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Homogenate extraction of total phenols from *Nelumbo nucifera* rhizome and evaluation of antioxidant activities

[Extracción de homogeneizado de fenoles totales del rizoma de *Nelumbo nucifera* y evaluación de actividades antioxidantes]

Dongmei Yang, Yan Wang & Guoping Ren

Department of Health Rehabilitation and Tourism, Hangzhou Wanxiang Polytechnic, Hangzhou, China

Reviewed by:
Zilda Cristiane Gazim
Universidade Paranaense
Brazil

Marcelo Luis Wagner
Universidad de Buenos Aires
Argentina

Correspondence:
Guoping REN
renguopingwx@163.com

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Abstract: This study applied the homogenate extraction method to extract total phenols (TP) from *Nelumbo nucifera* rhizome, and evaluated the antioxidant activities of TP. After the single-factor experiments and optimization using response surface methodology, the optimized homogenate extraction parameters for obtaining TP from *Nelumbo nucifera* rhizome were as follows: ethanol concentration, 37%; liquid-material ratio, 15 mL/g; extraction time, 27 s. With these conditions, the TP yield was 0.36 ± 0.04%. When the concentration was 30 mg/mL, the 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazyl and hydroxyl radical scavenging rates of TP were 80.15 ± 5.12% and 67.88 ± 6.34%, respectively. Compared with the traditional extraction, the homogenate extraction has the higher TP yield and shorter extraction time. In addition, the TP obtained by homogenate extraction have the higher antioxidant activities than traditional extraction. To sum up, the homogenate extraction is an efficient and rapid method for extracting TP from *Nelumbo nucifera* rhizome.

Keywords: Total phenols; *Nelumbo nucifera*; Rhizome; Homogenate extraction; Antioxidant

Resumen: Este estudio aplicó el método de extracción homogeneizada para extraer fenoles totales (TP) del rizoma de *Nelumbo nucifera* y evaluó las actividades antioxidantes de los TP. Después de realizar experimentos de un solo factor y optimizar utilizando metodología de superficie de respuesta, los parámetros optimizados para la extracción homogeneizada de TP del rizoma de *Nelumbo nucifera* fueron los siguientes: concentración de etanol, 37%; relación líquido-material, 15 mL/g; tiempo de extracción, 27 s. Con estas condiciones, el rendimiento de TP fue del 0.36 ± 0.04%. Cuando la concentración fue de 30 mg/mL, las tasas de eliminación de radicales 2,2-difenil-1-(2,4,6-trinitrofenil)-hidrazilo y radicales hidroxilos fueron del 80.15 ± 5.12% y 67.88 ± 6.34%, respectivamente. En comparación con la extracción tradicional, la extracción homogeneizada mostró un mayor rendimiento de TP y un tiempo de extracción más corto. Además, los TP obtenidos mediante extracción por homogeneizado presentaron actividades antioxidantes superiores a las obtenidas por extracción tradicional. En resumen, la extracción homogeneizada es un método eficiente y rápido para extraer TP del rizoma de *Nelumbo nucifera*.

Palabras clave: Fenoles totales; *Nelumbo nucifera*; Rizoma; Extracción por homogeneizado; Antioxidante.

