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Original Article

Ultrasonic-assisted extraction of phenolic and antioxidative compounds from lizard tail (*Houttuynia cordata* Thunb.)

Trakul Prommajak[1]*, Suthat Surawang[2], and Nithiya Rattanapanone[1]

[1] *Division of Food Science and Technology,*

[2] *Division of Product Development Technology,*
Faculty of Agro-Industry, Chiang Mai University, Mueang, Chiang Mai, 50100 Thailand.

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Abstract

Lizard tail (*Houttuynia cordata* Thunb.) is an Asian herb which has many biological activities, including antioxidative property from polyphenolic compounds. Response surface methodology and Box-Behnken design were employed to study the effect of extraction temperature (30 to 70°C), extraction time (10 to 30 min), ethanol concentration (30 to 70%), and solvent to sample ratio (2 to 6 ml/g) on ultrasonic-assisted extraction of phenolic compounds from lizard tail and antioxidant capacity of the herb extract. Extraction temperature was the most relevant factor on the responses. Optimal condition was the extraction temperature of 70°C for 30 min, using 60% ethanol concentration at the solvent to sample ratio of 5 ml/g. Model adequacies were confirmed by extraction at the optimal condition and normality of standardized residuals.

Keywords: ultrasonic-assisted extraction, *Houttuynia cordata* Thunb., phenolic compounds, antioxidant capacity

1. Introduction

Lizard tail (*Houttuynia cordata* Thunb.) is an indigenous herb in East and Southeast Asia (Pawinwongchai and Chanprasert, 2011; Xu *et al.*, 2005). In traditional medicine, it has been used for treatment of fever, chills, headaches, muscular pain, malaise, diarrhea, dry cough, phlegm, lung abscess, dyspnea leucorrhea and ureteritis (Lau *et al.*, 2008; Lu *et al.*, 2006). Scientifically, it was proved to possess antibacterial (Kim *et al.*, 2008), antiviral (Chiang *et al.*, 2003), anti-inflammatory (Lu *et al.*, 2006), antileukemic (Pawinwongchai and Chanprasert, 2011), anticancer (Kim *et al.*, 2001), immunomodulatory and anti-severe acute respiratory syndrome (SARS) (Lau *et al.*, 2008) activities. Bioactive phenolic compounds found in lizard tail include procyanidin B, catechin, chlorogenic acid, neo-chlorogenic acid, cryptochlorogenic acid, quercetin hexoside, rutin, hyperin, quercitrin, piperolactam A, aristolactam B, and cepharadione B (Meng *et al.*, 2009; Nuengchamnong *et al.*, 2009). Health benefits of neochlorogenic acid were anticarcinogenicity and antigenotoxicity. Catechin had antiproliferative, hypolipidemic and immunomodulatory activities (Crespo *et al.*, 2006). The flavonoids quercetin, quercitrin and hyperin had biological activities including inactivation of carcinogen, antiproliferation, cell cycle arrest and induction of apoptosis in human cancer cells (Pawinwongchai and Chanprasert, 2011).

Conventional methods for the extraction of bioactive compounds from plants are Soxhlet, hydrodistillation and maceration. Among these methods, Soxhlet is a standard extraction method that has been used for a long time (Wang and Weller, 2006). However, the main disadvantages of Soxhlet extraction are (1) decomposition of thermolabile compounds caused by high operating temperature, (2) the extraction process cannot be accelerated by agitation which results in long extraction time, (3) and a large amount of solvent is required for extraction, which requires a large

* Corresponding author.
Email address: tpromjak@gmail.com

amount of energy for the evaporation/concentration step (Luque de Castro and Garcýa-Ayuso, 1998). Therefore, an alternative extraction method is required to overcome these problems.

Ultrasonic-assisted extraction (UAE) is an alternative extraction process that can decrease extraction time and increase extraction yield in many plants (Ma *et al.*, 2008a). Ultrasound energy is a mechanical energy with a frequency higher than 18 kHz which is the upper limit that human typically hear. Ultrasound with frequency more than 1 MHz and power less than 1 W/cm^2 is used for food and medical diagnosis, while ultrasound with frequency between 20 to 100 kHz with power more than 5 W/cm^2 is used for food processing (Mason *et al.*, 2005). Ultrasound wave creates cavitation bubbles in the solvent which cause microjet impacts and shockwave-induced damage to plant cell wall and release cell content into the solvent (Esclapez *et al.*, 2011). The main advantages of UAE are its effectiveness, simplicity and low cost (both instrument and operation cost) (Ghafoor *et al.*, 2009). UAE could also be operated at moderate temperature which is suitable for heat-sensitive compounds (Kimbaris *et al.*, 2006).

