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Response surface optimization of enzyme-assisted extraction of total flavonoids from wisteria and study on hydroxyl radicals scavenging effect

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ABSTRACT

The response surface methodology (RSM) and Box-Benhnken Design (BBD) were employed to optimize the extraction conditions of total flavonoids from Wisteria. The hydroxyl radicals scavenging and inactivity scavenging and inhibiting effect of Wisteria total flavonoids were also studied in this paper. The total flavonoids were extracted from Wisteria neck, leaf, flower and fruit clip by using enzyme-assisted ethanol refluxing. According to the single-factor experimental results, Enzyme concentration of 0.15~0.25mg·mL^{-1}, enzymatic pH of 5 ~ 7, enzymatic temperature of 45 ~ 65! and enzymatic times of 1.5 ~ 2.5h were selected as the independent variables and scope for BBD. The preferred extract conditions optimized by RSM. Hydroxyl radicals scavenging and inhibiting effect of Wisteria extracts were also measured. The optimum extract conditions optimized by RSM and BBD were enzyme concentration of 0.23 mg·mL^{-1}; enzymatic pH of 6, enzymatic temperature of 64 ! and times for 2.0h with responding extraction ratio of 0.94% for Wisteria neck, 4.60% for Wisteria leaf, 3.32% for Wisteria flowers and 5.16% for Wisteria fruit clip. The experimental extraction ratio matched well with the calculated values by solving the multiple regression equation. It confirms that the fitted quadratic model has a predictive effect on target extracts. The scavenging effect on hydroxyl radicals displayed a significant dose-effect relationship for Wisteria flavonoids, but it showed a weaker scavenging effect compare to BHT with the same concentration.

KEYWORDS

Response Surface Methodology;
Box-Benhnken Design;
Enzyme-assisted extraction;
Wisteria;
Total flavonoids; scavenging effect.

INTRODUCTION

Flavonoids compounds, refers a series of compounds including two benzene rings connected by the middle three carbon atoms. Flavonoids have significant antioxidant, anti-cancer, anti-inflammatory, bactericidal, anti-virus and regulating body immunity, and etc. It is a kind of Potential natural medicine with great prospects[1-4]. The Extraction technology of flavonoids reported in the literature includes organic solvent extraction, ultrasonic extraction, microwave extraction, supercritical fluid extraction and Enzyme-assisted extraction[5-6]. The dis-

solution of total flavonoid were bounded by the plant cell wall, Cellulase could degrade the cellulose backbone of plant cell walls and increase the dissolution of flavonoids. Cellulase Enzyme-assisted extraction of total flavonoids from plants has received widespread attention as their characteristics of simple operation, proposed extraction ratio. Yao X L reported Cellulase Enzyme-assisted extraction of flavonoids from hawthorn and the extraction ratio up to 2.67 ± 0.06% in the literature[7].

Wisteria, Wisteria genus, Legumes, which was widely cultivated in the east, central, south, northwest and southwest regions of china. Fu M R reported ultrasonic extraction phenols and total flavonoids from Wisteria flowers by using 70% methanol and the antioxidant effect of wisteria extract was also studied[8]. Jiang Y H reported acetone extraction of active ingredients from wisteria leaf, and the inhibitory effect on Melon Fusarium oxysporum and cabbage soft rot bacteria of the extract from Wisteria leaf was studied [9]. Up to now, there was no report pertaining to Enzyme-assisted extraction of total flavonoids from Wisteria.

Response Surface Methodology (RSM) was demonstrated an effective statistic technique for optimizing complex processes, which has been successfully used to optimize the total Flavonoids compounds from many medicine plants [10-11]. The total Flavonoids extraction ratio was greatly influenced by extraction conditions, Box-Behnken Design was performed to predict the optimal extraction conditions and analyze the sensitivity of the total flavonoids extraction rate to corresponding factors[12]. Here, cellulose Enzyme-assisted and ethanol reflux extraction of total flavonoids from Wisteria neck, leaf, flowers and fruit clip were reported. The Box-Benhnken Design (BBD) combines with Response Surface Methodology (RSM) were used to optimize the extraction conditions. The distribution of total flavonoids content in different parts of Wisteria was analyzed. The scavenging effect of Wisteria flavonoids on hydroxyl radicals was also studied. This research would provide valuable experimental data towards the extraction of flavonoids and comprehensive utilization of Wisteria flavonoids.

MATERIALS AND METHODS

Materials

Wisteria (collected in Chuxiong Normal University) → separation → dry → crush → Spare.

Experimental methods

Response Surface Optimization cellulose Enzyme-assisted and ethanol reflux extraction of total flavonoids from Wisteria and their scavenging effect on hydroxyl radicals were illustrated in Figure 1. Extraction of total flavonoids from Wisteria, Qualitative experiments of flavonoids compounds, and obtains of linear equations from Rutin standard curve were performed according to the literature[13-14].

Optimization of the extraction process by RSM

Optimization of extraction of Flavonoids by Response surface methodology was operated as References[13]. Box-Benhnken Design combining with quadratic response model of three factors and three levels were performed to optimize extraction. First

