Enrichment and Antioxidant Properties of Flavones from Honeysuckle Leave using Macroporous Absorption Resin Followed by Column Chromatography

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***Abstract* — In this study, a simple and efficient procedure for the preparation of flavones from honeysuckle leaves is developed using macroporous absorption resin followed by column chromatography. Among six macroporous absorption resins, D101 resin was chosen to enrich flavones because of its good enrichment efficiency in static and dynamic tests. The results showed that D101 resin had a good adsorption and desorption performances of flavones from honeysuckle leaves. The optimal adsorption conditions were: 3.831 mg/mL of flavones in feed solution, 2.0 mL/min of flow rate, the best adsorption time was 3 h and loading volume no less than 100 mL. Elution conditions were 70 % ethanol with flow rate of 1.0 mL/min. After the D101 treatment, the purity of flavones increased from 7.14 % to 64.24 % with a recovery of 69.34 %. A sample taken after being treated by D101 was found to be a very effective scavenger against ABTS·+ radical compared with a sample without the treatment.**

Keywords - Flavones; Honeysuckle leaves; macroporous absorption resins; enrichment and purification; antioxidant capacity

I. INTRODUCTION

Honeysuckle is an important traditional Chinese medicine prepared from Lonicera japonica Thund which also called double flower. It is widely used for the treatment of tumors, bacterial dysentery, cold, pain, sores, carbuncles, furuncles, swelling and fever disease caused by influenza virus [1]. Studies have been found that honeysuckle contains many kinds of antioxidant and anti-tumor compounds such as chlorogenic acid, flavonoid, triterpenoid saponins, polysaccharides and volatile oil. As the by-product of honeysuckle, honeysuckle leaves have been regarded as waste material and not been used for a long time, its output is about tenfold that of honeysuckle [3]. Flavonoid, as the major bioactive constituent of honeysuckle leaves, has been shown the function of anti-bacterial, anti-inflammatory, liver-protection, hypolipidemic effect, anti-mutagenic and it is benefit to the immune system pharmacological activities [4]. So it is important to develop a convenient and efficient method for enrichment and purification of flavones from honeysuckle leaves.

The preparative enrichment of active components from plant extracts is an important step in the manufacture of photochemical products. Recently, high-speed counter-current chromatography, a support-free liquid chromatography technique, was considered to be an alterative for separation of flavones from plant materials. Nevertheless, this application is not effective to obtain these compounds concerning regents, labor, energy consumption and environment protection [5]. Alternatively, growing interest has been focused on employing macroporous absorption resins to enrich and purify bioactive constitutes from traditional Chinese herbs. For example, macroporous resins have been successfully used in the enrichment of rare madecassoside and asiaticoside from centella asiatica; hesperidin; flavone C-glycosides from troll flower extracts [6]. Macroporous absorption 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 [7].

It is considered that oxidative damage is attributable to excess active oxygen species generated in the body. It is well known that reactive oxygen, as well as being naturally generated, is also generated by factors such as strong ultraviolet, radial ray irradiation, and stress [8]. In order to reduce damage to the human body and prolong the storage stability of foods, synthetic antioxidants are often used for industrial processing. However, the use of synthetic antioxidants in food products is being questioned [9]. Consumers have also become more cautious about the nutritional quality and safety of food additives. In response to the growing consumer demand, investigations on antioxidants from natural sources have gained interest. In general, the natural antioxidants mainly consist of many compounds including phenolic, nitrogen compounds and carotenoids. As a kind of phenolic compound, antioxidant activities of flavones have attracted extensive attention [10].

In the present study, various macroporous absorption resins with different chemical and physical properties were employed to investigate the absorption and desorption process. It can develop a convenient and efficient method for enrichment and separation of antioxidant flavones from honeysuckle leaves with the optimal resin. Various parameters influencing the absorption and desorption processes were optimized. After this process, the purity and antioxidant activity of flavones was higher than that without treatment. The developed procedure in this study boasts easily reusable solvents, production of high-purity product as well as high recoveries. Besides, separation process provides a method for the large-scale separation and purification of flavones for its practical use. Our research can lay the foundation for a theoretical basis of flavones adsorption from

honeysuckle leaves on adsorption resin for industry application.

