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Optimization Extraction of Total Flavonoids from Walnut Branch by Response Surface Methodology and Antioxidant Activity Research

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ABSTRACT

In this research, the optimization extraction technology of total flavonoids (TF) from walnut branch was studied by response surface methodology (RSM). And the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radical, total reducing power were researched to reveal the antioxidant activity of TF from walnut branch. Using total flavonoids content (TFC) as evaluating indicator, the optimum conditions for TF extraction were: solid-solvent ratio 1:25 (g/mL), extraction temperature 60°C, and ethanol concentration 70%. Under these conditions, the TFC was 12.97 mg/g (mg rutin/g). The antioxidant activity test showed that TF could strongly eliminate DPPH and hydroxyl radical, And had obviously total reducing power too. Then the conclusion could be drawn: optimization of TF extraction from walnut branch by RSM was reliable and the TF of walnut branch had strong antioxidant activity.

Keywords: Walnut branch; Total flavonoids; RSM; Antioxidant activity

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INTRODUCTION

Fresh walnut branch as Chinese traditional medicine has been used for detoxification and relieving itching for a long history [1]. Modern medicine research revealed it could be used in adjuvant therapy of esophageal cancer, breast cancer, gastric cancer, cervical cancer, and the lymphatic system tumors [2]. Previous research showed that walnut leaves, green peel and fructus diaphragm were rich in flavonoids, and exhibited perfect antioxidant activity. However, there are few studies on the extraction and activity of flavonoids from walnut branch [3]. RSM can be used in the field of biomedical research with the most economical, the minimum number of trials and the shortest time of the selecting factors and testing parameters for a comprehensive study [4]. In this paper, the extraction technology of TF from walnut branch was studied by RSM optimization test, and the antioxidant activity of TF extracted from walnut branch was investigated.

EXPERIMENTAL SECTION

Materials and Methods

Fresh walnut branches were collected from Weifang, Shandong, China in March 2019 and kept under -40°C. The sample was ground into powder and passed through a 50-mesh sieve until use. Rutin (≥99.9%) and Vitamin C (100%) were obtained from National Institute for Food and Drug Control of China (Beijing, China). DPPH was obtained from Sigma Chemical (St. Louis, MO, USA). The other chemicals used were of regent grade.

Draw Standard Curve

The TFC was determined according to the method described in reference using UV-Vis spectrophotometer [5]. Standard rutin was dried to constant weight at 105°C and prepared to 0.1 mg/mL as standard solution. Taken above solution 0.0 mL, 0.5 mL, 1.0 mL, 2.0 mL, 3.0 mL, 4.0 mL into 10 mL volumetric flask, added 0.3 mL 5% $NaNO_2$, kept for 6 min. Then added 0.3 mL 5% $Al(NO_3)_3$, shaken up and kept for 6 min. Finally 2.0 mL 4% NaOH was added in and confirmed volume with 60% ethanol, kept for 15 min. The absorbance (*A*) was measured at 510 nm using a UV-spectrophotometer as ordinate in the standard curve and concentration C as abscissas. The matched regression equation: A=14.023 C-0.0042 (R^2=0.9995).

Extraction of TF

Weight 2.0 g walnut branch powder accurately into glass bottles and extracted with certain solid-solvent ratio, ethanol concentration, temperature and extraction time. The extracted solution was filtrated and diluted to 100 mL in volumetric flask. 1.0 mL of extracted solution was mixed with 0.3 mL 5% $NaNO_2$ in 10 mL volumetric flask. The mixture was placed at room temperature for 6 min, then added 0.3 mL 5% $Al(NO_3)_3$, shaken up and kept for 6 min. Finally 2.0 mL 4% NaOH was added in and confirmed volume with 60% ethanol, kept for 15 min. The absorbance was measured at 510 nm and the mixture solution without extract was used as the blank. TFC was calculated by the following equations: TFC (mg/g)=weight of TF (mg, rutin) /weight of plant (g).