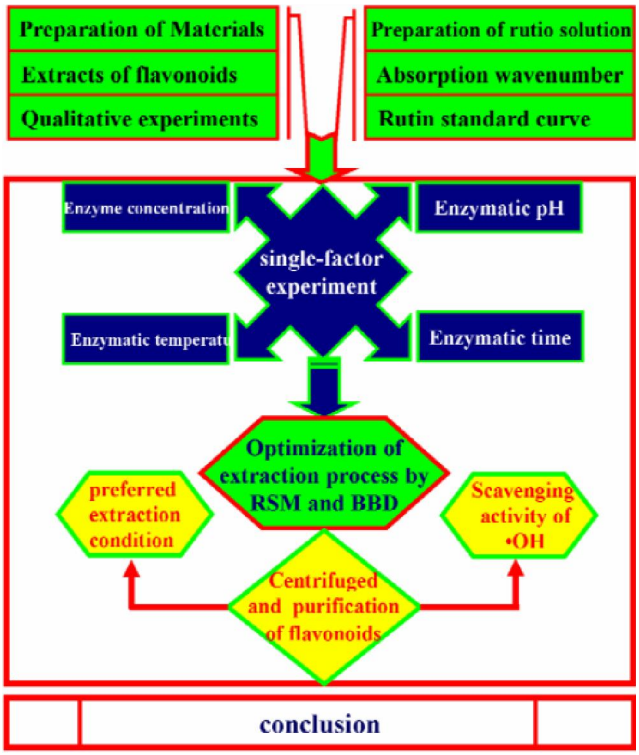

Figure 1 : The experimental illustration of RSM optimization of Wisteria flavonoids extraction and radical scavenging effect

TABLE 1 : Factors and levels of response surface methodology

Code levels of variables	variables			
	X_1: Enzyme concentration ($mg \cdot mL^{-1}$)	X_2 Enzymatic pH	X_3 Enzymatic temperature (℃)	X_3: enzymatic time (h)
-1	0.15	5	45	1.5
0	0.20	6	55	2.0
+1	0.25	7	65	2.5

determine the independent variables of three-factors, the level of variables were coded by -1, 0, 1 based on the results of single-factor experiment as shown in TABLE 1. A total of 31 points were designed, including points 16 factorial, 8 star points and 7 central points to ensure the precision of experiment.

Study on hydroxyl radical inhibition activity

Total flavonoids were extracted from Wisteria under the preferred conditions by RSM. The extracts were centrifuged, purification by macroporous resin, ethanol elution (ethanol volume fraction 78%), solvent evaporation, freeze-dried to obtain the total flavonoids powder. Wisteria total flavonoids solutions with different concentrations were prepared. Hydroxyl radical scavenging activity were operated as reference[13-15], Wisteria total flavonoids and BHT solutions with different concentrations were added, The absorbance were measured under 510nm, the average of absorbance were collected by parallel experiments. The scavenging ratio was calculated as:

The scavenging ratio of hydroxyl radical(%)

$$= [A_0 - (A_x - A_{x0})] / A_0 \times 100$$

A_0 is the absorbance of control solution, A_x was the absorbance of extract; A_{x0} was background absorbance of the extract without H_2O_2.

RESULTS AND DISCUSSION

Chromogenic reaction of Wisteria extracts was listed in TABLE 2, which was consistent with Rutin. It confirms that Wisteria contains total flavonoids.

The absorption spectra of Rutin were shown in Figure 2, 510nm was determined as the maximum absorption wavelength of flavonoids. The linear regression equation was formulated as A = 11.27857C-0.00136 R^2 = 0.99985 by the standard curve (Figure 2).

Result of single-factor experiment

Influence of each single factor on Wisteria flavonoids extraction ratio was shown in Figure 3. Extraction ratio was significantly increased with the in increasing of enzyme concentration, when the enzyme concentration was higher than 0.20$mg \cdot mL^{-1}$, extraction ratio began to decrease. The preferred enzyme concentration was 0.20$mg \cdot mL^{-1}$, lower than

TABLE 2 : Chromogenic reaction of flavonoids extracts and rutin solution

Reagents	HCl-Zn	$Al(NO_3)_3$	$FeCl_3$
Rutin	Pink	Yellow	Dark-green
Experimental phenomena	Pink	Yellow	green

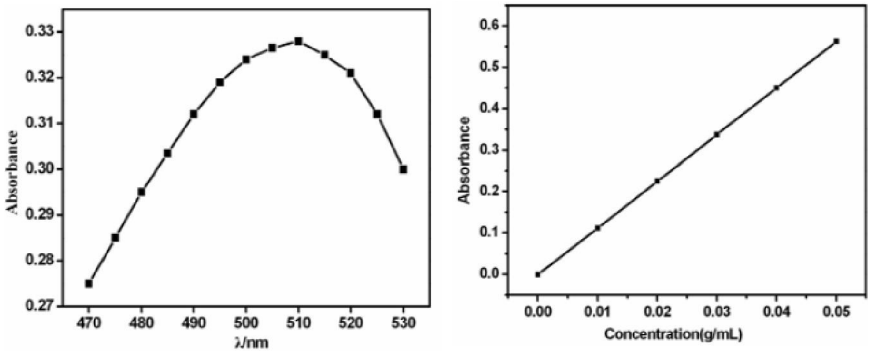

Figure 2 : The absorption spectrum of diagram of extracts and the standard curve of Rutin solution

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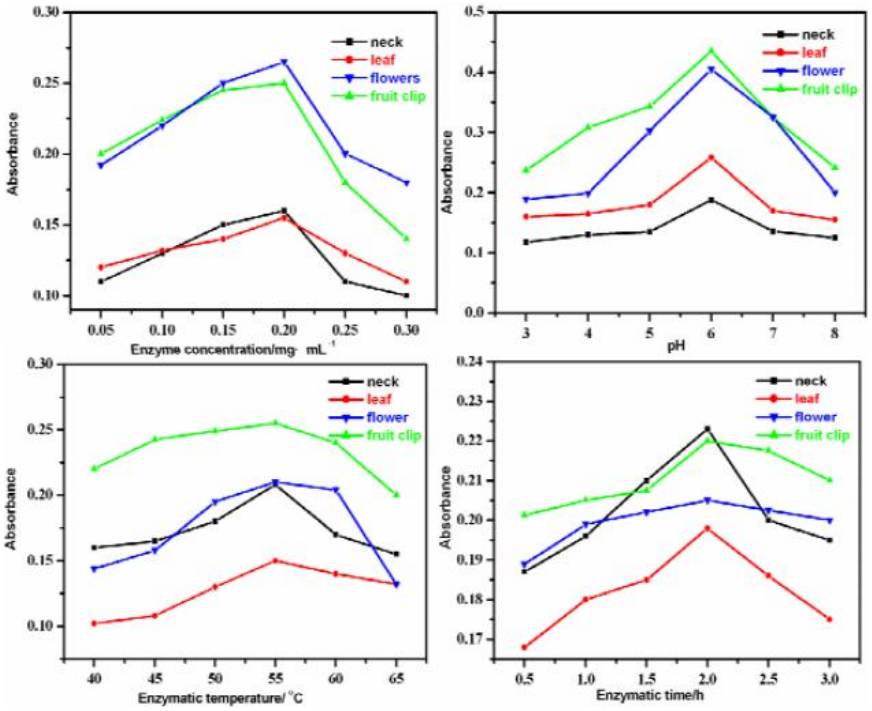

