The Journal of Animal & Plant Sciences, 25 (3 Suppl. 1) 2015 Special Issue Page: 254-259
ISSN: 1018-7081

OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION CONDITIONS OF ANTIOXIDATIVE COMPONENTS FROM *ARUNDINA GRAMINIFOLIA* BY RESPONSE SURFACE METHODOLOGY BASED ON DPPH RADICAL SCAVENGING ASSAY

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ABSTRACT

Response surface methodology(RSM) was employed to optimize conditions for ultrasound-assisted extraction(UAE) of antioxidantive components from Arundinagraminifolia. 1,1-diphenyl-2-picryl-hydrazyl(DPPH) free radical scavenging assay was used to evaluate the activity of antioxidative components. Based on the single-factor test, we identified extraction time, ethanol volume fraction, extraction temperature and liquid-solid ratio as the main variables that influence DPPH free radical scavenging activity of *A. graminifolia* extract. The optimal conditions to achieve the maximum activity were determined as follows: extraction time 35 min, extraction temperature46ºC, ethanol volume fraction 74%and liquid-solidratio27 $mL.g^{-1}$. The scavenging rateof 78.71%was achieved under the optimal extraction conditions, which was well in agreement with the optimal predicted values (79.16%). This extraction method was simple and efficient and provided a method of sample preparation to determine DPPH radical scavenging activity of total antioxidative components from *A. graminifolia*. The research also provided a reference for full utilization of *A. graminifolia* and identified a technique to extract antioxidative components.

Keywords: *Arundinagraminifolia*, antioxidative components, ultrasound-assisted extraction, response surface methodology, DPPH radical scavenging gassay.

INTRODUCTION

*Arundinagraminifolia (D.Don) Hochr*is a plant belonging to the family Arundina Bl of Orchidaceae and is distributed mainly in the tropics and subtropics(Jiangsu New Medical College Dictionary of Chinese Material Medical.,1986). It has traditionally been used by Dai people as a folk medicine for the treatment of food and drug intoxication. Recent studies have demonstrated that *A. graminifolia* extract exhibits significant antioxidant activity because of the high content of flavonoids and polyphenols (Liu *et al*., 2004; Li *et al*., 2000; Nakbi *et al*., 2010; Alén-Ruiz *et al*., 2009). Nature antioxidants are becoming attractive because of synthetic antioxidantsmay have cumulative toxic effects (Zhang *et al*., 2011; Anna, 2007). Therefore, it is necessary to develop efficient extraction techniques for the active ingredients in *A.graminifolia*.

Ultrasound-assisted extraction(UAE) has become a useful methodin the processing of plant materials (Kimbaris *et al*., 2006; Kamaljit *et al*., 2008; Yang and Zhang., 2008) because it is highly efficiency and uses low levels of energy and solvent. In recent years, there have been many reports on the application of UAE to extract active constituents from plant, such as polyphenols, flavanoids, saponins and anthocyani (Sun *et al*., 2007; Gao *et al*., 2012; Kwon *et al*., 2003). However, there have been no reports using UAE to extract antioxidative components from *A. graminifolia*.

The DPPH free radical is a stable synthetic free radical with an unpaired valence electron at one atom of the nitrogen bridge (Eklund *et al*., 2005; Om *et al*., 2009).The DPPH radical scavenging assay has been extensively applied in evaluating the general radical scavenging capabilities of various antioxidants (Tuanjai *et al*., 2011; Zheng *et al*., 2013).Response surface methodology (RSM) is an effective statistical technique for optimizing complex processes and has been successfully used to model and optimize biochemical and biotechnological processes related to food systems (Deniz *et al*., 2007; Zhang *et al*.,2009).

In thisstudy, ultrasound-assisted extractionof antioxidative components from *A.graminifolia* was investigated. This method adopts DPPH radical scavenging rate as the response value. UAE parameters, such as extraction time, ethanol volume fraction, extraction temperature and liquid-solid ratio were optimized with RSM to obtain the optimalextraction conditions forantioxidative components from *A.graminifolia*.