II. MATERIALS AND METHODS

The materials purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), Rutin (J&K, purity is 98 %) was dissolved in 95 % ethanol and stored at -20 ºC. Other reagents were analytical grade and purchased from Tianjin Chemical Reagent Factory (Tianjin, China). Distilled water was used throughout the whole experiment.

Honeysuckle leaves used in this study were harvested from Xintai Tai'an (Tai'an China), separated carefully from the stem, sun-dried, pulverized by a laboratory knife mill (FW100, Taisite Instrument Co., Ltd., Tainjin, China) and sieved. Particles sized 0.45 mm (mean diameter) were collected in sealed bags, and stored in a glass desiccator over silica at room temperature before further processing.

Six macroporous absorption resins including AB-8, D101, NAK-9, S-8, D4020 and HPD100A were purchased from Bonherb Technology Company（Hebei China）.The physical properties of these resins are summarized in Table.1. These resins were pretreated by soaking in 95 % ethanol for 24 h to swell adequately prior to use. Subsequently the resins were eluted by 95 % ethanol and then the resins were washed with pure water until there was no residue after distillation to remove the monomers and porogenic agents trapped inside the pores during the synthesis process. The resins were finally washed with deionized water until the liquor had no alcoholic odor [11]. The pre-treated resins were then placed in the pure water at room temperature.

The dried honeysuckle leaves were extracted with 70 % (v/v) aqueous ethanol in an ultrasonic cleaner (2510E-DTH ， Branson Ultrasonic Corporation in USA) with power of 500 w for 30 min at 60 ° C. The solvent to powder mass ratio was 30:1. Then the supernatant was obtained by filtering (X1R High Speed Multifuge from Aoheng technology co., Ltd., Beijing) the extracting solution and the residue was used for further extraction. The extraction process was repeated 3 times and all the supernatants were combined and condensed by using a rotary evaporator (RE52AA, Yarong Equipment Co. Ltd., Shanghai, China) under reduced pressure at 50 ºC to complete removal of ethanol. Then, the obtained crude extract was stored in a refrigerator at 4 ºC prior to further use.

The total flavones content in the supernatant was determined using Rutin–standard curve method with a slight modification [12]. 1 mL of diluted solution containing flavones and 0.5 mL of 5 % (w/w) NaNO2 were mixed for 5 min, and then 0.5 mL of 10 % Al(NO3)3 (w/w) was added and mixed；after 5 min, 2.5 mL of 5 % NaOH (w/w) was added. With 15 min standing, the absorbance of the solution was measured at 511 nm with UV spectrophotometer against the same mixture, without the sample as a blank. The calibration curve ranged 0-20μg/mL (R^2=0.9994). And then the percentage of flavones yield is calculated as follows:

$$Y = \frac{\frac{A + 0.0201}{18.358} \times V \times N \times 10^{-3}}{M} * 100\% \quad (1)$$

In the above formula: Y is percentage flavones yield (%), A is absorbance value of sample solution, V is volume (mL) of extract supernatant, N is dilution ratio, and M is the quality of honeysuckle leaves (g).

Six marcroporous resins including AB-8, D101, NKA-9, S-8, D4020 and HPD100A were used in this study. To chose optimum resin for the adsorption of flavones, static adsorption and desorption tests were performed. Pre-weighted quantities of hydrated resins 2 g were added into 100 mL Erlenmeyer flasks containing 30 mL of sample solutions of honeysuckle leaves obtained in Section 2.3. The Erlenmeyer flasks were sealed with stoppers and placed in an oscillator (THZ-82A, Changzhou Aohua Instrument Co., Ltd., Jiangsu, China). The whole process of adsorption was done at room temperature with a shaking speed of 120 r/min for 24 h. Subsequently, the content of flavones in sample solution was analyzed by UV spectrophotometry (Shimadzu Instruments Co., Ltd., Japan.). Then the resins were desorbed using 30 mL 95 % aqueous ethanol. The flasks were continually shaken at room temperature and 120 r/min. After 24 h desorption, desorption solutions were separated from the resins and analyzed by UV spectrophotometry. The adsorption properties of resins were evaluated by their adsorption capacity and desorption ratio which were calculated according to the following equations