Figure 3 : Effect of enzyme concentration, enzymatic pH, enzymatic temperature and time on the extraction ratio of total flavonoids from Wisteria

this value, Enzymatic balance could not be achieved; Higher than this value, the concentration gradient of solid-liquid phase was two small, which was not conducive to the dissolution of total flavonoids. The addition of excessive cellulase enzyme resulted in a waste of materials. The impact of enzymatic pH on flavonoids extraction ratio of Wisteria was shown in Figure 4, with the pH value increasing, extraction ratio increased significantly, the maximum of extraction ratio was achieved as pH of 6.0. The reasons was analyzed as that enzyme activity was greatly influenced by the pH value, the maximum enzymatic activity was achieved at a neutral pH. Acid or alkaline system reduced enzymatic activity and resulted lower enzymatic efficiency. The optimum cellulase enzymatic pH was 6.0. The impact of enzymatic temperature on flavonoids extraction ratio of Wisteria was shown in Figure 4, with the temperature increasing, extraction ratio increased significantly, the maximum of extraction ratio was achieved at 55 !. The high temperature will lead to ethanol evaporation, reduce of enzyme activity and oxidative degeneration of Flavonoids. The lower temperature decreases the dissolution rate of flavonoids. So the optimum enzymatic temperature was 55 ! for extraction total flavonoids from Wisteria. The optimum enzymatic time for extraction total flavonoids from Wisteria was 2.0h. Lower than 2.0h, the dissolution balance could not be reached, higher than 2.0h, the dissolution of other fat-soluble impurities complicated the post-separation and purification of flavonoids.

According to the results of single factor experiments, response surface analysis to determine the factors and scope of enzyme concentration of 0.15~0.25mg·mL^{-1}; enzymatic pH of 5 ~ 7, enzymatic temperature of 45 ~ 65 ! and times for 1.5 ~ 2.5h.

Response surface optimization of extraction process

Multiple regression model and analysis of variance (ANOVA)

The extraction process of total flavonoids from Wisteria was further optimized by RSM. According to the single-factor experimental results of 3, Enzyme concentration of 0.15~0.25mg·mL^{-1}, enzymatic pH of 5 ~ 7, enzymatic temperature of 45 ~ 65! and enzymatic times of 1.5 ~ 2.5h were selected as the actual levels of variables to maximize the extraction ratio of total flavonoids by Box-Benhnken design, as listed in TABLE 1. A total of 31 experi-

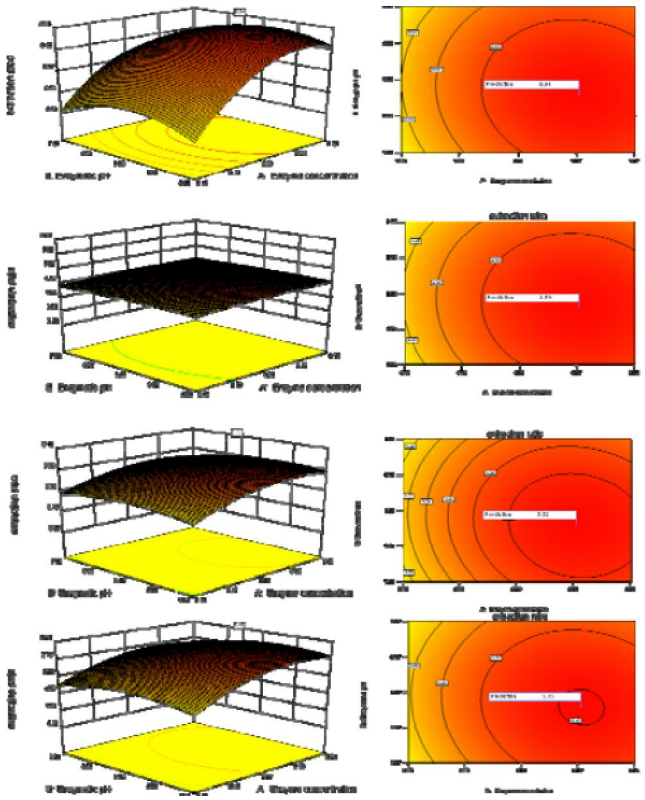

Figure 4 : RSM analyses for interactive effects of enzyme concentration and enzymatic pH