MATERIALS AND METHODS

Apparatus, reagents and chemicals: An AS10200AD ultrasound cleaning bath (China Tianjin Autoscience instrument Co., Ltd.) with internal dimensions of 330 cm × 270 cm ×290 cm, a volume of 10.0 L and with 4 transducers was used in the experiment, operating at a frequency of 60 kHz with an input power of 320 W. The extraction vessel was placed at the center of the bath during the experiment and the temperature was controlled and maintained at 35 ± 1 °C by circulating external water from a water bath with a thermostat. 8453UV-Vis spectrophotometer (Agilent Technologies, USA); Eyelan-1100 Rotary Evaporator (Shanghai Eyelan Instrument Co., Ltd.); thermostat-controlled water bath (W201, Shensheng Biotechnology Co., Ltd., Shanghai, China); AR224CN electronic balance (Ohaus instrument Co., Ltd.) and methanol (HPLC-grade) from Tedia (Fairfield, OH) were used. DPPH (1,1-diphenyl-2-picryl-hydrazyl, purity>99.0%) was obtained from Sigma-Aldrich (St. Louis, MO). Fresh DPPH solution was prepared in ethanol at a concentration of 0.02 mg.mL^{-1}and stored at 4 °C. All other reagents used were of analytical reagent grade. Twice-distilled water was used throughout. *A. graminifolia* was provided from Yunnan Xishuangbanna Dai Hospital, which was ground into a powder and then sieved (80 mesh).

Extraction of total antioxidative components from *A. graminifolia:* Dried *A. graminifolia* powder (0.500 g) was soaked in an ethanol solution (varying ethanol volume fraction from 50 to 90%(V/V) and liquid-solid ratio from 10 to 50) for 2 h and then placed in an ultrasound bath and sonicated at various temperatures (20 to 60°C) for various lengths of time (10 to 50 min).The solution was filtered through 0.45 µm microporous membrane. The filtrate was extracted with 25 mL of petroleum ether to remove fats, oils and chlorophyll, and then analyzed for antioxidant activity. The scavenging rate was measured by DPPH radical scavenging assay.

Assay to measure DPPH radical scavenging activity: The free radical scavenging activity of the antioxidative componentsextract from *A.graminifolia*wasdetermined by DPPH testdescribed byMiliauskas*et al*. (2004) with slightly modified. Briefly, the antioxidative extract (4.0 mL) was mixed with DPPH (2.0 mL) in ethanol(0.02 mg.mL^{-1}). The mixture was left to react in the dark for 60 minat25°C, and then the absorbance was measured at a wavelength of 517 nm by a spectrophotometer. Anhydrous ethanol was used as the positive controls. The experiment was performed in triplicate and averaged. The scavenging activity of DPPH free radicals was calculated with the following equation:

$E\ (\%) = (1 - A_{sample}/A_{blank}) \times 100$.

Experimental design: The central composite design (CCD) with four factors and five levels was employed to optimize the extraction condition to obtain the maximum DPPH free radical scavenging activity of *A. graminifolia* extract. Central composite design in the experiment consists of sixty factorial points and five replicates of the central point. Ultrasound extraction time (X_1), ethanol volume fraction (X_2), temperature(X_3) and liquid-solid ratio (X_4) were chosen for independent variables and the dependent variable was the DPPH free radical scavenging activity. The symbols and levels are shown in tab. 1.

The single-factor experiment indicated that the DPPH radical scavenging rate increased with an increase in extraction time (from 10 to 30 min), extraction temperature(from 30 to 40 ºC), ethanol volume fraction (from 40% to 70% (V/V)) and liquid-solid ratio from (10 to 30mL.g^{-1}). However, the scavenging rate decreased slightly as extraction time increased above 30 min, extraction temperature beyond 40 ºC, ethanol volume fraction beyond 70% (V/V) and liquid-solid ratio beyond 30 mL.g^{-1}. Therefore, the central point of the central composite design was 30 min for extraction time, 40 ºC for extraction temperature,70% (V/V) for ethanol volume fraction and 30 mL.g^{-1} for liquid-solid ratio.