$$Q = \frac{(C_0 - C_1)V_0}{W} \quad (2)$$

$$D = \frac{C_2 V_1}{(C_0 - C_1)V_0} \quad (3)$$

Where Q is the adsorption capacity, which represents the mass of adsorbed on 2 g of hydrated resin at absorption equilibrium (mg/g hydrated resin); C_0, C_1 and C_2 were the initial, absorption equilibrium and desorption concentrations of flavones in the sample solutions, respectively (mg/mL); V_0 and V_2 were the volume of the initial sample and desorption solution (mL), W was the weight of the hydrated resin (g); and D was the desorption ratio (%).

The static adsorption curve of flavones on optimum resin was studied by adding pre-weighed amounts of resin into 30 mL crude flavones extracts from honeysuckle leaves solution (concentration of flavones was 3.831 mg/mL), then it was shaken 120 r/min in oscillator at room temperature. One milliliter of liquid extract was withdrawn at time points of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 240, 360 and 720 min. The concentration of flavones in the solution was determined every certain time by spectrophotometry.

All kinetic absorption and desorption experiments were carried out in a glass column (250 mm×20 cm) wet-packed with the pretreated hydrated selected resin. The bed volume (BV) of the resin was 20 mL. All kinetic absorption and desorption experiments were performed at room temperature.

Five sample solutions with different initial concentrations (1.254 mg/mL, 2.237 mg/mL, 3.831 mg/mL, 4.734 mg/mL

and 5.741 mg/mL) 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 solution, sample flow rate, elute flow rate and so on. During dynamic adsorption, the concentrations of flavones in the aliquots of 5 mL effluents collected at 1 min interval were monitored by spectrophotometry to get dynamic leakage curve.

During the adsorption test, the concentration of flavones was set as 3.831 mg/mL. Over-loading test was carried out to investigate the best among a series of flow rate (1.5 mL/min, 2.0 mL/min, 2.5 mL/min, 3.0 mL/min and 3.5 mL/min). The kinetic absorption capacities were compared when the absorb processes were finished. The concentrations of flavones in the aliquots of 5 mL effluents collected at 1 min interval were detected at intervals by spectrophotometry to get dynamic leakage curve.

The purpose of preliminary optimization was to screen out the eluents which could elute flavones more completely. The experimental operations were as follows: once reaching adsorption equilibrium, the loading of the sample solution was stopped. The adsorbate-laden column was washed firstly by 100 mL deionized water and then eluted with a series of ethanol (50%, 60%, 70%, 80% and 90%) successively. The volume of each ethanol eluent was 100 mL and the flow rate was controlled at 1.0 mL/min. The eluted aliquots were collected at 5 mL intervals, and flavones in each ethanol eluates were detected by spectrophotometry.

The desorption flow rate of the optimal elution solution was evaluated by comparing the elution flow rate of 1.0 mL/min, 1.5 mL/min, 2.0 mL/min, 2.5 mL/min and 3.0 mL/min, respectively. The eluted aliquots were collected at 5 mL intervals, and the concentration of flavones in each part of desorption solution was determined by spectrophotometry.

After each purification cycle on the D101 adsorbent, for next run, the column was subjected to a cleaning-in-place (CIP) procedure using downward flow. The cleaning protocol consisted of: 4 sedimented bed volumes of 95 % ethanol at a flow of 1.5 mL/min and 4.5 sedimented bed volumes of deionized water at 2.0 mL/min. Then it soaked in 3 % - 5 % hydrochloric acid and 3 % - 5 % sodium hydroxide solution, respectively. Finally, it washed with deionized water until the liquor had no alcoholic odor. To verify the function of the adsorbent after repeated use, the D101 column was subjected to 3 subsequent purification cycles, each followed by the CIP protected described above.

Recovery of flavones with D101 resin column chromatography was determined by calculating the amount of flavones in powdered extract before and after purification on column packed with D101 resin to evaluate the efficiency of the method. The equation of recovery was described by the following mathematical formula:

$$\text{Recovery yield} = N_1/N_2 \quad (4)$$

Where N_1 is the amount of flavones after column; N_2 is the amount of flavones before column.

