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Response Surface Optimization of Extraction of Polyphenols and Carotenoids from *Sesbania grandiflora* Leaves with Ethanol-water System

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Authors' contributions

This work was carried out in collaboration between all authors. Authors KDPPG, KKDSR and HPVR designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors ODANP and HPSJ managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Response surface methodology was employed to optimize the extraction parameters for the extraction of total phenolics and carotenoids from leaves of *Sesbania grandiflora* with ethanol-water based system.
Method: The effects of solvent concentration (30-100%), extraction temperature (30-60°C) and

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extraction time (30-90 min) on the recovery of total phenolics and carotenoids were investigated. **Results and Discussion:** The constructed models were adequate to explain the behavior of the extraction system and predict the responses, total phenolics (R^2 = 88.53%) and carotenoid (R^2 = 90.60) contents. The optimum extraction conditions of ethanol concentration, extraction temperature and extraction time for phenolics, were 46.6%, 70.2°C, and 110.5 min and for carotenoids, the optimum parameters were 100%, 70.2°C and 110.5 min, respectively. The optimal predicted contents for total phenolics (7.74 mg Gallic Acid Equivalent (GAE)/ g DW) and carotenoids (4.32 mg/g DW) values in the extracts were agreed with the experimental values obtained with optimum extraction conditions for each response.

Keywords: Sesbania grandiflora leaves; phenolics, carotenoids; response surface methodology.

1. INTRODUCTION

Natural antioxidants such as polyphenolics and carotenoids are the secondary metabolites widely distributed in plants and have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory and anticarcinogenic [1]. Polyphenols and carotenoids have antioxidant activities as they could neutralize or quench free radicals or reactive oxygen species (ROS) which are responsible for the initiation of many chronic diseases such as cardiovascular diseases and cancers [2]. Furthermore, they are much effective antioxidant in scavenging singlet molecular oxygen and peroxyl radicals [3]. Currently, research and development activities that are aimed at bioactive rich dietary sources have become a global interest. It is well known fact that natural antioxidants extracted from medicinal herbs have high antioxidant potency and are used in many food applications such as functional food formulations. It has been established that the antioxidant effects are due to presence of polyphenolics, carotenoids and other bioactive compounds.

Sesbania grandiflora (Fabaceae) is a medicinal herb and leafy vegetable commonly known as Kathurumurunga in Sri Lanka. Leaves of this plant reported to be rich sources of polyphenols (10.98 mg GAE/g dry weight-DW) and carotenoids (2.29 mg/g DW) and possess antioxidant activity [4,5]. The leaf extracts of the *S. grandiflora* have been reported to have anxiolytic and anticonvulsant, anthelmintic, demulcent, expectorant, antipyretic, in treatment of bronchitis, cough, vomiting, wounds ulcers, diarrhoea, and dysentery [6]. Nowadays the trend is investigating natural dietary sources of antioxidants to be used in the functional foods and nutraceutical industry. Extraction is the initial and most vital step in the recovery and purification of bioactive compounds from plant sources [7]. Various factors such as solvent concentration, extraction temperature, solvent-to-solid ratio and extraction duration may affect the extraction efficiency and bioactive concentration [8]. Therefore, it is necessary to optimize the extraction conditions to obtain the highest bioactives recovery from plant sources. Response surface methodology (RSM) was introduced and widely used nowadays as a useful tool to evaluate effects of multiple factors and their interactions in one or more response variables. RSM is one of the most popular optimisation techniques in the area of food science and technology and has been applied for extraction of antioxidant bioactives from a number of dietary sources [9,10]. However, there are no studies reported to optimize the extraction conditions for polyphenols and carotenoids from leaves of *S. grandiflora*. Hence, the objective of the present study was to optimize the extraction conditions for *S. grandiflora* leaves to obtain the highest polyphenols and carotenoids content.

2. MATERIALS AND METHODS

2.1 Plant Materials

The leaves of *S. grandiflora* were collected at the site of Makandura, Sri Lanka. The leaves samples were taxonomically identified by a botanist and the voucher specimens of the samples have been deposited in the herbarium of the Department of Food Science and Technology of Wayamba University of Sri Lanka. These leaves were washed thoroughly with water and oven dried at 48°C for 48 hours and then dried leaves were pulverized and preserved in plastic sachets at -18°C until use.

2.2 Chemicals

Chemicals such as Gallic acid, folin ciocalteu reagent, sodium carbonate; were obtained from Sigma Ltd., USA, through Analytical Instrument

Pvt Ltd, Colombo, Sri Lanka. All other chemicals used were of analytical grade.

