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**Original Research Article**

# Optimization of isolation and purification of total flavonoids from *Ardisia mamillata* Hance roots using macroporous resins, and determination of their antioxidant activity

**Yongqiang Ma[1], Jinhua Shao[1,2], Xianzhe Fan[2], Xingguang Zhuang[1], Fulin He[2]**
*[1]Key Laboratory of Food Science and Engineering of Heilongjiang Province, College of Food Engineering, Harbin University of Commerce, [2]School of Chemical and Biological Engineering, Hunan University of Science and Engineering, Harbin 150076, China*

**For correspondence:* ***Email:*** *of0738@163.com*

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## *Abstract*

***Purpose:*** *To isolate, purify and determine the antioxidant property of total flavonoids from the roots of Ardisia mamillata, so as to provide a theoretical basis for development of natural antioxidants.*
***Methods:*** *Macroporous resin was used to optimize the isolation and purification of total flavonoids, taking adsorption rate and resolution rate as evaluation indices. The antioxidant property of the purified total flavonoids was determined using 1,1-diphenyl-2-picrylhydrazyl radical 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) radical scavenging activity.*
***Results:*** *The best conditions for separation and purification of total flavonoids from Ardisia mamillata roots were: use of ADS-7 resin, loading total flavonoid concentration of 0.8896 mg/mL, loading buffer flow rate of 1.5 mL/min, loading buffer pH of 4.48, elution ethanol concentration of 60 %, and flow rate of 2.5 mL/min. Under these conditions, the degree of purification of total flavonoids of Ardisia mamillata root was 76.43 ± 0.36 %, adsorption rate was 96.52 ± 0.19 %, while resolution rate was 99.31 ± 0.27 %. When the concentration of the purified total flavonoids was 4.0 mg/mL, its DPPH radical scavenging activity was stronger than that of the standard, butylated hydroxytoluene (BHT), but lower than that of vitamin C.*
***Conclusion:*** *ADS-7 resin is the best macroporous resin for the purification of total flavonoids from the radix of Ardisia mamillata Hance, under the optimized conditions. The purified total flavonoids of Ardisia mamillata root have stronger DPPH radical scavenging ability than the standard, BHT.*

***Keywords:*** *Szechwan raspberry root, Flavonoids, Macroporous adsorption resin, ADS-7 resin, Purification, Antioxidant*



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## INTRODUCTION

*Ardisia mamillata* Hance belongs to plants of Myrsinaceae, also called fairy red clothes, gold red beads, scarlet woollen blanket, fur bicolor and baoding red. It is native to China, especially in Hunan, Guangxi, Sichuan and Jiangxi [1]. This plant is used for medicinal purposes e.g. for

*clearing away heat, removing dampness*, stopping cough, resolving phlegm, resisting cancer, reducing swelling, detoxifying poisons, and stopping bleeding [2]. So far, studies on *Ardisia mamillata* Hance have mainly focused on essential oils [3], alkaloids [4], flavonoids [5,6], and polysaccharides [7].

Flavonoid compounds have multiple functions of regulating immunity, preventing cardiovascular and cerebrovascular diseases, preventing and resisting cancer, resisting bacteria and viruses, anti-aging, easing pain and stopping bleeding [8-11]. At present, the separation methods for flavonoids include extraction method, precipitation methods and column adsorption method [12,13]. Coarse pore adsorption is a new type of separation method for flavonoids. Compared with traditional methods, this method has advantages including long-time usage period, low cost and low toxicity [14-16]. However, studies on coarse pore resin for separation and purification of total flavonoids from *Ardisia mamillata* root have not been reported.

Therefore, the present study used coarse pore resin adsorption to do separation and purification for total flavonoids from *Ardisia mamillata* roots and study its antioxidant properties. The purpose was to provide theoretical basis for synthetic use of *Ardisia mamillata* roots and development of natural antioxidants for use in health care.

## EXPERIMENTAL

### Materials

*Ardisia mamillata* roots were collected from the Hunan Timesun Medicine Co., Ltd (Product Number 160712, Changsha City, China) The root bark was washed and cut with scissors. Then the roots were dried in a vacuum drying oven and ground into power in a vegetable grinder. The powder was sieved through a 0.33 mm diameter sieve prior to use.