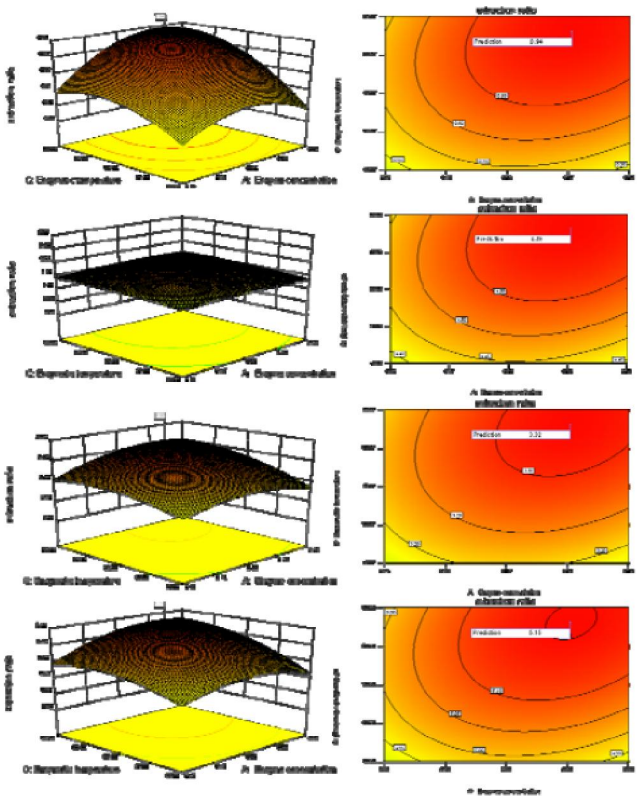

Figure 5 : RSM analyses for interactive effects of enzyme concentration and enzymatic temperature

ments were designed, including 16 factorial experiments, 8 star experiments and 7 central experiments to estimate the errors.

The RSM experimental design and results of total flavonids extraction ratio from Wisteria were shown in TABLE 3. Total flavonids extraction ratio ranged from 0.78 to 0.94 for Wisteria neck, from 3.82 to 4.58 for Wisteria leaf, from 2.76 to 3.30 for Wisteria flower and from 4.29 to 5.13 for Wisteria fruit clips. The maximum of Total flavonids extraction ratio was recorded under the experimental conditions of enzyme concentration of 0.15mg·mL^{-1}, enzymatic pH of 6, enzymatic temperature of 55! and enzymatic times of 1.5h. The experimental data was analyzed by RSM using Design-Expert8.0 software, the response variable of total flavonoids extract ratio and the four factors were related by the following multiple regression equation:

Extract ratio = 0.93+0.0076*A+0.00080* B+0.01085* C+0.0032*D-0.0018*A*B+0.0066*A*C+0.0048*A*D -0.0042*B*C+1.25E-06*B*D-0.013*C*D-0.012*A^2-0.0081*B^2-0.010*C^2-0.032*D^2 (for Wisteria neck by code)

Extract ratio = 4.56+0.037*A+0.0039*B+0.053*C+0.016*D-0.0088*A*B+0.032*A*C+0.024*A*D-0.021*B*C+6.25E-07*B*D-0.065*C*D-0.061*A^2-0.040*B^2-0.049*C^2-0.15*D^2 (for Wisteria leaf by code)

Extract ratio = 3.29+0.027*A+0.0028*B+0.038*C+0.011*D-0.0064*A*B+0.023*A*C+0.017*A*D-0.015*B*C+6.25E-0

7*B*D-0.047*C*D-0.044*A^2-0.029*B^2-0.035*C^2-0.11*D^2 (for Wisteria flower by code)

Extract ratio =5.12+0.042*A+0.0044*B+0.060*C+0.018*D-0.0099*A*B+0.036*A*C+0.026*A*D-0.023*B*C-0.073* C*D-0.068*A^2-0.045*B^2-0.055*C^2-0.17*D^2 (for Wisteria fruit clip by code)

TABLE 4 shows the analysis of variance (ANOVA) for the multiple regression equation, the linear term (except for B and D) and quadratic term were significant for response variable. While in the interaction terms, only the interaction of enzymatic temperature and times terms (CD) was significant, indicating the response variable (the extraction ratio of total flavonoids) and the four test factor were not a simply linear relationship. The lack of fit for

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TABLE 3 : Experimental design and results for extraction ratio from Wisteria by using box-behnken and RSM

		Variables				Response 1 extraction ratio (%)			
Std	Run	X_1: Enzyme concentration ($mg \cdot mL^{-1}$)	X_2 Enzymatic pH	X_3 Enzymatic temperature (℃)	X_3: enzymatic time (h)	neck	leaf	flower	Fruit clip
20	1	0.20	6.00	55.00	2.00	0.93	4.55	3.29	5.11
27	2	0.15	7.00	65.00	1.50	0.88	4.29	3.10	4.82
23	3	0.20	6.00	75.00	2.00	0.92	4.48	3.23	5.03
12	4	0.15	7.00	45.00	2.50	0.88	4.32	3.12	4.84
18	5	0.25	5.00	65.00	2.50	0.90	4.39	3.17	4.92
17	6	0.25	7.00	65.00	1.50	0.88	4.32	3.12	4.84
24	7	0.20	6.00	35.00	2.00	0.86	4.22	3.05	4.74
28	8	0.20	6.00	55.00	2.00	0.93	4.55	3.29	5.11
11	9	0.20	4.00	55.00	2.00	0.90	4.39	3.17	4.92
5	10	0.20	6.00	55.00	2.00	0.94	4.60	3.32	5.16
4	11	0.10	6.00	55.00	2.00	0.88	4.29	3.10	4.82
21	12	0.15	5.00	65.00	2.50	0.86	4.22	3.05	4.74
19	13	0.25	7.00	45.00	2.50	0.90	4.39	3.17	4.92
22	14	0.25	5.00	65.00	1.50	0.91	4.46	3.22	5.00
14	15	0.25	5.00	45.00	1.50	0.83	4.08	2.95	4.58
6	16	0.20	6.00	55.00	2.00	0.93	4.55	3.29	5.11
7	17	0.20	6.00	55.00	2.00	0.94	4.58	3.30	5.13
9	18	0.15	5.00	45.00	2.50	0.87	4.27	3.08	4.79
1	19	0.15	5.00	45.00	1.50	0.83	4.06	2.93	4.55
15	20	0.25	7.00	45.00	1.50	0.84	4.13	2.98	4.63
29	21	0.25	5.00	45.00	2.50	0.89	4.36	3.15	4.90
8	22	0.15	7.00	45.00	1.50	0.85	4.15	3.00	4.66
16	23	0.25	7.00	65.00	2.50	0.91	4.43	3.20	4.97
30	24	0.30	6.00	55.00	2.00	0.88	4.32	3.12	4.84
25	25	0.15	5.00	65.00	1.50	0.87	4.25	3.06	4.76
13	26	0.20	6.00	55.00	3.00	0.78	3.82	2.76	4.29
3	27	0.20	6.00	55.00	2.00	0.93	4.53	3.27	5.08
26	28	0.15	7.00	65.00	2.50	0.85	4.15	3.00	4.66
2	29	0.20	6.00	55.00	2.00	0.94	4.58	3.30	5.13
10	30	0.20	6.00	55.00	1.00	0.82	4.03	2.91	4.52

