EXTRACTION PROCESS OPTIMIZATION OF TOTAL PHENOLIC AND TOTAL FLAVONOID FROM LEAVES OF PHYLLANTHUS URINARIA L. USING THE RESPONSE SURFACE METHODOLOGY

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TÓM TẮT

TỐI ƯU HÓA QUÁ TRÌNH CHIẾT XUẤT TỔNG HÀM LƯỢNG PHENOLIC VÀ FLAVONOID TỪ LÁ CÂY DIỆP HẠ CHÂU BẰNG PHƯƠNG PHÁP ĐÁP ỨNG BỀ MẶT

Sử dụng phương án thiết kế thí nghiệm tại tâm của phương pháp đáp ứng bề mặt để tối ưu hóa điều kiện chiết xuất tổng hàm lượng phenolic và flavonoid từ lá cây Diệp hạ châu (Phullanthus urinaria.L) với 3 yếu tố ảnh hưởng là nhiệt độ chiết xuất, thời gian chiết xuất và nồng độ ethanol. Các điều kiện tối ưu từ phương pháp đáp ứng bề mặt được xác định là nhiệt độ chiết xuất ở 70^{0}C, thời gian chiết xuất 80 phút và nồng độ ethanol 80%. Giá trị thực nghiệm tại điều kiện tối ưu của tổng hàm lượng phenolic và flavonoid là 126.83±0.5mgGAE/g và 13.36±0.2 mg QE/g.

Từ khóa: *Diệp hạ châu, tổng hàm lượng phenolic, tổng hàm lượng flavonoid, đáp ứng bề mặt, chiết xuất.*

1. INTRODUCTION

Phyllanthus urinaria L. belongs to the castor family Euphorbiaceae. In Chinese, The *Phyllanthus urinaria* L., also called ‘‘pearls under the leaves”, is widely used as a traditional folk medicine. Leaves of *P. urinaria* are widely used as a traditional folk medicine for inflammatory relief [1]. It was reported that boiling water extracts of *P. urinaria* exhibited cytotoxic activity against Lewis lung carcinoma cells and human cancer cells such as HL-60, Molt- 3, HT 1080, K-562, Hep G2, and NPC-BM1 [2]. The boiling water extracts of *P. urinaria* were also reported to exhibit antitumor and antiangiogenic effects against Lewis lung carcinoma in mice [3]. All parts of the *P. urinaria* plant especially leaves of contain high amounts of phenolic and flavonoid compounds with potential antioxidant properties. The organic solvent (including acetone, ethanol, and methanol) extracts of *P. urinaria* were able to inhibit HSV-2 infection [4]. The ethanolic extracts of *P. urinaria* were reported to have antioxidant and cardioprotective effects against doxorubicin-induced cardiotoxicity [5].

Response surface methodology (RSM) is a very powerful tool for optimization works, As a package of statistical and mathematical techniques employed for developing, improving, and optimizing process, RSM can be effectively used to evaluate the effects of

multiple factors and their interaction on one or more responses [6,7]. With an appropriate experimental design, RSM can reduce the number of experiments and provide a mathematical model. Thus, RSM is a useful tool for optimizing the technology process over the conventional one factor at a time approach, which is relatively expensive and time-consuming. In this study, we have optimized the extraction conditions of total phenolic and total flavonoid from leaves of *P. urinaria*.

2. MATERIAL AND METHODS

2.1. Material

Leaves of *Phyllanthus urinaria* were collected in Con Cuong District of Nghe An Province, Vietnam in September 2019 and identified by Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. The material is dried, crushed and stored at 4ºC for further experiments

2.2 Methods

2.2.1. Total Phenolic Content (TPC)

TPC of the *P. urinaria* leaves extracts were determined colorimetrically using Folin–Ciocalteu reagent, as established by [8]. Thirty microliters of the prepared extract with 600 µl of Folin-Ciocalteu reagent (10%) mixed with 90 µl of distilled water and let to remain for 10min. Then, 480 µl of sodium carbonate was added and the mixture was kept in a dark place for 2 h. Color change of extracts was determined colorimetrically at a wavelength of 765 nm by UV-8453 spectrophotometer (Agilent). Gallic acid equivalent (GAE) was used as a standard and results were expressed as milligrams gallic acid equivalents per gram dry matter basis.

