Journal of Medicinal Plants Research Vol. 6(37), pp. 5038-5047, 26 September, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR11.1596
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Optimization of total flavonoid extraction in *Callicarpa nudiflora* Hook. Et Arn. using response surface methodology

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Accepted 16 May, 2012

***Callicarpa nudiflora* Hook. Et Arn. is one of the most important medicinal plants, but no information is available on optimal extraction of its total flavonoids. Effects of four single-factor treatments on total flavonoids during extraction of *C. nudiflora* and optimization of extraction condition, namely time, ethanol concentration, liquid-solid ratio, and temperature on the extracting rate of total flavonoids of *C. nudiflora* were studied in this paper. The optimization processes were conducted using response surface methodology. The results showed that the optimal conditions by central composite experimental design for extracting rate of total flavonoids was at a temperature of 90°C, liquid-solid ratio 41:1, ethanol concentration 73%, and time 3.6 h. Under these conditions, the maximal observed extracting rate (5.378%) of total flavonoids of leaves was obtained. Analysis of variance for quadratic polynomial regression model indicated that the model was extremely significant (*P*<0.0001) and the determination coefficient (R^2) was 0.9613, which implied that the model of extraction technology is simple, reliable and highly efficient. At the same time, it can forecast the changes of extracting rate of total flavonoids during the extraction, and provide theoretical basis for practical production.**

Key words: *Callicarpa nudiflora* Hook. Et Arn., total flavonoids, response surface methodology, extraction technology.

INTRODUCTION

Callicarpa nudiflora Hook. Et Arn. is one of the most important members of the *Callicarpa* tribe, within the Verbenaceae family (Wu, 2008). *C. nudiflora* is widely distributed in tropical regions including India, Vietnam, Malaysia, China and Singapore (Wu, 2008). *C. nudiflora* is a deciduous shrub or small tree reaching a height of 3 to 4 m (Liang et al., 2009). It has been used for the treatment of the antibacterial, anti-inflammatory detoxification, stasis swelling, dispels wind and dampness, as well as an effect of analgesic and hemostatic in the medicinal materials of China and its surrounding region (Ana et al., 2003). *C. nudiflora* mainly is used for luohuazizhu tablet, luohuazizhu suppository and luohuazizhu capsule in main raw material for medicine. The leaves of *C. nudiflora* have flavonoids, aetheroleas, phenolics, condensed tannins, polysaccharides and other compounds (Tellez et al., 2000; Kobaisy et al., 2002; Liang et al., 2009).

Several studies have been conducted to examine the chemical constituents, clinical curative effect and pharmacology of *C. nudiflora* (Tellez et al., 2000; Kobaisy et al., 2002; Wang et al., 2007; Liang et al., 2009; Zhang et al., 2010a, 2010b), but no information concerning total flavonoids extraction from leaves of *C. nudiflora* using response surface methodology has been published. Would there be any ways to increase the concentration of total flavonoids and improve the efficacy of value of *C. nudiflora* through different extraction methods? To develop a process for the maximum extraction of total flavonoids, standardization of ethanol concentration, liquid-solid ratio, temperature, and time condition is crucial.

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Table 1. Independent variables and their coded and actual values using a CCD-RSM for optimization of total flavonoids extraction.

Independent variable	Symbol	Code levels				
		-2	-1	0	1	2
Alcohol (%)	X_1	50	60	70	80	90
Liquid-solid ratio (/)	X_2	20	30	40	50	60
Time (h)	X_3	1	2	3	4	5
Temperature (°C)	X_4	60	70	80	90	100

Currently, several design methods which contains orthogonal design method, uniform design and response surface methodology design (RSM) were applied in the extraction of flavonoids of herbal (Wang et al., 2007; Liao et al., 2008; Yang et al., 2010). Orthogonal design method is a better combination only in the established factors and levels, but the setting values are at the discrete level and the variation law of the data has uncertainty; uniform design is less work but with poor flexibility.

The RSM is an empirical statistical technique employed for multiple regressionn analysis using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously and carrying out only a limited and fixed number of experiments (Cochran and Cox 1957; Box and Hunter, 1978; Nabeena et al., 2005; Su et al., 2009). Thus, the main objective of this study was to find a way to improve the extracting rate of total flavonoids in *C. nudiflora* and reduce cost by applying RSM.

MATERIALS AND METHODS

The fresh leaves of *C. nudiflora* were obtained from Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences (Danzhou, China). The materials were stored at 4°C until used. Rutin standard sample was purchased from Chinese National Institute for the Control of Pharmaceutical and Biological Products. A series of rutin standards were prepared by diluting a stock solution of rutin (0.239 mg/ml) with ethanol to obtain 0, 23.9, 47.8, 71.7, 95.6 and 119.5 μg/ml. The stability of these standards was investigated at indoor temperature. The other chemicals and reagents used were of analytical grade. The equation of calibration curve was y = 0.960 x + 0.056, with the R^2= 0.9994, where y stands for the concentration of rutin standard solution and x for the corresponding absorbency. The results of content and absorbance have a good linear relationship.

