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Optimization of Extraction of Phenolic Compounds from *Avicennia marina* (Forssk.) Vierh using Response Surface Methodology

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***Abstract*—**Optimization of extraction of phenolic compounds from *Avicennia marina* using response surface methodology was carried out during the present study. Five levels, three factors rotatable design (CCRD) was utilized to examine the optimum combination of extraction variables based on the TPC of *Avicennia marina* leaves. The best combination of response function was 78.41 °C, drying temperature; 26.18°C; extraction temperature and 36.53 minutes of extraction time. However, the procedure can be promptly extended to the study of several others pharmaceutical processes like purification of bioactive substances, drying of extracts and development of the pharmaceutical dosage forms for the benefit of consumers.

Keywords*—Avicennia marina*, Central Composite Rotatable Design (CCRD), Response Surface Methodology, Total Phenolic contents (TPC)

I. Introduction

MANGROVE forests have been utilized for many functions including wood production, firewood and charcoal [1]. Besides, mangroves also provided many non-timber products such as tannin, fish poison, medicine, food, fodder, etc [2]. They have been used as traditional medicine in South Asian countries including India. Recently, it has been strongly recommended that mangroves should be considered as a valuable source for chemical constituents with potential medicinal and agricultural values [3]. *Avicennia marina* (Forssk.) Vierh. (Avicenniaceae) has received some attention in determining its important chemical constituents. Phenolic compounds are secondary plant metabolites and are involved in a wide range of specialized physiological functions. They are very important for the normal growth, development and defense mechanisms of plants [4]. These compounds are capable of inhibiting free radicals, and hence can retard the aging process [5].

Traditional medicinal plants are often cheaper, locally available, and easily consumable and have simple medicinal preparations [6]. Although their efficacy and mechanisms of action have not been tested scientifically in most cases, these simple medicinal preparations often mediate beneficial responses due to their active chemical constituents [7]. The standardization of plant extractive solutions should be the first step during the technological development of phytopharmaceuticals. The influence of several parameters such as extractive method and technology, type and concentration of solvent, as well as plant concentration, and their influence in the physical-chemical properties of the extractive solutions should be evaluated and quantified [8]. In the extraction processes, where there are multiple independent variables affecting the responding factors, it is likely to use an optimization method that can determine all the factors. In addition, the possibility of interactions between the independent variables should be considered in order to determine the optimal experimental conditions [9].

The response surface methodology (RSM) not only is all factors at the time of approach allow accounting for possible interaction effects between variables. If adequately used, this powerful tool can provide the optimal conditions that improve a process [10]. With this kind of approach, it is possible to create response surfaces that allow the ranking of each variable according to its significance on the studied responses. Therefore, with reduced time and experimental effort, it may be possible to predict what extractive condition will produce a desired or optimum response [11-14]. However to the best of our knowledge, optimization of extraction of phenolic antioxidants from *Avicennia marina* leaves using RSM has not been reported yet. Therefore the present work was an innovative step towards evaluating the best extraction conditions for *Avicennia marina* leaves to maximize simultaneously the yield of total phenolic content (TPC) by using response surface methodology.

II. MATERIALS AND METHODS

A. Plant Materials and Chemicals

Two kg of fresh and matured leaves of *Avicennia marina* were collected from Punnaikayal estuary of Tuticorin, Tamilnadu, India in the month of April-2011. Bright coloured leaves without pigmentation and disease were chosen for this experiment. All chemicals were of analytical grade, obtained from Merck and SD fine and used without any further purification.

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B. Sample preparation

The leaves were removed by picking from the stem upon picking and washed thoroughly with tap water. The leaves were dried in an oven under different temperatures based on the experimental design. The dried leaves were powdered and packed in polyethylene bag stored in room temperature for a period of one month.