To assess the ABTS·+ scavenging activity, an improved ABTS·+ method was used [13]. Briefly, ABTS·+ was produced by mixing ABTS·+ (7 mmol/L; Sigma-Aldrich, St.Louis, MO, USA) and potassium persulfate (140 mmol/L), the mixture was kept in the dark at room temperature for 12 h before use. The ABTS + solution was diluted with ethanol to obtain an absorbance of 0.70+0.02 at 734 nm. Sample solution (100 μL，0.05-3.00 mg/mL) in ethanol was added to 4.0 mL ABTS • + solution. Absorbance at 734 nm was measured after reaction of 30 min. VC was used as a positive control. All measurements were in triplicate and averaged.

All tests were performed in triplicate and the results were reported as means and standard deviation of three parallel measurements. Significance of the differences between variables was tested by one-way ANOVA, using SPSS 10.0. The mean values were considered significantly different at $P<0.05$.

III. RESULTS AND DISCUSSION

A. Absorption Capacity and Ratio of Desorption

Six macro absorption resins with different physical properties were employed to enrich and purify flavones in honeysuckle leaves, and the results were shown in Table 1. It was observed that the adsorption and desorption performances of different resins were distinct. The adsorption capacity and desorption ratio of flavones on D101 resins towards flavones were relatively higher than those of others. Adsorption properties of resin correlate with its physical and chemical capabilities and the chemical features of the adsorbed substance [14]. Resins with similar polarity to solute exhibited better adsorption ability, such as this paper in the literature tested that chromatography column packed with D101 was used to separate and purify flavonoids [15]. Moreover, in general, higher surface area contributed to the process of adsorption. Hence, D101 was employed in the process of enrichment and purification of flavones in the following study. The polar NAK-9 and weak-polar D101 resins exhibited better adsorption capabilities due to their similar polarities with flavones and their higher surface area. However, NAK-9 possessed a strong affinity for solute so that the desorption capacities of flavones on it was actually not notable. The desorption ratios of D101 resin toward three flavones were higher than those of other resins. With respect to D101 resin, owing to its approximately similar average pore diameter with the target molecule of flavones, high specific surface area and moderate polarity, it showed better performance on adsorption and desorption capacities than another five resins. Finally, D101 was selected for further investigations. This finding was in agreement with a previous study showing that D101 resin exhibited notably higher adsorption capacity towards flavones than other five resins [16].

B. Static Adsorption Curve of Flavones on D101 resin

Static adsorption curve was studied to acquire the most suitable adsorption time. The result was presented in Figure1 It indicated that D101 resin has excellent ability of

TABLE Ⅰ. PHYCICAL PROPERTITES AND RESULT OF STATIC ABSORPTION TESTS ON SIX MACRO ABSORPTION RESINS

Name	Polarity	Particle diameter （mm）	Surface area （m^2/g）	Average pore diameter (nm)	Moisture content（%）	Absorption capacity（mg/g）	Desorption ratio （%）
AB-8	Weak-polar	0.31-1.25	480-520	130-140	60-70	28.01	79.51
D101	Weak-polar	0.30-1.25	500-550	90-100	65-75	28.41	80.33
NAK-9	Polar	0.31-1.25	250-290	155-165	50-66	29.01	79.89
S-8	Polar	0.31-1.25	100-120	280-300	60-70	26.25	70.01
D4020	No polar	0.30-1.25	540-580	100-105	70-80	23.12	56.92
HPD100A	No polar	0.30-1.25	650-700	85-90	65-70	22.88	60.13

flavones adsorption, and the adsorption reached equilibrium when time was 3 h. Therefore, the best adsorption time was 3h.