ANOVA was significant ($p=0.0014<0.001$), indicating that the model could adequately fit the experimental data. The adequate precision value of 12.485, greatly higher than the desirable 4.00, which presented a higher "signal (response) to noise (deviation)" and indicated that the model was significant for the extraction process of total flavonoids from Wisteria. The value of R^2(0.916) and R_{Adj}^2(0.843) for the multiple regression equation was approaching and close to 1, indicated a high degree of cor-

TABLE 4-1 : The ANVOA analysis results of response surface quadratic model for Wisteria neck

Source of deviation	Sum of Squares	DF	Mean Square	F Value	P-value Prob > F	Significant
Model	1.235276	14	0.088234	12.48045	<0.0001	***
A	0.04213	1	0.04213	5.959111	0.0266	*
B	0.000467	1	0.000467	0.066021	0.8005	
C	0.085076	1	0.085076	12.03368	0.0032	**
D	0.007469	1	0.007469	1.056536	0.3193	
AB	0.001575	1	0.001575	0.222821	0.6433	
AC	0.02118	1	0.02118	2.995911	0.1027	
AD	0.011203	1	0.011203	1.584655	0.2261	
BC	0.008578	1	0.008578	1.213399	0.287	
BD	6.66E-16	1	6.66E-16	9.42E-14	1	
CD	0.084725	1	0.084725	11.98406	0.0032	**
A^2	0.131789	1	0.131789	18.64114	0.0005	***
B^2	0.057219	1	0.057219	8.093525	0.0117	*
C^2	0.085424	1	0.085424	12.08292	0.0031	**
D^2	0.863115	1	0.863115	122.0851	< 0.0001	***
Residual	0.113117	16	0.00707			
Lack of Fit	0.109116	10	0.010912	16.36429	0.0014	**
Pure Error	0.004001	6	0.000667			
Cor Total	1.348393	30				
R-Squared	0.916		Adj R-Squared	0.843		
Adeq Precision	12.485		C.V. %	1.732		

relation between the experimental and predicted values and the model is suitable. The lower value of coefficient of the variance (C.V.=1.732%) indicated a good reproducibility of the model.

The result of analysis of variance (ANOVA) showed that significant levels of the four factors were sorted by > enzyme concentration> enzymatic times > enzymatic pH. The quadratic terms of enzyme concentration and enzymatic times were extremely significant for response variable; the linear and quadratic terms of enzymatic temperature, interaction term of enzymatic temperature and time, had a very significant impact on the response variable; while the linear term of enzyme concentration and quadratic term of enzymatic pH were significant for response variable.

RSM analysis and research on the optimum extract process

The multiple regression models could be vividly reflected by the three-dimentional response surface and Contour lines plots, as shown in Figure 4-9. The 3D response surface plots intuitively reflected the effects of multiple independent variables on the response variable, the sensitivity of response variable to different arguments could be further analyzed. In the Contour lines plots, the closer the curve to the center, the greater of the value corresponding response variable; contour lines with circular indicated weak interactions between independent variables, contour lines with oval indicated strong interaction between independent variables. An increase of enzyme concentration (A) and enzymatic

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TABLE 4-2 : The ANVOA analysis results of response surface quadratic model for Wisteria leaf

Source of deviation	Sum of Squares	DF	Mean Square	F Value	P-value Prob > F	Significant
Model	0.981689	14	0.070121	12.48009	<0.0001	***
A	0.033483	1	0.033483	5.959277	0.0266	*
B	0.000371	1	0.000371	0.066015	0.8005	
C	0.06761	1	0.06761	12.03327	0.0032	**
D	0.005935	1	0.005935	1.056301	0.3193	
AB	0.001252	1	0.001252	0.222817	0.6433	
AC	0.016833	1	0.016833	2.995964	0.1027	
AD	0.008903	1	0.008903	1.584619	0.2261	
BC	0.006817	1	0.006817	1.213215	0.287	
BD	6.25E-12	1	6.25E-12	1.11E-09	1	
CD	0.067331	1	0.067331	11.98363	0.0032	**
A^2	0.104736	1	0.104736	18.6409	0.0005	***
B^2	0.045476	1	0.045476	8.09375	0.0117	*
C^2	0.067889	1	0.067889	12.08291	0.0031	**
D^2	0.685925	1	0.685925	122.0811	< 0.0001	***
Residual	0.089898	16	0.005619			
Lack of Fit	0.086718	10	0.008672	16.36217	0.0014	**
Pure Error	0.00318	6	0.00053			
Cor Total	1.071586	30				
R-Squared	0.916		Adj R-Squared	0.843		
Adeq Precision	12.485		C.V. %	1.732		

temperature (C) resulted in a monotonous increase of response variable to a maximum at a certain levels; while an increase of enzymatic pH (B) and enzymatic times (D) resulted in an initial increase and then decrease of response variable.