2.3 Preparation of extracts

One gram of powdered dried leaves were extracted with aqueous ethanol at desired concentrations and extraction was carried out using a rotary shaker (Unimax 1010, Heidolph, Kelheim, Germany) at 400 rpm, at specified temperature as dictated by the experimental design. The optimization procedure was designed based on a three-factor inscribed central composite design (CCD) consisting of ethanol concentration (30–100%), temperature (30–60°C) and extraction time (30-90 min) as shown in Table 1. The obtained extract was further filtered with Whatman filter paper and then the filtrates were used to determine the total phenolic content and carotenoid contents.

2.4 Determination of Total Phenolic Content

Total phenolic content of aqueous ethanol extract of *S. grnadiflora* was determined using Folin–Ciocalteu based assay [11] with some modification, as described by Gunathilake *et al*. [12]. The blue colour formed due to the polyphenol present in the extract was measured at 760 nm using UV spectrophotometer (Optima, SP-3000, and Tokyo, Japan). The extract (0.5 mL) was mixed with the 5 N Folin-Ciocalteu phenol reagent (0.1 mL) and were mixed and incubated at room temperature for 15 minutes at dark. Then, sodium carbonate (15% w/v, 2.5 mL) was added to the mixture and further incubated for 2 hours at dark at room temperature and then the absorbance was measured at 760 nm using UV/VIS spectrometer (Optima, SP-3000, and Tokyo, Japan). All the experiment was performed in triplicate. The total phenolic content is expressed as mg of gallic acid equivalents (GAE) per gram DW.

2.5 Total Carotenoids Content

The carotenoid content was analyzed according to the method described by Şükran et al. [13] with slight modifications. According to this method, total carotenoids content in the extracts were determined after having subtracted the concentration of chlorophyll A and B in all extracts, using wavelengths 661.6 and 644.8 nm, respectively. Carotenoid contents were reported as mg/g DW.

2.6 Response Surface Optimization Design

RSM was used for investigating the influence of three independent variables, ethanol concentration, extraction temperature, and extraction time; and the response variables were total phenolic and total carotenoid contents. A three-factor inscribed central composite design (CCD) was used to identify the relationship existing between the response functions and the process variables, as well as to determine those conditions that optimized the extraction process of total phenolics and carotenoids contents of the extracts. The independent variables and the range studied were ethanol concentration (30–100%), temperature (30–60°C) and extraction time (30-90 min). The selection and range of these three factors were based on previous studies. Each variable to be optimized was coded at three levels 1, 0, +1 (Table 1). Twenty randomized experiments including six replicates as the center points were assigned based on CCD and the values of independent process variables considered, as well as measured total phenolic content and carotenoid content, are given in Table 2.

2.7 Statistical Design

For data analysis, Minitab15 software was used. The assumptions of normality and constant variance were checked and confirmed. A response surface analysis and analysis of variance (ANOVA) were employed to determine the regression coefficients, the statistical significance of the model terms and to fit the mathematical models of the experimental data that aimed to optimize the overall region for both response variables. A second-order polynomial model was applied to predict the response variables as given below:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_1^2 X_1^2 + \beta_2^2 X_2^2 + \beta_3^2 X_3^2 + \beta_1\beta_2 X_1 X_2 + \beta_1\beta_3 X_1 X_3 + \beta_2\beta_3 X_2 X_3$$

where Y is the predicted dependent variable; β_0 is a constant that fixes the response at the central point of the experiment; β_1, β_2 and β_3 are the regression coefficients for the linear effect terms; β_1^2, β_2^2 and β_3^2 are the quadratic effect terms; and $\beta_1\beta_2$, $\beta_1\beta_3$ and $\beta_2\beta_3$ are the interaction effect terms, respectively. X1, X2, and X3 are the independent variables (Table 1). The adequacy of the model was predicted through the regression analysis (R^2) and the ANOVA analysis. The relationship between the

Table 1. Levels of extraction variables for experimental designs

Independent variables	Level	Total	Phenol	Content/Carotene	Content
	+1	0	-1	+1.682	-1.682
X1: Ethanol (%)	100	65	30	123.86	6.137
X2: Temperature (°C)	60	45	30	70.23	19.773
X3: Time (min)	90	60	30	110.45	9.546

independent variables and the response variables (Phenolic and carotenoids contents) was demonstrated by the response surface plots. Multiple graphical and numerical optimizations of the experimental data were done to identify the optimum extraction conditions to achieve the maximum recovery of polyphenols and carotenoids. For the verification of predicted extraction conditions that would give higher levels of phenolics and carotenoids, experimental data for the contents of phenolics and carotenoids in *S. grandiflora* leaf samples were determined based on the best extractions conditions obtained with RSM.