### Reagents

Meletin and campheral (98 % pure) and DPPH (96 % pure) were bought from Shanghai Yuanye Biotech Co. Ltd (Shanghai, China); AB-8, NKA-Ⅱ, X-5, D3520, D101 and ADS-7 coarse pore resin were products of Tianjin Guangfu Fine Chemical Engineering Institute; absolute ethyl alcohol, sodium nitrite, aluminum nitrate, sodium hydroxide and hydrochloric acid were from Shanghai Sinopharm Chemical Reagents Factory.

### Main instruments used

Shimadzu LC-20A HPLC was bought from Japanese Shimadzu Koito; JA3003 electronic scale was from Shanghai Sunny Hengping Instrument Co. Ltd; R201D-Ⅱ rotary evaporator was product of Zhengzhou Greatwall Scientific Industrial and Trade Co. Ltd; new-type electro-thermostatic blast oven was supplied by Ningbo Jiangnan Instruments Factory, while XH-MC-1 microwave irradiation equipment was a product of Beijing Xianghu Science and Technology Co. Ltd.

### Extraction of total flavonoids

The *Ardisia mamillata* root powder was suspended in 65 % alcohol in the ratio of 1:12 (w : v). The mixture was refluxed three times, each for 2 h, and the pooled extract was subjected to evaporation in a rotary evaporator and vacuum-dried.

### Measurement of total flavonoids

#### *Preparation of standards*

Meletin (3.7 mg) and campherol (5.4 mg) were weighed and dissolved in 50 mL of 80 % methyl alcohol to yield the complex standard stock solution.

#### *Chromatographic conditions*

Shimadzu LC-20A HPLC, Shimadzu C18 chromatographic column (4.6 × 250 mm and 5 µm) was used. The mobile phase was 50:50 volume ratio of methyl alcohol and 0.4 % phosphoric acid. Ultraviolet wavelength of detection instrument was 360 nm. Sample size was from 10 to 20 µL. Flow rate was 1.0 mL/min, while column oven temperature was 40 $^{o}$C. Total flavonoids in the root extract of *Ardisia mamillata* was assayed by hydrolyzing the flavonoid glycosides into meletin and campherol [17]. Then HPLC was used to measure the levels of meletin and campherol. The total flavonoid content (M) was calculated Eq 1.

$$M = (m_1.1.65) + (m_2.1.74) \quad \text{..... (1)}$$

where M is total flavonoids glycosides, $m_1$ is meletin level, $m_2$ is campheral level

### Static adsorption test

After soaking in ethanol for 24 h, macroporous resins AB-8, ADS-7, D101, D3520, NKA-Ⅱ and X-5 were washed in ethanol until the ethanol was

clear. Finally, each macroporous resin was rewashed in distilled water until no flavor of alcohol remained. The resins were then filtered using a Buchner funnel, and dried. For each resin, 1.5 g was placed in a conical flask, and then 35 mL crude extract of total flavonoids from *Ardisia mamillata* Hance radix (0.8896 mg/mL) was put into each flask, and the flasks were placed on a 25 ℃ constant temperature shaker for static adsorption. Samples were taken from each conical flask every 0.5 h for determination of total flavonoid content. When the content of flavonoids was stable, the adsorption reached equilibrium. At this moment, the content of flavonoids was in post-adsorptive state.

After adsorption, each macroporous resin was filtered with a Buchner funnel, and after air pump filtration, the macroporous resins were put into six conical flasks and then washed three times in distilled water, followed by addition of 45 mL of 70 % ethanol. Each flask was placed in a constant temperature shaker at 25 $^{o}$C. Ten hours later, the content of total flavonoids in the solution of each conical flask was determined. The quantity of adsorption (Q) and desorption rate (W) of each macroporous resin at static adsorption was calculated separately. The higher the adsorption, the greater the desorption rate, and the better the corresponding macroporous resin. The quantity of adsorption (Q) is related to the mass of total flavonoids (M) by Eqs 2 and 3

$$Q = \frac{M - M_d}{M_P(1-\alpha)} \quad \text{..... (2)}$$

$$W = \frac{M_e}{M - M_d} \times 100 \quad \text{..... (3)}$$

where $Q$ is the quantity of adsorption of resin (mg/g), M is the mass of moist resin (g), $\alpha$ is the moisture content of resin (%), $M$ is the mass of total flavonoids in the pre-purified solution (mg), $M_d$ is the mass of total flavonoids in the post-adsorptive solution (mg), and $M_e$ is the mass of total flavonoids in eluent (mg).