Experimental Design of RSM

On the basis of single-factor experiment, the relevant factors for the extraction of TF and the proper range for each factor were determined. In single-factor experiment, the influence of TFC including extraction time, extraction temperature, solid-solvent ratio, and ethanol concentration were investigated. Firstly, the extraction time was investigated by considering five levels (30, 60, 90, 120, 150; min), and the other parameters were fitted as: extraction temperature 60°C, solid-solvent ratio 1:25 (g/mL), and ethanol concentration 70%. Secondly, extraction temperature was investigated at 30°C, 40°C, 50°C, 60°C, 70°C and 80°C. The other parameters were fitted as: extraction time 90 min, solid-solvent ratio 1:25 (g/mL), and ethanol concentration 70%. Then the solid-solvent ratio was investigated by considering five ratios (1:15, 1:20, 1:25, 1:30, 1:35; g/mL), and the other parameters were: extraction time 90 min, extraction temperature 60°C and ethanol concentration 70%. Finally, the ethanol concentration was investigated as 30%, 40%, 50%, 60%, 70% and 80%. The other parameters were: extraction time 90 min, extraction temperature 60°C and solid-solvent ratio 1:25 (g/mL).

Based on the results of single-factor experiment, RSM was then employed to determine the optimal combination of variables for TF extraction by Box-Behnken central composite design principle.

Assay of DPPH Radical Scavenging Activity

The scavenging activity of DPPH radical was studied according to the method mentioned in the reference [6]. The TF extract were added into DPPH ethanol solution, mixed thoroughly and incubated for 30 min at 25°C in dark. Then the absorbance was measured at 571 nm as A_i. The TF extract was replaced by equal volume of ethanol, and the absorbance was measured by the same method mentioned above as A_0. The DPPH solution was replaced by equal volume of ethanol, and the absorbance was measured by the same method mentioned above as A_{i0}. Vitamin C was used as positive control. The scavenging capacity of the DPPH radical was calculated by the fowling formula:

$$\text{Scavenging ratio (\%)}=[1-(A_i-A_{i0})/A_0] \times 100$$

Assay of Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity was studied according to the method mentioned in the reference [7]. In a 10 mL test tube with stopper, 1.0 mL $FeSO_4$ solution (4.5 mM) and 1.0 mL sodium salicylate ethanol solution (4.5 mM) were mixed thoroughly. Then added certain amount of TF extract and supplemented to the same scale with deionized water. Finally 1.0 mL H_2O_2 (4.4 mM) was added and incubated at 37°C for 30 min to measure the absorbance at 510 nm. The absorbance of test tube with 0.0 mL TF extract considered as A_0, the other considered as A_i. The absorbance with 1.0 mL H_2O_2 replaced by deionized water considered as A_{i0}. Vitamin C was used as positive control. The scavenging capacity of the hydroxyl radical was calculated by the fowling formula:

$$\text{Scavenging ratio (\%)}=[1- (A_i-A_{i0})/ A_0] \times 100$$

Assay of Total Reducing Power

The total reducing power of the TF extract was determined by the method in reference [8]. The TF extract (0.5 mL) with different concentration was mixed with 1.0 mL phosphate buffer (pH 6.6) and 2.0 mL [$K_3Fe(CN)_6$] (1%, w/v), followed by incubating at 50°C by water bath for 20 min. The reaction was stopped by fast cooling. Then added 1.0 mL trichloroacetic acid (10%, w/v) and 0.5 mL $FeCl_3$ (0.1%, w/v), incubated at room temperature for 10 min. The absorbance was measured at 700 nm as the reducing power. Higher absorbance indicated greater reducing power [9]. Vitamin C was used as positive control.

RESULTS AND DISCUSSION

Effect of Extraction Time on TFC

Extraction time is an important factor on extraction efficiency and energy cost. The effect of extraction time on TFC was investigated for the first step. The results (Figure 1) revealed that TFC in extract increased with the extraction time increasing from 30 min to 90 min. Over 90 min the TFC decreased with the time prolonged. This may be due to the time increasing led to some flavonoids were oxidized. Thus selected extraction time as 90 min in the following experiments.