3. RESULTS AND DISCUSSION

The uncoded coefficient values for the experimental designs for total phenolics and carotenoids of *S. grandiflora* leaves are given in Table 2. The obtained data were used for the prediction of an optimum set of extraction parameters from leaf extract with high phenolics and carotenoids contents. A number of phenolics and carotenoids in the extracts were employed in a multiple regression analysis, performed using RSM to fit the second-order polynomial equations given in Table 3 for both phenolics and carotenoids, respectively. The ''fitness'' of the model was studied through the lack-of-fit test ($p > 0.05$), which indicated the adequacy of models to accurately predict the variation [14]. The quality of fit to the second-order polynomial models for leaf extracts of *S. grandiflora* was established based on the coefficients of determination (70% $>R^2$), regression p-value ($p<0.1$) and lack of fit ($p>0.05$) indicating that the models could be used to predict the responses. The software generated the quadratic equations from estimated regressions coefficients for RSM (Table 3 and Table 4). These findings demonstrate the empirical relationship between extraction parameters (solvent concentration, extraction temperature and extraction time) and response variables (phenolics and carotenoids).

3.1 Model Fitting of Parameters Based on Total Phenolic and Carotenoid Content

The responses, phenolics and carotenoids yields, of each run of the experimental design, were presented in Table 2. Total phenolics content of S*. grandiflora* leaf extracts varied from 1.02 to 7.12 mg GAE/g dry sample. Total carotenoids contents varied from 0.22 to 3.68 mg/g DW. The software generated the estimated regression coefficients of the second-order polynomial equations for RSM analysis of total phenolics and carotenoids extraction as shown in Table 3. The ANOVA table for phenolics and carotenoids extractions from *S. grandiflora* leaves is shown in Table 4. Regression for phenolics showed that the models were significant ($p < 0.05$) with R^2 and p-values of 0.89 and 0.000, respectively (Table 4). There was no significance in the lack of fit ($p = 0.215$) in the model indicating that the model could be used to predict the responses. The quadratic regression models for carotenoids extraction showed that the models were significant ($p < 0.05$) with R^2 and p-values of 0.91 and 0.00, respectively (Table 4). The lack of fit ($p = 0.268$) in the model was not significance ($p>0.05$) and this indicated that the model could be used to predict responses.

3.2 Effect of Extraction Parameters on Total Phenolic Content

The responses demonstrated that the ethanol concentration significantly ($p<0.05$) affect the recovery of phenolics from *S. grandiflora* leaves (Fig. 1). Many researchers have used aqueous alcohols particularly ethanol for the extraction of various bioactive antioxidants from plants sources when used for food uses [15,16]. Phenolic extraction from *S. grandiflora* prefers ethanol-water solvent combinations than use of pure ethanol. Generally, higher recovery of phenolics was observed at lower ethonolic concentration in the range used (Fig. 1). As the extraction and separation of phenolics depend greatly on the polarity of the extraction

solvent [7], a combination of alcohol with water is more effective in extracting phenolics than pure ethanol. A similar observation was made in the extraction of leaves of *Passiflora edulis* [17], ginger [9,18] and *Olax zeylanica* [19]. However, the effects of extraction temperature and duration of the extract on total phenolics extraction were insignificant ($p>0.05$) as shown in Table 3.

3.3 Effect of Extraction Parameters on Carotenoids Content

Solvent extraction method is universally acceptable for carotenoids extraction [20] and ethanol is also a good solvent that can be used for carotenoids extraction. Extraction of carotenoids is highly influenced by procedural variables including solvent concentration, extraction temperature and time [21]. However, many researchers have used non polar solvent for carotenoid extraction. The Influence of three extraction conditions towards total carotenoids extraction was reported through the significant ($p < 0.05$) coefficient of the second-order polynomial regression equation in Table 3. Results showed that the ethanol concentration positively significant ($p<0.05$), whereas extraction temperature and extraction duration negatively affect carotenoid extraction. As there are polar carotenoids (e.g. Lutein) as well as non-polar carotenoids (e.g. β-carotenoids), the extraction and separation of carotenoids depend largely on the polarity of the solvents [21]. However, for *S. grandiflora*, higher carotenoids extractions were observed when 100% ethanol was used (Fig. 2). When ethanol concentration increased from 30% to 100% while keeping extraction temperature and time at 30°C and 30 min, respectively, increase in the carotenoids content from 1.04 to 2.59 mg /g DW was observed (Table 2). This may be due to the presence of more non-polar carotenoids in *S. grandiflora* and hence could extract more carotenoids towards decreasing polarity as the solvent polarity is decreased with increasing solvent concentration. Extraction temperature and extraction duration showed some influence on carotenoids contents from *S. grandiflora* leaves (Fig. 2). This may be due to the degradation of carotenoids at higher temperatures. Meléndez-Martínez et al. [22] have reported that carotenoids are degraded at elevated temperatures.

