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Optimization of ultrasound assisted extraction method for polyphenols from *Desmodium triquetrum* (L.) DC. with response surface methodology (RSM) and *in vitro* determination of its antioxidant properties

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Abstract: The response surface method was used to study the ultrasonic extraction of traditional Chinese medicine *Desmodium triquetrum* (L.) DC. phenolic acid. By measuring the total phenolic content, the liquid/solid ratio, ultrasonic power, temperature, time and ethanol solubility were determined to be the significant influencing factors. The total phenolic content reached the highest value (30.3708 mg g^{-1}) under the conditions of the liquid/solid ratio 30%, ultrasonic power 160 w, temperature 40 °C, time 20 min, and ethanol solubility 60%, compared with the traditional boiling method. The total phenolic content was improved, and it was close to the predicted value (29.6548 mg g^{-1}), which proves that the scheme is feasible. After testing, the phenolic acid extracted under these conditions has a good antioxidation effect. The study suggests that ultrasonic extraction methods have the potential to extract antioxidants from traditional Chinese medicines. Also, the influence parameters affecting the process can be further optimized for industrial production.

Keywords: ultrasonic extraction; antioxidant activity; phenolic acids; Chinese herbal medicine

Desmodium triquetrum (L.) DC. is a traditional Chinese herb, mainly distributed in southern China. It has the functions of clearing away heat and toxic material, removing dampness through diuresis, insecticidal and anticorrosion properties. It can be used to prevent heatstroke, treat cold, fever, sore throat, nephritis, etc. However, there are few relevant reports found around the world (Jupudi et al. 2019).

Currently, reports focus on its chemical composition. The chemical constituents are mainly flavonoids, phenols, saponins, phenylpropanoids, and triterpenoids. According to the current literature, there are more than 50 compounds found (Nong et al. 2015). Among them, antioxidant activity mainly comes from phenolic acids and flavonoids. These antioxidant-resistant phenolic acids can be used in functional foods (Pejin et al. 2013; Tarun et al. 2019; Vedpal et al. 2019).

The traditional method can extract the active ingredient from *Desmodium triquetrum* (L.) DC. by boiling. At the same time, the influence of process parameters

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on the extract considers only a single factor, this does not maximize its antioxidant properties. However, these techniques are time-consuming, requiring large amounts of solvent compounds in the process due to hydrolysis, ionization, and oxidation. Most of the active ingredients of Chinese herbal medicines are present in plant cells. It is difficult to destroy the cell wall and cell membrane by the traditional method so that the active component completely diffuses the cavitation effect of the ultrasonic wave in the solvent. The mechanical effect and the thermal effect can effectively destroy the cell, accelerate the release, diffusion, and dissolution of the effective substance in the cell, and improve the extraction rate (Tatake et al. 2002). The effect of sonication on the total phenolic content (TPC) and antioxidant capacity of defatted cannabis, flaxseed, and rapeseed cakes has been reported. Compared with traditional methods, the extraction rate and antioxidant capacity of ultrasonic treatment of polyphenols at room temperature are their double (Teh et al. 2014). At the same time, the application of ultrasonic extraction of the active ingredients of Chinese herbal medicines contributes to their industrial production because it reduces the use of organic solvents and shortens the extraction time (Shirsath et al. 2017).

The response surface methodology (RSM) comes from a combination of mathematics and modern statistics. It is used to study the functional relationship between response variables and independent variables (one or more influence factors). The functional relationship is displayed by graphical techniques, and further visual analysis can be performed to determine the best conditions or the best areas (Qadir et al. 2019). There have been many reports on the use of response surface methodology to optimize experimental conditions, but to the best of the author's knowledge, no research has so far involved the use of response surface methods to optimize the extraction of polyphenols from *Desmodium triquetrum* (L.) DC.