Extraction and measurement of total flavonoids

Powered sample (0.3 g) was added to 35% alcohol at 30 times of sample volume, and then refluxed at 40°C for 6 h. The content of total flavonoids was measured at 510 nm, corresponding to maximum absorbance by ultraviolet (UV) spectrophotometer (Hongshen yiqi Co. Ltd, Shenzhen, China) (Liao et al., 2010).

The extraction rates were obtained according to the standard curve (y = 0.960x + 0.056).

Experimental design

The experimental design was performed using Stat-Ease software (Design-Expert version 6.0.10 Trial, Delaware, USA Echip, 1993). Central composite design of RSM (CCD-RSM) was employed to optimize the extraction for total flavonoids in *C. nudiflora*. A four-factor five-level central composite design with four replicates was chosen to evaluate the combined effects of four independent variables. These factors were code at five levels starting from -2, -1, 0, 1, and 2 defined by Equation 1:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad i = 1, 2, 3, \ldots k \tag{1}$$

Where x_i is the dimensionless coded value of the variable X_i, X_0 is the value of the X_i at the center point and ΔX_i the step changes. On the basis of single factor experiments, alcohol (X_1), liquid-solid ratio (X_2), time (X_3), and temperature (X_4) were determined (Table 1). Extracting rate of total flavonoids (Y) was taken as response value. CCD-RSM is provided in Table 2. The complete experimental design consisted of 30 experimental points.

Data analysis

For statistical calculations, the variables Xi were coded as x_i following transformation according to Equation 1. The experimental results should be the same with a second-order polynomial equation (Equation 2) by a multiple regression technique:

$$Y = \beta_0 + \sum_{i=1}^{k} B_i X_i + \sum_{i=1}^{k} B_{ii} X_i^2 + \sum_{i>j}^{k} B_{ij} X_i X_j \tag{2}$$

where Y stands for extracting rate of total flavonoids, β_0 denotes the model intercept; X_i and X_j are the coded independent variables; B_i, B_{ii} and B_{ij} represent the regression coefficients of variables for linear, quadratic and interaction regression terms, respectively; k equals to the number of the tested factors (k = 4). All the trials were carried out four times. The experimental results obtained were expressed as mean ± SD. An analysis of variance (ANOVA) table is generated to determine individual linear, quadratic and interaction regression coefficients and the means separated by Duncan's multiple range test (Jeong et al., 2009). The significances of polynomial relations are examined statistically by computing the F value at a probability (*P*) of 5%, 1%, or 1‰ level, respectively. The regression coefficients are then used to make statistical analyses and to generate contour maps of the regression models.

Table 2. Central composite design of response surface methodology and experiment data for total flavonoids from the leaves of *C. nudiflora.*

Treatment no.	X_1	X_2	X_3	X_4	Extracting rate of total flavonoids (%)	
	Alcohol (%)	Liquid- solid ratio (*l*)	Time (h)	Temperature (°C)	Observed value	Predicted value
1	60	30	2	70	4.06 ± 0.37	3.92
2	80	30	2	70	4.24 ± 0.32	4.23
3	60	50	2	70	4.69 ± 0.11	4.50
4	80	50	2	70	4.92 ± 0.22	4.94
5	60	30	4	70	4.34 ± 0.23	4.39
6	80	30	4	70	4.60 ± 0.39	4.68
7	60	50	4	70	4.71 ± 0.13	4.80
8	80	50	4	70	4.90 ± 0.25	4.95
9	60	30	2	90	4.74 ± 0.45	4.68
10	80	30	2	90	5.08 ± 0.40	5.13
11	60	50	2	90	5.12 ± 0.16	5.20
12	80	50	2	90	4.98 ± 0.29	5.01
13	60	30	4	90	5.11 ± 0.21	5.15
14	80	30	4	90	5.16 ± 0.26	5.20
15	60	50	4	90	5.21 ± 0.31	5.25
16	80	50	4	90	5.35 ± 0.34	5.30
17	50	40	3	80	4.87 ± 0.35	4.90
18	90	40	3	80	5.02 ± 0.20	5.00
19	70	20	3	80	4.59 ± 0.16	4.54
20	70	60	3	80	4.98 ± 0.15	4.87
21	70	40	1	80	4.45 ± 0.33	4.56
22	70	40	5	80	5.05 ± 0.14	5.12
23	70	40	3	60	4.10 ± 0.15	4.15
24	70	40	3	100	5.25 ± 0.12	5.27
25	70	40	3	80	5.17 ± 0.14	5.26
26	70	40	3	80	5.17 ± 0.13	5.34
27	70	40	3	80	5.16 ± 0.46	5.23
28	70	40	3	80	5.17 ± 0.37	5.16
29	70	40	3	80	5.17 ± 0.36	5.21
30	70	40	3	80	5.17 ± 0.34	5.23

RESULTS

Effect of ethanol concentration on extracting rate of total flavonoids

The extraction condition used from 20 to 90% ethanol at 30 times of sample volume, refluxing at 80°C for 3.0 h according to Liao et al. (2008). The results showed that the extracting rate of total flavonoids increased with the rise of ethanol concentration. The extracting rate of total flavonoids was the highest using 70% ethanol and subsequently decreased from 70 to 90%. Therefore, the extracting concentration of ethanol, which is from 50 to 90%, was chosen for further experiment (Figure 1).

