doi.org/10.36721/PJPS.2021.34.2.REG.621-627.1

# The optimization of total flavonoids from rhizoma drynariae by response surface methodology and the metal ion chelation activity assay

**Li Guan*, Xingyu Huang, Huiru Zhao and Weize Li**
Department of Natural Medicinal Chemistry, Institute of Medicine, Xi'an Medical University, Xi'an, PR China

**Abstract**: Rhizoma Drynariae, the dried rhizome of *Drynaria fortunei* (Kunze), is rich in flavonoids and has varieties of pharmacological activities. To optimize the extract conditions for bioactive flavonoids, a response surface methodology (RSM) was utilized to assess the effects of three independent variables (liquid-to-solid ratio (mL/g), extract temperature (°C) and ethanol concentration (%) on the total flavonoids content (TFC). To test the chelation with metal ion, the UV-visible spectrophotometer was used to detect metal ion chelation of extracted flavonoids. Regression analysis displayed a good fit of the experimental data. The optimal condition was liquid-to-solid ratio with 50:1, extract temperature with 80 °C and ethanol concentration with 40.22%. The total flavonoids had a better chelation with metal ions $Cu^{2+}$, $Fe^{2+}$, $Fe^{3+}$ than $Zn^{2+}$. These results suggested that the model employed is suitable and the application of RSM in optimizing the extract conditions is successful. The experimental values were in fine agreement (the yield 24.05±0.69mg/g) with predicted values. The total flavonoids from the extract presented good chelation against four metal ions ($Cu^{2+}$, $Zn^{2+}$, $Fe^{2+}$ and $Fe^{3+}$), which provided a good evidence for Alzheimer's disease treatments.

**Keywords**: Alzheimer's disease, rhizoma drynariae, total flavonoids, response surface method, metal ion chelation.

## INTRODUCTION

Alzheimer's disease (AD), clinically characterized by memory and cognitive dysfunctions, is a progressive and fatal degenerative nervous system disease (Skovronsky *et al.*, 2006). It mainly occurs in the elderly and is the main cause of dementia (Scharfenberg *et al.*, 2019). The pathological manifestations of AD are neurofibrillary tangles (NFTs) by hyperphosphorylated tau protein and senile plaques (SPs) composed of β-amyloid (Aβ) (Hanseeuw *et al.*, 2019; Cieplak, 2019). In recent years, various studies have shown that metal ions are critically related in the pathogenesis of nervous system diseases (Budimir, 2011; Nam *et al.*, 2019). Metal ion chelation might be a potential treatment for Alzheimer's disease involving metal ion imbalance (Zatta *et al.*, 2009).

Rhizoma Drynariae is the dried rhizome of the perennial pteridophyte *Drynaria fortunei* (Kunze) J. Sm (Guo *et al.*, 2019), which was firsted in <BEN CAO SHI YI> over 1000 years (Song *et al.*, 2017). In the Chinese Pharmacopoeia, it is listed for the treatment of bone fractures or related diseases (Song *et al.*, 2017; Zhang *et al.*, 2017). Recently, Rhizoma Drynariae gained increasing attention as an important resource for new drug discovery (Wang *et al.*, 2008; Wang *et al.*, 2016; Li *et al.*, 2013). Total flavonoids are the main active constituents of Rhizoma Drynariae and naringin (4', 5, 7-trihydroxyflavanone 7-rhamnoglucoside), a well-known flavanone glycoside, is the main component (Wang *et al.*, 2011). It was demonstrated that naringin played a neuroprotective role by modulating endogenous biomarkers and down regulation of free radical and so on (Kandhare *et al.*, 2012). Anand Kamal Sachdeva et al. found that naringin improved memory deficit in Alzheimer's disease by relieving mitochondrial dysfunction (Sachdeva *et al.*, 2014). However, no metal ion chelation activity of total flavonoids in Rhizoma Drynaria or narigin has been explored in these years.

Extraction is the first important step to recover bioactive compounds from plants for natural product research (Mahugo *et al.*, 2009). Many factors such as extraction temperature, liquid-to-solid ratio, extraction time, solvent composition, among others, may significantly influence the extraction efficacy (Fan *et al.*, 2019). In general, optimization of a process can be determined by an empirical or statistical method. However, empirical methods have the limitations in perfect optimization. The Response Surface Methodology (RSM) could optimize complex extraction procedures by establishing a less laborious and time-consuming mathematical model (Yoo *et al.*, 2018; Li *et al.*, 2017; Chen *et al.*, 2017). The Box-Behnken design (BBD), as a more easier and efficient RSM, is widely used for arranging and interpreting experiments (Mao *et al.*, 2018).