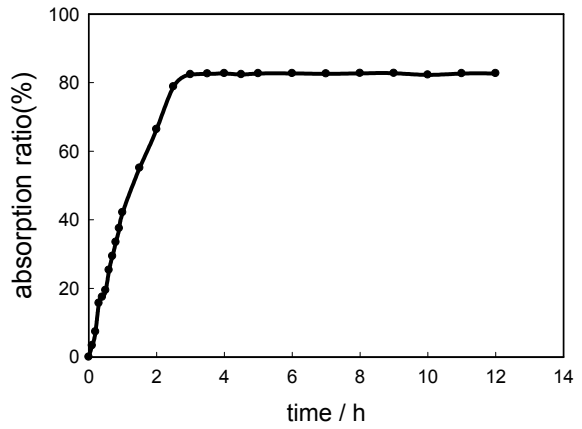

Figure 1. Static Adsorption Curve of Flavones on D101 Resin

C. Adsorption Kinetics

The results of static adsorption and desorption in the batch experiments can be applied to predict the behavior of the resins in dynamic systems [17]. Adsorption kinetics can describe the adsorption rate of the adsorbate on an adsorbate at specific initial concentration, flow rate, best eluent and the time required for the adsorption from the beginning to the equilibrium can be known for the kinetics.

D. Optimization of Initial Concentration C_0

The initial concentration of the sample solution has a great influence on adsorption efficiency [18]. However, the adsorption equation can only, theoretically, reveal the relationship between the C_0 and C_1. During the industrial process, the amount of resin and crude herb extract are usually foreseeable. Therefore, the optimal C_0 became a determinant in achieving the best adsorption.

The experiment was designed aiming at verifying the correlation between C_0 and C_1. In order to investigate the initial concentration of loading sample solution, five concentrations (1.254 mg/mL, 2.237 mg/mL, 3.831 mg/mL, 4.734 mg/mL and 5.741 mg/mL) of sample solution were selected. Shown in Figure 2A, when initial concentration is 1.254 mg/mL, the absorption capacities did not increase significantly. On the contrary, with the concentration increasing to 5.741 mg/mL, the leakage point appeared early than others, so mass of flavones not be absorbed increased inevitably. Due to these reasons, the optimal C_0 was eventually set at 3.831 mg/mL, corresponding to about 1.0 g crude drugs/mL.

E. Optimization of Loading Flow Rate

In general, increasing flow rate has a negative effect on dynamic adsorption capacity of adsorbate on resins because adsorbate molecules have no sufficient time to undergo interactions with active sites at the surface of resins and vice versa. The effect of flow rate on dynamic adsorption capacity is shown in Figure 2B. Breakthrough point was defined as 1 % ratio of the exit to the inlet solute concentration [19]. As can be seen from it, at the 1% breakpoint, breakthrough volume of flavones on D101 resin was 20 mL, 30 mL, 40 mL, 50 mL, 60 mL at flow rates of 3.5 mL/min, 3.0 mL/min, 2.5 mL/min, 1.5 mL/min, 2.0 mL/min respectively; corresponding breakthrough adsorption capacities were calculated as 29.01 mg/g, 30.4 mg/g, 31.2 mg/g, 33.81 mg/g, 34.95mg/g resin. Because breakthrough adsorption capacities differed little at the flow rates of 1.5 mL/min and 2.0 mL/min and a lower flow rate prolonged process time, therefore, 2.0 mL/min was used as the proper adsorption flow rate in further experiments.

F. Optimization of Eluent

As a kind of good solvent which can be easily removed from the solution and recycled, ethanol has the advantages of both low cost and no toxicity to the samples. Experiments have confirmed that, different solvent have great influence on the flavones extraction, with ethanol as the extraction solvent, the extraction rate was higher than other extraction solvent such as water, ethyl acetate and methanol [20]. Therefore, ethanol was chosen as an appropriate desorbent for flavones. The kinetic desorption curve on D101 was obtained based on a gradient elution program. As depicted in Figure 2C, the flavones absorbed by D101 were desorbed almost completely after eluted by 50 %, 60 %, 70 %, 80 % and 90 % ethanol. Preliminary optimization demonstrated that when the ratios of the ethanol reached 50 %, detectable flavones

were found in ethanols elute, and more flavones were detected as the ratio of the ethanol increased. On the other hand, minimal amount of flavones was detected in the eluate of 50 % ethanol. Therefore, the ethanol eluents with ratios ranging from 50 % to 90 % were selected to perform the regular optimization. The elution capacity increased before the ratio of ethanol reached 70 %. Whereas the ethanol concentration increased to 80 %, other substances were washed off so the desorption ratio became decreased. Hence,