INTRODUCTION

Nelumbo nucifera Gaertn (family Nelumbonaceae), a water plant, has a long cultivation history in Eastern Asia, particularly in China (Hu & Skibsted, 2002). *Nelumbo nucifera* rhizome belongs to the enlarged rhizome of *Nelumbo nucifera* Gaertn. It not only contains rich nutrients such as carbohydrates, dietary fiber, and vitamins, but also contains abundant phenolic compounds (Hu & Skibsted, 2002; Tu *et al.*, 2015; Chen *et al.*, 2018). Phenolic compounds are the secondary metabolites widely present in plant tissues and are considered as catalytic reaction products of phenylalanine enzymes. They play an important therapeutic role in antioxidant (Katalinic *et al.*, 2006), anti-inflammatory (Suzuki *et al.*, 2023), anticancer (Maheshwari & Sharma, 2023), antiviral (Montenegro-Landívar *et al.*, 2021), anti-hyperglycemic (Golovinskaia & Wang, 2023) and other aspects. Phenolic compounds constitute the main secondary metabolites found in *Nelumbo nucifera* rhizome (Tsuruta *et al.*, 2011). The methods of extracting phenolic compounds consist of conventional heat extraction, microwave-assisted extraction, ultrasound-assisted extraction, pressurized liquid extraction, supercritical CO_2 extraction and others (Alara *et al.*, 2021). Homogenate extraction is an extraction procedure in which the chemical compositions are extracted from materials in solvent by high-speed mechanical shearing, mixing, fluid cutting action and smashing without heating and pressure (Shi *et al.*, 2009; Liu *et al.*, 2013). The homogenate extraction has been widely used in the extraction of various plant active ingredients. Compared with traditional extraction methods, the homogenate extraction has the advantages of short extraction time, high extraction efficiency, and easy operation (Zhu *et al.*, 2014; Zhang *et al.*, 2015; Zhang *et al.*, 2020). There are reports on application of homogenate extraction for obtaining phenolic compounds (Zhang *et al.*, 2015; Cao *et al.*, 2018). However, until now the extraction of TP from *Nelumbo nucifera* rhizome using this method has not been reported. In this study, the homogenate extraction method was applied to extraction of total phenols (TP) from *Nelumbo nucifera* rhizome. The extraction parameters were explored, and the antioxidant activities of TP were investigated. The purpose is to provide a basis for further developing efficient extraction methods of TP from *Nelumbo nucifera* rhizome.

MATERIALS AND METHODS

Materials and apparatus

Nelumbo nucifera rhizome was purchased from Hangzhou Gouzhuang Vegetable Market (Hangzhou, China), and was identified as *Zhehu Er Hao*. The edible parts of rhizome were washed by tap water, freeze-dried, and ground to pass a 100 mesh sieve to obtain the sample powder. JHBE-50A homogenate extractor was provided by Golden Star Technology, Inc., Ltd. (Zhengzhou, China). Ethanol was purchased from Hangzhou Shengli Chemical Co., Ltd., (Hangzhou, China). Other reagents were purchased from Sigma-Aldrich Inc. (Saint Louis, USA).

Homogenate extraction of TP

Nelumbo nucifera rhizome powder and ethanol solution were put into the homogenate extractor for extraction at room temperature. After extraction, the mixture was centrifuged at 256 × g for 10 min. The supernatant was concentrated by vacuum and freeze-dried to obtain the crude extract. The selective separation of TP was performed using the macroporous resin adsorption. The D101 macroporous resin column was prepared. The crude extract solution was loaded on the adsorption resin column, followed by standing for 24 h. The deionized water was added to dilute the impurity until the effluent was clear. The ethanol solution with concentration of 70% was added to dilute the product, and the eluents were collected in different portions. The TP in each eluent portion were identified and quantified by Folin-Ciocalteu assay using gallic acid as standard (Martins *et al.*, 2021). The eluent portions with rich TP were combined, and were concentrated by vacuum and freeze-dried to obtain the final extract. The TP content in extract was analyzed again by above method. The TP yield was calculated as follows: TP yield (%) = (m / M)*100%, where m (g) was the mass of TP calculated by UV-Vis spectrophotometry, and M (g) was the mass of *Nelumbo nucifera* rhizome powder. The extraction parameters were investigated by single-factor experiments and optimization using response surface methodology (RSM).

Traditional extraction of TP

Based on the optimized conditions, *Nelumbo nucifera* rhizome powder and ethanol solution were put into the reflux apparatus, followed by for heat extraction.

After extraction, the mixture was centrifuged at 256 × g for 10 min. The supernatant was concentrated by vacuum and freeze-dried to obtain the crude extract. The selective separation of TP was performed using the macroporous resin adsorption, and the operations were basically the same with the homogenate extraction.