This study aimed to optimize the UAE of phenolic compounds with antioxidative property from lizard tail using response surface methodology. Extraction temperature, extraction time, ethanol concentration, and solvent to sample ratio were factors to be examined.

2. Materials and Methods

2.1 Materials

Lizard tail (*Houttuynia cordata* Thunb.) was purchased from local market in Chiang Mai province of Thailand. Lizard tail leaves without bruise was selected and homogenized with a blender (Moulinex, France).

Ethanol (J.T. Baker, Netherlands), Folin-Ciocalteu phenol reagent (Merck, Germany), gallic acid, 2,2-diphenyl-1-picryl,hydrazyl (DPPH) (Sigma-Aldrich, USA), ascorbic acid and sodium carbonate (Fisher, USA) were analytical grade.

2.2 Experimental design

Box-Behnken design was used to study the effects of ethanol concentration, solvent ratio, extraction time and temperature on total phenolic content and antioxidant capacity of lizard tail extracts. The design contained 29 experimental run which had 5 replicates at the center point, as shown in Table 1.

2.3 Extraction of antioxidant phenolic compounds

The sample (5 g) was placed into glass bottle with the solvent (30 to 70% ethanol, 2 to 6 solvent to sample ratio) and extracted in an ultrasonic bath (Tru-Sweep 1875DAE, 45 kHz, Crest Ultrasonics, Malaysia). Extraction temperature and time were used according to the experimental design (Table 2). After extraction, the sample was filtered through muslin cloth and centrifuged at 4,500 rpm for 10 min (Hermle Z200A, Hermle, Germany). The supernatant was subjected to total phenolics and antioxidant assays.

2.4 Determination of total phenolic content

Total phenolic content was analysed by Folin-Ciocalteu assay (Dudonné *et al.*, 2009). In brief, 30 µl of appropriate dilution (5x) of the aliquot was mixed with 150 µl of 10% Folin-Ciocalteu reagent in microtiter plate. Then, 120 µl of 7.5% (w/v) sodium carbonate solution was added. After 60 min, absorbance at 755 nm was recorded by Anthos Zenyth 200RT microplate reader (Biochrom, England). Total phenolic content was calculated from calibration curve and expressed as gallic acid equivalent (GAE).

Total phenolic content (mg GAE/g db)

$$= \frac{P \times V \times D}{W \times (100 - M) \times 10}$$

where

P = Total phenolic content calculated from calibration curve (mg GAE/l)
V = volume of extraction solvent (ml)
D = dilution factor
W = fresh weight of sample (g)
M = moisture content of sample (%)

2.5 Determination of DPPH radical scavenging capacity

An aliquot (30 µl) was mixed with 270 µl of DPPH solution (prepared by dissolving DPPH in 80% v/v ethanol to obtain $OD_{515} = 0.7$) in 96-wells microtiter plate. After incuba-

Table 1. Variables and factor levels for optimization of lizard tail extraction.

Independent variables	Symbol	Levels		
		-1	0	1
Temperature (°C)	X_1	30	50	70
Time (min)	X_2	10	20	30
Ethanol concentration (% v/v)	X_3	30	50	70
Solvent to sample ratio (ml/g)	X_4	2	4	6

tion for 1 h in dark condition, absorbance at 515 nm was recorded (Surveswaran *et al.*, 2007). DPPH radical scavenging capacity was calculated from a calibration curve and expressed as ascorbic acid equivalent (AAE).

DPPH radical scavenging activity (mg AAE/g db)

$$= \frac{A \times V \times D}{W \times (100 - M) \times 10}$$

where

A = DPPH radical scavenging activity calculated from calibration curve (mg AAE/l)

2.6 Statistical analysis

Statistical analysis was performed by R version 2.15.1 with 'RcmdrPlugin.DoE' and 'desirability' packages. Multiple regression analysis was used to fit the result with polynomial quadratic equation, as shown in the following equation:

$$Y_i = b_0 + \sum_{i=1}^{4} b_i X_i + \sum_{i=1}^{4} b_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} b_{ii} X_i X_j$$

where Y_i were the dependent variables, b_0 was a constant, b_i, b_{ii}, b_{ij} were the regression coefficients and X_i, X_{ij} were the independent variables (Prommajak and Raviyan, 2010). Some terms were removed from the equations to obtain the highest correlation between actual and predicted values. Optimal condition was calculated for the maximum total phenolic content and DPPH-radical scavenging capacity. Models validation was conducted by Shapiro-Wilk normality test of standardized residuals and extraction at the optimal conditions.