C. Solvent extraction

Ten gram sample was transferred into a 250ml conical flask wrapped with aluminum foil. In this flask, 40 ml of extracting solvent (Methanol) was added in 1:4 ratio. Then the conical flask was placed in the water bath shaker under different temperatures at various time based on experimental design and the extracts were filtered using Whatmann No: 1 filter paper and the filtrates were transferred in to test tubes. Then the test tubes were dried at 40ºC for solvent evaporation.

D. Total phenolic content (TPC) assay

The TPC of *Avicennia marina* leaves extracts was determined spectrophotometrically using Folin-Ciocalteu's reagent according to the method described by Lim et al [15] with slight modifications. Approximately 0.3 ml sample (15x dilutions) was added into the test tubes followed by 1.5 ml of Folin-Ciocalteau reagent (10% v/v) and 1.2 ml of sodium carbonate (7.5% w/v). The test tubes were covered with parafilm and aluminum foil, mixed for 10 seconds using vortex and allowed to stand at room temperature for 30 minutes in dark environment. Absorption was measured at 765 nm using spectrophotometer (Model Systronics 106). Blank sample was prepared by adding 0.3 ml solvent without the extract. Gallic acid was used as standard and TPC were expressed in gallic acid equivalents, mg GgAE / 100g DW analysis was done in triplicate.

E. Selection of Relevant Variables and Experimental Ranges

Before the development of the study through Response Surface Methodology (RSM), a first set of tests were performed to select the relevant factors such as drying temperature, extraction temperature and extraction time which are effective on phenolic extraction yield (dependent variable) and the experimental ranges for these independent variables.

In general, efficiency of the extraction of a compound is influenced by such multiple parameters as temperature, time and solvent polarity, and their effects may be either independent or interactive [16]. According to the previously published papers on the extraction of phenolic compounds from different samples; in this study, effect of varying drying temperatures of 65 to 75°C to dry the leaves, extraction temperatures of 25 to 35°C and extraction time of 25 to 45 min were investigated on the total phenolic extraction (Table 1).

TABLE I
THE CODED AND ACTUAL VALUES OF THE VARIABLES IN CENTRAL COMPOSITE DESIGN

Variables	Unit	- α	-1	0	+1	+ α
Drying temperature (X_1)	°C	61.59104	65	70	75	78.40896
Extraction temperature (X_2)	°C	21.59104	25	30	35	38.40896
Extraction time (X_3)	min.	18.18207	25	35	45	51.81793

A five level, three factors rotatable central composite rotatable design (CCRD) (Minitab version 15; Minitab Ltd., Coventry CV3 2TE, UK) was utilized to examine the optimum combination of extraction variables based on the TPC of *Avicennia marina* leaves samples. The CCRD design comprised of 20 experimental runs with eight factorial points, six axial points (two axial points on the axis of each design variable at a distance of 1.68179 from the design center) and six replicates at the centre point (Table 2). The CCRD was proceeded to obtain a quadratic model, consisting of factorial trails and star points to estimate quadratic effects and central points to estimate the pure process variability with TPC content as response. The response variable was fitted by a second order model in order to correlate the response variable to the independent variables.

The linear quadratic model with 4 variables expressed as:

$$Y=\beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_{12} + \beta_{22}X_{22} + \beta_{33}X_{32} + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 \quad (1)$$

Where Y is the measured response, β_0 is the intercept term, β_1, β_2 and β_3 are linear coefficient of drying temperature of leaves, extraction temperature and extraction time respectively, β_{11}, β_{22}, β_{33} are quadratic coefficient, β_{12}, β_{13}, β_{23} are interaction coefficient and X_1, X_2 , X_3, X_4 are coded independent variables.

F. Verification of model

Optimal extraction conditions on TPC of *Avicennia marina* leaves samples crude extract were obtained using the predictive equations generated by RSM. TPC was tested using Folin-Ciocalteu method after solvent extraction under specific optimal conditions. Each set of experiment was conducted in two replicates, and the experimental and predicted values were compared in order to examine the validity of the model.