The range and center point values for the four independent variables in Table 1 were based on the results of preliminary experiments. The variables were coded according to Eq. (1):

$x_i = (X_i - X_0)/\Delta X$ (1)

Where x_i is the coded value of the variable, X_i, X_0 is the value of X_i at the centre point, and ΔX is the step change. The behavior of the system was explained by the following second degree polynomial equation:

$$Y = \sum A_0 + \sum_{i=1}^{4} A_i X_i + \sum_{i=1}^{4} A_{ii} X^2_i + \sum_{i=1}^{4} \sum_{j=i+1}^{4} A_{ij} X_i X_j \quad (2)$$

Where Y is the response variable (DPPH scavenging rate of total antioxidative components), A_0 is constant, and A_i, A_{ii} and $A_{ij}$are coefficients estimated by the model. X_i and X_j are the levels of the independent variables. A_i, A_{ii} and A_{ij} represent the linear, quadratic and cross-product effects of the factors (X_1, X_2, X_3 and X_4) on the response, respectively.

Analysis of the experimental design and calculation were performed using Design Expert software (version7.0, Stat-Ease Inc., Minneapolis, MN, USA). Analyses of variance were performed. Mean values are considered significantly different when $P < 0.05$.

Table 1. Independent variables and their levels used for central composite design

Variables	Levels				
	-2	**-1**	**0**	**+1**	**+2**
X_1extraction time /min	10	20	30	40	50
X_2 ethanol volume fraction / %	50	60	70	80	90
X_3 extraction temperature / ºC	20	30	40	50	60
X_4 liquid-solidratio/ (mL.g^{-1})	10	20	30	40	50

RESULTS AND DISCUSSION

Optimization of ultrasound-assisted extraction for total antioxidative components from *A. graminifolia*: Based on the results of preliminary experiments, the value of responses (DPPH radical scavenging rate of total antioxidative components) under various experimental conditions for coded variables is given in Table 2. The response variable and the test variables are related by the following second-order polynominal equation:

$$E=78.9-0.11X_1+0.29X_2+0.26X_3+0.26X_4-0.26X_1X_2-0.018X_1X_3-0.39X_1X_4+0.069X_2X_3+0.29X_2X_4-0.032\ X_3X_4-0.67X_1^2-0.45\ X_2^2-0.30X_3^2-0.71X_4^2 \quad (3)$$

Table 2.Results of response surface analysis of total antioxidative components from *A. graminifolia* with factors

Test NO	X_1	X_2	X_3	X_4	Scavenging rate (E, %)
1	-1	-1	+1	+1	76.86
2	-1	-1	-1	-1	75.72
3	0	0	-2	0	76.51
4	0	0	0	0	79.25
5	0	0	0	0	78.32
6	-1	+1	-1	-1	76.13
7	0	0	0	0	79.40
8	-1	+1	-1	+1	78.06
9	0	0	0	0	78.59
10	+1	-1	-1	+1	76.06
11	+1	-1	-1	-1	77.13
12	+1	+1	+1	-1	76.56
13	0	0	0	+2	75.63
14	+1	+1	-1	-1	76.33
15	+2	0	0	0	75.53
16	0	0	0	-2	75.77
17	0	0	0	0	79.34
18	0	-2	0	0	76.06
19	+1	-1	+1	-1	77.39
20	0	0	0	0	78.52
21	-2	0	0	0	76.23
22	+1	-1	+1	+1	76.90
23	0	0	+2	0	78.19
24	-1	+1	+1	+1	78.99
25	-1	-1	-1	+1	77.41
26	0	+2	0	0	77.49
27	-1	+1	+1	-1	77.00
28	-1	-1	+1	-1	76.06
29	+1	+1	-1	+1	77.29
30	+1	+1	+1	+1	77.26

As shown in Table 3, high significant level ($P<0.0001$) and good correlation coefficient ($r=0.9364$) of the model indicate that the deduced model can predict the DPPH scavenging rate of total antioxidative components.