3. Results and Discussion

Experimental and predicted response values are shown in Table 2. Mathematical models for both total

Table 2. Experimental design and response values

Order	Independent variables				Total phenolic content (mg GAE/g db)		DPPH-radical scavenging capacity (mg AAE/g db)	
	Temperature (°C)	Time (min)	Ethanol (%)	Solvent to sample ratio	Actual	Predicted (ml/g)	Actual	Predicted
1	30	10	50	4	12.53	13.69	4.23	3.47
2	70	10	50	4	18.58	18.44	9.18	8.89
3	30	30	50	4	12.58	13.35	7.20	8.37
4	70	30	50	4	35.76	35.24	12.63	13.79
5	50	20	30	2	20.88	18.20	8.06	9.45
6	50	20	70	2	19.59	22.52	8.47	8.45
7	50	20	30	6	24.39	22.73	7.74	8.40
8	50	20	70	6	24.37	27.05	13.71	13.00
9	30	20	50	2	11.11	8.91	4.55	4.46
10	70	20	50	2	27.10	25.57	9.64	9.88
11	30	20	50	6	16.51	16.79	6.68	6.21
12	70	20	50	6	25.81	26.76	11.85	11.63
13	50	10	30	4	13.73	15.15	6.21	6.09
14	50	30	30	4	27.59	27.12	13.05	12.94
15	50	10	70	4	24.00	23.22	10.08	9.84
16	50	30	70	4	30.38	27.70	12.99	12.79
17	30	20	30	4	11.57	12.95	5.70	5.02
18	70	20	30	4	20.22	22.22	10.39	9.19
19	30	20	70	4	14.61	13.23	4.69	5.57
20	70	20	70	4	31.36	30.60	11.87	12.24
21	50	10	50	2	16.39	16.67	7.01	7.13
22	50	30	50	2	21.69	24.90	13.60	12.03
23	50	10	50	6	23.15	21.21	7.63	8.88
24	50	30	50	6	29.75	29.44	14.29	13.78
25	50	20	50	4	24.86	26.20	12.18	12.18
26	50	20	50	4	26.38	26.20	11.72	12.18
27	50	20	50	4	27.42	26.20	13.42	12.18
28	50	20	50	4	26.88	26.20	12.35	12.18
29	50	20	50	4	25.45	26.20	11.34	12.18

phenolic contents and DPPH-radical scavenging capacity were significant ($p<0.0001$). The lack-of-fit for both models was insignificant. These results indicate adequacy of the models. Coefficient of determination (R^2) is used to determine the variance of the response as influenced by the factor variables. In this study, 93.90% of the variance of total phenolic content and 93.92% of the variance of DPPH-radical scavenging capacity of lizard tail extract can be explained by extraction temperature, extraction time, ethanol concentration and solvent to sample ratio (Table 3).

3.1 Total phenolic contents

Effects of independent variables in total phenolic content were shown in Figure 1. Coefficients for the coded factors showed the effects of independent variables (Table 3). Extraction temperature (range from 30 to 70°C) had the most influences on total phenolic content, followed by extraction time (10 to 30 min). Concentration of extracted phenolic compounds increased with the elevated temperature due to higher solubility of phenolic compounds and lower viscosity

Table 3. Coefficients estimated and *p*-value of regression models.

