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Enrichment and purification of flavones from rhizomes of *Abacopteris penangiana* by macroporous resins

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[ABSTRACT] AIM: To investigate the enrichment and purification of flavones from the rhizomes of *Abacopteris penangiana* (RAP) by macroporous resins. **METHODS**: Static adsorption and desorption tests were performed to select the appropriate resin. The kinetic adsorption and desorption experiments were carried out on selected HPD500 resin to optimize the separation process of flavones. Additionally, the effects of four parameters including adsorption flow rate, elute flow rate, volume of water and ethanol solution for elution were explored by a L4/3 orthogonal experiment. Finally, the ABTS (2, 2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activities of samples before and after being treated by HPD500 were compared. **RESULTS**: The results showed that the optimal parameters were initial concentration of 2.86 mg·mL^{-1}, elute solution of 70% ethanol, absorb flow rate of 1 mL·min^{-1}, elute flow rate of 2 mL·min^{-1}, 5 BV of water for elution and 5 BV of ethanol solution for elution. **CONCLUSION**: The content of flavones is above 60% in RAP after being treated by HPD500, indicating that macroporous resins could be successfully applied to enrich and purify flavones in RAP.

[KEY WORDS] *Abacopteris penangiana*; Flavones; Macroporous resins

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1 Introduction

The rhizomes of *Abacopteris penangiana* (Hook.) Ching (RAP), called *jixuelian*, is a folk medicine used by Tujia people to promote blood circulation and remove blood stasis in the western region of Hubei Province, China [1]. Previous phytochemistry researches suggested that novel flavan-4-ol compounds were the main kind of constituents in RAP [2-4]. Moreover, those compounds were proved to present powerful antioxidant and neuroprotective activities [2, 5].

Conventional methods including liquid–liquid extraction and gel chromatography are not effective to obtain these compounds concerning regents, labor, energy consumption and environmental protection. Alternatively, growing interest has been focused on employing macroporous resins to enrich and purify bioactive constitutes from traditional Chinese herbs [6-14]. Macroporous resins can be used to selectively absorb targeted phytochemicals because of their unique properties, including ideal pore structure and various surface functional groups available, low operation expense, less solvent consumption and easy regeneration [10].

In the present study, various macroporous resins with different chemical and physical properties were employed to investigate the adsorption and desorption process and to develop a convenient and efficient method for enrichment and separation of antioxidant flavones from RAP with the optimal resin. Various parameters influencing the adsorption and desorption processes were optimized.

2 Materials and Methods

2.1 *Chemicals and regents*

The dried rhizomes of *Abacopteris penangiana* were collected in June 2009 from Jiujiang, Jiangxi province, China and authenticated by Prof. TAN Ce-Ming, Jiujiang Forest Plants Specimen Mansion. The voucher specimen (PZX0311) has been deposited in College of Pharmacy, Tongji Medical

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Center, Huazhong University of Science and Technology. All chemicals and regents used in the experiments were of analytical or chromatographic grade. Abacopterin I, triphyllin A, eruberin B and 6″-*O*-acetyl eruberin B were isolated from RAP in our laboratory and purity of each compound was more than 98% by HPLC.

2.2 *Absorbents*

Macroporous resins including HPD100, HPD400, HPD450, HPD500, HPD600, HPD722 and D101 were purchased from Bonherb Technology Company (Hebei, China). These resins were soaked with 95% ethanol for 24 h to swell adequately. Subsequently the resins were eluted by 95% ethanol until white casse disappeared when the eluting reagent was mixed with pure water (1 : 5, *V*/*V*), and then the resins were washed with pure water until the liquor had no alcoholic odor. The pre-treated resins were then placed in the drying oven at 70 °C over 24 h until constant.