In this study, we used BBD to optimize the liquid-to-solid ratio, extraction time and ethanol concentration for maximum yield of total flavonoids in Rhizoma Drynariae. In deep, the ultraviolet absorption of extract without or with metal ion ($Cu^{2+}$, $Zn^{2+}$, $Fe^{2+}$, $Fe^{3+}$) was measured for the metal ion chelation activity.

**Corresponding author:* e-mail: guanli0246@163.com

## MATERIALS AND METHODS

***Plant materials***

The Rhizoma Drynariae was bought by our lab from Changsha, Hunan province, which was also provided by a certificate of identity and quality. The dried Rhizoma Drynariae was crushed into fine powder for use.

***Reagents and apparatus***

All organic solvents, such as ethanol, methanol, used for extraction and content determination were of analytical grade and were purchased from Tianjin Tianli Chemical Reagent Co., Ltd. Narigin was purchased form Jianglai Biology; $CuSO_4$, $ZnSO_4$, $FeSO_4$ and $FeCl_3$ were analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd.

***Crude sample extraction***

The water bath was performed in an intelligent digital display thermostatic water bath device (Jiangsu Kewei Experimental Equipment Co., Ltd., China). For single-factor tests, Sample (1.0g) was extracted under these various conditions: extract time from 0.5h to 2.5h, liquid-to-solid ratio from 10:1 to 50:1, extract time from 40°C to 80°C and ethanol concentration from 30% to 70%. For extraction using RSM, fixed extract time 2.0 h was used, liquid-to-solid ratio from 30:1 to 50:1, extract time from 60°C to 80°C and ethanol concentration from 30% to 50%. After extraction, the mixtures were vacuum filtered to remove the insoluble materials and the supernatants were collected for TFC determination and metal ion chelation activity assay.

***Determination of total flavonoids content***

Quartz colorimetric method was used for the TFC determination with narigin as a standard. Briefly, accurately weigh 4.8mg of naringin standard in a 50mL volumetric flask, dilute with methanol to a fixed volume, and shake well to obtain a reference solution (0.096 mg/mL). Accurately draw reference solution 0, 2.5, 3.0, 3.5, 4.5, 5.0, 6.5mL in 25mL volumetric flask add methanol to the mark, using blank methanol as a control, the absorbance of all solutions was measured at 283 nm by UV-visible spectrophotometer. Calculate the linear regression equation as A=24.19C+0.010, r=0.9998, in the range of 0.0096-1024496mg/mL. The TFC was expressed as milligram of narigin equivalent per gram of dry weight material (mg/g) (Equation 1).

TFC=C*V*dilute factor/m

Where C is the concentration of determination of sample (mg/mL), V is the filtered volume (mL) and m is the weight of material (g)

***Experimental design***

RSM was applied to optimize the extraction conditions for total flavonoids from Rhizoma Drynariae. Liquid-to-solid ratio ($X_1$, mL/g), extract temperature ($X_2$, °C) and ethanol concentration ($X_3$, %) were preferred for independent variables related to the response values of total flavonoids content. A three-level three-variables BBD was applied in this study. table 1 displayed the variables and their levels, with both coded and natural values. Regression analysis was performed based on experimental data from BBD and fitted to a second-order polynomial model (Equation 2) (Md Yusof *et al.*, 2019).

$$Y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} A_{ij} X_i X_j$$

Where Y is the response o TFC, $A_0$ is intercept, $A_i$ is coefficient of variable for linear, $A_{ii}$ is coefficient of variable for quadratic and $A_{ij}$ is coefficient of variable for interaction term. Xi and $X_j$ are independent variables. Design-Expert version 8.0.6.1, (Stat-Ease Inc., Minneapolis, MN, USA) was used as a statistical package for experimental data analyse. The adequacy of the model was evaluated by the lack of fit, coefficient of determination ($R^2$), Fisher's test value (F-value) generated from the analysis of variance (ANOVA) analysis ($P< 0.05$).

***Verification of model***

To confirm the predictive value of the model, the optimum conditions with the maximum yield for total flavonoids were determined and used in the extract test. The precision of the fitted model was verified by comparing the predicted value of the experimental value obtained from the three replicates.