**Dynamic adsorption test**

The preparation procedure for each macroporous resin was similar to that outlined in static adsorption test. Each dry macroporous resin was weighed (10.00 g), moistened with distilled water and loaded in a chromatographic column. Then, 40 mL of crude extract sample of total flavonoids from *Ardisia mamillata Hance* radix (0.8896 mg/mL) was added to each chromatographic column, which was eluted at a flow rate of 1.0 mL/min. Thereafter, each column was washed with 150 mL distilled water and eluted with 450 mL of 70 % ethanol. After elution, a part of eluent was collected under vacuum at 65 $^{o}$C, dried into powder, and the content of total flavonoids in the powder was determined as outlined earlier. The desorption rate (W), the adsorption rate (Q) and purity (P) of each macroporous resin were calculated. The higher the adsorption rate, the greater the desorption rate, and the better the corresponding macroporous resin. Desorption rate (F) was calculated as in Eqs 4 and 5.

$$F = \frac{S - S_d}{S} \times 100 \quad \text{..... (4)}$$

$$W = \frac{H_e}{H} \times 100 \quad \text{..... (5)}$$

where $S$ is the mass of total flavonoids in the sample (mg), $S_d$ is the mass of total flavonoids in the effluent (mg); $H_e$ is the mass of total flavonoids in the dried eluent (mg); and $H$ is the mass of the dried solution (mg).

**Purification of total flavonoids by macroporous resin ADS-7**

From the results of adsorption and desorption rates, ADS-7 was considered the optimum macroporous resin.

Therefore, the crude extract of total flavonoids from *Ardisia mamillata* Hance radix was weighed (40 mg) and applied on a chromatographic column packed with ADS-7. The indices listed below were used successively: concentration of crude extract of flavonoids (0.2214, 0.4428, 0.625, 0.8896, 1.106 and 1.345 mg/mL); pH (2.48, 3.48, 4.48, 5.48 and 6.48); flow rate of elution of crude extract with ethanol (1.0, 1.5, 2.0, 2.5, 3.0 mL/min); and concentration of ethanol for elution (30, 40, 50, 60, 70 and 80 %). In line with the methods used in dynamic adsorption test, the rate of adsorption and desorption rates were determined under the various conditions (concentration of crude flavonoid extracts, pH and flow rate). When the absorption or desorption rate reached its peak, the corresponding parameter was the optimum. When the parameter changed, the value of its front parameter was set as the optimum one obtained.

With the optimum parameter, 40 mL crude extract of total flavonoids from *Ardisia mamillata* Hance radix was put into the chromatographic column with 10 g ADS-7 for dynamic absorption based on the procedures in the dynamic adsorption test above. The content of total flavonoids in eluent, desorption rate (*W*), adsorption rate (*F*) and purity (*P*) were measured.

### Further purification of total flavonoids

The sample of the total flavonoids from *Ardisia mamillata* Hance radix which was purified with ADS-7 (5 g), was dissolved in 100 mL methanol, and then mixed with silica gel (size: 100-200 mesh). The mixture was placed in a ventilated place for drying. The silica gel (65g) was packed dry in a 2.4 mm × 600 mm chromatographic column. The dry silica gel powder was packed in the chromatographic column to a level 5 cm from the top of the column, and the mixture was applied to the top part of the packed column. After eluting with pure chloroform, graded volume ratios of chloroform-methanol i.e. 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1 and 1:1, and pure methanol, were used for gradient elution, and a portion of each eluent was collected. The content of total flavonoids in each eluent was determined by high-performance liquid chromatography. Some eluents containing most total flavonoids were merged and then concentrated slightly, crystallized and subjected to silica gel column chromatography again to obtain a higher purity total flavonoids.