G. Statistical analysis for central composite design (CCD)

The statistical software package Minitab version 15 (Minitab Ltd., Coventry CV3 2TE, UK) was used for regression and Sigma plot (Systat Software, Inc.501 Canal Blvd, Suite E, Richmond, CA 94804-2028) graphical analyses of the data obtained. The optimal concentrations of the critical variables were obtained by analyzing contour plots. The statistical analysis of the model was represented in the form of analysis of variance (ANOVA).

TABLE II
CENTRAL COMPOSITE DESIGN (CCD) OF FACTORS IN CODED VALUE FOR OPTIMIZATION OF PROCESS VARIABLES IN TPC CONTENT OF *AVICENNIA MARINA* LEAVES

Trails	Type	Drying temperature (X_1)	Extraction temperature (X_2)	Extraction time (X_3)	Experimental TPC content (mg GAE/g. extract)	Predicted value of TPC content (mg GAE/g. extract)	Residue
1	Factorial	-1	-1	-1	63.550	66.5344	-2.98442
2	Factorial	1	-1	-1	72.600	68.9771	3.62293
3	Factorial	-1	1	-1	69.300	70.8272	-1.52722
4	Factorial	1	1	-1	48.720	57.2999	-8.57988
5	Factorial	-1	-1	1	60.090	51.2460	8.84400
6	Factorial	1	-1	1	80.800	79.0087	1.79134
7	Factorial	-1	1	1	29.800	33.1588	-3.35881
8	Factorial	1	1	1	48.200	44.9515	3.24853
9	Axial	-1.68179	0	0	76.900	77.3515	-0.45154
10	Axial	1.681793	0	0	89.400	89.3220	0.07803
11	Axial	0	-1.68179	0	65.350	71.9261	-6.57614
12	Axial	0	1.681793	0	53.100	46.8974	6.20263
13	Axial	0	0	-1.68179	56.540	50.7826	5.75739
14	Axial	0	0	1.681793	21.412	27.5429	-6.13090
15	Center	0	0	0	86.980	87.6177	-0.63765
16	Center	0	0	0	88.140	87.6177	0.52235
17	Center	0	0	0	87.680	87.6177	0.06235
18	Center	0	0	0	87.440	87.6177	-0.17765
19	Center	0	0	0	87.590	87.6177	-0.02765
20	Center	0	0	0	87.940	87.6177	0.32235

III. RESULTS AND DISCUSSION

The experimental design of five-level, three-variable CCRD and the predicted and experimental results of extraction are shown in Table 3. The maximum content of total phenolics (89.400 mg GAE/ g DW) was recorded under trail No.10 with experimental parameters of leaves drying temperature of 62°C, extraction temperature of 30°C, and extraction time of 35 minutes. The lowest TPC (24.412 mg GAE/ g DW) was found at 70°C leaves drying temperature, 52 minutes extraction time, and 30°C extraction temperature under 14th trail. The larger the magnitude of the t-value and the smaller the p-value, indicate more significant of the corresponding coefficient and its effect on extraction of TPC. The p-values are used as a tool to check the significance of each of the coefficients and to understand the interactions between the best variables [17]. Linear effect of X_2 and X_3 , quadratic of X_2^2 and X_3^2 and interaction effect of $X_1 * X_3$ and $X_2 * X_3$ a was highly significant ($p < 0.05$), which showed the existence of the optimal value within the experimental area. This suggested that the change in either factor will influence TPC distinctly as shown in Table 3. Joglekar and May [18] have suggested for a good fit of a model, regression coefficient R^2 should be at least 80%.