***Metal ion chelation assay***

Metal chelation activity assay was performed in methanol using UV-vis spectrophotometer with wavelength ranging from 200 to 500 nm (Li *et al.*, 2014; Ozil *et al.*, 2019; Guan *et al.*, 2016). The extract obtained after the verification test was diluted to 50μg/mL with methanol. The four metal ion salts of $CuSO_4$, $ZnSO_4$, $FeCl_3$ and $FeSO_4$ were respectively arranged into a solution containing a concentration gradient of metal ions of 2, 10, 20, 50, 100μM and then mixed with the extract in a volume of 1:1, diluted into The total flavonoid solution of Rhizoma Drynariae (25μg/mL) with a metal ion concentration of 1, 5, 10, 25, 50μM. Full-wavelength scanning of the total flavonoids without or with different metal ions was operated in the range of 200-500 nm. The data were processed using Origin 8.

## STATISTICAL ANALYSIS

The values are represented as mean±S.E.M, and Statistical analysis were performed using Design Expert 8.0. The Confidence level was 95% for all parameters and the differences were considered significant at $p\leq0.01$.

## RESULTS

***Single-factor test***

We first evaluated the effects of each single factor on the extraction yield. As shown in fig. 1, the yield of TFC

**Table 1**: Code and uncoded levels of independent variables used for Box-Behnken design (BBD)

| Independent variable | Code units | code levels | | |
|---|---|---|---|---|
| | | -1 | 0 | 1 |
| Liquid-to-solid ratio (mL/g) | $X_1$ | 30:1 | 40:1 | 50:1 |
| Extraction temperature (°C) | $X_2$ | 60 | 70 | 80 |
| Ethanol concentration (%) | $X_3$ | 30 | 40 | 50 |

**Table 2**: Experimental design and responses of the dependent variables to extraction conditions

| Standard order[a] | Run order[b] | Coded variables | | | Responses of TFC (mg/g) | |
|---|---|---|---|---|---|---|
| | | $X_1$ | $X_2$ | $X_3$ | experimental value | Predicted value |
| 3 | 1 | -1 | 1 | 0 | 22.22 | 22.85 |
| 17 | 2 | 0 | 0 | 0 | 17.43 | 17.83 |
| 2 | 3 | 1 | -1 | 0 | 18.89 | 18.26 |
| 8 | 4 | 1 | 0 | 1 | 18.56 | 18.42 |
| 15 | 5 | 0 | 0 | 0 | 17.71 | 17.83 |
| 4 | 6 | 1 | 1 | 0 | 25.27 | 25.26 |
| 14 | 7 | 0 | 0 | 0 | 19.12 | 17.83 |
| 7 | 8 | -1 | 0 | 1 | 16.70 | 15.94 |
| 5 | 9 | -1 | 0 | -1 | 15.47 | 15.61 |
| 10 | 10 | -1 | -1 | -1 | 23.82 | 23.04 |
| 16 | 11 | 0 | 0 | 0 | 17.13 | 17.83 |
| 9 | 12 | 0 | -1 | -1 | 15.60 | 15.47 |
| 13 | 13 | 0 | 0 | 0 | 17.76 | 17.83 |
| 11 | 14 | 0 | -1 | 1 | 16.06 | 16.83 |
| 1 | 15 | -1 | -1 | 0 | 16.27 | 16.26 |
| 6 | 16 | 1 | 0 | -1 | 16.80 | 17.56 |
| 12 | 17 | 0 | 1 | 1 | 22.73 | 22.87 |

**Table 3**: The ANOVA of TFC for response surface quadratic model analysis of variance table

| Source | Sum of square | Degree of freedom | Mean surares | *F* | *P* |
|---|---|---|---|---|---|
| Model | 139.13 | 9 | 15.46 | 19.41 | 0.0004*** |
| $X_1$ | 9.83 | 1 | 9.83 | 12.35 | 0.0098** |
| $X_2$ | 92.57 | 1 | 92.57 | 116.25 | <0.0001*** |
| $X_3$ | 0.71 | 1 | 0.71 | 0.89 | 0.3777 |
| $X_1X_2$ | 0.044 | 1 | 0.044 | 0.056 | 0.8203 |
| $X_1X_3$ | 0.072 | 1 | 0.072 | 0.09 | 0.7731 |
| $X_2X_3$ | 0.6 | 1 | 0.6 | 0.75 | 0.4156 |
| $X_1^2$ | 0.027 | 1 | 0.027 | 0.034 | 0.8593 |
| $X_2^2$ | 31.69 | 1 | 31.89 | 40.05 | 0.0004*** |
| $X_3^2$ | 4.44 | 1 | 4.44 | 5.58 | 0.0502 |
| Residual | 5.57 | 7 | 0.8 | | |
| *Lack of Fit* | 3.23 | 3 | 1.08 | 1.84 | 0.2803 |
| *Pure Error* | 2.34 | 4 | 0.59 | | |
| Cor Total | 144.71 | 16 | | | |
| $R^2$=0.9615, $R_{Adj}^2$=0.9120 | | | | | |