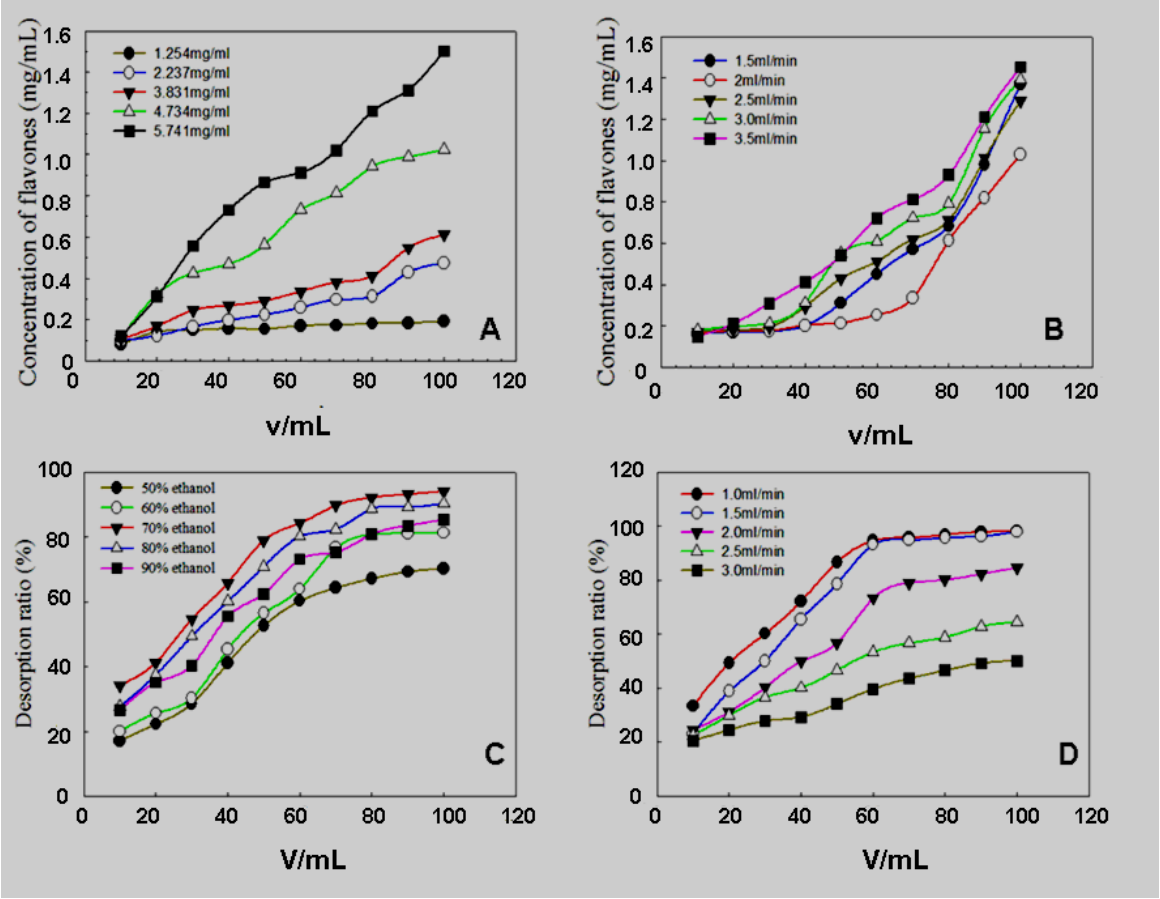

Figure 2. Adsorption and desorption behaviors of the flavones from honeysuckle leaves on D101 resin. Effect of different initial concentration (A) and different loading flow rate (B) on adsorption capacity Effect of different ethanol concentration (C) and different desorption flow rate (D) on desorption ratio.

the appropriate and economic ethanol concentration of elution solution can be optimized as 70 %.