Effect of liquid-solid ratio on extracting rate of total flavonoids

The extraction condition used 60% ethanol at 10, 20, 30, 40, 50, and 60 times of sample volume, respectively, refluxing at 80°C for 3.0 h according to Liao et al. (2008). The results shown in Figure 2 indicated that the extracting rate of total flavonoids increased significantly ($P<0.05$) when the solid-liquid ratio were from 10:1 to 40:1. The extracting rate of total flavonoids was non-significant ($P>0.05$) when the liquid-solid ratio were from 40:1 to 60:1. The extracting rate of total flavonoids may be caused by degradation of high liquid-solid ratio. Medical Components are almost totally extracted when certain liquid-solid ratio as reported in recent studies in the field of the optimization conditions (Zheng et al., 2009; Liao et al., 2008, 2010). Hence, it was chosen from 20:1 to 60:1 for further experiment.

Effect of extracting time on extracting rate of total flavonoids

The extraction condition involved 60% ethanol at 30

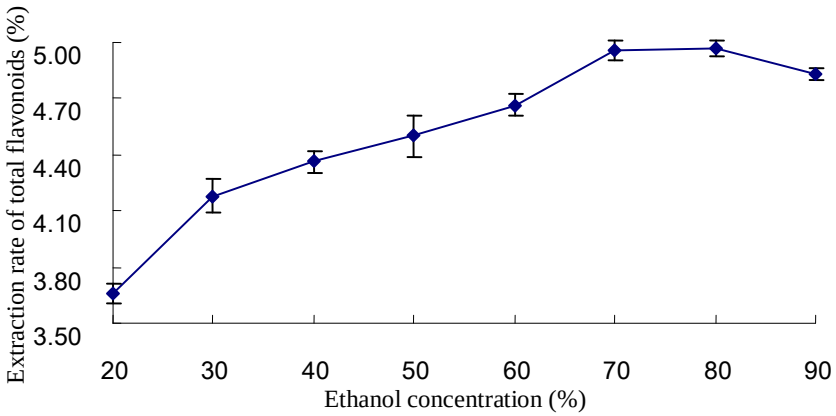

Figure 1. Effect of alcohol concentration on the extracting rate of total flavonoids.

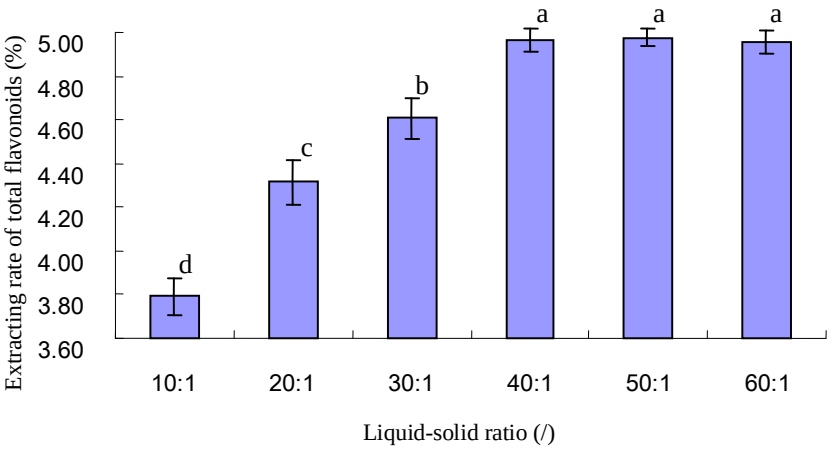

Figure 2. Effect of liquid-solid ratio on the extracting rate of total flavonoids.

times of sample volume, refluxing at 80°C for 1, 2, 3, 4, 5, and 6 h, respectively, according to Liao et al. (2008). The results showed that the extracting rate of total flavonoids increased significantly (P<0.05) with the extracting time between 1 and 3 h. It decreased non-significantly (P>0.05) with the extraction time from 3 to 6 h. Thus, the extracting time with 1 to 5 h, was chosen for further experiment.

Effect of extracting temperature on extracting rate of total flavonoids

The extraction condition used 60% ethanol at 30 times of sample volume and refluxing from 40 to 80°C for 3 h according to Liao et al. (2008). The results showed that the extracting rate of total flavonoids was highest when temperature increased to 90°C. It, however, increased insignificantly (P>0.05) when the temperature rose from 80 to 100°C. Therefore the extracting temperature, between 60 and 100°C, was chosen for further experiments.