increased as extract time increases at the begining and it reached to the highest when the extract time was 2.0h. Thereafter the yield decreased. Therefore, extract time of 2.0 h was appropriate. When liquid-to-solid ratio was up to 40:1 from 30:1, The yield of TFC increased to the highest. This might be the reason that within a certain range, flavonoids can be better dissolved with increasing solvents. However, the continuously increasing solvent lead to the content stable or slightly lower. As a result, 40:1 was picked as the center point for BBD experiment. The yield of TFC was continuously increased with the increasing extract temperature until the boiling point of ethanol (80°C). We chose 70°C as the center temperature for BBD experiment. When the ethanol concentration was up to 40%, the total flavonoids content was highest.

**Table 4**: Predicted and experimental values of response variables under optimal conditions

| Parameters | optimum values | |
|---|---|---|
| | Predicted values[a] | Experimental values[b] |
| Liquid-to-solid ratio (mL/g) | 50:1 | 50:1 |
| Extract temperature (°C) | 80.00 | 80 |
| Ethanol concentration (%) | 40.23 | 40 |
| Total flavonoids content (mg/g) | 25.28 | 24.05±0.69 |

[a]Predicted using ridge analysis of response surface quadratic mode, [b]Mean±SD of triplicate determinations from different experiments.

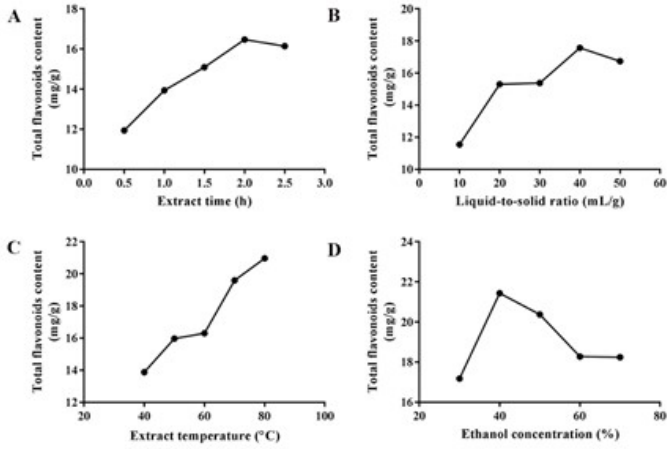


**Fig. 1**: On the yields of total flavonoids in single factor experiments: (A) extract time, (B) liquid-to-solid ratio, (C) extract temperature and (D) ethanol concentration

***Statistical analysis and model fitting***

Seventeen experiments were designed and carried out by BBD of RSM, which was operated for the optimization of extract conditions (Lee *et al.*, 2013). The results were listed in table 2. The TFC value varied from 15.47mg/g to 25.26mg/g. Among these 17, experiment 6 (liquid-to ratio 50:1, extract temperature 80°C, ethanol concentration 40%) represented the highest TFC value while experiment 9 (liquid-to ratio 30:1, extract temperature 70°C, ethanol concentration 30%) represented the least TFC value.

The ANOVA of TFC values for the model was displayed in table 3, the *F*-value was 19.41 and the p-value <0.001, which discripted that the model was significant (Cui *et al.*, 2013). The high value of coefficient of determination $R^2$=0.9615 for TFC as well as $R^2_{adj}$ =0.9120 indicated a good correlation between the experimental and predicted values (Kumar *et al.*, 2019). In addition, no significance of lack of fit (*P*=0.2803>0.05) for the model indicated the well reproducibility of the experimental data (Ren *et al.*, 2008).

***Response surface analysis of TFC value***

The experimental data were analyzed through multiple regression analysis. The regression equation asserted the relationship between liquid-to-solid ratio ($X_1$), extract temperature ($X_2$) and ethanol concentration ($X_3$).