G. Optimization of Dynamic Desorption Flow Rate

To effectively elute flavones from the resin, a proper flow rate is necessary. During industrial process, both eluents and time consumptions require careful consideration. Thus, the accumulative desorption ratio-volume of eluents curve Figure 2D and accumulative desorption ratio-time curve Figure 3 were employed in this study instead of the commonly used desorption curve. The 70 % ethanol was used to elute flavones with flow rates set at 1.0 mL/min, 1.5 mL/min, 2.0 mL/min, 2.5 mL/min and 3.0 mL/min.

As shown in Figure 2D, the desorption processes under different flow rates successively reached their plateaus when the consumption of the eluent grown up to about 1.0 mL/min. It was obvious that the flow rate of 1.0 mL/min could afford the best desorption capacity per unit volume of eluent. In Figure 3, the higher flow rate would provide a higher desorption efficiency in a short time. However, as desorption time increased, the accumulative desorption ratio using this higher flow rate remained approximately constant. Therefore, it was assumed that lower flow rate possibly brought better desorption ratio. Nevertheless, it was time-consuming for the elution with a lower flow rate. It was evident from Figure 2D, the flow rate of 1.0 mL/min gave a desirable desorption time and accumulative desorption ratio.

The dynamic adsorption and desorption tests were repeated for three times under optimal conditions, and percent recovery of flavones was calculated using formula [21]. After the D101 resin treatment, the purity of flavones in the powdered extract increased from 7.51 % to 64.76 %, and the overall recovery of flavones was 68.34 %.

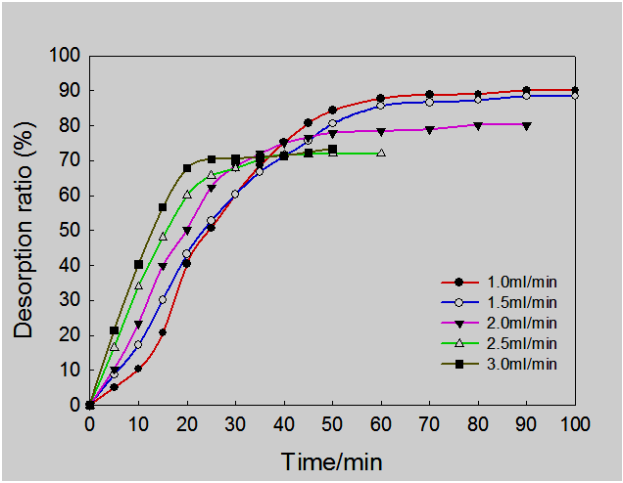

Figure 3. Accumulative desorption ratio-time curve

H. Reusability of D101 Resin

The reusability of adsorbent resin has a significant impact on process economy in downstream processing [22]. At the 1 % breakpoint, adsorption capacity of flavones on the regenerated resin was determined before cycle 1 and after cycles 2, 3, 4 and 5. The results were summarized in Figure 4. It indicated that the adsorbent could be reused for less than 3 cycles without compromising its function, which is encouraging for flavones production. After 3 cycles, the resin should be regenerate, which used 3 %-5 % hydrochloric acid and 3 %-5 % sodium hydroxide solution.

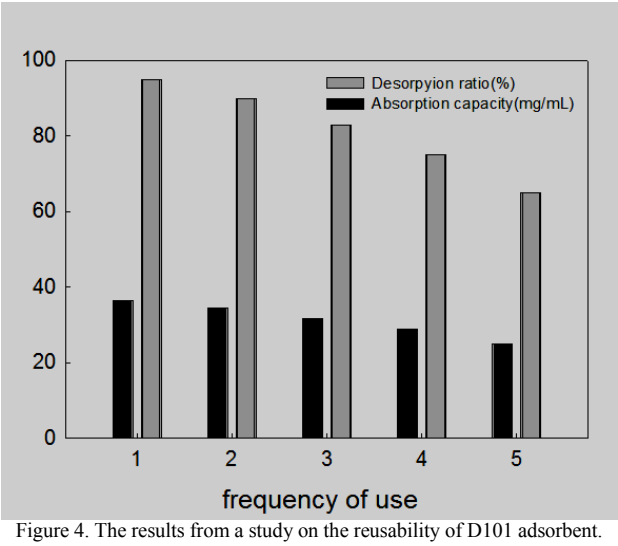

Figure 4. The results from a study on the reusability of D101 adsorbent.