2.2.2. Total Flavonoid Content (TFC)

TFC was determined by a colorimetric assay [9]. Five hundred microliters of extracts described above were added to a 15 ml tube containing 2ml of deionized water. Then, 150 µl of 5 % sodium nitrite was added to this mixture and remain at room temperature for 5 min. After that, 300 µl of 10 % aluminum chloride ($AlCl_3.6H_2O$) was added and allowed to further react for 6 min, and then 1 ml of 1 mol/l sodium hydroxide was added. Distilled water was added to bring the final volume of the mixture to 5 ml. The absorbance of the solution was measured immediately at 510 nm. The results are expressed as quercetin equivalents using a standard curve prepared from authentic quercetin.

2.2.3. Experimental design

RSM was used to determine the optimum levels of extraction time (min), temperature (°C) and ethanol concentration (%) as extraction medium on two responses TPC and TFC in the *P. urinaria* leaves extracts. These three factors, namely extraction temperature (X_1), extraction time (X_2) and ethanol concentration (X_3) were coded into three levels (-1, 0, +1). Ranges of extraction temperature, extraction time and ethanol concentration and the central point were selected based on preliminary experimental results. Statistical analysis on the means of triplicate experiments was carried out using the ANOVA procedure of the design expert software, version 7.0.

3. RESULTS AND DISCUSSION

3.1. Fitting the response surface models

The central composite design (CCD) was used to identify the relationship between the response functions and process variables as well as to find out the conditions that optimized the extraction process total phenolic content and total flavonoid content for *P. urinaria* leaves. The experimental design and corresponding three response variables are presented in Table 1. This design consisted of 20 experimental points with six replicates at the central point. In the present study, according to the sequential model sum of squares, the highest order polynomials were utilized to select the models where the additional estimated coefficient was significant and the models are not aliased.

Table 1. The experimental data obtained for the three responses based on the CCD matrix

RUN	X_1 (°C)	X_2 (min)	X_3 (%)	TPC Y_1 (mgGAE/g)	TFC Y_2 (mgQE/g)
1	50.00(-)	120.00(+)	90.00(+)	117.15	11.95
2	60.00(0)	100.00(0)	80.00(0)	116.95	12.52
3	60.00(0)	100.00(0)	80.00(0)	117.48	12.57
4	70.00(+)	120.00(+)	70.00(-)	118.76	12.58
5	60.00(0)	100.00(0)	80.00(0)	117.16	12.53
6	76.82(+1.68)	100.00(0)	80.00(0)	130.61	13.86
7	43.18(-1.68)	100.00(0)	80.00(0)	110.98	11.90
8	60.00(0)	100.00(0)	96.82(+1.68)	114.01	12.23
9	60.00(0)	66.36(-1.68)	80.00(0)	110.16	11.84
10	60.00(0)	133.64(+1.68)	80.00(0)	117.11	12.53
11	50.00(-)	80.00(-)	70.00(-)	93.65	10.17
12	70.00(+)	80.00(-)	70.00(-)	116.65	12.48
13	60.00(0)	100.00(0)	80.00(0)	117.08	12.52
14	50.00(-)	120.00(+)	70.00(-)	111.62	11.96
15	70.00(+)	120.00(+)	90.00(+)	117.27	12.54
16	70.00(+)	80.00(-)	90.00(+)	126.83	13.50
17	60.00(0)	100.00(0)	80.00(0)	117.39	12.56
18	60.00(0)	100.00(0)	63.18(-1.68)	99.93	10.79
19	60.00(0)	100.00(0)	80.00(0)	117.09	12.53
20	50.00(-)	80.00(-)	90.00(+)	110.67	11.89

The final empirical regression model of their relationship between responses and the three tested variables for phenolic and flavonoid contents could be expressed by the following quadratic polynomial equation [Eqs. (1–2)]:

$$Y_1 = 115.89 + 7.80X_1 + 1.61X_2 + 5.48X_3 - 2.06X_1X_2 - 1.61X_1X_3 - 1.38X_2X_3 + 1.34X_1^2 - 0.30X_2^2 - 3.54X_3^2 \quad (1)$$

$$Y = 12.41 + 0.78X + 0.16X + 0.56X - 0.21X_1X_2 - 0.17X_1X_3 - 0.14X_2X_3 + 0.13X_1^2 - 0.029X_2^2 - 0.36X_3^2 \quad (2)$$