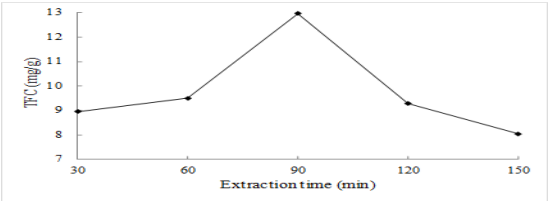

Figure 1. Effect of extraction time on TFC

Effect of Extraction Temperature on TFC

The effect of extraction temperature on TFC was investigated from 30°C to 80°C. The results (Figure 2) showed that the TFC increased with the temperature rising up to 60°C. TFC decreased over 60°C, possibly due to the high temperature destroying some thermo-sensitive flavonoids. Thus the centre point of extraction temperature chosen for RSM was 60°C.

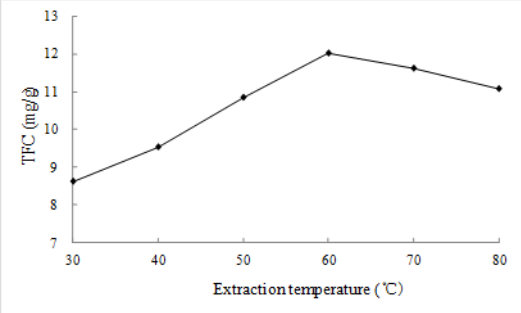

Figure 2. Effect of extraction temperature on TFC

Effect of Solid-Solvent Ratio on TFC

The different solid-solvent ratio (1:15-1:35) was studied to reveal the effect on TFC. The results (Figure 3) indicated the yield of total flavonoids increased with the solid-solvent ratio growing from 1:15 to 1:25 and got to maximum at 1:25. After that, with the increase of solvent dosage, the yield of total flavonoids leveled out and decreased slightly, indicating that the increase of solvent could no longer promote the dissolution of total flavonoids. Although high solid-solvent ratio with high yield, but led to resource waste and environmental pollution. So the solid-solvent ratio 1:25 was selected as centre point for RSM.

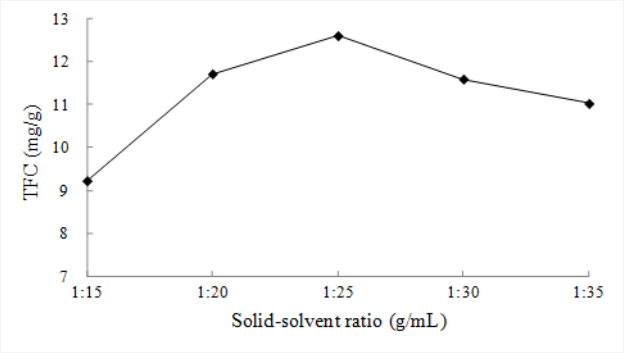

Figure 3. Effect of material to liquid ratio on TFC

Effect of Ethanol Concentration on TFC

To investigate the effect of ethanol concentration on TFC, the concentration of ethanol from 30% to 80% were studied. The results (Figure 4) showed that the yield of total flavonoids appeared a sharp increase from 30% to 40% of ethanol concentration. After that, the TFC still kept increasing obviously and reached to the maximum when the ethanol concentration was 70%, after which the TFC decreased. The reason might be related to the influence of ethanol polarity on TF solubility in walnut branch. So the ethanol concentration 70% was selected as centre point for RSM.

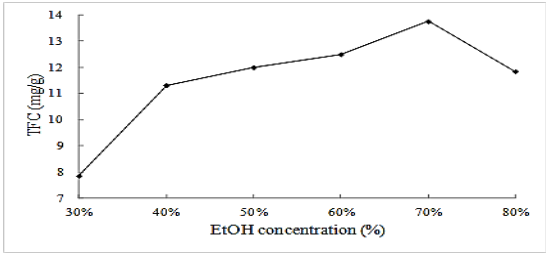

Figure 4. Effect of ethanol concentration on TFC

Optimization by RSM

The results of response surface design: Employed Design Expert 8.0, on the base of single-factor experiment, solid-solvent ratio (A), extraction temperature (B) and ethanol concentration (C) were selected as response value (Table 1). The extraction time was fixed in 90 min. Seventeen experiments were carried out to optimize the parameters for highest yield and the results were showed in Table 2. The design expert software generated the second-order polynomial equation to demonstrated the relationship between the factors and the predicted response: TFC (mg rutin/g)=12.87+0.68*A*+0.44*B*-0.35*C*-0.38*AB*+0.29*AC*+0.84*BC*-1.14A^2-0.67B^2-0.92C^2.