In this study, ethanol solvents were used as a more environmentally friendly method to optimize the ultrasonic extraction process of polyphenols in *Desmodium triquetrum* (L.) DC. The effects of liquid-solid ratio (L/S), temperature, treatment time, ultrasonic power, and ethanol concentration on the total phenolic compounds were studied, and their antioxidant properties were determined. Compared with the traditional boiling method, the extraction efficiency of ultrasonic treatment was studied, which laid the foundation for the industrialization of *Desmodium triquetrum* (L.) DC. polyphenol extraction.

MATERIAL AND METHODS

Desmodium triquetrum (L.) DC. was purchased from Taihua Pharmacy (China). After grinding, it was passed through a 50 mesh sieve and sealed at 4 °C for use. Chemicals were obtained such as Azabis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH) purchased from Tokyo chemical industry (Japan). Gallic acid, rutin, and Folin-Ciocalteu phenol reagents were obtained from Beijing Solarbio Biotechnology (China). Sodium nitrite, aluminium nitrate, sodium hydroxide, ethanol, sodium carbonate, potassium persulfate, iron (II) sulphate heptahydrate, and hydrogen peroxide were obtained from Tianjin Biotechnology (China). Salicylic acid comes from Shanghai Macklin Biochemical (China).

Grinding machine (800Y) was from Huangdai (China). Digital ultrasonic equipment (KQ-250DE, temperature control, adjustable power, frequency 40 kHz) was from Shumei (China) and a spectrophotometer (WFJ 2100) from Shanghai Unico (China). Digital thermostat water bath (HH-S4) came from Jintan Medical Equipment (China) and Eppendorf AG 22331 Hamburg (5418) centrifuge thermocycler was made in Germany.

Determination of total phenolic content (TPC)

The TPC of the sample was measured by the improved colorimetric Folin-Ciocalteu method (Wolfe et al. 2003). The extract of 0.1 mL was accurately taken in a 10 mL volume bottle, distilled water was added to the total volume of 3.9 mL, and 0.2 mL of the Folin phenol reagent was also added and shook well. After that, 2 mL of Na_2CO_3 (60%) solution was added right after 5 min, and distilled water was added to a constant volume of 10 mL. After thoroughly shaking, the extract was let stand for 30 min, and the absorbance was measured at 760 nm wavelength. The standard curve was drawn with gallic acid as a standard sample, and the total phenol content was expressed by milligram gallic acid equivalents per gram of dry sample:

$$\omega\ (\text{m mg}^{-1}) = \frac{GA \times V \times N}{1\ 000\ M} \quad (1)$$

where: GA – gallic acid content (μg mL^{-1}), V – volume (mL) of the extract added, N – dilution factor, and M – sample amount (g).

Traditional extraction method. In order to compare with the ultrasonic treatment, phenolic acid in *Desmodium triquetrum* (L.) DC. was extracted by a conventional boiling method. A 50-gram sample was mixed in 150 mL water, placed on an electric stove, boiled for 60 min and

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Table 1. Range of different factors investigated with Plackett-Burman design (PBD)

Variable	Low (–)	High (+)
X_1 (%)	20	30
X_2 (w)	160	240
X_3 (°C)	40	70
X_4 (%)	30	50
X_5 (min)	20	30
X_6, X_7, X_8, X_9, X_{10}, X_{11}	–	–

X_1 – liquid/solid ratio; X_2 – ultrasonic power; X_3 – temperature; X_4 – ethanol concentration; X_5 – extraction time; X_6, X_7, X_8, X_9, X_{10}, X_{11} – virtual factors

then filtered. After filtration, 150 mL of water were added again, boiled, filtered, and then mixed the filtrate, which aims to extract effective components as much as possible. The extract was centrifuged for 10 min (12 000 rpm min^{-1}), the supernatant was taken and concentrated to 150 mL and stored at 4 °C.

Plackett-Burman design (PBD). Ultrasonic extraction experiments were performed on PBD using Design-Expert software (Stat-Ease Inc., USA) (Table 1). The five main factors of the extraction process were screened: L/S, temperature, time, ultrasonic power and ethanol concentration, plus six dummy variables. Each variable was determined at two levels (+) and (–), and a total of 12 experiments was performed to determine the importance for each factor.