Table 1 The parameters tested in the orthogonal experiment

Absorb flow rate/mL·min^{-1}	Elute flow rate/ mL·min^{-1}	Volume of water for elution/BV	Volume of ethanol solution for elution /BV
1	2	3	3
2	3	5	5
4	4	7	10

2.3 *Preparation of sample solutions of RAP*

Three hundred grams of dry and minced RAP was extracted with 1 800 mL of ethanol–water (80 : 20, *V*/*V*) solution by hot reflux extraction for 90 min. Extract of RAP was obtained by concentrating the extracting solution to dryness by removing the ethanol solvent in a rotary evaporator (Shanghai SENCO Science & Technology Co., Ltd., China) at 60 °C. Pure water was added into the extract, and then the liquor was centrifuged by a centrifugal machine (Jintan Xinkang instrument factory, China) with 2 500 r·min^{-1} for 15 min to obtain the sample solutions.

2.4 *Determination of flavones content by UV spectrophotometry*

On account of no chromogenic structure of flavones, content of flavones-4-ol compounds in RAP cannot be determined based on the conventional color reaction of rutin. Alternatively, concerning the flavones-4-ol compounds in RAP have same skeleton, abacopterin I isolated from RAP was employed as a standard compound to determine the content of flavones by UV spectrophotometry in this study. The working curve of abacopterin I was: A = 3.820 3 C + 0.138 2 (r = 0.999 8, 0.017 1−0.220 mg·mL^{-1}, detective wavelength of 276 nm), where A is absorbance of the tested sample and C is concentration of the tested sample.

2.5 *Static absorption and desorption tests*

The static absorption test for screening the best resins were performed as follows: pre-weighted quantities of hydrated resins (equal to 250 mg in dry weight) were added into 250 mL Erlenmeyer flasks containing 50 mL of sample solutions of RAP obtained in Section 2.3. The Erlenmeyer flasks were shaken at 120 r·min^{-1} for 12 h at room temperature; and then standing for 12 h. Subsequently, the content of flavones in sample solution was analyzed by UV spectrophotometry.

The static desorption experiments were performed on three types of resins which had largest absorption capacity by placing the resins that had completed the absorption process described previously in 250 mL Erlenmeyer flasks containing 50 mL of 95% ethanol. The Erlenmeyer flasks were shaken at 120 r min^{-1} for 12 h at room temperature; and then standing for 12 h. The total concentration of flavones in desorbed solution was determined.

2.6 *Absorption and desorption kinetics*

All kinetic adsorption and desorption experiments were carried out in a glass column (20 mm × 300 mm) wet-packed with the selected HPD500 resin. The bed volume (BV) of the resin was 23 mL. All kinetic adsorption and desorption experiments were performed at room temperature.

The Kinetic-Tandem assay was employed to investigate kinetic adsorption capacity of HPD500 resins. The Kinetic-Tandem assay was performed as follows: 350 mL extract sample solution (the content of flavones was 1.467 8 mg·mL^{-1}) flowed through the glass column at the flow rate of 3 mL·min^{-1}. The content of flavones (C_1) in elution (E_1) was detected by UV analysis when the sample solution drained away through the column. Subsequently the elution flowed through the column again at the same flow rate. Concentration of flavones (C_2) in elution (E_2) was analyzed. Repeated the above steps until C_{n+1} changed little comprised with C_n. At this moment the kinetic absorption can be seen reaching the equilibrium point. Then the leached fraction and kinetic absorption mass of HPD500 were calculated.

Five sample solutions with different initial concentrations (0.53, 1.47, 2.86, 4.29 and 6.70 mg·mL^{-1}) were employed to investigate effect of the initial concentration of sample solution on the enrichment and purification process. These five samples were dealt with same parameters including loading mass of extract, absorb flow rate, elute flow rate and so on. The kinetic absorption capacities were compared when the absorb processes were finished.

The resin column that reached absorb equilibrium was used to do kinetic desorption tests based on a gradient elution program. The column was eluted by pure water, 30% ethanol (*V*/*V*), 50% ethanol (*V*/*V*), 70% ethanol (*V*/*V*), 80% ethanol (*V*/*V*) for 5 BV, respectively, at a flow rate of 1.5 mL·min^{-1}. The concentration of flavones in desorption solution collected at 1 BV internals was monitored.