The interaction effects of enzyme concentration and enzymatic pH on the extraction ratio of Wisteria flavonoids were shown in Figure 4. In the 3D response surface plots, the corresponding surfaces of enzyme concentration were steeper, indicated its significant impact on the response value of extraction ratios. Contour lines plots were circular-like, indicated the interaction effects of enzyme concentration and enzymatic pH on the extraction ratio were not obvious P = 0.6433. When the enzymatic pH of 5.8 was fixed, the maximum of extraction ratios were obtained as the enzyme concentration of 0.23 mg·mL^{-1}. Figure 5 showed the interaction effect of enzyme concentration and enzymatic temperature on the extraction ratios of Wisteria flavonoids, the corresponding surfaces of enzymatic temperature were steeper, indicated its extremely significant impact on the response value of extraction ratio. Contour lines plots were close to Oval, indicated the interaction effect of enzyme concentration and enzymatic temperature on the response values were a little significant P = 0.1027. When the enzyme concentration of 0.23 mg·mL^{-1} was fixed, the maximum of extraction ratios were obtained as the enzymatic temperature of 63°C.

The 3D response surface and Contour lines plots of the interaction effect of enzyme concentration and enzymatic time on the response values of extraction ratio were shown in Figure 6, the corresponding sur-

TABLE 4-3 : The ANVOA analysis results of response surface quadratic model for Wisteria flower

Source of deviation	Sum of Squares	DF	Mean Square	F Value	P-value Prob > F	Significant
Model	0.511374	14	0.036527	12.48028	< 0.0001	***
A	0.01744	1	0.01744	5.958971	0.0266	*
B	0.000193	1	0.000193	0.066004	0.8005	
C	0.03522	1	0.03522	12.03379	0.0032	**
D	0.003092	1	0.003092	1.056295	0.3193	
AB	0.000652	1	0.000652	0.222829	0.6433	
AC	0.008768	1	0.008768	2.995807	0.1027	
AD	0.004638	1	0.004638	1.584675	0.2261	
BC	0.003551	1	0.003551	1.213382	0.287	
BD	6.25E-12	1	6.25E-12	2.14E-09	1	
CD	0.035075	1	0.035075	11.98419	0.0032	**
A^2	0.054555	1	0.054555	18.64001	0.0005	***
B^2	0.023687	1	0.023687	8.093197	0.0117	*
C^2	0.035362	1	0.035362	12.08247	0.0031	**
D^2	0.35731	1	0.35731	122.0842	< 0.0001	***
Residual	0.046828	16	0.002927			
Lack of Fit	0.045172	10	0.004517	16.36321	0.0014	**
Pure Error	0.001656	6	0.000276			
Cor Total	0.558202	30				
R-Squared	0.916		Adj R-Squared	0.843		
Adeq Precision	12.485		C.V. %	1.732		

face of enzyme concentration was steeper, indicated its impact on the response value was more significant than enzymatic time. Contour lines plots were close to Oval, indicated the interaction effect of enzyme concentration and enzymatic temperature on the response values were a little significant P = 0.2261. When the enzyme concentration of 0.23 $mg \cdot mL^{-1}$ was fixed, the maximum of extraction ratios were obtained as the enzymatic temperature of 1.96 h. The interaction effects of enzymatic pH and enzymatic temperature on the extraction ratio were shown in Figure 7. The impact of enzymatic temperature on the extraction ratio were more significant than that of enzymatic pH, as the corresponding surfaces of enzymatic temperature were steeper in the 3D response surface plots. From Contour lines plots, the maximum of extraction ratios were obtained as the enzymatic pH of 5.8 and enzymatic temperature of 63°C.

The effects of enzymatic pH and enzymatic time on the response values of extraction ratios were not significant as shown in Figure 8. the interaction effects of the two independent variables on the extraction ratios were also not obvious from the Contour lines plots. The 3D response surface and Contour lines plots of the interaction effect of enzymatic temperature and enzymatic time on the response values were shown in Figure 9, the corresponding surface of enzymatic temperature was more steeper, indicated its impacts on the response value were more significant than enzymatic time. Contour lines plots were Oval-like, indicated the interaction effect of enzyme concentration and enzymatic temperature on the response values were extremely significant with

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TABLE 4-4 : The ANVOA analysis results of response surface quadratic model for Wisteria fruit clip

Source of deviation	Sum of Squares	DF	Mean Square	F Value	P-value Prob > F	Significant
Model	1.235276	14	0.088234	12.48045	< 0.0001	***
A	0.04213	1	0.04213	5.959111	0.0266	*
B	0.000467	1	0.000467	0.066021	0.8005	
C	0.085076	1	0.085076	12.03368	0.0032	**
D	0.007469	1	0.007469	1.056536	0.3193	
AB	0.001575	1	0.001575	0.222821	0.6433	
AC	0.02118	1	0.02118	2.995911	0.1027	
AD	0.011203	1	0.011203	1.584655	0.2261	
BC	0.008578	1	0.008578	1.213399	0.287	
BD	6.66E-16	1	6.66E-16	9.42E-14	1	
CD	0.084725	1	0.084725	11.98406	0.0032	**
A^2	0.131789	1	0.131789	18.64114	0.0005	***
B^2	0.057219	1	0.057219	8.093525	0.0117	*
C^2	0.085424	1	0.085424	12.08292	0.0031	**
D^2	0.863115	1	0.863115	122.0851	< 0.0001	***
Residual	0.113117	16	0.00707			
Lack of Fit	0.109116	10	0.010912	16.36429	0.0014	**
Pure Error	0.004001	6	0.000667			
Cor Total	1.348393	30				
R-Squared	0.916		Adj R-Squared	0.843		
Adeq Precision	12.485		C.V. %	1.732		

Noteÿ*P< 0.001ÿ**P< 0.01ÿ*P< 0.05**

P = 0.0032. The maximum of extraction ratios were obtained as enzymatic temperature of 63!and enzymatic time of 1.96 h.