Analysis of CCD-RSM and determination of the regression equation

The CCD-RSM design and the corresponding experimental data of four single-factor experiment (Figures 1 to 4) were shown in Table 2. To understand an empirical relationship between the extracting rate of total flavonoids and test variables (Table 1), we found that the model of CCD-RSM design were consistent with the second-order polynomial equation referred to in Equation (2). The second-order polynomial model describing the correlation between extracting rate of total flavonoids and the four variables in this study (Table 1) was obtained in Equation (3):

$$Y = 5.17 + 0.068 \times X_1 + 0.14 \times X_2 + 0.11 \times X_3 + 0.27 \times X_4 - 0.024 \times X_1 \times X_2 + 1.750E\text{-}003 \times X_1 \times X_3 - 0.030 \times X_1 \times X_4 - 0.041 \times X_2 \times X_3 - 0.087 \times X_2 \times X_4 + 0.017 \times X_3 \times X_4 - 0.056$$

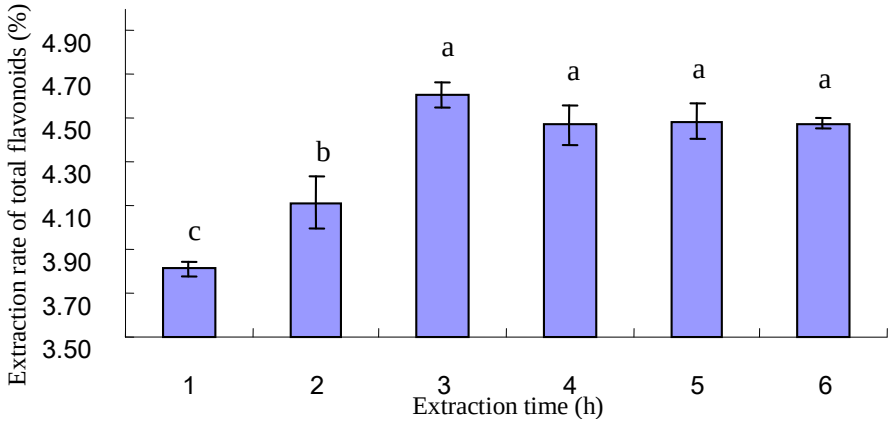

Figure 3. Effect of extracting time on the extracting rate of total flavonoids.

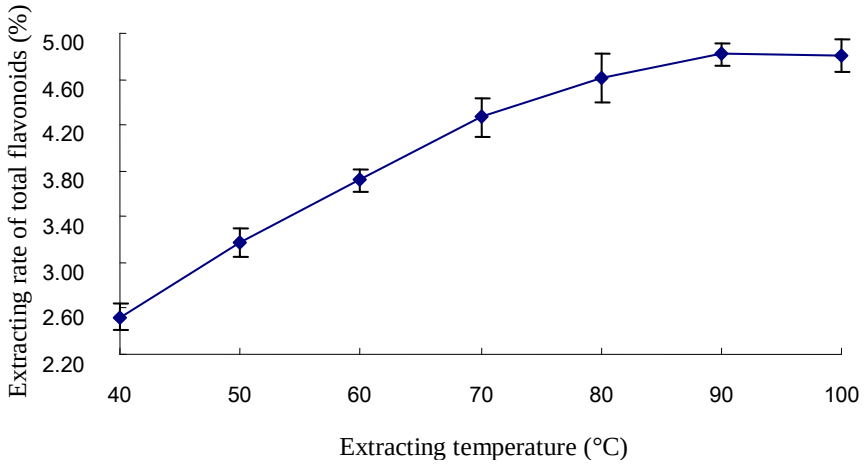

Figure 4. Effects of extracting temperature on the extracting rate of total flavonoids.

$\times X_1^2 - 0.089 \times X_2^2 - 0.098 \times X_3^2 - 0.12 \times X_4^2$ (3)

The comparison of the observed values of extracting rate of total flavonoids with the predicted values (Figure 5) showed that the two sets of values were very close, indicating that the experimental model was valid. The calculated coefficient of determination (R^2) was 0.9627 (Figure 5). By ANOVA for Equation (3), we obtained an F-value of 29.304, which implied that the model was very significant (P<0.0001). A coefficient of determination (R^2) of 0.9613 indicated a close agreement between experimenttal and predicted extracting rate of total flavonoids (Table 3). These results also proved the validity of the experimental model.

Analysis of optimization of extracting conditions by CCD-RSM

Extracting rate of total flavonoids at 80°C for 3 h was non-significant between ethanol concentration and liquid-solid ratio (P>0.05) as shown in Figure 6. However, ethanol concentration and liquid-solid ratio had significant quadratic effect (P<0.0029) or most significant linear and quadratic effects (P<0.0001) on the extracting rate of total flavonoids (Table 3). Extracting rate of total flavonoids increased with increase of ethanol concentration and liquid-solid ratio under fixed time and temperature. The extracting rate of total flavonoids reached a maximum of 5.17% when ethanol concentration and liquid-solid ratio was 70% and 40:1, respectively. Figure 7 shows the relationship between the ethanol concentration (50 - 90%) and extracting time (1 - 5 h) (very significant independent variables) and extracting rate of total flavonoids (dependent variable) when the temperature and liquid-solid ratio were set. When the ethanol concentration was 73%, the extracting rate of total flavonoids was at its peak, indicating that 73% was the optimal temperature for total flavonoids extraction. At the same time, when extracting time was 3.6 h, the extracting rate of total flavonoids was at the maximum value.