2.7 *L4/3 orthogonal experiment*

In order to fully study the best parameters in process of purification of flavones by macroporous resin, a L4/3 orthogonal experiment was performed in a glass column (20 mm × 500 mm) wet-packed with HPD500 resin. The bed volume (BV) of the resin was 50 mL. The parameters to be

tested and their values are listed in Table 1. After each test was finished, the yield mass of extract, yield mass of flavones, yield ratio and content of flavones were investigated. The L4/3 orthogonal experiment was performed at room temperature.

Table 2 Results of the orthogonal experiment

Order	Yield mass of extract /mg	Yield mass of flavones /mg	Loading mass of extract/mg	Yield ratio /%	Content of flavones /%
1	231.8	138.78	981.5	14.14	59.87
2	215.1	132.35	1 022.4	12.95	61.53
3	295.1	148.17	1 022.4	14.49	50.21
4	251.0	144.70	1 034.0	13.99	57.65
5	232.7	113.81	1 034.0	11.01	48.91
6	223.9	125.12	1 034.0	12.10	55.88
7	264.5	131.32	1 034.0	12.70	49.65
8	218.7	118.80	1 022.4	11.62	54.32
9	263.0	137.65	1 034.0	13.31	52.34

2.8 *Enrich and purify flavones by HPD500 with optimized parameters and HPLC analysis of flavones in RAP*

The Enrichment and purification process with optimized parameters was performed in a 37 mm × 300 mm column with the parameters optimized in the experiments above and content of flavones in the obtained sample was determined. Subsequently, Chromatography of obtained flavones was achieved on a Hitachi pump L-2130 equipped with a Hitachi UV Detector L-2400 (Hitachi, Japan) with a detective wavelength of 276 nm. The chromatographic separation was performed on an amethyst C_{18}-p column (Sepax-tech, 4.6 mm × 200 mm, 5 μm) with column temperature of 25 °C and flow rate of 1 mL·min^{-1}. The injection volume of each sample was 20 μL. The solvents were filtered through a 0.45 μm nylon filter membrane and degassed in a sonicator bath prior to use. The solvents were water containing 0.1% phosphate acid (*V*/*V*, solvent A) and acetonitrile (solvent B). The gradient elution program was follow: 0.00 min, 82.0% A (18.0% B); 3.00 min, 82.0% A (18.0% B); 15.00 min, 79.0% A (21.0% B); 50.00 min, 55.0% A (45.0% B); 60.00 min, 55.0% A (45.0% B).

2.9 *ABTS radical scavenging activity*

To assess the ABTS scavenging activity, an improved ABTS method [15] was used. Briefly, $ABTS^{\bullet+}$ was produced by mixing ABTS (7 mmol·L^{-1}; Sigma–Aldrich, St.Louis, MO, USA) and ammonium persulphate (2.45 m mol·L^{-1}), the mixture was kept in the dark at room temperature for 16 h before use. The $ABTS^{\bullet+}$ solution was diluted with ethanol (1 : 12, *V*/*V*) to obtain an absorbance of 0.7 ± 0.02 at 734 nm. Sample solution (100 μL, 0.04−2.27 mg·mL^{-1}) in ethanol was added to 3.9 mL $ABTS^{\bullet+}$ solution. Absorbance at 734 nm was measured after reaction of 6 min. Trolox (Sigma–Aldrich, St.Louis, MO, USA; purity ≥ 98%) was used as a positive control. All measurements were made in triplicate and averaged.

3 Results and Discussion

3.1 *Absorption capacity and ratio of desorption*

The capacity of adsorption and desorption ratios are calculated as follows.

Adsorption capacity: $Q = \dfrac{(C_0 - C_e)V_0}{W}$

where Q is the adsorption capacity, which represents the mass of adsorbate adsorbed on 1 g of dry resin at adsorption equilibrium; C_0 and C_e is the initial and equilibrium concentration of flavones in the sample solutions, respectively; V_0 is the initial volume of sample solution, and W is the weight of the dry resin.