Determination of 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazyl (DPPH) radical scavenging capacity

TP solutions with concentration of 5-30 mg/mL were prepared, respectively. The TP solution was mixed with equal volume of ethanol solution of DPPH (2.0×10^{-4} mol/L). After shaking vigorously, the mixture was placed at room temperature for reaction for 30 min. Then, the absorbance of reaction mixture was detected in the UV-Vis spectrophotometer, with detection wavelength of 517 nm. The water was used as the blank control. The DPPH radical scavenging rate of TP was calculated as follows: DPPH radical scavenging rate (%) = $[1 - (A_1 - A_2) / A_0] \times 100$ (A_1, absorbance of reaction mixture with TP and DPPH; A_2, absorbance of reaction mixture without DPPH; A_0, absorbance of reaction mixture without TP) (Ruiz-Ruiz *et al.*, 2018).

Determination of hydroxyl radical scavenging capacity

TP solutions with concentration of 5-30 mg/mL were prepared, respectively. The 0.2 mL of 7.5 mmol/L orthophenanthroline solution, 0.2 mL of 7.5 mmol/L $FeSO_4$ solution, 1 mL of pH 7.47 tris-HCl buffer solution, 1 mL of 7.5 mol/L H_2O_2 solution and 0.1 mL of TP solution were added to a 10 mL colorimetric tube. After incubating at 37 ºC for 1 h, the absorbance of the reaction mixture was detected in UV-Vis spectrophotometer, with detection wavelength of 536 nm. The water was used as the blank control. The hydroxyl radical scavenging rate of TP was calculated as follows: hydroxyl radical scavenging rate (%) = $[(A_2 - A_1) / (A_0 - A_1)] \times 100$ (A_0, absorbance of reaction mixture without H_2O_2 or TP; A_1, absorbance of reaction mixture with H_2O_2 and without TP; A_2, absorbance of reaction mixture with H_2O_2 and TP) (Rahman *et al.*, 2015).

Statistical analysis

Each experiment or test was carried out for three times. Data were presented as mean±standard deviation. In the single-factor experiments, the comparison among difference groups was performed by analysis of variance (AOV) using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The optimization of extraction parameters was performed by RSM using Design-Expert 8.0.6 software (Stat-Ease, Inc., MN, USA). The comparison between two extraction methods was performed using t test. $p<0.05$ and $p<0.01$ were considered as statistically significant and highly statistically significant, respectively.

RESULTS AND DISCUSSION

Results of single-factor experiments

Successful completion of an extraction is affected by many factors. The homogenate extraction is generally carried out at room temperature, so the extraction temperature does not need to be considered in investigating the extraction parameters (Shi *et al.*, 2009; Liu *et al.*, 2013). Our preliminary study showed that, the ethanol concentration, liquid-solid ratio, extraction time and extraction frequency could affect the TP yield in homogenate extraction, so we investigated these parameters in single-factor experiments. As shown in Figure No. 1, the ethanol concentration of 40%, liquid-solid ratio of 25 mL/g, extraction time of 50 s and extraction frequency of 3 obtained the highest TP yield, respectively. However, after liquid-solid ratio of 15 mL/g, extraction time of 30 s and extraction frequency of 1, the increase of TP yield was not obvious. Therefore, considering the efficiency of extraction, the ethanol concentration of 40%, liquid-solid ratio of 15 mL/g, extraction time of 30 s and extraction frequency of 1 were chosen for the next optimization experiments.

Optimization of extraction parameters

RSM is an empirical modeling technique used to estimate relationships between a set of experimental control factors and observed results (Kleijnen, 2008). In this study, Box-Benhnken design (BBD) was applied to optimize extraction parameters by using RSM. According to preliminary experiment results, the ethanol concentration, liquid-solid ratio and extraction time were chosen as key parameters and were designated as Z_1, Z_2 and Z_3, respectively, and their low, middle and high levels for RSM design were shown in Table No. 1. After conversion, the variables for BBD were obtained as follows: $A = (Z_1 - 40)/10$; $B = (Z_2 - 15)/5$; $C = (Z_3 - 30)/10$. Using A, B and C as independent variables and TP yield as the

response value, the experimental design and experimental results were shown in Table No. 2.