The optimum values of the selected variables were obtained by solving the multiple regression equation. The values obtained were A=0.23 mg·mL^{-1}, B=5.79, C=63.09 ℃ and D=1.96 h, with the corresponding response variable value of 0.94% for Wisteria neck, 4.59% for Wisteria leaf, 3.32% for Wisteria flowers and 5.15% for Wisteria fruit clip, calculated by design-expert 8.0 software. In the experiment, the preferred extract conditions were determinated as enzyme concentration of 0.23 mg·mL^{-1}; enzymatic pH of 6, enzymatic temperature of 64 ℃ and times for 2.0h. Three triplicate experiments were performed under the preferred extract conditions to confirm the experimental data, the average values of total flavonoids extraction ratio were 0.94% for Wisteria neck, 4.60% for Wisteria leaf, 3.32% for Wisteria flowers and 5.16% for Wisteria fruit clip. The experimental data were listed in TABLE 5, the experimental and calculated values of response variable matched each other very well, which indicated that the model were reliable for the extraction process of total flavonoids from Wisteria.

Study on hydroxyl radical scavenging activity of Wisteria extracts

The Flavonoids compounds had a scavenging and inhibiting effect on hydroxyl radical as the Prodihydroxy from the structural Benzene ring. As operated in the literature, Hydroxyl radical scavenging activities of Wisteria flavonoids and BHT with different concentrations were measured. The result

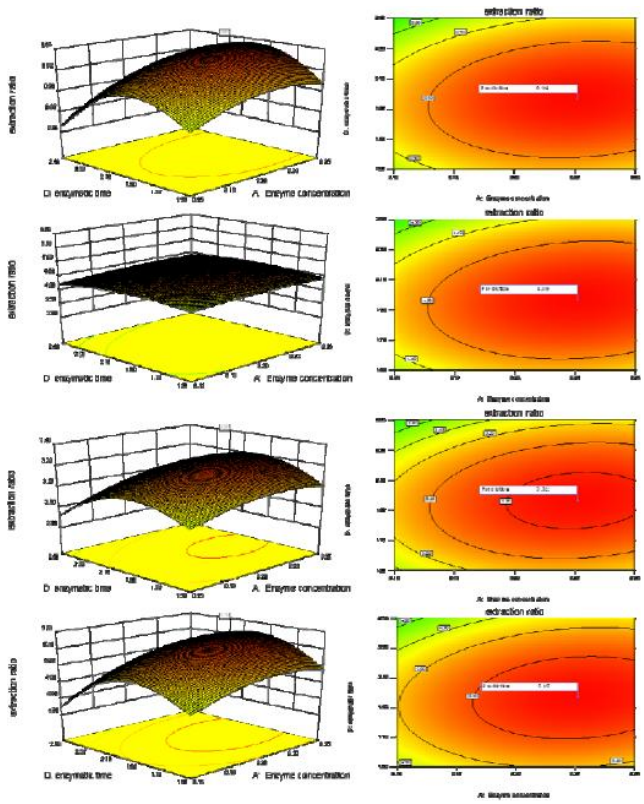

Figure 6 : RSM analyses for interactive effects of enzyme concentration and enzymatic time

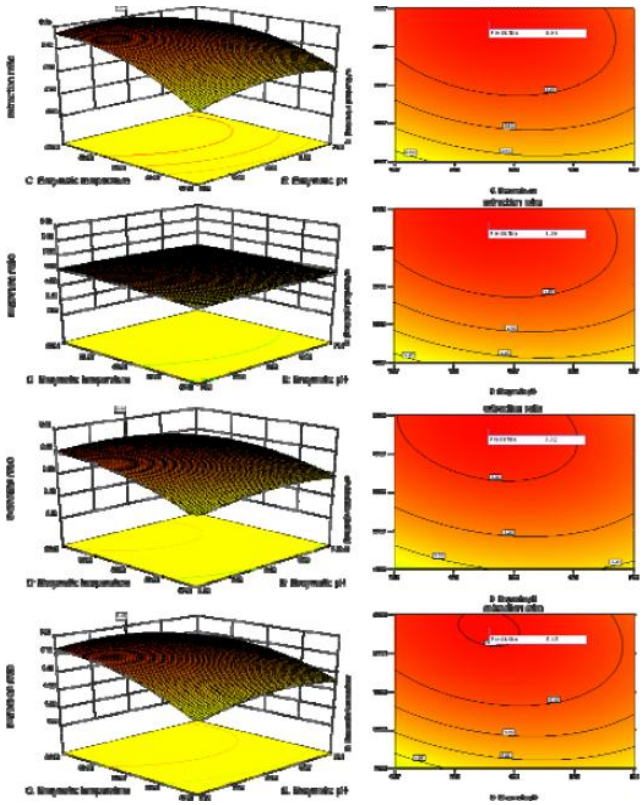

Figure 7 : RSM analyses for interactive effects enzymatic pH and enzymatic temperature

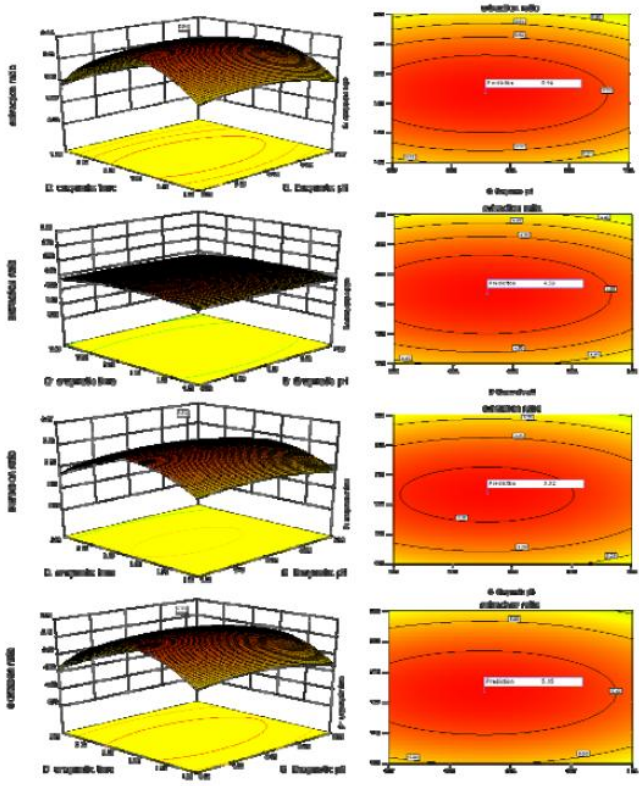

Figure 8 : RSM analyses for interactive effects of enzymatic pH and enzymatic time