Terms	Total phenolic content		DPPH-radical scavenging capacity	
	Estimated coefficients	*p*-value	Estimated coefficients	*p*-value
Coded factors:				
(Intercept)	26.20	<0.0001	12.18	<0.0001
X_1	6.66	<0.0001	2.71	<0.0001
X_2	4.11	<0.0001	2.45	<0.0001
X_3	2.16	0.0030	0.90	0.0056
X_4	2.27	0.0021	0.88	0.0068
X_1X_2	4.28	0.0010		
X_1X_3	2.03	0.0765	0.63	0.2212
X_1X_4	-1.67	0.1374		
X_2X_3	-1.87	0.0995	-0.98	0.0640
X_3X_4			1.40	0.0112
X_1^2	-4.78	<0.0001	-2.98	<0.0001
X_2^2	-1.23	0.1608	-0.57	0.1591
X_3^2	-1.66	0.0649	-1.19	0.0066
X_4^2	-1.91	0.0372	-1.16	0.0082
Actual factors:				
(Intercept)	-45.1391	0.0101	-30.4600	0.0003
Temp	1.0148	0.0032	0.8027	0.0000
Time	0.3022	0.5614	0.7164	0.0023
Ethanol	0.4581	0.1123	0.2229	0.1165
Solvent	7.0420	0.0051	1.0010	0.3300
Temp×Time	0.0214	0.0010		
Temp×Ethanol	0.0051	0.0765	0.0016	0.2212
Temp×Solvent	-0.0418	0.1374		
Time×Ethanol	-0.0094	0.0995	-0.0049	0.0640
Ethanol×Solvent			0.0350	0.0112
$Temp^2$	-0.0120	0.0000	-0.0075	0.0000
$Time^2$	-0.0123	0.1608	-0.0057	0.1591
$Ethanol^2$	-0.0042	0.0649	-0.0030	0.0066
$Solvent^2$	-0.4771	0.0372	-0.2892	0.0082
Model		<0.0001		<0.0001
Lack of fit		0.0605		0.3201
R^2		0.9390		0.9392
Adjusted R^2		0.8932		0.8998

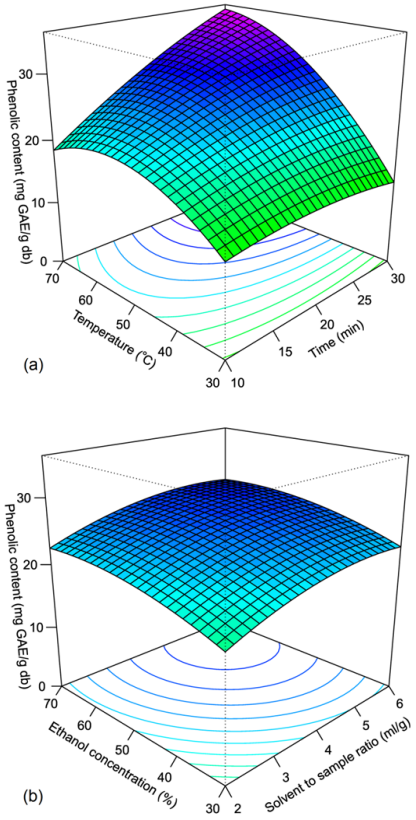

Figure 1. Surface plot of total phenolic content as a function of (a) extraction temperature and extraction time, and (b) ethanol concentration and solvent to sample ratio. The other 2 factors in each graphs were fixed at the mid level.

of the mixture (Ghafoor and Choi, 2009). Increasing of contact time between sample and solvent improve the diffusion of the compound. Similar result was also found in UAE of citrus peel (Ma *et al.*, 2008b). In case of grape seeds, although study parameters (40 kHz ultrasound frequency, temperature of 33 to 67°C, 16 to 34 min, 33 to 67% ethanol) were similar to those used in this study (45 kHz ultrasound frequency, temperature of 30 to 70°C, 10 to 30 min, 30 to 70% ethanol), the total phenolic content of the extract was more influenced by the extraction time than the temperature (Ghafoor *et al.*, 2009). Extraction time and temperature had the highest interactive and synergistic effect on extraction of phenolic compounds, as shown by coefficient of X_1X_2 term. Extraction time had a small effect at low extraction temperature, but at high temperature, its effect was highly increased (Figure 1a). Ethanol concentration (30 to 70%) and solvent per sample ratio (2 to 6 ml/g) had minor, but significant effects on total phenolic content. Ethanol is a suitable solvent for food industry. The presence of water in the solvent caused swelling of plant sample and enhanced the extraction. The appropriate ethanol concentration offered a suitable polarity for the target compounds. Increasing the solvent to sample ratio accelerate mass transfer between solvent and sample (Huang *et al.*, 2009). Ethanol concentration and solvent to sample ratio had no interaction effect, as described by the absence of ethanol×solvent (X_3X_4) term in the model. A similar result was found in the ultrasonic-assisted extraction of flavonoid from *Folium eucommiae* which showed insignificant interaction between ethanol concentration and solid-to-liquid ratio (Huang *et al.*, 2009).