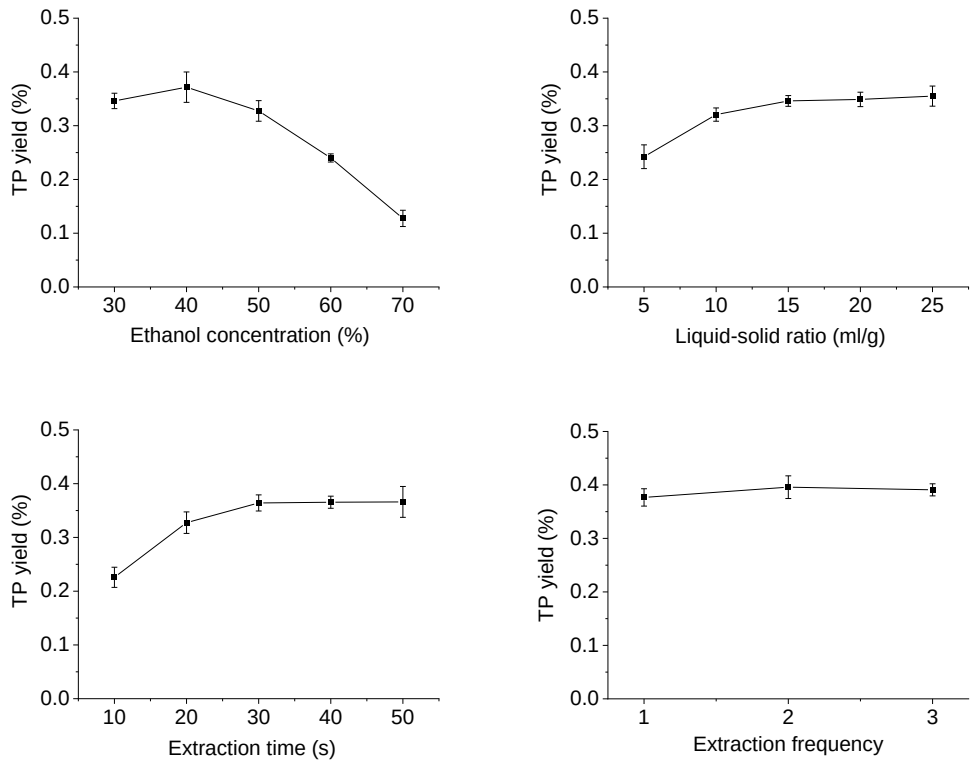

Figure No. 1
Effects of ethanol concentration, liquid-solid ratio, extraction time and extraction frequency on TP yield in single-factor experiments. TP, total phenols

Table No. 1
Variables and levels of Box-Benhnken design

Variable	Level		
	-1	0	1
Ethanol concentration (Z_1)	30	40	50
Liquid-solid ratio (ml/g) (Z_2)	10	15	20
Extraction time (s) (Z_3)	20	30	40

Table No. 2
Box-Benhnken design and experimental results

No.	Variable			TP yield (%)
	A	B	C	
1	-1	-1	0	0.21 ± 0.02
2	1	-1	0	0.10 ± 0.01
3	-1	1	0	0.22 ± 0.02
4	1	1	0	0.05 ± 0.01
5	-1	0	-1	0.25 ± 0.02
6	1	0	-1	0.25 ± 0.02
7	-1	0	1	0.31 ± 0.03
8	1	0	1	0.08 ± 0.01
9	0	-1	-1	0.19 ± 0.04
10	0	1	-1	0.28 ± 0.03
11	0	-1	1	0.17 ± 0.02
12	0	1	1	0.09 ± 0.02
13	0	0	0	0.35 ± 0.05
14	0	0	0	0.34 ± 0.04
15	0	0	0	0.40 ± 0.03
16	0	0	0	0.38 ± 0.04
17	0	0	0	0.33 ± 0.05

TP=total phenols

A summary of ANOVA for the selected regression model was shown in Table No. 3. The F value of model was 27.86, with Prob > *F* of 0.0001. This indicated that the quadratic multiple regression model can accurately reflect the relationship of TP yield with ethanol concentration, liquid-solid ratio and extraction time. The *F* value of Lack of fit was 0.736, with Prob > *F* of 0.5832. This indicated that there was no significant influence of other factors in this experiment, so this model can be used to analyze and predict the TP yield. The R^2 was 0.9728. This indicated that 97.28% of variation in the model response value came from the selected dependent variables, and there was a high correlation between the predicted values and the measured values. The order of variables affecting TP yield was as follows: A > C > B. The A, C, AC, A^2, B^2 and C^2 had highly significant effect on TP yield ($p<0.01$), and the BC had significant effects on TP yield ($p<0.05$). The final polynomial equation, describing TP yield as a simultaneous function of A, B and C was obtained as follows: TP yield = $0.36-0.064\times A-0.003516\times B-0.039\times C-0.015\times AB-0.059\times AC-0.043\times BC-0.087\times A^2-0.13\times B^2-0.050\times C^2$.