Table 1. Factors and levels of RSM

Level	Factor		
	A: solid-solvent ratio (g/mL)	**B: extraction temperature (°C)**	**C: ethanol concentration (%)**
-1	01:20	50	60
0	01:25	60	70
1	01:30	70	80

Table 2. Design and results of RSM

S No.	A: solid-solvent ratio (g/mL)	B: extraction temperature (°C)	C: ethanol concentration (%)	TFC（mg/g）
1	-1	0	-1	10.71
2	0	-1	1	9.73
3	-1	1	0	11.36
4	0	0	0	13
5	-1	-1	0	9.68
6	0	-1	-1	11.99
7	1	-1	0	11.53
8	0	1	1	12.24
9	0	1	-1	11.14
10	0	0	0	12.55
11	0	0	0	12.97
12	1	1	0	11.68
13	-1	0	1	9.3

14	1	0	1	11.5
15	0	0	0	12.84
16	0	0	0	13.01
17	1	0	-1	11.74

The influence of the interaction among with factors: The statistical significance of regression equation was checked by *F*-test and analysis of variance as showed in Tab. 3. The analysis results indicated model first term A, B, C and quadratic term A^2, B^2, C^2 had significant effect ($P<0.01$) on the extraction rate of TF from walnut branche. The *P*-values of interaction term AB and BC were less than 0.01, indicated a high interaction significance of the TF extraction. According to *F*-value in Tab. 3, the influence extent of three factors on TF extraction was: solid-solvent ratio (A) >extraction temperature (B) >ethanol concentration (C).

The *F*-value 53.03 and $P<0.0001$ implied the model was highly significant, and consistent with statistical analysis. The lack-of-fit was insignificant ($P=0.3324>0.05$) relative to the pure error due to noise. The determination coefficients ($R^2=0.9855$) was close to 1, represented the satisfactory correlation between actual values and predicated one. The R^2_{Adj} value (0.9670) indicated most variation could be predicted by the model. Linear relation was significant between response variable and response value. Therefore, the model can be used for prediction of TF extraction (Table 3).

Table 3. Analysis of variance for fitted quadratic polynomial model

Term	d_f	Sum of squares	Mean square	F-value	P-value
Model	9	22.08	2.45	53.03	<0.0001**
A	1	3.65	3.65	78.8	<0.0001**
B	1	1.52	1.52	32.91	0.0007**
C	1	0.99	0.99	21.34	0.0024**
AB	1	0.59	0.59	12.65	0.0093**
AC	1	0.34	0.34	7.4	0.0298*
BC	1	2.82	2.82	61.02	0.0001**
A2	1	5.44	5.44	117.68	<0.0001**
B2	1	1.92	1.92	41.41	0.0004**
C2	1	3.6	3.6	77.8	<0.0001**
Residual	7	0.32	0.046		
Lack of fit	3	0.17	0.058	1.55	0.3324
Net error	4	0.15	0.037		
Total deviation	16	22.4			

*indicate significant ($P<0.05$), ** indicate highly significant ($P<0.01$).

Analysis of contour and response surface: The 3D response surface and 2D contour plots are the graphical representation of regression equation. The 3D response surface can illustrate the sensitiveness of response value toward the change of variable and the 2D contour plots can describe significant coefficients between different variables [10]. The 2D contour plots in Figures 5-7 indicated that the interaction between ethanol concentration and extraction temperature was significant, the interaction between solid-solvent ratio and extraction temperature was the second, and the plots of solid-solvent ratio and ethanol concentration were approximately circular, indicating that the interaction between the two groups was not significant. The 3D response surfaces in Figures 5-7 showed the surface changes of ethanol concentration and extraction temperature were relatively steep, indicated the most significant factors on TFC. The surface change of solid-solvent ratio was smooth, indicated it was the less significant factors.