Table 2. Analysis of variance (ANOVA) for the model

Source	Y_1 – Total phenolic content		Y_2 – Total flavonoid content	
	F- value	p- value	F- value	p-value
Model	1592.49	0.0001***	1589.37	0.0001***
X_1 (temperature)	7011.75	< 0.0001***	6947.96	< 0.0001***
X_2 (time)	873.17	0.030*	847.45	0.031*
X_3 (solvent ratio)	3461.01	< 0.0001***	3533.14	< 0.0001***
X_1X_2	1629.34	0.0001***	1626.00	0.0001***
X_1X_3	248.94	0.015*	267.95	0.013*
X_2X_3	739.19	0.026*	713.92	0.021*
X_1^2	313.05	0.012*	283.49	0.01*
X_2^2	245.02	0.017*	234.08	0.014*
X_3^2	2180.32	0.0001***	2178.11	0.0001***
Lack of Fit	4.04	0.0757	4.11	0.0736
R^2	0.9993		0.9963	
C.V%	0.25		0.23	

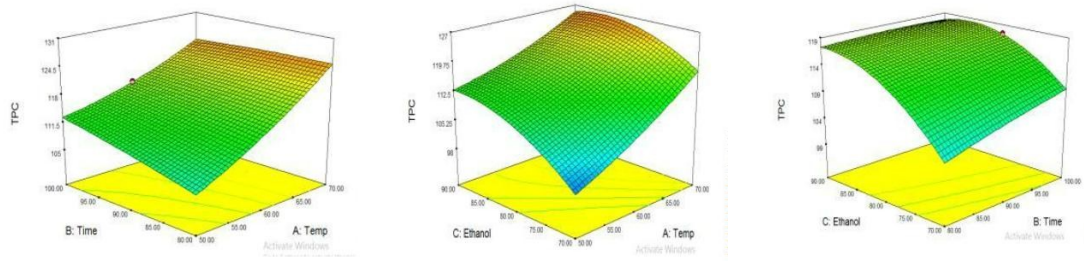
*p< 0.05; **p< 0.01; ***p< 0.001; NS: non-significant.

The ANOVA analysis results for multiple regression and response surface quadratic model of Y_1 and Y_2 were evaluated using the corresponding p and R^2 values (Table 2). F-values of Y_1 and Y_2 were calculated to be 1592.49 and 1589.37, both leading to a p-value <0.05, suggesting both the models were statistically significant. The models' coefficient of determination (R^2) were 0.9993 and 0.9963, indicating that more than 99.93%; and 99.63% of the response variability were explained, and supporting a good accuracy and ability of the established model within the range limits used. The F-values of Lack of Fit of Y_1 and Y_2 were 4.04 and 4.11, respectively, implying that the Lack of Fit was not significant relative to the pure error.

3.2. Response surface analysis

Effects of extraction temperature, extraction time and ethanol concentration to the extraction condition of the maximum total phenolics and total flavonoids content. Three-dimensional model graphs were plotted as shown in the respective figures. The response surface plots of the model were done by varying two variables, within experimental range under investigation and holding the other variables at its central level.

3.2.1. Response surface analysis of total phenolic content



a b c

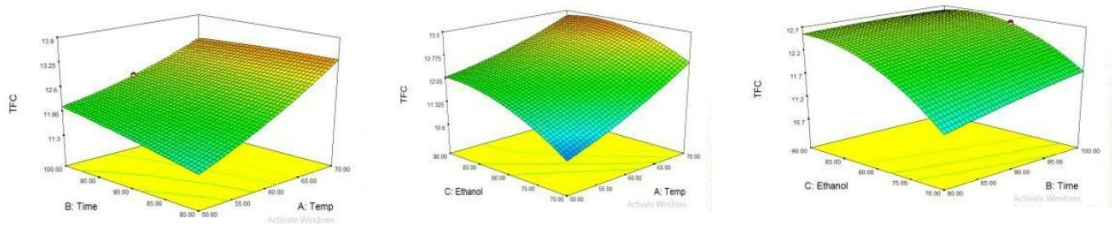
Figure 1. The response surface plot of TPC

The response surface plots for total phenolic extraction of *P. urinaria* leaves extract are shown in Fig. 1 demonstrating the effect and interaction of independent variables on the yields of total phenolics. As shown in Fig. 1 and Table 2, all three factors (extraction temperature, extraction time and ethanol concentration) have shown negative quadratic effects ($p<0.01$). In fig. 1a, The urface plot demonstrates the function of extraction temperature versus time effect on TPC at fixed ethanol concentration (80%). The yields of total phenolic content increased with the increase of extraction temperature from 50°C to 70°C and the maximum amount of phenolics can be achieved at the highest temperature of 65÷70°C. Higher solubility and diffusion coefficient of polyphenols were observed with increased temperature [10].