Optimization of experimental design – Box-Behnken Design (BBD). Using the Design-Expert software, the BBD method was used to experimentally design the three significant influencing factors selected by PBD (Table 2), while fixing other non-critical factors. Using the total phenolic content as a response, the response surface and contour plots of the model predictive response are used to determine the variables that produce the maximum (Myers et al. 1989).

Hydroxyl radical scavenging assay. According to the principle of Fenton reaction, the method is as follows: 1 mL of test solution was mixed with 1 mL of ferrous sulphate solution ($FeSO_4 \cdot 7H_2O$, 9 mmol L^{-1}), after that 1 mL of hydrogen peroxide solution (10 mmol L^{-1}), which was incubated at 37 °C for 10 min. After adding 1 mL of salicylic acid solution (9 mmol L^{-1}), it was mixed and incubated at 37 °C for 30 min, and then the absorbance of the reaction solution was measured at 510 nm. Water was used as a blank.

Table 2. Range of different factors investigated with PBD

Variable	Level		
	–1	0	1
A (%)	20	25	30
B (w)	160	200	240
C (°C)	40	55	70

PBD – Plackett-Burman design; *A* – liquid/solid ratio; *B* – ultrasonic power; *C* – temperature

ABTS radical scavenging assay. Refer to the test of Re et al. (1999) and others as follows: prepare a mixed solution containing ABTS (7 mmol L^{-1}) and potassium persulfate (2.45 mmol L^{-1}) in pure water and incubate in the dark at 23 °C for 16 h to prepare an ABTS radical cationic base solution. The base solution was diluted with pure water to an absorbance of 0.700 ± 0.005 at a wavelength of 734 nm to obtain an ABTS radical cation working solution. Take 0.1 mL of different concentrations of the test solution and mix with 3.9 mL of the working solution, incubate at 23 °C for 6 min, measure the absorbance of the reaction mixture at 734 nm, and use pure water as a blank control.

Statistical analysis. The Design-Expert software is used to examine immediate optimization of numerous responses. Duncan's multiple comparisons were used to determine the difference between the means. All data were analysed by one-way ANOVA (analysis of variance) and $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Screening of significant factors. The results of the PBD are documented in Table 3. In this study, the effects of five variables on TPC were evaluated using the PBD in the Design-Expert software (Hongxia et al. 2019). Extraction parameters indicating the statistically significant effects were separated by ANOVA. The L/S was the most important factor, followed by ultrasonic power and temperature; the remaining parameters were not significant. This was consistent with the findings of other authors, increasing the L/S while achieving high bioavailability and antioxidant activity, as it promoted solvent penetration into plant cells to dissolve target components (Vinatoru et al. 2017).

The ANOVA shows that the obtained regression equation is significant ($P = 0.0020$), *i.e.* the model fits well in the whole regression region studied. The correlation coefficient (R^2) was 0.8276, indicating a significant correlation. The coefficient of determination of adjusted $R^2 = 0.7630$ showed that the 76.30% variability of the experimental data can be explained by the regression model.

In general, the lower the coefficient of variation (*CV*), the higher the experimental credibility and accuracy.

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Table 3. Plackett-Burman experimental design and response values

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	TPC (mg g^{-1})*
1	–	–	+	–	+	+	–	+	+	+	–	20.6841
2	–	+	+	–	+	+	+	–	–	–	+	20.0023
3	+	–	+	+	+	–	–	–	+	–	+	19.5705
4	+	–	+	+	–	+	+	+	–	–	–	20.4341
5	–	–	–	–	–	–	–	–	–	–	–	20.9795
6	–	+	–		+	–	+	+	+	–	–	20.9568
7	+	+	+	–	–	–	+	–	+	+	–	19.3886
8	+	+	–	+	+	+	–	–	–	+	–	19.8659
9	+	+	–	–	–	+	–	+	+	–	+	20.0250
10	–		+	+	–	–	–	+	–	+	+	19.7750
11	–	–	–	+	–	+	+	–	+	+	+	21.1386
12	+	–	–	–	+	–	+	+	–	+	+	20.3659