Table No. 3
Results of variance analysis of regression model

Source	df	Sum of squares	Mean square	F value	Prob > F
Model	9	0.19	0.02	27.86	0.0001
A	1	0.03	0.03	43.146	0.0003
B	1	1.125E-004	1.125E-004	0.15	0.7107
C	1	0.01	0.01	16.99	0.0045
AB	1	9.000E-004	9.000E-004	1.199	0.3106
AC	1	0.01	0.01	17.55	0.0041
BC	1	0.01	7.225E-003	9.59	0.0174
A^2	1	0.03	0.03	42.78	0.0003
B^2	1	0.07	0.07	90.83	< 0.0001
C^2	1	0.01	0.01	13.97	0.0073
Residual	7	5.275E-003	7.536E-004		
Lack of fit	3	1.875E-003	6.250E-004	0.736	0.5832
Pure error	4	3.400E-003	8.500E-004		
Cor total	16	0.19	$R^2 = 0.9728$		

Df = degree of freedom

In order to more intuitively reflect the effect of interaction among A, B, and C on TP yield, a 3D response surface graph of regression model was created (Figure No. 2). The variable A represented the steepest curved face, and it was the most significant factor affecting the TP yield, followed by C. B had little effect on yield of total flavonoids, of which the curved face was stepless. By solving the inverse matrix, the parameters for maximum response value were obtained as follows: A = -0.28, B = 0.04, C = -0.26. After conversion, the ethanol concentration was 37.20%, with liquid-solid ratio of 15.2 ml/g and extraction time 27.4 s. Under these optimal conditions, the predicted TP yield was 0.37%.

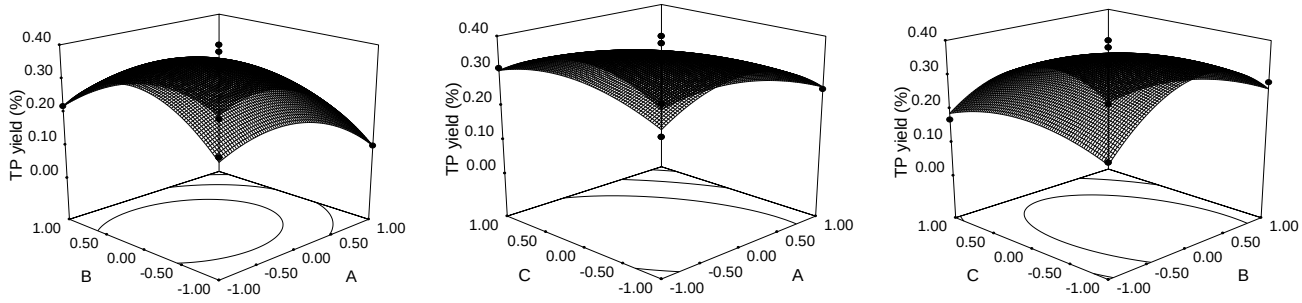

Figure No. 2
Response surfaces plots of ethanol concentration (A), liquid-solid ratio (B) and extraction time (C) on TP yield. TP, total phenols

Validation of extraction parameters

Considering the feasibility of experiment operation, the actual homogenate extraction parameters were appropriately adjusted from optimized values as follows: ethanol concentration, 37%; liquid-material ratio, 15 mL/g; extraction time, 27 s. With these parameters, the TP yield was 0.36±0.04%, with only a 2.70% variation with predicted value of 0.37%. This indicated that the equation of model fit well with the actual situation, fully verifying the correctness of

the model. Therefore, the parameters obtained by RSM optimization had good accuracy and reliability, with certain practical value.