Although some terms, including X_1X_3, X_1X_4, X_2X_3, X_2^2 and X_3^2 were insignificant, but removing each of these terms from the model caused a decline of coefficient of determination.

3.2 DPPH-radical scavenging capacity

Figure 2 shows the effect of independent variables on DPPH-radical scavenging capacity of herbal extract. The antioxidant capacity increased with an increasing extraction temperature until about 60°C, and thereafter decreased due to degradation of antioxidative compounds (Ma *et al.*, 2008b). A correlation (R=0.8449, $p<0.001$) was found between total phenolic content and DPPH-radical scavenging capacity, as illustrated in Figure 3. The correlation between total phenolic content and antioxidant activity was also found in the extracts of citrus peel (R=0.775), basil (R^2=0.71), Asian

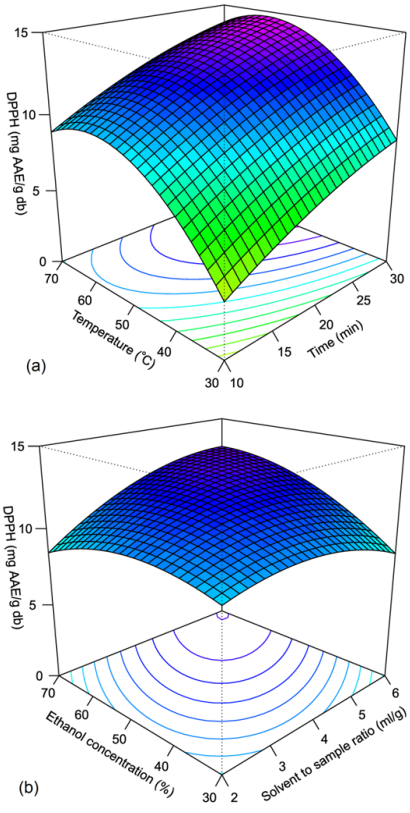

Figure 2. Surface plot of DPPH radical scavenging capacity as a function of (a) extraction temperature and extraction time, and (b) ethanol concentration and solvent to sample ratio. The other 2 factors in each graphs were fixed at the mid level.

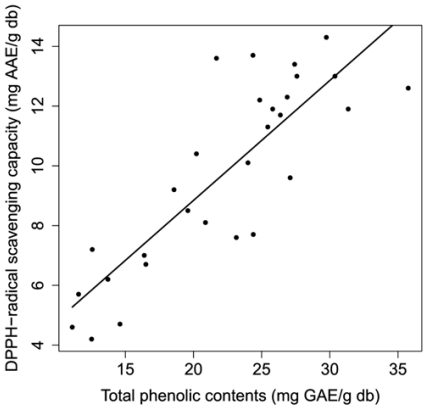

Figure 3. Correlation plot between total phenolic content and DPPH-radical scavenging capacity (R=0.8449, p<0.001).

vegetables (R^2=0.6578) and Chinese medicinal plants (R^2= 0.9580) (Cai *et al.*, 2004; Javanmardi *et al.*, 2003; Kaur and Kapoor, 2002; Ma *et al.*, 2008b). Antioxidant c apacity increased with prolonged extraction time. Similarly, ethanol concentration and solvent per sample ratio also had small effects as same as the total phenolic content model.

3.3 Optimal conditions and model validation

The optimal condition for the extraction of antioxidant compounds from lizard tail extract was found to be at 70°C for 30 min using 5 ml 60% ethanol per g of sample. At this optimal condition, the predicted response values were 35.80 mg GAE/g db for total phenolic content and 14.26 mg AAE/g db for DPPH-radical scavenging capacity. External validation can be conducted by extraction at this optimal condition. It was found that the actual response values were within 95% confidence interval of predicted values (Table 4).

This optimal condition was both similar and different from other studies on optimization of ultrasonic-assisted extraction of phenolic compounds from plant materials, as shown in Table 5. Optimal extraction temperature varied from 55°C (*Folium eucommiae*) to 79°C (*Prunella vulgaris* L.), indicating that each plant contains different types of phenolic

Table 4. Predicted and actual response value at optimal conditions

Response variables	Predicted value	95% confidence interval		Actual value[1]
		Lower	Upper	
Total phenolic content	35.80	31.76	39.84	36.40±0.48
DPPH	14.26	12.80	15.73	14.89±0.14

[1]means ± standard deviation (n=3)

Table 5. Optimal conditions for ultrasonic-assisted extraction of phenolic compounds from various plant materials.