TFC value was obtained according to (Equation 2) as given below:

$Y_{TFC}=17.83+1.11*X_1+3.40*X_2+0.30*X_3+0.11*X_1*X_2+0.13*X_1*X_3-0.39*X_2*X_3+0.080*X_1^2+2.75*X_2^2-1.03*X_3^2$

The significant of each coefficient was checked by F values and P values and the results were shown in table 3. The yield of TFC was significantly affected by two linear ($X_1$, $X_2$) and one quadratic ($X_2^2$) parameters as P<0.05 and the other coefficients not significant (p>0.05). Based on the yield of TFC, factors were ranked by significance in the following order: $X_2>X_1>X_3$.

To investigate the interactive effects of variables for the TFC value, three-dimensional surface plots were constructed in accordance with Equation 2. fig. 2 showed the effect of liquid-to-solid ratio, extract temperature, ethanol concentration and their mutual interaction on TFC value. As shown in fig. 2A, when ethanol concentration was fixed at the center point (40%), TFC values were increased by increasing the liquid-to-solid ratio from 30:1 to 50:1 and steadily reached the maximum value by increasing extract temperature from 60°C to 80°C. fig. 2B showed the correlation between liquid-to-solid ratio and ethanol concentration on the TFC value at a fixed temperature 70°C. TFC value increased when ethanol concentration increased from 30% to 40%; however, the TFC value decreased when ethanol concentration exceeded 40%. TFC value also increased when liquid-to-solid ratio increased from 30:1 to 50:1mL/g. fig. 2C showed the correlation between extract temperature and

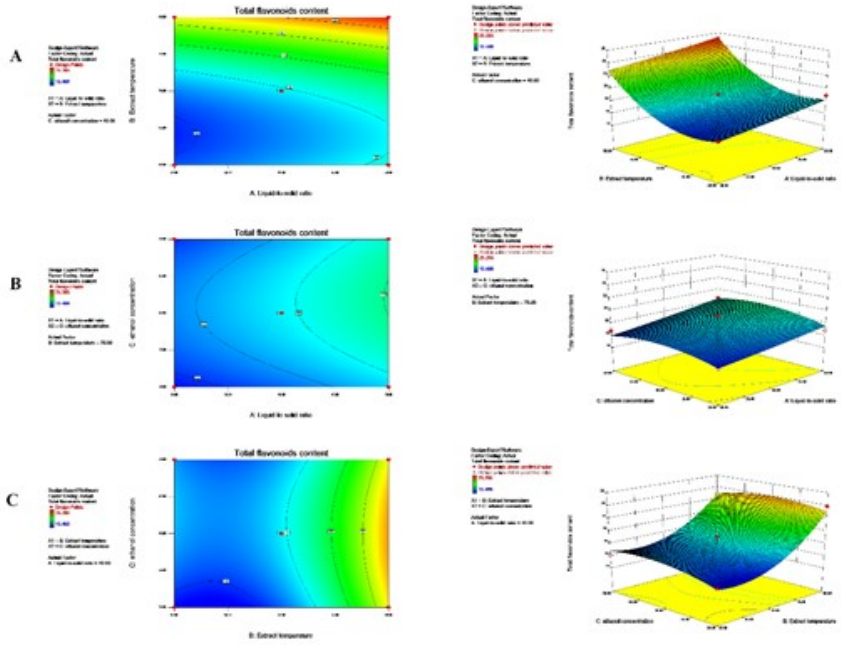

**Fig. 2**: Response surface plot for the effects of extraction conditions for total flavonoids from Rhizoma Drynariae, (A) liquid-to-solid ratio and extract temperature, (B) liquid-to-solid ratio and ethanol concentration, (C) extract temperature and ethanol concentration.