Statistical analysis shows that quadratic terms, such as the liquid-solid ratio ($P<0.0001$) and ethanol volume fraction ($P<0.0001$) for the DPPH scavenging rate of total antioxidative components are highly significant ($P<0.01$), and liner term, such as extraction time ($P=0.0136$), ethanol volume fraction ($P=0.0108$) and extraction temperature ($P=0.0164$) and quadratic terms,

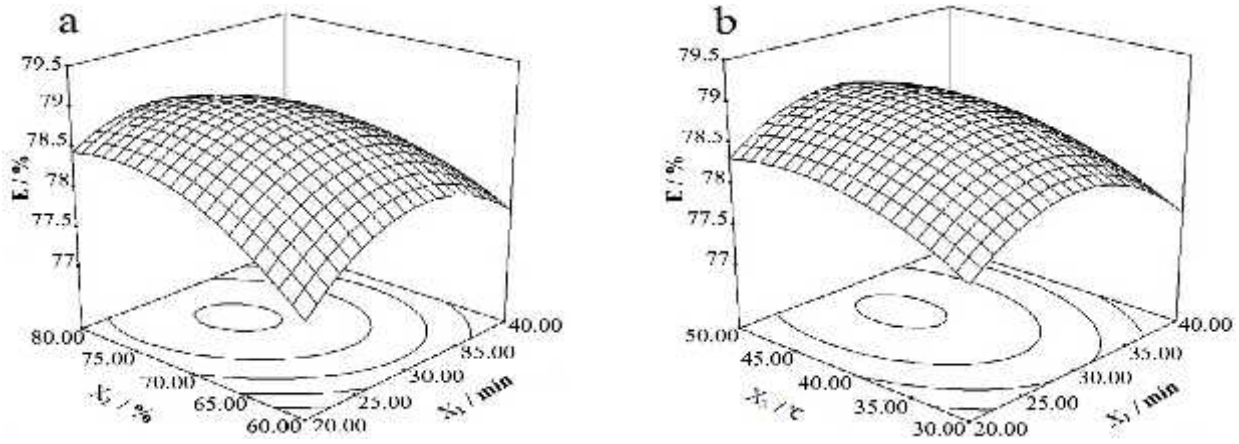
such as extraction time(P=0.0022) and extraction temperature(P=0.0005) are significant (P<0.05).

The interaction between ethanol volume fraction and extraction temperature(P=0.0126), extraction temperature and liquid-solid ratio (P=0.0264) and liquid-solid ratio and ethanol volume fraction (P=0.0097) are significant (P<0.05).Quadratic terms, such as liquid-solid ratio(P=0.4017)and the interaction between extraction time and ethanol volume fraction (P=0.9917), extraction time and extraction temperature (P=0.8354) and extraction time and liquid-solid ratio (P=0.6049) are non-significant terms (P>0.05).

Table 3. Analysis of variance for the fitted regression model

Source	df	Sum of Squares	Mean Square	FValue	PValue
Model	14	35.97	2.57	11.66	0.0001
X_1	1	0.31	0.31	8.46	0.0136
X_2	1	2.01	2.01	7.30	0.0108
X_3	1	1.63	1.75	7.82	0.0164
X_4	1	1.62	0.17	0.74	0.4017
X_1X_2	1	1.09	2.5×10^{-5}	1.117×10^{-4}	0.9917
X_1X_3	1	5.256×10^{-3}	0.010	0.045	0.8354
X_1X_4	1	2.49	0.062	0.28	0.6049
X_2X_3	1	0.077	1.80	8.03	0.0126
X_2X_4	1	1.35	1.96	8.76	0.0097
X_3X_4	1	0.016	1.36	6.07	0.0264
X_1^2	1	12.28	3.04	13.57	0.0022
X_2^2	1	5.44	13.93	62.27	< 0.0001
X_3^2	1	2.49	4.45	19.88	0.0005
X_4^2	1	13.99	13.99	60.11	< 0.0001
Residual	15	5.05	0.34		
Lack of Fit	10	3.91	0.39	1.28	0.4147
Pure Error	5	1.14	0.23		
Cor Total	29	41.02			