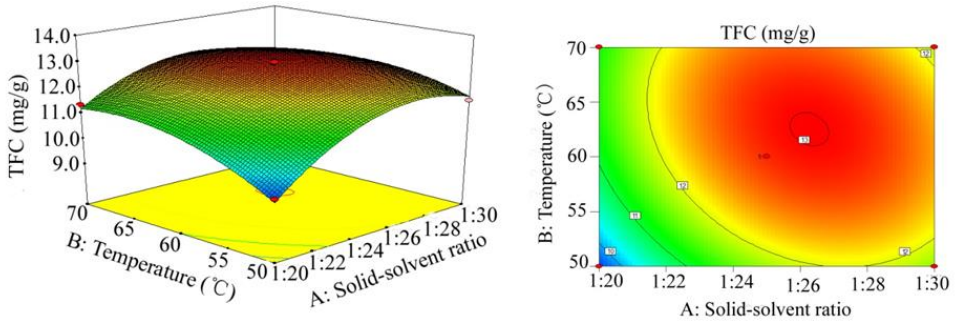

Figure 5. Response surface and contour plots for the effects of solvent-solid ratio and extraction temperature on flavonoids extraction efficiency

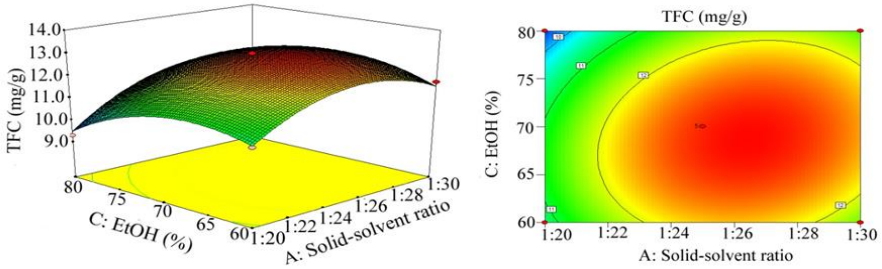

Figure 6. Response surface and contour plots for the effects of solvent-solid ratio and ethanol volume fraction on flavonoids extraction efficiency

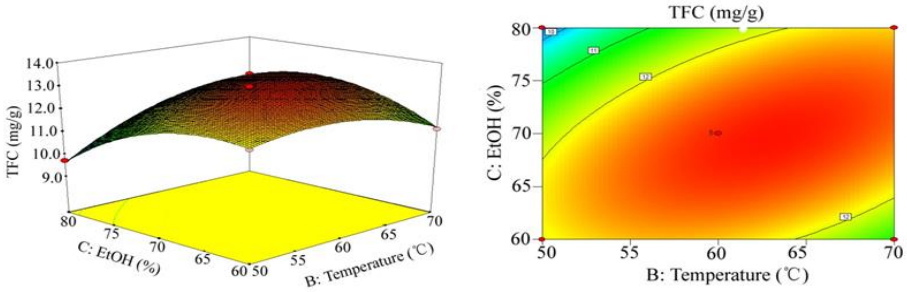

Figure 7. Response surface and contour plots for the effects of ethanol volume fraction and extraction temperature on flavonoids extraction efficiency

Optimization for process variables and verification: The optimum levels of variables were obtained by analyzing the response surface contour plots using Design-Expert 8.0. The optical extraction conditions were: 1:26.27 (g/mL) for solid-solvent ratio, 62.20°C for extraction temperature, 69.51% for ethanol concentration. On this station the predicted TFC was 13.02 mg/g. In order to test the predicated value and the theoretical value were consistent, considering the actual operating conditions, setting the solid-solvent ratio as 1:25 (g/mL), extraction temperature as 60°C, ethanol concentration as 70%, the TFC was 12.97 mg/g. Compared with the predicted value, the results showed that the response surface method was accurate and reliable.