I. Dynamic Desorption Curve on D101 Resin

To choose a proper flow rate (1.0 mL/min) of desorption solution, the dynamic desorption curves Figure 5 were drawn on the basis of the volume of desorption solution and the flavones concentrations in the desorption solution. The dynamic desorption curves suggested that, at the 1.0 mL/min flow rate, flavones began to elute when the volume reached 20 mL; with the increasing of the volume of desorption solution, the concentration of flavones became larger, the high saturation point appeared to 40 mL. The volume of desorption solution was approximate 60 mL by which flavones can be completely desorbed from D101 resin.

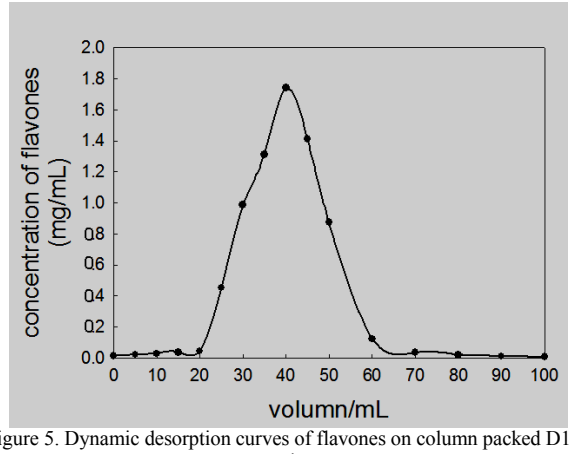

Figure 5. Dynamic desorption curves of flavones on column packed D101 resin.

J. ABTS·+ radical scavenging activity of samples before and after treated by D101

As can be seen from Figure 6, sample after treated by D101 was found to be a very effective scavenger against ABTS·+ radical with the sample without the treatment, and its activity increased in a concentration-dependent manner [23]. After treated by D101, ABTS·+ radical scavenging activity reached 80 % when the concentration of flavones was 1.0 mg/mL. On the contrary, only the concentration increased to 2.0 mg/mL, the ABTS·+ radical scavenging activity can reached 80 %. The antioxidant compound, VC, was acted as the reference. The ABTS·+ radical scavenging activity of sample treated by D101 was moderately higher than that of VC, indicating the sample presented powerful antioxidant activity.

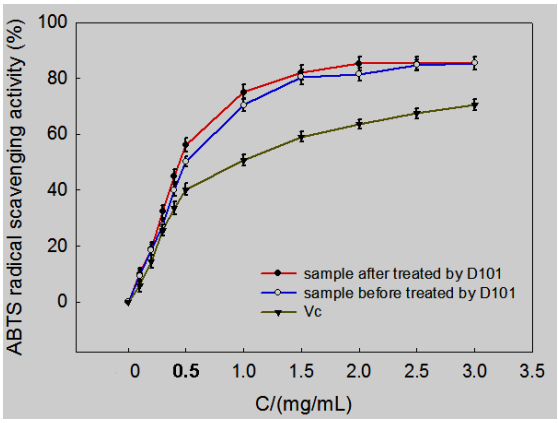

Figure 6. The ABTS·+ radical scavenging activities of samples before and after treated by D101.

IV. CONCLUSIONS

In this study, the enrichment and purification process of flavones of honeysuckle leaf with macro absorption resin D101 has been successfully developed. D101 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, initial concentration of 3.831 mg/mL, and elution solution of 70 % ethanol, absorb flow rate of 2.0 mL/min, elute flow rate of 1.0 mL/min, 60mL of ethanol for elution, content of flavones in honeysuckle leaf was increased significantly after dealt by D101. Besides, sample after treated by D101 was found to be a very effective scavenger against ABTS·+ radical with the sample without the treatment. In conclusion, the result of our study suggested that macro absorption resin adsorption method was applied successfully to enrich and purify flavones in honeysuckle leaves. Besides, this method was highly efficient, economic and environmental friendly, which showed good potential for industrial production of these ingredients for functional food and pharmaceutical applications.

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

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