The surface plot in Fig. 1b show the function of temperature versus ethanol concentration effect on TPC at extraction time (120 min). The yields of TPC increased with the increase of ethanol concentration from 70 % v/v to 90%v/v and the maximum phenolic content in *P. urinaria* leaves can be achieved at highest ethanol concentration (90 %). The higher phenolic content could be explained by the natural polarity of the solvents used [11]. The maximum total phenolic content in *P. urinaria* leaves can be obtained with optimum ethanol concentration of approximately 80÷90 %.

In Fig. 1c. The surface plots revealed that the higher TPC in *P. urinaria* leaves can be obtained when conducted at increasing ethanol concentration at fixed extraction time. Based on the result at constant extraction time of 120 min, 90 % of ethanol concentrations yielded the most TPC as compared with 70 % ethanol concentrations. These overall results of phenolic content indicate a similar trend as observed in the phenolic content in other study [12].

3.2.2. Response surface analysis of total flavonoid content



a b c

Figure 2. The response surface plot of TFC

The 3D surface plots in Fig.2a shows the response surface plot of temperature (X_1) and time (X_2) at fixed extraction solvent ratio (80 %). Response surface plot showed that extraction temperature exhibited a weaker efect whereas extraction time represented a relatively signifcant effect on the flavonoids yield. An increase in the yield of flavonoid could be signifcantly achieved with the increase of extraction time, at any level of extraction temperature. Therefore, the optimum amount of flavonoid was achieved in this study at 50÷55°C and 110÷120 min of extraction time.

Fig. 2b shows the interaction between extraction temperature (X_1) and solvent ratio (X_3) at the fixed 100 min. It was observed that the value of TFC in *P. urinaria* leaves increased when ethanol concentration was increased from 70 to 90 % at fixed 60°C extraction temperature. In contrast, increasing the extraction temperature at the highest ethanol concentrations resulted in the decrease of TFC values.

At Fig. 2c, An increase in ethanol concentration promoted the breakdown of the cell membrane that enhanced the permeability of the solvent into a solid matrix. In this study, highest flavonoids content can be achieved when conducted at highest ethanol concentration 80-90 % when extraction time was increased.

3.3. Optimization and model verification

The final result for the simultaneous optimization using the desirability function approach suggested that the optimal ethanolic extraction conditions for *P. urinaria* leaves extract were at 70°C with 80 min and 88% of ethanol concentration to achieve the best combination for highest total phenolic and flavonoids content. Table 3 shows the predicted and experimental values for the extraction of target compounds from *P. urinaria* leaves.

The actual values obtained from the experimental gave the extraction yields of total phenolic and total flavonoid as 126.83±0.5 mgGAE/g and 13.36±0.2 mgQE/g. These experimental values were close to the predicted values (TPC = 127.129 mgGAE/g, TFC = 13.525 mgQE/g) derived from the respective regression models with the CV ranging from 0.24 % to 1.21 %.

Table 3. Optimum conditions, predicted and experimental values of responses on extraction of P. urinaria leaves extract[a].

Independent variables			**Dependent variables (Response)**	**Experimental[b]**	**Predicted**	**% Difference (CV, %)**
X_1	X_2	X_3				
70°C	80min	88%	Y_1 (mgGAE/g)	126.83±0.5	127.129	0.24
			Y_2 (mgQE/g)	13.36±0.2	13.525	1.21

a

X_1, extraction temperature (°C); X_2, extraction time (min); X_3, ethanol concentration (%); Y_1, TPC (mgGAE/g); Y_2, TFC (mgQE/g); [b]Mean ± standard deviation (SD) of three determinations (n= 3) from two crude extract replications.

4. CONCLUSION

Response surface methodology was successfully used to optimized the process of extracting *Phyllanthus urinaria* leaves extract with the optimal parameters: extraction temperature 70°C, extration time 80 min and ethanol concentrations 88 %. Under this optimum conditions, the experimental values of TPC and TFC were 126.83±0.5 mgGAE/g and 13.36±0.2 mgQE/g.

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