Antioxidant assay results: The antioxidant assay results were shown in Figures 8-10. In the DPPH radical scavenging ability assay as showed in Figure 8, the TF and vitamin C both had perfect scavenging activity, and the scavenging ratio increased with the increasing of concentration. At the same concentration, the scavenging ratio of TF was significantly higher than that of vitamin C control group; the difference between the two groups was statistically significant ($P < 0.05$). The result indicates that the radical scavenging ability of TF was higher than the natural antioxidant vitamin C. The reducing power analysis found that the reducing power increased with the concentration of TF and Vitamin C, and indicated both of them had good reducing power. The Figure 9 showed, TF exhibited higher reducing power than the commercial antioxidants vitamin C significantly ($P<0.05$). In Figure 10, the TF and vitamin C both had hydroxyl radical scavenging activity, but the scavenging ability of TF was lower than that of the control group vitamin C ($P < 0.05$). These results implied that the TF of walnut branch had high antioxidant activity, even higher than vitamin C in some respects.

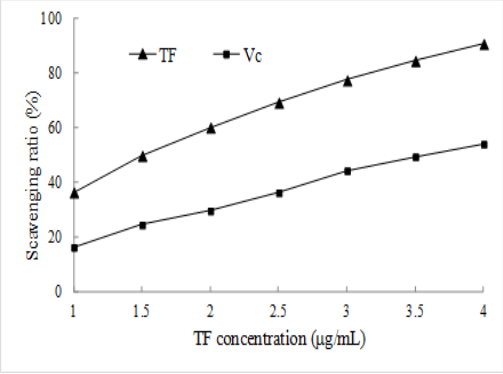

Figure 8. DPPH free radical scavenging capacity of walnut flavonoids

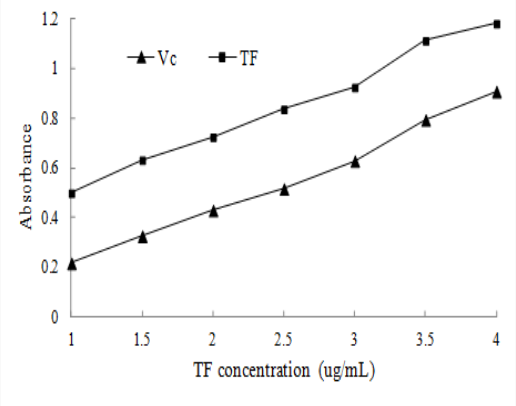

Figure 9. Total reducing power of walnut flavonoids

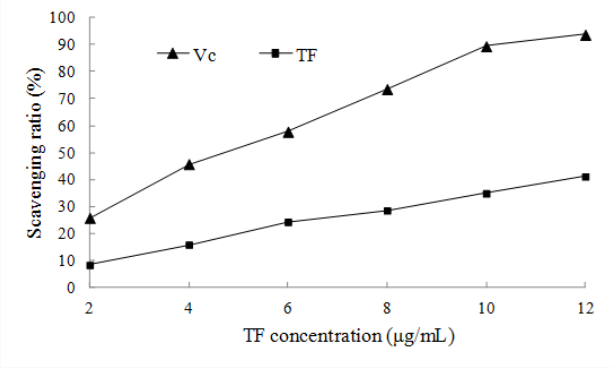

Figure 10. Hydroxyl free radical scavenging capacity of walnut flavonoids

CONCLUSION

In the present study, response surface methodology was used to optimize the optical condition for extraction of TF from walnut branch. Optimal conditions were found to be solid-solvent ratio 1:25 (g/mL), extraction temperature 60°C, ethanol concentration 70%, which gave a maximum TFC of 12.97 mg/g, similar to the predicted yield 13.02 mg/g.

From antioxidant assay, the TF showed strong DPPH radical scavenging activity, reducing power and hydroxyl radical scavenging activity.

This study has provided essential information for TF from walnut branch which is expected to be used as natural antioxidant.

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