X_1 – liquid/solid ratio; X_2 – ultrasonic power; X_3 – temperature; X_4 – ethanol concentration; X_5 – extraction time; X_6, X_7, X_8, X_9, X_{10}, X_{11} – virtual factors; TPC – total phenolic content; *mean of triplicate determinations

The *CV* value was 1.40%, satisfying the consistency and precision of the PBD experiment. The accuracy (Adeq Precision) was the ratio of the effective signal to the noise, and its value greater than 4.0 was considered reasonable, and the precision of the experiment reached 10.695.

Determination of the best extraction process. The corresponding quadratic equation model was obtained by performing the BBD on L/S, ultrasonic power and temperature (also see Table 4):

$$Y = 24.05 + 4.24A - 0.34B - 0.41C + 0.25AB - 0.38AC + + 0.31BC - 0.1A^2 + 0.20B^2 + 0.13C^2 \quad (2)$$

where: Y – the response value that showed TPC in *Desmodium triquetrum* (L.) DC.; A – liquid/solid ratio; B – ultrasonic power; C – temperature.

The quadratic model variance in the RSM analysis of the experimental results is shown in Table 5. The F value was 30.01, and the multivariate correlation coefficient (R^2) was 0.9748, indicating that the model was tailored. The model is considered extremely significant ($P < 0.0001$), which elucidated that the model could be used to predict response values.

The significance test of the regression coefficient in the quadratic model (Table 6) showed that the linear effect of factor A on the extraction of phenolic acid was extremely significant, while the factors B and C and the surface effects of the factors A^2, B^2 and C^2 were not significant. No significant difference was shown in the interaction effects of *AB*, *AC*, and *BC* on the extraction.

Figures 1, 2, and 3 were response surface plots and their contour plots from the multiple regression equation. This allowed the analysis and evaluation of TPC after extraction of any two factors to determine the optimal factor level range.

Figure 1 shows the interaction of L/S and ultrasonic power on the phenolic acid extraction rate at an op-

Table 4. Box-Behnken Design (BBD) experiment and response values

Run	A	B	C	TPC_{Actual}* (mg g^{-1})	$TPC_{Predicted}$ (mg g^{-1})
1	–1	–1	0	20.29	20.45
2	1	–1	0	28.51	28.43
3	–1	1	0	19.18	19.26
4	1	1	0	28.40	28.25
5	–1	0	–1	19.52	19.81
6	–1	0	–1	28.54	29.05
7	1	0	–1	20.27	19.75
8	1	0	1	27.76	27.48
9	0	–1	–1	25.87	25.44
10	0	1	–1	24.49	24.13
11	0	–1	1	23.64	24.00
12	0	1	1	23.50	23.94
13	0	0	0	23.30	24.05
14	0	0	0	23.39	24.05
15	0	0	0	25.11	24.05
16	0	0	0	23.84	24.05
17	0	0	0	24.57	24.05

A – liquid/solid ratio; B – ultrasonic power; C – temperature; TPC – total phenolic content;*mean of triplicate determinations

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Table 5. Analysis of variance (ANOVA) for the regression quadratic model equation of BBD

Type	*df*	Sum of squares	MS	*F*	*P* > *F*
Regression	9	147.79	16.42	30.01	< 0.0001
Residual	4	2.43	0.61		
Loss fault	3	1.39	0.46	0.76	0.5713
Sum	13	150.22			

BBD – Box-Behnken design; *df* – degree of freedom; MS – mean square

timum temperature of 40 °C. We recognized that the ultrasonic power was either at a low (109.37 w) or high level (230.83 w), the TPC was increased by increasing the L/S. From the secondary model it was recognized that there was an interaction between the ultrasonic power and the L/S. However, the three-dimensional map showed no significant effect. It could be concluded that the ultrasonic power was low, and the L/S was high to achieve a higher TPC.