### Determination of antioxidant activity

About 1 mL of 0.1 mmol/L DPPH (1,1-Diphenyl-2-picrylhydrazyl radical 2,2-diphenyl-1- (2,4,6-trinitrophenyl) hydrazyl) in ethanol, was mixed separately with 3 mL each of different concentrations of the purified total flavonoids from *Ardisia mamillata* Hance radix (1.0, 2.0, 3.0, 4.0, and 5.0 mg/mL). The mixtures were shaken and kept in a dark room at room temperature for 30 min. The absorbance values of total flavonoids from *Ardisia mamillata* Hance radix, crude extract, and DPPH were measured separately against a blank made up of 3 mL of absolute ethanol. Vitamin C and BHT were used as standards. The OD value (B) of total flavonoids from *Ardisia mamillata Hance* radix, crude extract and DPPH in the blank group was determined. OD value (C) of total flavonoids from *Ardisia mamillata Hance* radix, crude extract and DPPH in the control group was also determined. BHT (butylated hydroxytoluene) and Vitamin C solution with same mass concentration were used for control, and the other steps followed those mentioned above. Antioxidant activity was calculated as in Eq 6.

$$S\ (\%) = \{1 - (A - C)/B\}100 \quad \ldots\ldots\ldots\ldots\ (6)$$

where S is antioxidant activity rate; A is the OD value of total flavonoids from *Ardisia mamillata Hance* radix, crude extract and DPPH in the BHT or Vitamin C group; C is the OD value of total flavonoids from *Ardisia mamillata Hance* radix, crude extract and DPPH in the control group; B is the OD value of total flavonoids from *Ardisia mamillata Hance* radix, crude extract and DPPH in the blank group.

### Statistical analysis

All data are presented mean ± standard deviation (SD). Comparisons between different groups were done with analysis of t test. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

## RESULTS

### Static adsorption capacity and desorption rate of total flavonoids

A comparison of the six macroporous resins showed that ADS-7 had the largest adsorption capacity (33.05 ± 0.22) and highest desorption rate (67.53 ± 0.21) for flavonoids (Table 1)

**Table 1:** Adsorption capacities and resolution ratios of different types of macroporous resins

| Resin type | Polar | Adsorption capacity | Resolution ratio |
|---|---|---|---|
| AB-8 | Weak polar | 28.88±0.15 | 36.03±0.33 |
| ADS-7 | Strongly polar | 33.05±0.22 | 67.53±0.21 |
| D101 | Nonpolar | 19.33±0.23 | 39.95±0.18 |
| D3520 | Polar | 20.97±0.31 | 23.79±0.27 |
| NKA-Ⅱ | Polar | 24.25±0.19 | 56.94±0.24 |
| X-5 | Nonpolar | 29.58±0.28 | 18.77±0.25 |

### Dynamic adsorption of total flavonoids by macroporous resins

Amongst the six macroporous resins, ADS-7 was the best resin for purification, with adsorption and desorption rates of 93.05 and 98.22 %, respectively (Table 2).

**Table 2:** Adsorption rates, desorption rates and purification effects of macroporous resins in dynamic adsorption

| Resin type | Purity of flavonoids | Adsorption (%) | Resolution ratio |
|---|---|---|---|
| AB-8 | 15.4±0.25 | 88.88±0.23 | 85.32±0.35 |
| ADS-7 | 18.6±0.32 | 93.05±0.27 | 98.22±0.26 |
| D101 | 9.6±0.38 | 79.33±0.36 | 71.22±0.18 |
| D3520 | 11.5±0.29 | 80.97±0.31 | 73.79±0.24 |
| NKA-Ⅱ | 13.9±0.23 | 84.25±0.29 | 75.36±0.28 |
| X-5 | 16.3±0.21 | 89.58±0.22 | 88.77±0.31 |

### Optimum conditions for purification of total flavonoids

From Figure 1, the % adsorption of total flavonoids was maximum (86.52 %) at sample

concentration of 0.8896 mg/mL. At pH 4.48, the adsorption of flavonoids reached a peak value of 77.37 %. When the flow rate was 1.5 mL/min, the adsorption of flavonoids reached it maximum value of 93.28 %, and at 60 % ethanol, the desorption rate peaked at 92.53 %. When the flow rate of eluent was 3 mL/min, the desorption rate of total flavonoids reached its climax.