As shown in Figure 8, it can be seen that the maximum

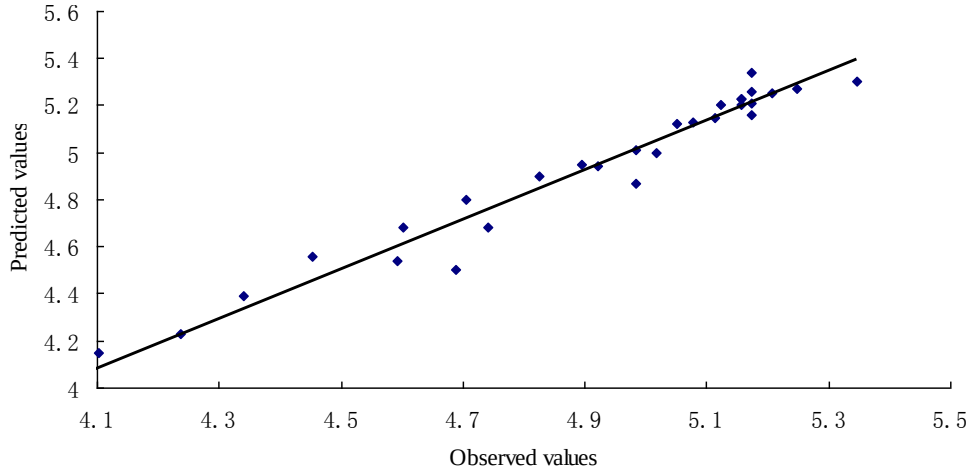

Figure 5. Regression plot for extracting rate of total flavonoids observed and predicted values.

Table 3. Analysis of variance (ANOVA) for response surface quadratic model analysis.

Source	Sum of squares	df	Mean square	F-Value	P-value
Model	3.5521	14	0.2537	29.304	< 0.0001*
X_1	0.1096	1	0.1096	12.661	0.0029*
X_2	0.4609	1	0.4609	53.237	< 0.0001*
X_3	0.3110	1	0.3110	35.919	< 0.0001*
X_4	1.8084	1	1.8084	208.868	< 0.0001*
X_1X_2	0.0095	1	0.0095	1.098	0.0311
X_1X_3	0.0000	1	0.0000	0.941	0.1016
X_1X_4	0.0143	1	0.0143	1.649	0.0218*
X_2X_3	0.0266	1	0.0266	3.069	0.1002
X_2X_4	0.1222	1	0.1222	14.108	0.0019*
X_3X_4	0.0045	1	0.0045	0.518	0.0483*
X_1^2	0.0851	1	0.0851	9.832	0.0068*
X_2^2	0.2183	1	0.2183	25.211	0.0002*
X_3^2	0.2632	1	0.2632	30.399	< 0.0001*
X_4^2	0.3760	1	0.3760	43.428	< 0.0001*
Residual	0.1299	15	0.0087		
Lack of fit	0.1292	10	0.0129	91.564	< 0.0001
Pure error	0.0007	5	0.0001		
Cor. Total	3.6819	29			

*p<0.05, R^2=0.9613.

response for extracting rate of total flavonoids is attained when ethanol concentration and extracting temperature are about 73% and 90°C, respectively, by setting the extraction time (3 h) and liquid-solid ratio (40: 1). These two factors have synergistic effects on extracting rate of total flavonoids (Table 3). Figure 9 depicted the combined effects of liquid-solid ratio and extraction time in the extraction rate of total flavonoids by fixed ethanol concentration (70%) and extracting temperature (90°C). From the elliptical nature of the contour plot, a prominent interaction between them was obvious. We noted a distinct increase in extracting rate of total flavonoids with the addition of liquid-solid ratio or prolonged extracting time. The extracting rate of total flavonoids was the highest when liquid-solid ratio and extracting time were 41: 1 and 3.6 h, respectively. Furthermore, as shown in Figure 10, the interaction between the two variables (liquid-solid ratio and extracting temperature) in this model was very significant (P<0.01) in extracting rate of total flavonoids (Table 3) at 70% ethanol and 3 h. There