The solubility of phenolic compounds was increased by increasing the L/S until the saturation of the extraction solvent, when the biologically active compound was reached. Ultrasonic power played an important role in the extraction of compounds due to a cavitation effect. Ultrasonic energy increased the pressure inside the plant cells, which accelerates the cell rupture and promotes the destruction of the sample surface, which is then out into the surrounding solvent (Hayat et al. 2009). Compounds other than phenolic acids are also released from the ruptured plant cells into the extraction solvent, which limits the permeability of the extraction solvent (Toma et al. 2001), therefore, the L/S should be higher. In addition, a probable cause of the lower extraction yield at the elevated influence is possibly the production of higher temperature and pressure with the extreme disintegration of cavitation bubbles in greater power of ultrasonication (Nipornram et al. 2018; Arruda et al. 2019).

Table 6. Coefficient estimates by the regression quadratic model

Source	Regression coefficient (mean ± SD)	*F*	*P* > *F*
Model	24.05 ± 0.33		
A	4.24 ± 0.26	263.91	< 0.0001
B	–0.34 ± 0.26	1.71	0.2319
C	–0.41 ± 0.26	2.43	0.1633
A^2	0.25 ± 0.37	0.46	0.5179
B^2	–0.38 ± 0.37	1.07	0.3361
C^2	0.31 ± 0.37	0.71	0.4279
AB	–0.15 ± 0.36	0.18	0.6845
AC	0.20 ± 0.36	0.31	0.5948
BC	0.13 ± 0.36	0.12	0.7344

A – liquid/solid ratio; *B* – ultrasonic power; *C* – temperature; *A*, *B*, *C* – linear terms; A^2, B^2, C^2, D^2 – quadratic terms; *AB*, *AC*, *AD*, *BC*, *BD*, *CD* – interaction terms; SD – standard deviation

Figure 2 shows the interaction between the temperature and L/S extraction effects, when the ultrasonic power was at an optimum value (160 w). It was shown that higher TPC was achieved when the L/S was at a high level, at the same time it was indicated that TPC was increased slightly when the temperature level was reduced.

The same indications were also reported by Belwal et al. (2019), who stated that the highest Cy3-gal extraction level can be obtained at a lower temperature. During sonication, the temperature had a significant effect on solvent polarity, density, surface tension and viscosity (Chemat et al. 2011; Huang et al. 2019). Fluctuation of temperature can interrupt the ultrasonic effect, which results in interference in the extraction yield (Fang et al. 2018). Some articles mentioned that higher temperatures can reduce the cavitation effect by adjusting the physical properties of the solvent, thereby reducing the extraction yield (Rawson et al. 2011; Fang et al. 2018). Applicable with the above-mentioned research results, it could be understood from Figure 3 that the high level of TPC was obtained with the higher level of L/S and lower level of ultrasonic power and temperature. Our results revealed that the optimal extraction process was determined as follows: the key factor L/S 30%, ultrasonic power 160 w, temperature 40 °C, theoretical calculation of TPC reached 29.6548 mg g^{-1}. With the traditional extraction method, the TPC of 2.4663 mg g^{-1} could only be achieved, and the ultrasonic extraction results greatly improve the extraction rate of total phenol compared with the traditional extraction method.