Sample	Temperature (°C)	Time (min)	Ethanol (%)	Solvent to sample ratio (ml/g)	References
Lizard tail	70 (30-70)[1]	30 (10-30)	60 (30-70)	5 (2-6)	This study
Grape seed	56 (33-67)	30 (16-34)	53 (33-67)	50	Ghafoor *et al.* (2009)
Grape peel	53 (23-57)	24 (11-29)	46 (33-67)	50	Ghafoor and Choi (2009)
Red grape jam	50 (33-67)	20 (11-29)	60 (43-77)	50	Morelli and Prado (2012)
Wheat bran	60 (33-67)	25 (11-29)	64 (43-77)	20	Wang *et al.* (2008)
Prunella vulgaris L.[2]	79 (60-80)	30.5 (25-35)	41 (30-50)	30 (25-35)	Zhang *et al.* (2011)
Folium eucommiae[2]	55	70 (35-85)	40 (23-57)	60 (45-79)	Huang *et al.* (2009)

[1]Numbers in parentheses were study ranges.
[2]A response was total flavonoids.

compounds with varying heat sensitivity. Optimal extraction time mostly varied between 20 and 30 min. Ethanol concentration varied from 40 to 64%, indicating that different target compounds are contained in different plant materials which require different polarity of extracting solvent. In other studies, solvent to sample ratio ranged from 20 to 60 ml/g for dried sample (Ghafoor and Choi, 2009; Ghafoor *et al.*, 2009; Huang *et al.*, 2009; Morelli and Prado, 2012; Wang *et al.*, 2008; Zhang *et al.*, 2011). However, in the present study, fresh samples (86.20% moisture content) were used and the required amount of solvent was 5 ml/g. If the moisture content is removed, the calculated solvent to sample ratio will be 42 ml/g dried sample which is within the range reported in other studies.

Standardized residual is used for identifying outliers (the value greater than 2.58 or smaller than -2.58) of the observed residual (Vogt and Johnson, 2011). As shown in Figure 4, standardized residuals of both models had no outlier. Internal validation of mathematical models can be evaluated by normality test of standardized residuals. Shapiro-Wilk test showed normality of standardized residuals for both total phenolic content and DPPH models (p-value = 0.7634 and 0.6148, respectively), confirming validity of the models.

4. Conclusions

Regression models for total phenolic content and DPPH-radical scavenging capacity were both significant, while the lack-of-fits were insignificant. Standardized residuals had normal distribution. Extraction temperature was the most relevant factor among the factors studied. Optimal condition for extracting phenolic compounds from lizard tail was found to be at 70°C for 30 min using 60% ethanol concentration at the solvent to sample ratio of 5 ml/g. Actual responses at optimal condition was within 95% confidence interval of the predicted values.

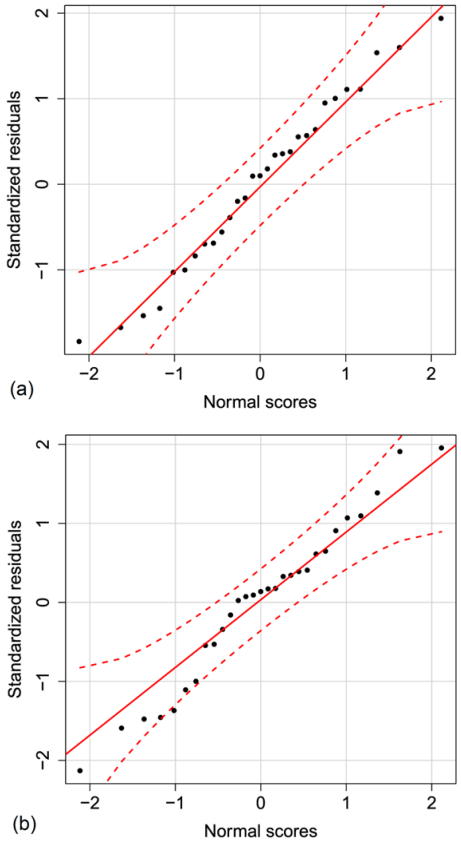

Figure 4. Normal quantile-quantile plots of standardized residuals for (a) total phenolic content and (b) DPPH models.

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