TABLE III
ESTIMATED REGRESSION COEFFICIENTS OF SECOND ORDER POLYNOMIAL MODEL FOR OPTIMIZATION OF TPC

Term	Coef	SE Coef	T	P
Constant	87.618	2.429	36.070	0.000*
Drying temperature (X_1)	3.559	1.612	2.208	0.052
Extraction temperature (X_2)	-7.441	1.612	-4.617	0.001*
Extraction time (X_3)	-6.909	1.612	-4.287	0.002*
Drying temperature* Drying temperature (X_1^2)	-1.514	1.569	-0.965	0.357
Extraction temperature* Extraction temperature (X_2^2)	-9.972	1.569	-6.356	0.000*
Extraction time* Extraction time (X_3^2)	-17.131	1.569	-10.919	0.000*
Drying temperature* Extraction temperature ($X_1 * X_2$)	-3.992	2.106	-1.896	0.087
Drying temperature* Extraction time($X_1 * X_3$)	6.330	2.106	3.006	0.013*
Extraction temperature * Extraction time ($X_2 * X_3$)	-5.595	2.106	-2.657	0.024*
R-Sq = 95.49% ; R-Sq(adj) = 91.44%				

SE- Standard error, t – student's test, p – corresponding level of significance, * Significant

The *R*2 value is the proportion of variation in the response attributed to the model was 0.9549. This means that this model fitted well with the experimental data. The R^2 value implies that the sample variation of 95.49% for TPC yield is attributed to the factors.

TABLE IV
ANALYSIS OF VARIANCE (ANOVA) OF SECOND ORDER POLYNOMIAL MODEL FOR OPTIMIZATION OF TPC

Source	DF	Seq SS	Adj MS	F	P
Regression	9	7517.64	835.29	23.55	0.000*
Linear	3	1581.09	527.03	14.86	0.001*
Square	3	5238.05	1746.02	49.22	0.000*
Interaction	3	698.50	232.83	6.56	0.010*
Residual Error	10	354.72	35.47		
Lack-of-Fit	5	353.90	70.78	432.18	0.000*
Pure Error	5	0.82	0.16		
Total	19	7872.37			

The statistical significance of the model was also determined by F-test for analysis of variance (ANOVA) and residuals analysis was performed to validate the model at 95% confidence level. The model fitted well with the TPC yield and the optimal values from the model was justified (p=0.05). The ANOVA given in Table 4 indicates that the Linear and quadratic terms in second order polynomial Model equation (1) were highly significant ($p<0.05$) and adequate to represent the relationship between TPC yield (mg GAE/g.extract) and drying temperature, extraction temperature and extraction time.

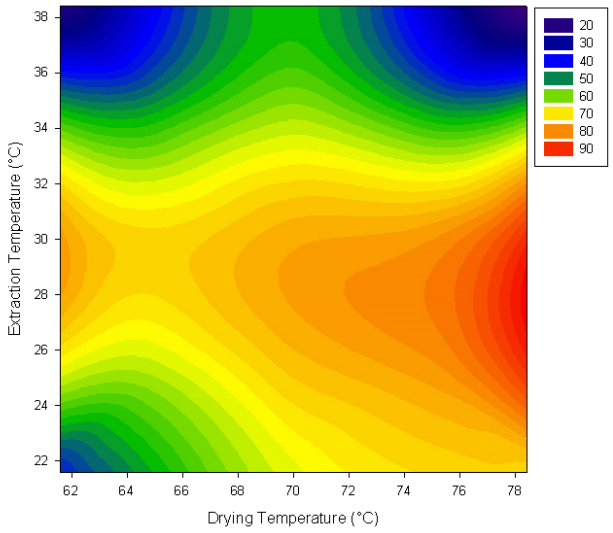

Fig.1 Contour plot of the combined effect of drying and extraction temperature on TPC

The contour plot describing combined effect between pair of factors on hydrolysis of potato starch were given in figure 1 to 3 by keeping other variable constant at their middle level. Fig.1 indicates that TPC yield as a function of drying temperature and extraction temperature. It was observed that middle to high level of temperature (68 to 74°C) and middle level of time (26 to 32 min.), the TPC significantly high.