Validation results. According to the optimized extraction conditions: L/S 30%, ultrasonic power 160 w, temperature 40 °C, ethanol concentration 50%, time 20 min, the verification experiment was carried out. The measured TPC reached 30.3708 mg g^{-1}, which was close to the predicted value of 29.6548 mg g^{-1}. It proved the feasibility and accuracy of PBD and BBD in optimizing the ultrasonic extraction of *Desmodium triquetrum* (L.) DC. phenolic acid. The statistical de-

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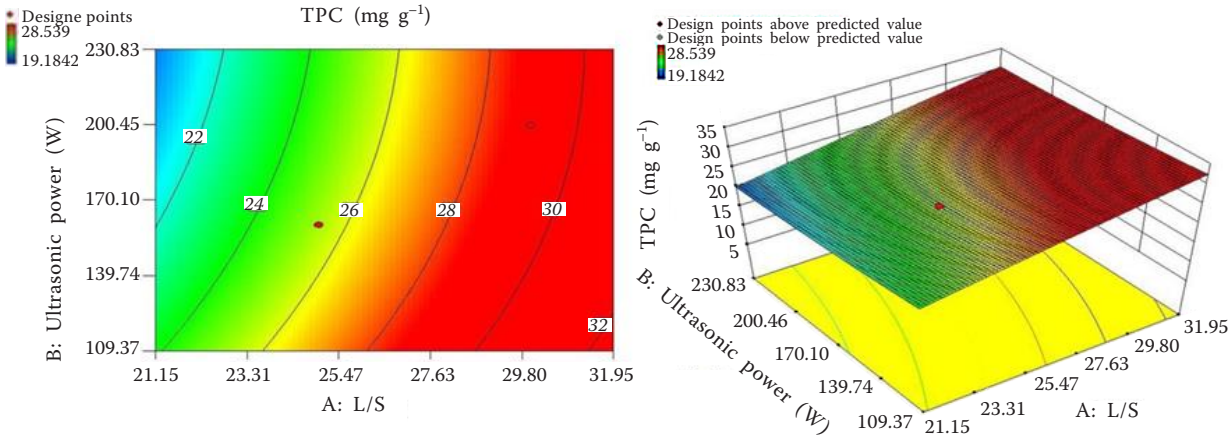

Figure 1. Response surface plots illustrating the interactive effects of L/S vs ultrasonic power on TPC
TPC – total phenolic content; L/S – liquid-solid ratio

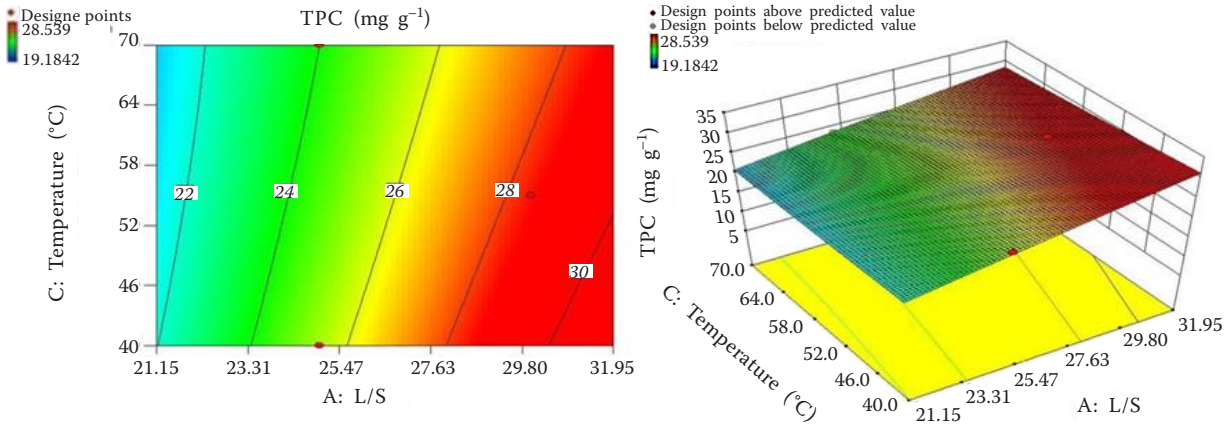

Figure 2. Response surface plots illustrating the interactive effects of L/S vs temperature on TPC
For abbreviations see Figure 1