Comparison of two extraction methods

Homogenate extraction method was compared with traditional extraction method for obtaining TP. Based on the optimization experiments, the traditional extraction parameters were as follows: ethanol concentration, 40%; extraction temperature, 70°C; extraction time, 2 h; extraction for once. The purification operation was the same with that of homogenate extraction. Results found that, the TP yield of traditional extraction was 0.17±0.01%, which was significantly lower than 0.36±0.04% of homogenate extraction (p<0.05). Zhu *et al.* (2014) has adopted the homogenate extraction method to extract gardenia yellow pigment from *Gardenia Jasminoides* Ellis and compared it with the traditional extraction method. Results show that, compared with the traditional extraction method, the color value of gardenia yellow pigment of homogenate extraction is higher and the extraction time is shorter. This finding is similar with our study

Antioxidant activities of TP

It is found that, the TP have the obvious antioxidant activities (Foti, 2007; Shahidi & Ambigaipalan, 2015; Zeb, 2020). The antioxidant activities of substance are often presented by its scavenging capacity on DPPH radical (Silva *et al.*, 2024) and hydroxyl radical (Milella *et al.*, 2014). Results of this study showed that, the TP obtained by two extraction methods had obvious scavenging capacity on DPPH hydroxyl and hydroxyl radical. The scavenging rate increased gradually with the increase of TP concentration. When the TP concentration was 30 mg/mL, the DPPH and hydroxyl radical scavenging rates of TP obtained by homogenate extraction were 80.15 ± 5.12% and 67.88 ± 6.34%, respectively, and those of TP obtained by traditional extraction were 70.43 ± 6.01% and 52.22 ± 4.88%, respectively. At each concentration, the DPPH and hydroxyl radical scavenging rates of homogenate extraction were significantly higher than those of traditional extraction, respectively. The reason may be that, although the TP content in product obtained by two extraction methods is the same, the content of effective antioxidant substances of homogenate extraction is higher than that of traditional extraction, because the homogenate extraction is performed at room temperature and with short extraction time, which can avoid the destruction and loss of effective antioxidant substances.

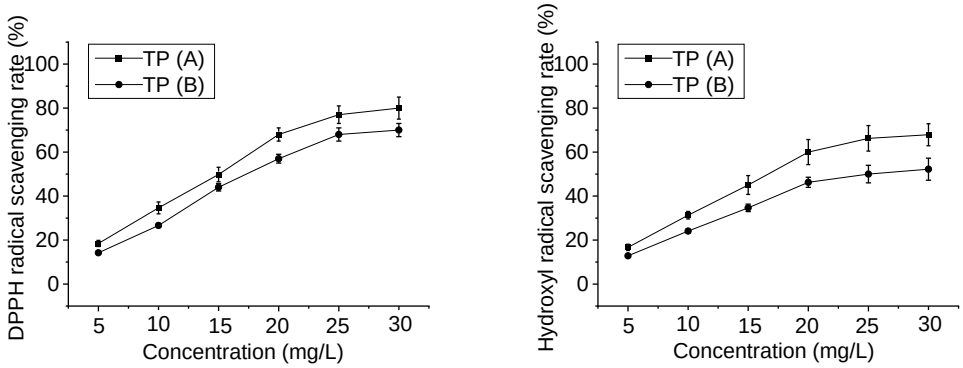

Figure No. 3
Scavenging capacity of TP on DPPH radical (A) and hydroxyl radical (B). TP (A), obtained by homogenate extraction; TP (B), obtained by traditional extraction. TP, total phenols; DPPH, 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazyl

CONCLUSION

Homogenate extraction is successfully applied to extract TP from *Nelumbo nucifera* rhizome. The optimal extraction parameters are as follows: ethanol concentration, 37%; liquid-material ratio, 15 mL/g; extraction time, 27 s. With these parameters, the TP yield was 0.36 ± 0.04%. Compared with traditional extraction, the homogenate extraction has the higher TP yield and shorter extraction time. In addition, the TP obtained by homogenate extraction have the

higher antioxidant activities than those obtained by traditional extraction. To sum up, the homogenate extraction is an efficient and rapid method for obtaining TP from *Nelumbo nucifera* rhizome.

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