Desorption ratio: $D = \dfrac{C_d V_d}{(C_0 - C_e)V_0}$

where D is the desorption ratio (%); C_d is the concentration of flavones in the desorption solutions (mg/ml); V_d is the volume of the desorption solution; C_0, C_e and V_0 are the same as those defined above.

Seven macroporous resins with different physical properties were employed to enrich and purify flavones in RAP, and the results were shown in Figure 1. The absorption capacity and desorption ratio of flavones on HPD500 resins were higher than other resins. Hence, HPD500 was employed in the process of enrichment and purification of flavones in the following study.

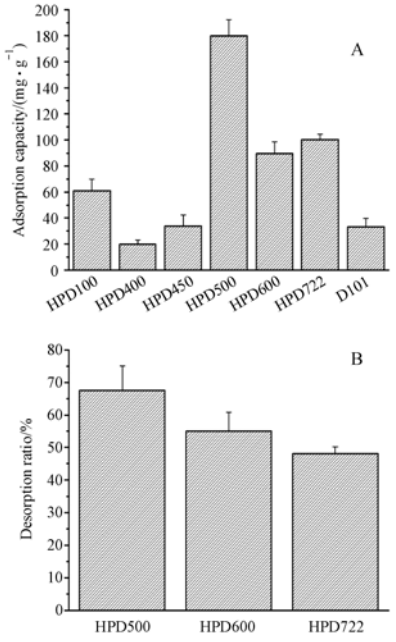

Fig. 1 (A) The result of static absorption tests on seven macroporous resins. (B) The result of static desorption tests on three macroporous resins

3.2 *Absorption and desorption kinetics*

The leached fraction is calculated as follows.

Leached fraction (LF): $LF = \frac{C_{n+1}}{C_n}$ (C_n and C_{n+1} are defined in 2.6)

The kinetic absorption process is different with the static absorption and may be influenced with several factors including absorb flow rate, the initial concentration of load solution and the size of columns. In our study, the kinetic-Tandem absorption test was carried out to determine the maximum absorption capacity when the selected resins reached the equilibrium point in kinetic process. As shown in Figure 2, the leached fraction raised rapidly when the second tandem absorption was performed. After sixth tandem absorption process, the leached fraction of resins almost reached 100% (97.9%), meaning the resins reached its equilibrium point in kinetic absorption process. According to the dates shown in Fig. 2, the maximum absorption capacity was calculated as 18.46 mg/(1 mL weighted resins), which can be used to determine the weight of macroporous resins on the basis of the mass of sample.

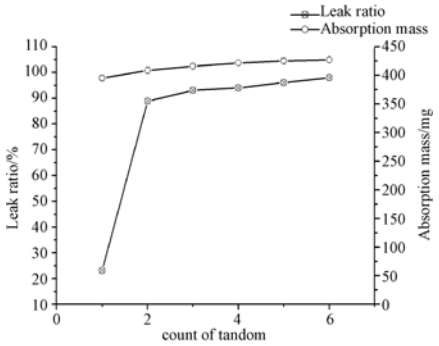

Fig. 2 The curves of leached fraction and absorption capacity in the Kinetic-Tandem assay

In order to investigate the initial concentration of loading sample solution, five concentrations (0.53, 1.47, 2.86, 4.29 and 6.70 mg·mL^{-1}) of sample solution were selected. Shown in Fig. 3, when initial concentration is above 2.86 mg·mL^{-1}, the absorption capacities did not increase significantly. On the contrary, the mass of flavones not be absorbed increased inevitably. Due to these reasons, 2.86 mg·mL^{-1} was chosen to be initial concentration of the sample solution.