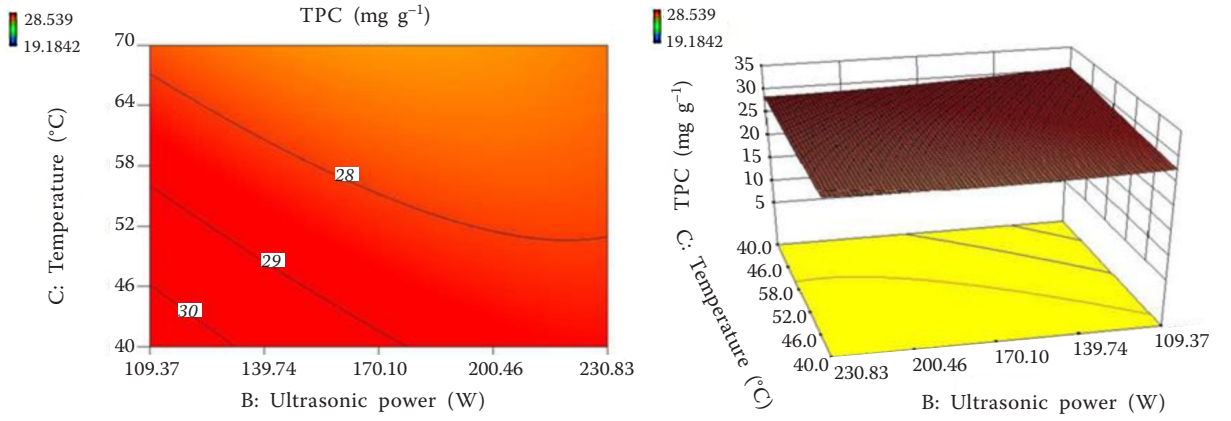

Figure 3. Response surface plots illustrating the interactive effects of temperature vs ultrasonic power on TPC
For abbreviations see Figure 1

sign and screening optimization method had a certain reference value for another Chinese herbal medicine extraction process.

Determination of antioxidant capacity of phenolic acids. The vitamin C solution having the same solubility as the polyphenol solution extracted under

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the optimum conditions was prepared as a reference, and the ABTS radical cation and hydroxyl radical scavenging effects were measured. Vitamin C is a nutrient in most foods, and it also has a good antioxidant effect. Therefore, it is an excellent and reliable indicator.

The ABTS radical cation scavenging experiment is based on the influence of different concentrations of the test substance on the absorbance of the ABTS radical cation solution to determine the ability of the natural product to remove the ABTS radical cation. This method was first proposed by Miller et al. (1993) and was widely used to test the antioxidant activity of natural products after being improved by Re et al. (1999). In the ABTS radical cation scavenging experiment, *Desmodium triquetrum* (L.) DC. polyphenols had the same ability as vitamin C, and the clearance rate was 100%. However, the phenolic acid hydroxyl radical scavenging effect of *Desmodium triquetrum* (L.) DC. was weaker than that of vitamin C. The hydroxyl radical scavenging rate of the phenolic acids of *Desmodium triquetrum* (L.) DC. was 69.30%, and the hydroxyl radical scavenging rate of vitamin C was 99.92%. However, under the traditional method, the clearance rates of the two free radicals were only 99.27% and 48.41%, respectively. This indicated that *Desmodium triquetrum* (L.) DC. polyphenols had a certain antioxidant capacity, and ultrasonic extraction could enhance their antioxidant performance.

CONCLUSION

The optimum process for ultrasonic extraction of *Desmodium triquetrum* (L.) DC. polyphenols is L/S 30%, ultrasonic power 160 w, temperature 40 °C, time 20 min, ethanol solubility 60%; under these conditions, the amount of polyphenol extraction is 30.3708 mg g^{-1}. The anti-oxidation performance of *Desmodium triquetrum* (L.) DC. polyphenols extracted under these conditions is better, the ultrasonic extraction process is simple, green as well as efficient, and the obtained *Desmodium triquetrum* (L.) DC. polyphenols can be used as a natural antioxidant in food, which has certain developmental prospects.

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