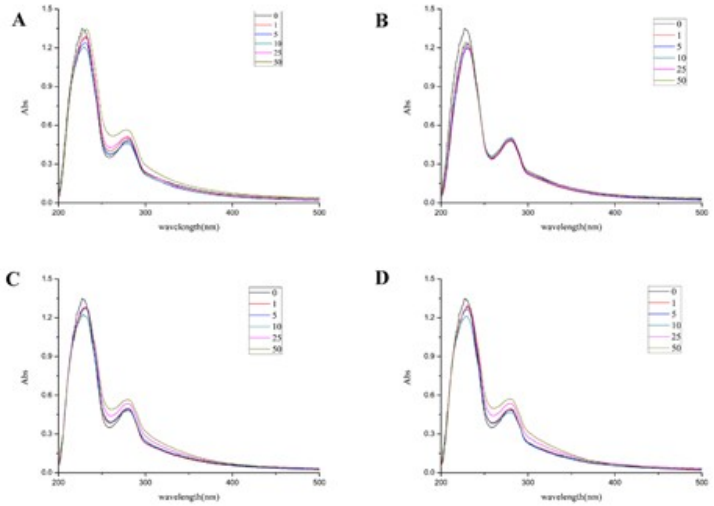

**Fig. 3**: UV spectrum of total flavonoids (25μg/mL) alone and in the presence of different concentration of metal ions (1, 5, 10, 25, 50μM) in methanol. (A) $Cu^{2+}$; (B) $Zn^{2+}$, (C) $Fe^{2+}$, (D) $Fe^{3+}$

ethanol concentration on the TFC value at a fixed liquid-to-solid ratio of 40:1mL/g. The maximum TFC value was obtained at a ethanol concentration of 30%-50% and the extract temperature of 80°C. As a result, the response surface analysis and statistical analysis showed that the TFC values were significantly affected by the liquid-to-solid ratio, extract temperature and ethanol concentration.

***Experimental validation of the optimum conditions***

Verification experiments were performed using the recommended optimal conditions derived from RSM (table 4). The optimal conditions for the maximum TFC value determined at a liquid-to-solid ratio 50:1mL/g, extract temperature of 80.00°C and ethanol concentration of 40.23%. This predicted an extraction of 25.28mg/g of TFC value. Under the liquid-to-solid ratio of 50:1mL/g, extract temperature of 80°C and ethanol concentration of 40%, the observed values were 24.05±0.69mg/g of TFC value. These results indicated that the actual experimental values are in good agreement with the predicted values in the regression model. Hence, the response surface modeling can be employed effectively to the prediction of extraction of total flavonoids from Rhizoma Drynariae.

***Metal ion chelation activity assay***

The metal chelation assay of total flavonoids was studied

by UV-vis spectrometry with wavelength ranging from 200 to 500nm (Li *et al.*, 2016). UV-vis spectra of total flavonoids (25μg/mL) without or with increasing $Cu^{2+}$, $Zn^{2+}$, $Fe^{2+}$ or $Fe^{3+}$ concentrations from 2 to 50μM were shown in fig. 3. Comparing the total flavonoid extract without metal ion solution with the addition, the greater the difference in absorbance between the curves indicates that the total flavonoid extract has stronger chelation ability with metal ions. It can be analyzed that the absorbance of flavonoids changed when it combined with metal ions, which may be related to the mutual chelation between total flavonoids and metal ion. $Cu^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ have a greater chelation activity than $Zn^{2+}$.Therefore, the chelation activity of total flavonoids to metal ions is expected to be for the treatment of Alzheimer's disease.

## DISCUSSION

In this paper, RSM was successfully employed to optimize the total flavonoids extraction from Rhizoma Drynaria. The three key parameters in BBD design included liquid-to-solid ratio, extract temperature and ethanol concentration. The $R^2$ value of the second order polynomial models for TFC value was 0.9615, which represented a well fit to the quadratic models. The optimal conditions for obtaining maximal TFC value of 25.28 mg/g, defined to be 50:1 of liquid-to-solid ratio, 80°C of extract temperature, and 40.23 of ethanol concentration. These results showed that extract temperature was the most important factor for total flavonoids extraction from Rhizoma Drynaria. Metal ion chelation assay showed that total flavonoids had been a better chelator for $Cu^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ than $Zn^{2+}$, which provided an evidence for total flavonoids applied to the treatment of Alzheimer's disease.

## CONCLUSION

This study confirmed RSM can improve the efficiency of optimizing the total flavonoids of Rhizoma Drynariae. Metal ion chelation assay can be used as a simple and effective method to evaluate the activity of compounds for AD.

## ACKNOWLEDGEMENTS

This research work was financially supported by Xi'an Medical University Ph.D. Research Startup Fund Project (2017DOC06) and Youth Innovation Team of Shaanxi Universities (Shaanxi 2019 No. 90) and Special Scientific Research Project of Shaanxi Provincial Department of Education (No. 20JK0895).

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