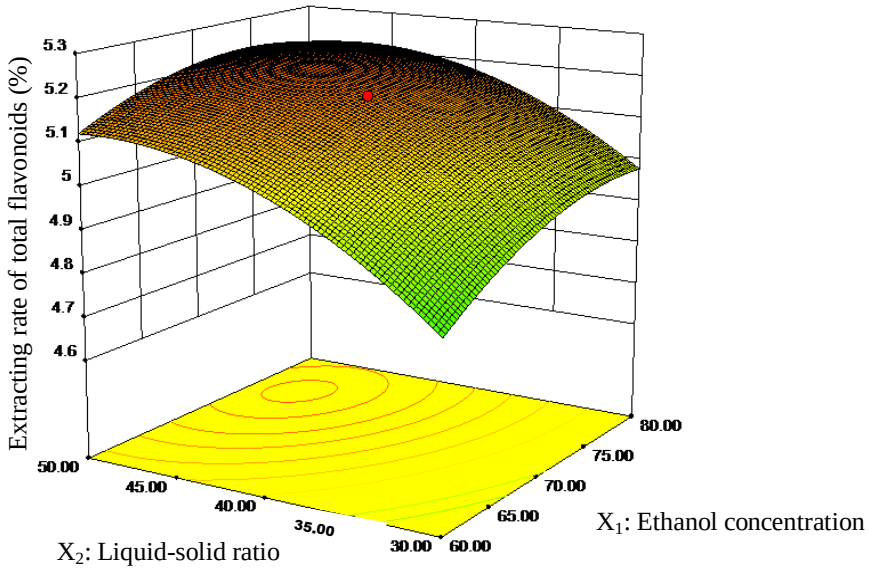

Figure 6. Response surface plots representing the effects of liquid-solid ratio and ethanol concentration on the extracting rate of total flavonoids.

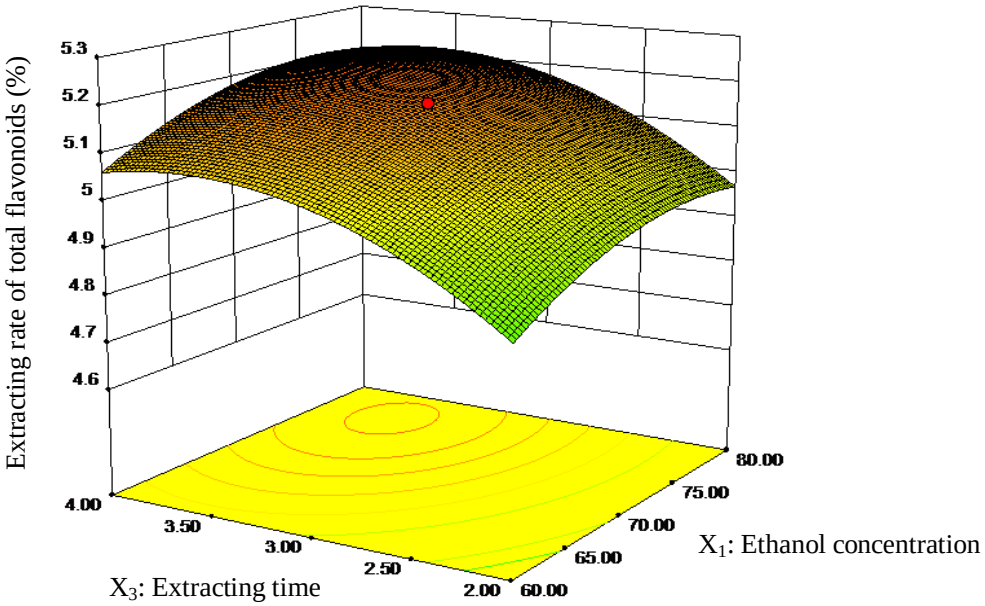

Figure 7. Response surface plot on the effects of extracting time and ethanol concentration on the extracting rate of total flavonoids.

are very significant linear relationship ($P<0.0019$) between liquid-solid ratio and extracting temperature. The extracting rate of total flavonoids increased when extracting temperature increased from 50 to 90°C. However, it did not continue to increase significantly when temperature was higher than 90°C. The optimization combination was as follows: liquid-solid ratio 41: 1 and temperature 90°C.

The roles of extracting rate of total flavonoids on extracting time and extracting temperature are illustrated in Figure 11. The experimental results demonstrate that total flavonoids increased with increasing extracting time and temperature. The extracting rate of total flavonoids was highest when extracting time and temperature were 3.6 h and 90°C, respectively. To sum up, according to CCD-RSM test results, the optimal condition obtained from the model was as follows: ethanol 73%, liquid-solid ratio 41: 1, time 3.6 h, and temperature 90°C. The model