Response surface analysis



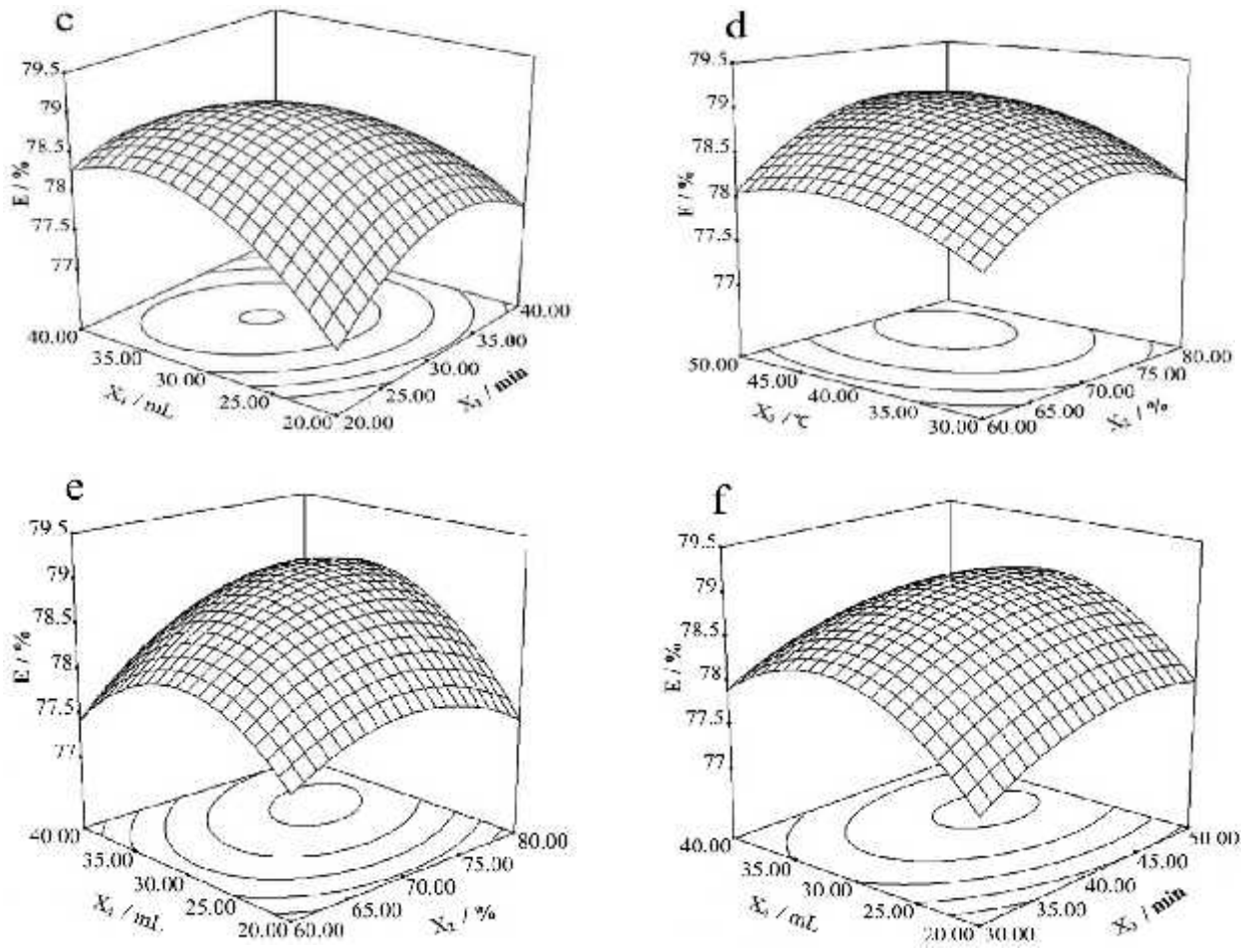


Fig.1 Response surface for effect of extraction time (X_1),ethanol volume fraction (X_2), extraction temperature (X_3) and liquid-solid ratio (X_4) on scavenging rate (E)