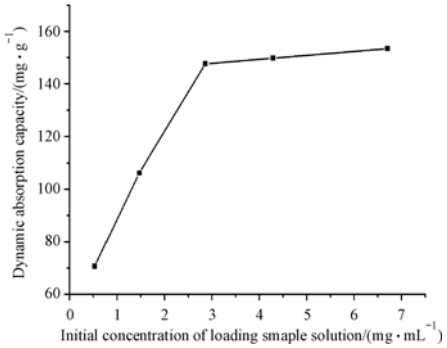

Fig. 3 The effect of initial concentration of loading sample solution on kinetic absorption capacity

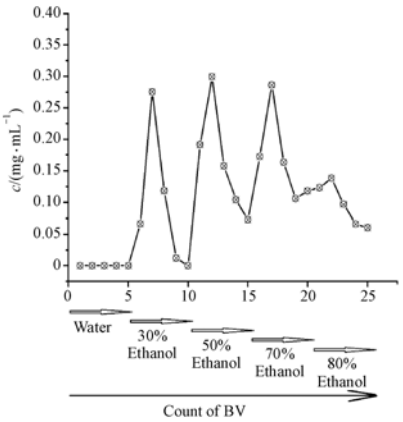

Fig. 4 The kinetic desorption curve based on the gradient elution program

The kinetic desorption curve on HPD500 was obtained based on a gradient elution program. As depicted in figure 4, the flavones absorbed by HPD500 were desorbed almost completely after eluted by 30%, 50% and 70% ethanol. Hence, the appropriate and economic ethanol concentration of elution solution can be optimized as 70%.

3.3 *The optimization of parameters by orthogonal experiment*

According table 2 and 3 the parameters of absorb flow rate, elute flow rate, volume of water for elution and volume of ethanol solution for elution were optimized as 1 mL·min^{-1}, 2 mL·min^{-1}, 5 BV and 5 BV, respectively. Furthermore, among these four parameters, volume of ethanol solution for elution and absorb flow rate were important factors in the enrichment and purification process of flavones in RAP according to Rj.

Table 3 Analysis of the orthogonal experiment on the basis of content of flavones in RAP

	Absorb flow rate/ (mL·min^{-1})	Elute flow rate/ (mL·min^{-1})	Volume of water for elution/BV	Volume of ethanol solution for elution/BV
Kj.1	57.203	55.723	53.707	56.690
Kj.2	54.147	54.920	55.687	57.173
Kj.3	52.103	52.810	54.060	49.590
Rj	5.100	2.913	1.980	7.583

3.4 *The optimal enrich and purify process and HPLC analysis of flavones in RAP*

After dealt by the optimal enrich and purify process on HPD500, the content of flavones in the sample is increased from 21.85% to 63.12%. The chromatograms of the tested samples before and after treatment with HPD500 resin were shown in Fig. 5. By comparison, it can be seen that some impurities in the crude extracts were removed and the relative peak area of three typical falvone-4-ol compounds, triphyllin A, eruberin B and 6″-*O*-acetyl eruberin B, increased significantly after treated by HPD500 resins.