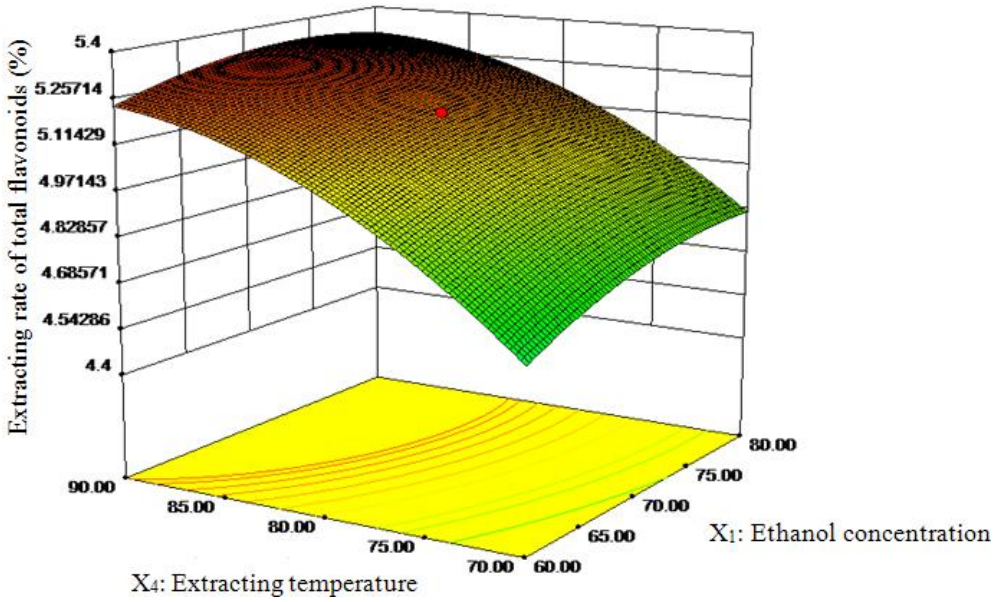

Figure 8. Response surface plot on the effects of extracting temperature and ethanol concentration on the extracting rate of total flavonoids.

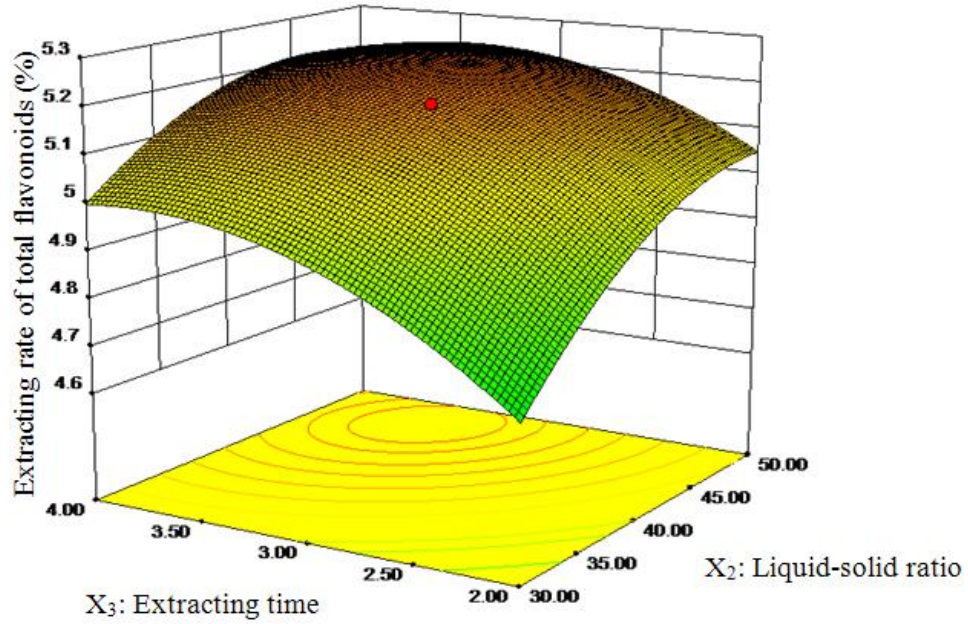

Figure 9. Response surface plot on the effects of liquid-solid ratio and extracting time on the extracting rate of total flavonoids.

predicted that the maximal extracting rate of total flavonoids was 5.374%, and the predicted desirability was attained as 0.976 as shown in Figures 6 to 11. Under these conditions, the observed extracting rate of total flavonoids was 5.380%, which was quite close to the predicted value. The observed results therefore proved that the model was valid.

DISCUSSION

The classical method of extraction medium optimization involves changing the level of one variable parameter at a time, while holding the other test variables constant. This single factor strategy is generally time-consuming and requires a large number of experiments to be carried out,

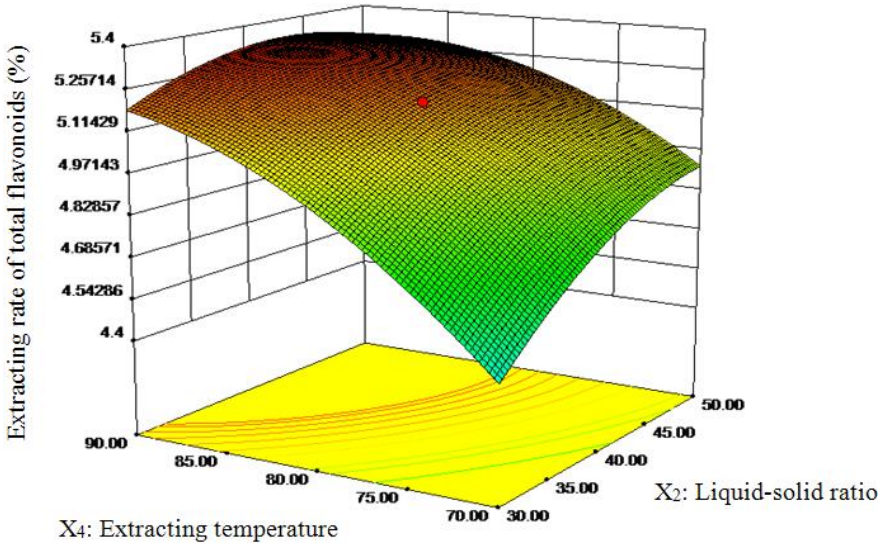

Figure 10. Response surface plot on the effects of liquid-solid ratio and extracting temperature on the extracting rate of total flavonoids.