Contour plots were obtained using statistical software according to each independent variable and responses. Contour plots were used to determine the optimum formulation parameters. Each contour plot had its own optimal area which represented the optimal formula condition for ideal response.

The three-dimensional response surfaces showing the interaction effects of the optimization parameters on the DPPH radical scavenging rate are shown in Fig. la-f. Fig.1a shows the simultaneous effect of extraction time and ethanol volume fraction on the scavenging rate. Scavenging rate increased with an increase of 20 to 35 min in extraction time and of 50% to 75%in ethanol volume fraction. However, the scavenging rate decreased with an increase in extraction time beyond 35 min and ethanol volume fraction beyond 75%. The effect of extraction time and extraction temperature on scavenging rate is shown in Fig.1b. This demonstrates that the scavenging rate increased with an increase in extraction time. While an increase in extraction temperature also led to an increase in scavenging rate, the initial increase in the scavenging rate was followed by a decrease at more than 35 min and above 50 ºC.Fig.1c shows the effect of extraction time and liquid-solid ratio on the scavenging rate, with an increase asthe liquid-solid ratio went from 20 to 30 at extraction time of below 35 min. However, a liquid-solid ratio of more than 30 at more than 35 min appeared to have a negative effect. From Fig.1a-c, it can be seen that an increase in extraction time from 20 to 35 min enhanced the extraction, while at more than 35 min a negative effect was observed. This may be because a longer extraction time results in the chemical decomposition of antioxidative compounds present in the extract.

According to the slope gradient in the 3-Dresponse surface plots (Fig.1) and the value of Prob>Fin Table.3, the importance of the independent variables on the DPPH radical scavenging rate can be

ranked in decreasing order: ethanol volume fraction>extraction time>extraction temperature>liquid-solid ratio. This is consistent with the results of variance analysis.

The experimental optimization and experimental verification: The validate experiment was carried out under the optimal conditions and the predicted value is 79.16% and the measured value is 78.71% (n=5, RSD=3.6%). The deviation between them is 0.45%, which demonstrates that the central composite design coupled with response surface methodology (CCD-RSM)is highly predictive in experimental design.

Conclusions: The response surface methodology (RSM) was successfully employed to optimize the ultrasonic-assisted extraction of the antioxidative components from *A. graminifolia*. The four variables chosen (extraction time, ethanol volume fraction, extraction temperature and liquid-solid ratio) all influence the DPPH free radical scavenging activity of *A.graminifolia* extract. Response surface analysis showed that the optimum extraction conditions were as follows: extraction time 35 min, ethanol volume fraction 74%, extraction temperature46ºC and liquid-solid ratio 27 ($mL.g^{-1}$). Under these conditions, the average DPPH radical scavenging rate of antioxidative components from *A. graminifolia*was 78.71 % (n=5, RSD=3.6%), which was close to the predicted values (79.16 %). In addition, the antioxidative components produced under the optimum conditions exhibited excellent DPPHfree radical scavenging activity. These findings can provide a reference for full utilization of *A.graminifolia* and a technique to extract antioxidative components.

Acknowledgement: The authors thank the director, for his encouragement and support. This study was supported by the National Natural Science Foundation of China (51062018 and 21205104), the Natural Science Foundation of Yunnan (2010FXW004), the Program for Innovative Research Team (in Science and Technology) at the University of Yunnan Province (2010UY08, 2011UYN09) and Program for Yunnan Provincial Innovation Team (2011HC008).

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