressurized solvent extraction [9] and supercritical fluid extraction [10]. But, it will still take a long time for the application of these new methods in industrial production, considering the cost and popularity of the equipment. So, traditional method, water extraction, is still being widely used at present. It costs less time and needs no additional explosion-proof heating devices.

The purification of flavonoids is also an important research subject. There are several conventional separation methods, such as two-phase solvent extraction [11], salting-out [12] and ion complex method [13] have been employed. However, these methods have some disadvantages, such as heavy metal residues, consumption of large amounts of solvent and low recovery.

Recently, macroporous resin adsorption technology has become a promising method for purifying natural products. The adsorption properties of macroporous resin are correlated with their surface area, diameter, and the molecular weight, polarity and shape of the target compounds. This method has some advantages, like easy operation, low-cost and easy recycling.

In this study, the enrichment of puerarin and flavonoids with macroporous resin was successfully achieved. The most effective resin (HPD200A) was successfully applied to obtain a product with high content of puerarin and flavonoids. Though it is a resin with non-polar, it has the largest surface area among all these resins, which may be helpful to the adsorption of the flavonoids. It suggests that, surface area is an important parameter when optimizing the resins.

Finally, the flavonoids content was up to 75 %, and the resins can be re-used more than 10 times before regeneration.

A product with higher purity may have higher medicinal value and economic value. So, further studies should be done to obtain a product with higher purity. New techniques, like ultrafiltration membrane technique[14,15] and activated carbon [16] are effective methods to purify natural products.

CONCLUSION

The method is useful for the extraction and purification of puerarin and flavonoids from *Radix puerariae*.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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