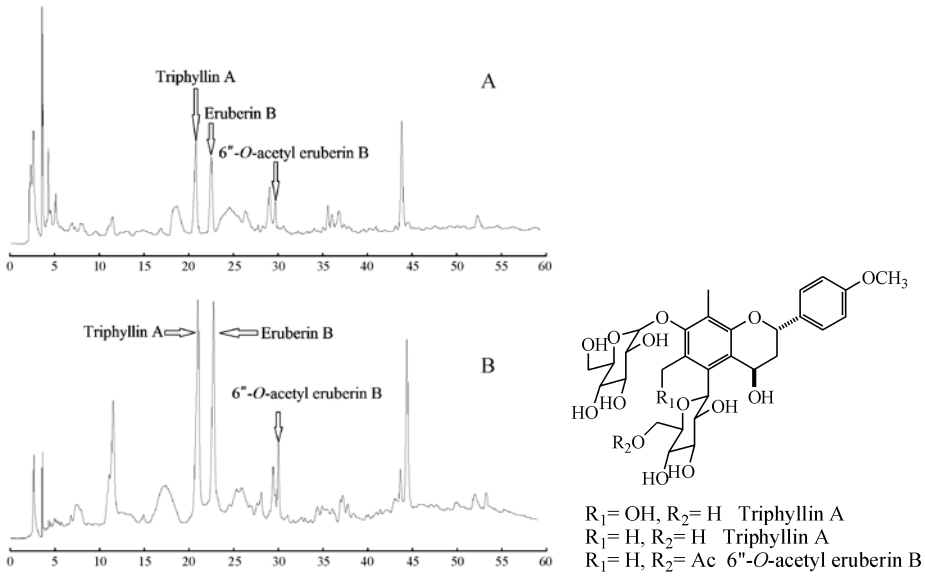

Fig. 5 The HPLC comparation of samples before and after treated by HPD500

3.5 *ABTS radical scavenging activity of samples before and after treated by HPD500*

As can be seen from Figure 6, sample after treated by HPD500 was found to be a very effective scavenger against ABTS radical compared with the sample without the treatment, and its activity increased in a concentration-dependent manner. The antioxidant compound, trolox, was acted as the reference. The ABTS radical scavenging activity of sample treated by HPD500 was moderately higher than that of trolox, indicating the sample presented powerful antioxidant activity.

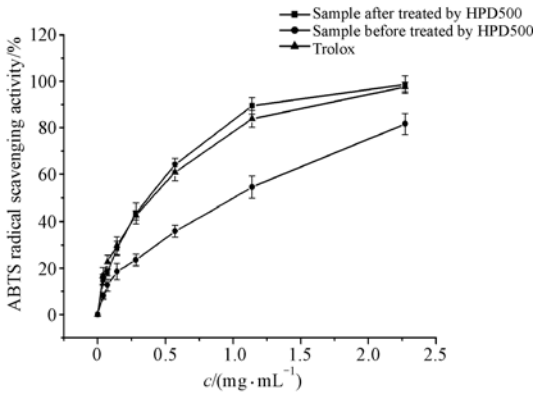

Fig. 6 The ABTS radical scavenging activities of samples before and after treated by HPD500

4 Conclusion

In our study, the enrichment and purification process of flavones of RAP with macroporous resin HPD500 has been successfully developed. HPD500 was selected because of the best performance in static absorption and desorption tests. Additionally, the effects of several factors were investigated to make optimization of the adsorption and desorption conditions. Our study showed that under the optimized conditions, *i.e.* initial concentration of 2.86 mg·mL^{-1}, elution solution of 70% ethanol, absorb flow rate of 1 m L·min^{-1}, elute flow rate of 2 mL·min^{-1}, 5 BV of water for elution and 5 BV of ethanol for elution, content of flavones in RAP was increased significantly after dealt by HPD500 according to HPLC comparison. In conclusion, the result of our study suggested that macroporous resin adsorption method was applied successfully to enrich and purify flavones in RAP.

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大孔树脂富集纯化披针新月蕨(*Abacopteris penangiana*)中的黄酮类成分

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【摘　要】 目的：研究使用大孔树脂富集纯化披针新月蕨根茎中的黄酮类成分。**方法：**通过静态吸附及解吸附试验筛选最优型号树脂; 动态吸附、解吸附试验和正交试验优化树脂最大上样量、最佳上样浓度、最佳洗脱醇浓度、吸附流速、解析流速、水洗体积和醇洗体积等工艺参数; 采用最佳参数富集纯化黄酮类成分后, HPLC 分析和 ABTS (2, 2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid)抗氧化试验比较样品经树脂处理前后的差别。**结果：**优化大孔树脂富集纯化披针新月蕨黄酮类成分的条件为: 上样浓度 2.86 mg·mL^{-1}, 醇洗浓度 70%, 吸附流速 1 mL·min^{-1}, 解吸流速 2 mL·min^{-1}, 水洗体积和醇洗体积均为 5 倍柱体积。样品经大孔树脂处理后黄烷-4-醇类黄酮含量明显增加。**结论**: 样品经大孔树脂处理后黄酮含量由 21.85%增加到 63.12 %, 表明大孔树脂可以应用于披针新月蕨中黄酮成分的富集纯化过程。

【关键词】 披针新月蕨; 黄酮; 大孔树脂

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