**Purified total flavonoids from *Ardisia mamillata* Hance radix**

Under the optimum conditions, the total flavonoids were purified three times with dynamic adsorption in ADS-7 column. The adsorption was 96.52 ± 0.19 %, and the mean desorption was 99.31 ± 0.27 %, while the mean purity of total flavonoids was 76.43 ± 0.36 %. The standards and total flavonoids from *Ardisia mamillata* Hance radix before and after HPLC purification are shown in Figure 2. It is evident that purifying effect of the ADS-7 macroporous resin was very significant.

**DPPH radical scavenging activity of total flavonoids purified by silica gel column**

The scavenging effect of the total flavonoids from *Ardisia mamillata* Hance radix purified by silica gel column on DPPH free radicals is shown in Figure 3. The purified total flavonoids showed strong DPPH radical scavenging activity in a dose-dependent manner. The DPPH scavenging activity of the unpurified total flavonoids from *Ardisia mamillata* Hance radix was less than that of the purified one. When the concentration of the total flavonoids was 1.0～4.0 mg/mL, the scavenging capacity of total flavonoid sample was weaker than that of BHT and vitamin C. However, when the concentration was above 4.0 mg/mL, the DPPH radical scavenging activity of the purified total flavonoid sample was slightly weaker than that of vitamin C, but stronger than that of BHT. Thus, the total flavonoids from *Ardisia mamillata* Hance radix possess strong antioxidant activity.