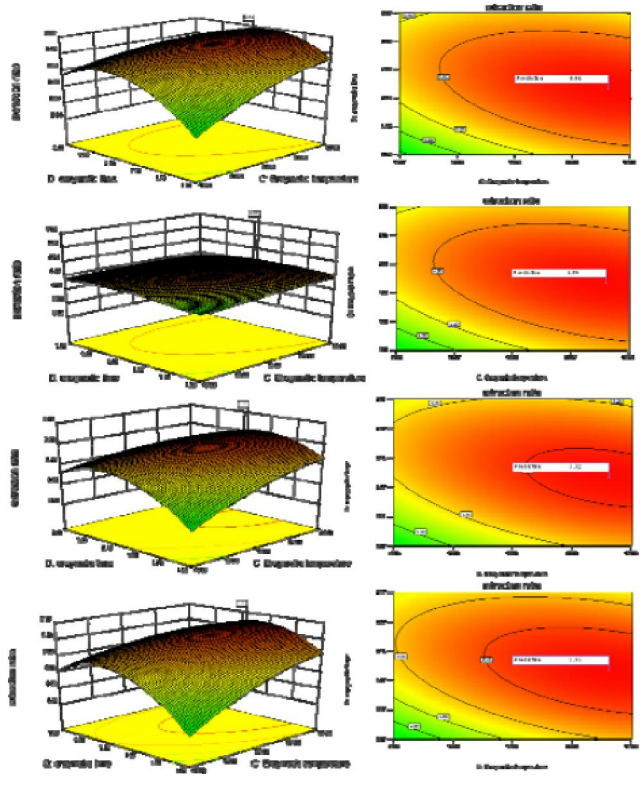

Figure 9 : RSM analyses for interactive effects of enzymatic temperature and enzymatic time

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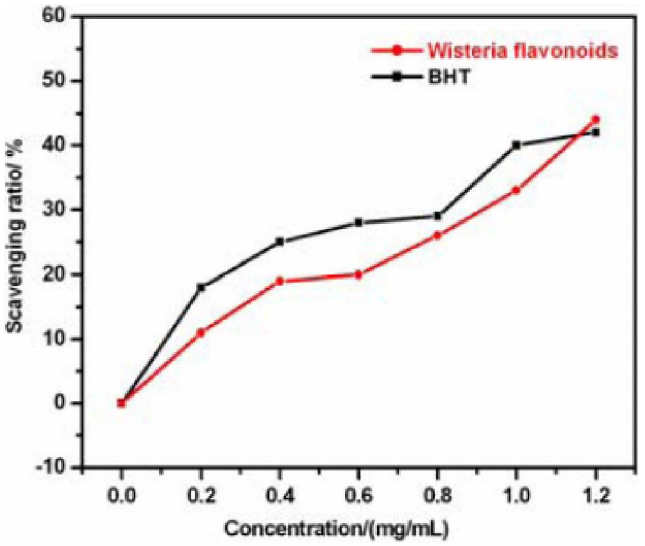

Figure 10 : Scavenging activity comparison of Wisteria extracts and BHT

was listed in Figure 10. As the concentration increasing of total flavonoids and BHT, the scavenging ratio for hydroxyl radicals increased, which showed a significant degree of dose-effect relationship. But the Wisteria flavonoids showed a weaker scavenging effect compare to BHT with the same concentration. The reasons were analyzed as follows: first, o-dihydroxy from benzene rings were partly methylated, leading to the reduction of inhibitory activity on hydroxyl radical[14-16]. Second, the lack of necessary separation and identification for Wisteria total flavonoids, and the presence of impurities also affected its scavenging and inhibiting effect on hydroxyl radical.

CONCLUSIONS

The response surface methodology (RSM) and Box-Benhnken design were successfully employed to optimize the extraction conditions of flavonoids from Wisteria. The enzyme concentration and enzymatic temperature significantly influenced the extraction ratio of total flavonoids form wisteria, while the impact of enzymatic ph value and enzymatic time for the extraction ratio were not significant. According to the single-factor experimental results, Enzyme concentration of 0.15~0.25mg·mL^{-1}, enzymatic pH of 5 ~ 7, enzymatic temperature of 45 ~ 65°C and enzymatic times of 1.5 ~ 2.5h were selected as the independent variables and scope for response surface analysis. The preferred extract conditions optimized by RSM and Box-Benhnken design were enzyme concentration of 0.23 mg·mL^{-1}; enzymatic pH of 6, enzymatic temperature of 64 °C and times for 2.0h with responding extraction ratio of 0.94% for Wisteria neck, 4.60% for Wisteria leaf, 3.32% for Wisteria flowers and 5.16% for Wisteria fruit clip. The experimental extraction ratio matched well with the calculated values by solving the multiple regression equation. The result of hydroxyl radicals scavenging activity experiment displayed a significant dose-effect relationship for Wisteria flavonoids, but Wisteria flavonoids showed a weaker scavenging effect compare to BHT with the same concentration. The isolation, purification and structure identification of Wisteria total flavonoids extracts, relationship between antioxidant activity and structure of flavonoids, and related research work is underway.

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