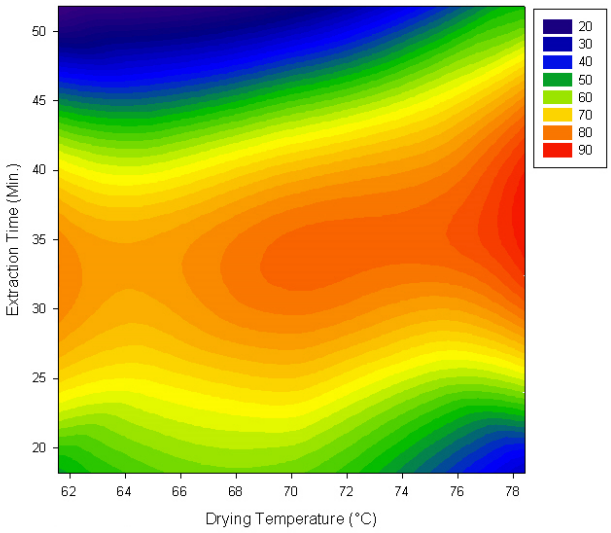

Fig.2 Contour plot of the combined effect of drying temperature and extraction time on TPC

As reflected in Figure 2, the predicted response surface showed the effect of drying temperature and extraction time on TPC. A higher amount of phenolic content approximately yielded in the region of drying temperature between 69 and 78°C and extraction time between 25 and 40 min.

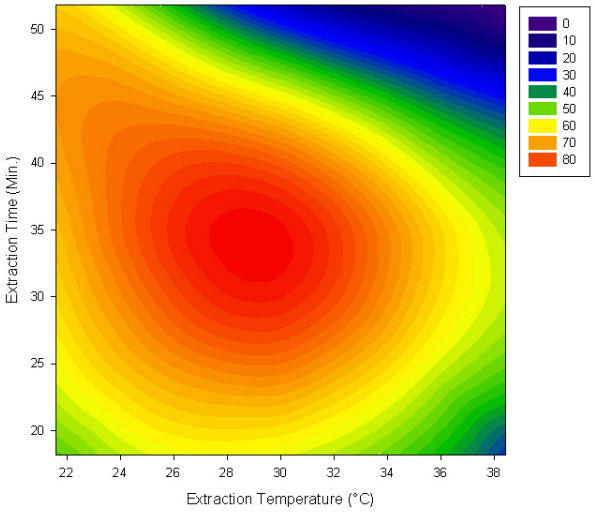

Fig.3 Contour plot of the combined effect of extraction temperature and time on TPC

Fig.3 shows relation between extraction temperature and extraction time with the TPC yield. TPC yield increases at middle level of extraction temperature and a linear increase was observed with increase in low to high level of time.

IV. VALIDATION OF THE MATHEMATIC MODEL

The optimal extraction conditions of TPC from *Avicennia marina* leaf extracts acquired using the model was as follows: drying temperature, 78.41 °C; extraction temperature, 26.18°C; and extraction time, 36.53 minutes. Under these optimal conditions, the model predicted a maximum response of 95.14 mg GAE/ g DW of *Avicennia marina* leave extracts.

A mean value of 94.18 ± 0.45059 (SD) mg GAE/ g DW of *Avicennia marina* leaf extracts was acquired from real experiments.

V. CONCLUSION

The optimal extraction condition to prepare *Avicennia marina* leaf extract with total phenolic was determined using CCD experimental design. The statistical analysis performed by the multiple coefficients of regression, *t*-test for the coefficients and analysis of variance showed the adequacy of the mathematical models, providing reliance to the surface generated. The regression coefficient and *p*-value indicated that extraction temperature ($p<0.01$) and extraction time ($p<0.01$) was the most significant factor affecting extraction of TPC, followed by drying temperature of leaves. The best combination of response function was 78.41 °C, drying temperature; 26.18°C; extraction temperature and 36.53 minutes of extraction time. However, the procedure can be promptly extended to the study of several others pharmaceutical processes like purification of bioactive substances, drying of extracts and development of the pharmaceutical dosage forms for the benefit of consumers. The regression model will help manufacturers optimize the extraction process of total phenol content of *Avicennia marina* leaf extract.

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