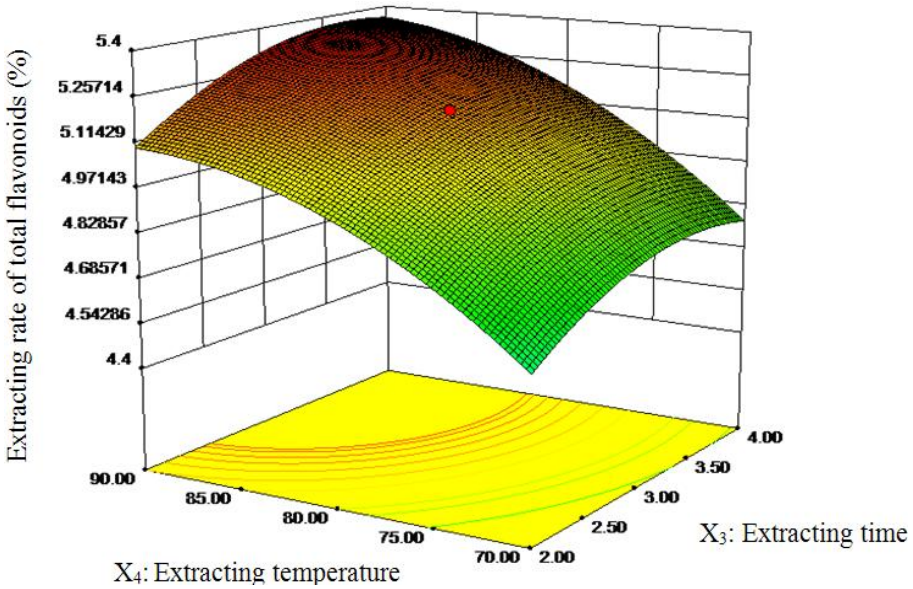

Figure 11. Response surface plot on the effects of extracting time and extracting temperature on the extracting rate of total flavonoids.

especially when the number of variables is large. An alternative and more efficient approach in extracting technology makes use of statistical experimental design methodology (Su et al., 2009). RSM has been used in the studies on extractions in *Setaria italica* L. (Bai et al., 2008), cereals (Cho et al., 2010), chestnut shell (Vázquez et al., 2010) and other plants (Liao et al., 2008, 2010). As for the application of RSM in the medicinal plants, the previous reports mainly focused on the preliminarily study of the relationships among different species including Chinese yam (Zu et al., 2011), *Hypericum perforatum* (Guo et al., 2011), *Arnebia euchroma* (Yang et al., 2010), *Flos Chrysanthemi Indici* (Fang et al., 2009), and other medicinal plants (Fang et al., 2009).

There are many methods which are related to total flavonoids extraction. As compared with ultrasonic extraction (Dong and Bai, 2008), microwave-assisted extraction (Xie et al., 2009), and supercritical fluid extraction (Ou et al., 2010), lixiviating method has advantage of low cost, simplified operation and wide application (Chen et al., 2011). Therefore, in this study total flavonoids of *C. nudiflora* were extracting using lixiviating method.

Based on our results, there are four factors affecting extraction of total flavonoids, which agreed with previous reports (Dorota, 1999; Wang et al., 2004; Li et al., 2006; Xu et al., 2009). It was demonstrated that the total flavonoids was increasingly or decreasingly extracted from leaves of *C. nudiflora* with the increase of certain factors (time, ethanol concentration, liquid-solid ratio, and temperature) and thawing (Figures 1 to 4). It may be that poor affinity of high concentration ethanol and medical components of Chinese Herbs (Zheng et al., 2009) or labile flavonoids were degraded with increasing temperature and time (Li et al., 2006). Extracting rate may also be decreased because of chemical content instability with increasing time (Li et al., 2006). Wang et al. (2004) and Li and Yu. (2009) showed that alcohol-soluble impurities was extracted with increasing ethanol concentration and then the bonding strength was decreased between total flavonoids and ethanol.

Herein, we conducted experiments using CCD-RSM to identify the optimal extraction factors for the extracting rate of total flavonoids on this study. Among the four factors, ethanol concentration affected the extraction slightly less than other three factors. By CCD-RSM, the optimized extracting conditions were ethanol 73%, liquid-solid ratio 41: 1, time 3.6 h, and temperature 90°C. Under these optimized conditions, the extracting rate of observed value (5.380%) was close to the predicted value (5.374%) and 17% higher than random conditions. The results showed that the model by four-factor five-level CCD-RSM was confirmed to be valid. This study indicates that *C. nudiflora* contains rich total flavonoids and provides good guiding role for industrial production.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (31060266) and the Hainan Provincial Natural Science Foundation (310029). The authors are grateful to the editors and referees for their valuable comments to improve our manuscript.

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