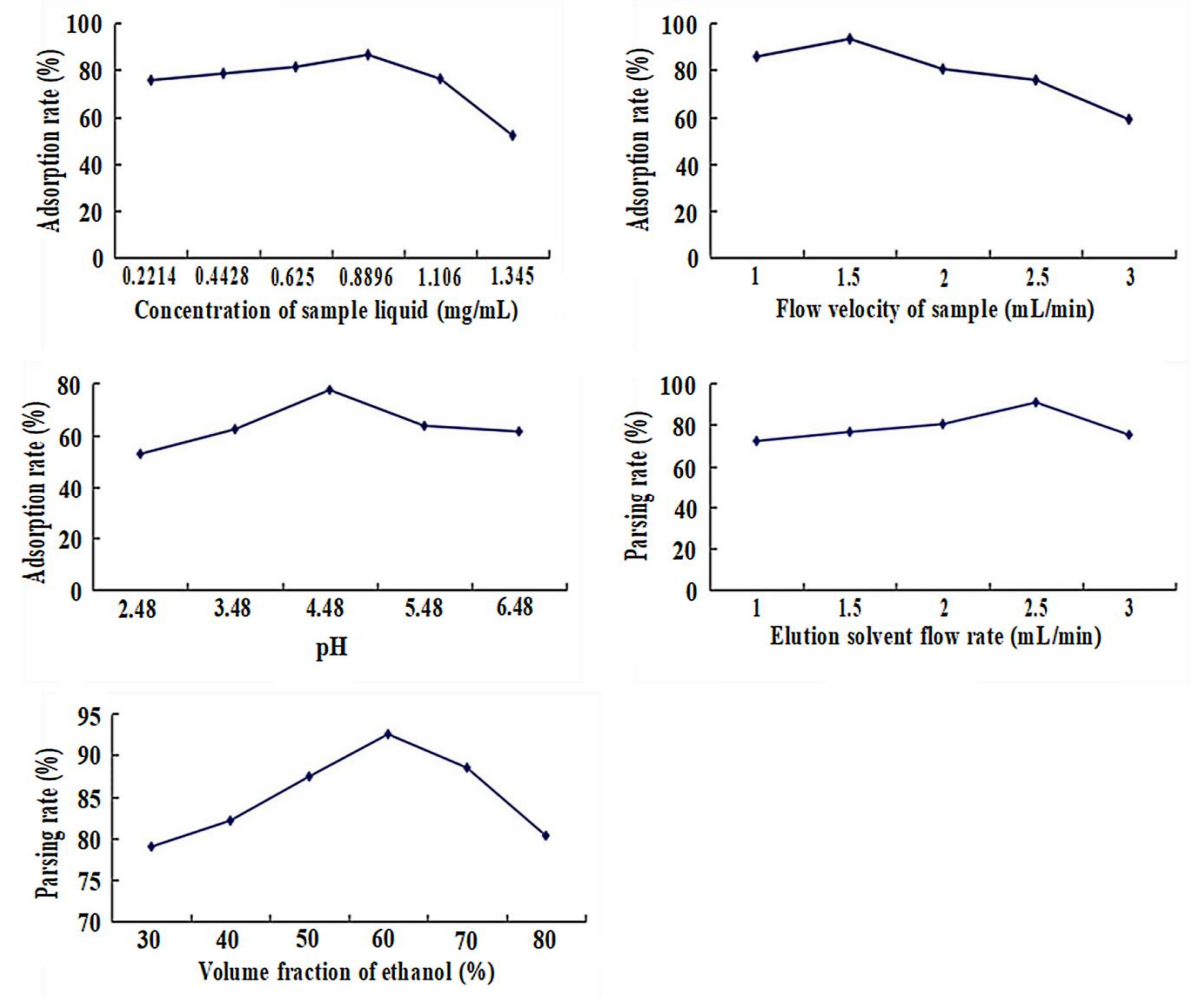


**Figure 1:** The best condition for purification of total flavonoids

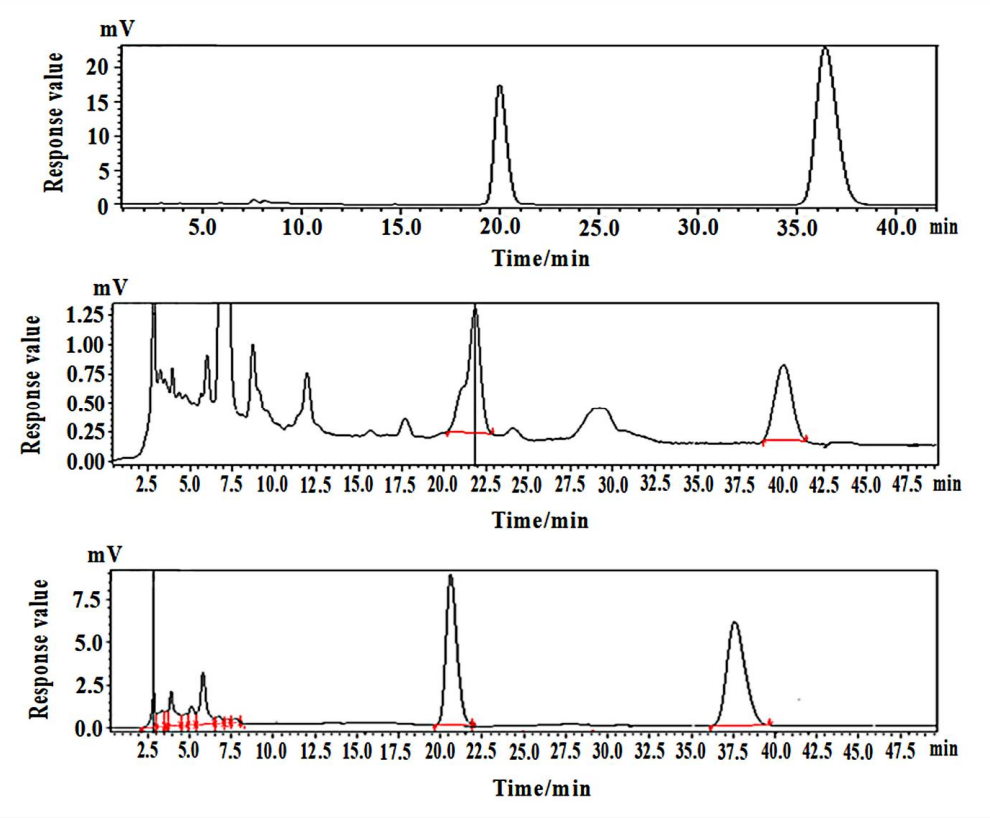


**Figure 2:** Results of HPLC purification using ADS-7. First panel, quercetin and kaempferol standards; Second panel, total flavonoids from *Ardisia mamillata* Hance radix before purification; third panel, HPLC peaks of total flavonoids from *Ardisia mamillata* Hance radix after purification

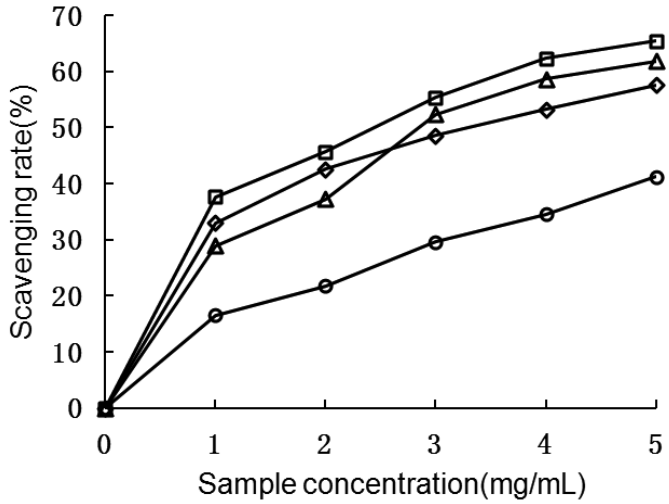


**Figure 3:** DPPH radical scavenging activity of the purified total flavonoids from *Ardisia mamillata* Hance radix. ***Note:*** *○-Scavenging rate of crude flavonoids, △-Scavenging rate of pure flavonoids, ◇-Scavenging rate of BHT , □-Scavenging rate of Vc*

## DISCUSSION

In this study, six types of commonly used macroporous resins were selected for the purification of total flavonoids from *Ardisia mamillata* Hance radix. Through static and dynamic adsorption tests, it was found that ADS-7 was the best resin for the adsorption and isolation of total flavonoids from *Ardisia mamillata* Hance radix. Resins have different purification properties for diverse compounds and have different separation effects under various isolation conditions [21]. The optimum pH for maximum purification of the total flavonoids

was 4.48. This might be due to the fact that flavonoids, being polyhydroxy compounds [22], are slightly acidic, and so they may be easily adsorbed by resins in a slightly acidic condition [23].

The principle involved in macroporous resin separation technique is that after the mixture to be purified is adsorbed to the resin, it is washed with some solvent to remove the adsorbed components. Thus, the concentration of eluent is a major influencing factor during the purification process. The present study found that when the concentration of ethanol was 70 %, its purifying effect was better, and it was much easier to resolve the total flavonoids from marcoporous resin than at any other ethanol concentration. The optimum flow rate for sample loading was 1.5 mL/min. Since macroporous resin chromatography is a dynamic adsorption process, changes in the sample loading flow rate directly impacts the diffusion of flavonoids to the internal surface of resin, thereby affecting the adsorption efficiency.
After sample purification, the resin column was washed with distilled water to remove most impurities like sugars and amino acids, thereby ensuring maximum purity of the effluent. The purity of the extract of flavonoids from *Ardisia mamillata* Hance radix was greatly increased after purification with the macroporous resin. In addition, resin treatment can effectively get rid of sugars, mineral salt, and proteins in the extract.

In this study, the total flavonoids from *Ardisia mamillata* Hance radix showed high antioxidant activity through scavenging of DPPH radicals. This is most likely due to the polyhydric phenolic hydroxyl groups in flavonoids.

## CONCLUSION

The DPPH radical scavenging activity of *Ardisia mamillata* is stronger than that of BHT standard, but slightly lower than that of ascorbic acid. The results of the present study show that ADS-7 resin is the best macroporous resin for the purification of total flavonoids from the radix of *Ardisia mamillata* Hance. The total flavonoids from the radix of *Ardisia mamillata* Hance exhibit good antioxidant activity.

## DECLARATIONS

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### *Conflict of interest*

No conflict of interest is associated with this work

### *Contribution of authors*

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors, all authors read and approved the manuscript for publication. Jinhua Shao and Yongqiang Ma conceived and designed the experiments; Jinhua Shao and Xianzhe Fan performed the experiments; Xingguang Zhuang and Xianzhe Fan analyzed the data; Yongqiang Ma contributed reagents/materials/analysis tools; Jinhua Shao wrote the paper.

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