Table 2. Central composite design arrangement for extraction of phenolics and carotenoids from *Sesbania grandiflora*

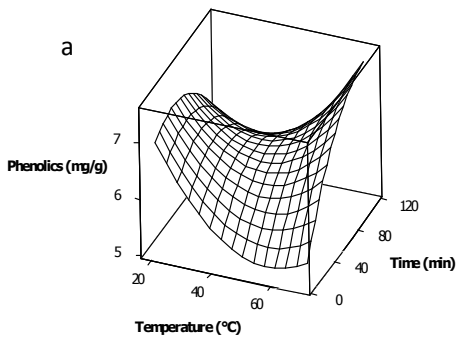
Run order	Ethanol %	Temperature (°C)	Time (min)	Phenolics (mg GAE/g DW)	Carotenoid (mg/g)
1	100	45	60	1.02	2.59
2	65	45	60	5.66	2.31
3	65	70	60	6.75	2.88
4	65	45	60	6.58	2.52
5	65	45	60	6.01	2.41
6	30	30	30	6.28	1.04
7	65	45	9.5	5.04	2.05
8	65	45	60	5.34	2.93
9	6.14	45	60	4.92	0.22
10	100	30	90	2.65	2.73
11	100	60	30	2.72	2.77
12	30	60	90	6.82	1.29
13	30	30	90	6.00	0.77
14	65	45	60	5.64	2.36
15	100	30	30	2.55	2.59
16	65	19	60	7.12	2.62
17	65	45	60	5.41	2.45
18	30	60	30	5.89	1.18
19	100	60	90	3.69	3.68
20	65	45	110.5	6.14	2.92

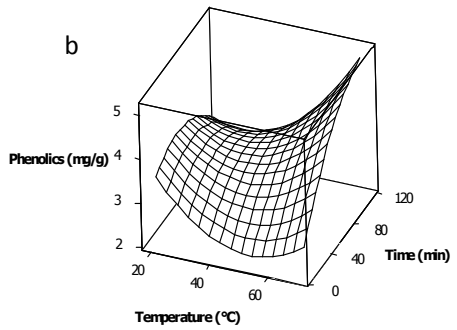
Table 3. Estimated regression coefficients for phenolics and carotenoids using data in uncoded units

Term	Phenolics		Carotenoids	
	Coefficient	P	Coefficient	P
Constant	8.18193	0.000	0.6508	.000
Ethanol %	0.0641701	0.000	0.0556	0.000
Temperature (°C)	-0.159971	0.640	-0.0354	0.046
Time (min)	0.00343137	0.123	-0.0121357	0.037
Ethanol %*Ethanol %	-9.045E-04	0.000	-3.478E-04	0.000
Temperature (°C)*Temperature (°C)	0.00130831	0.079	0.00022	0.494
Time (min)*Time (min)	-2.013E-04	0.257	-4.89E-05	0.543
Ethanol %*Temperature (°C)	0.000192	0.630	0.0001	0.549
Ethanol %*Time (min)	5.179E-05	0.794	0.00014	0.139
Temperature (°C)*Time (min)	0.000579167	0.227	0.00032	0.155
R^2	88.53%		90.60%	

Table 4. ANOVA table for response surface for total phenollics and carotenoids analysis of variance for phenolics (mg GAE/g DW)

Analysis of variance for phenolics (mg GAE/g DW)						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	50.9955	50.9955	5.6662	17.29	0.000
Linear	3	30.0992	30.0992	10.0331	30.62	0.000
Square	3	20.2483	20.2483	6.7494	20.60	0.000
Interaction	3	0.6481	0.6481	0.2160	0.66	0.596
Residual Error	10	3.2765	3.2765	0.3276		
Lack-of-Fit	5	2.2182	2.2182	0.4436	2.10	0.218
Pure Error	5	1.0583	1.0583	0.2117		
Total	19	54.2720				
Analysis of variance for carotenoids (mg/g DW)						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	13.5253	13.5253	1.50281	21.36	0.000
Linear	3	10.4096	10.4096	3.46988	49.31	0.000
Square	3	2.7404	2.7404	0.91347	12.98	0.001
Interaction	3	0.3753	0.3753	0.12509	1.78	0.215
Residual Error	10	0.7037	0.7037	0.07037		
Lack-of-Fit	5	0.4522	0.4522	0.09044	1.80	0.268
Pure Error	5	0.2515	0.2515	0.05031		
Total	19	14.2291				





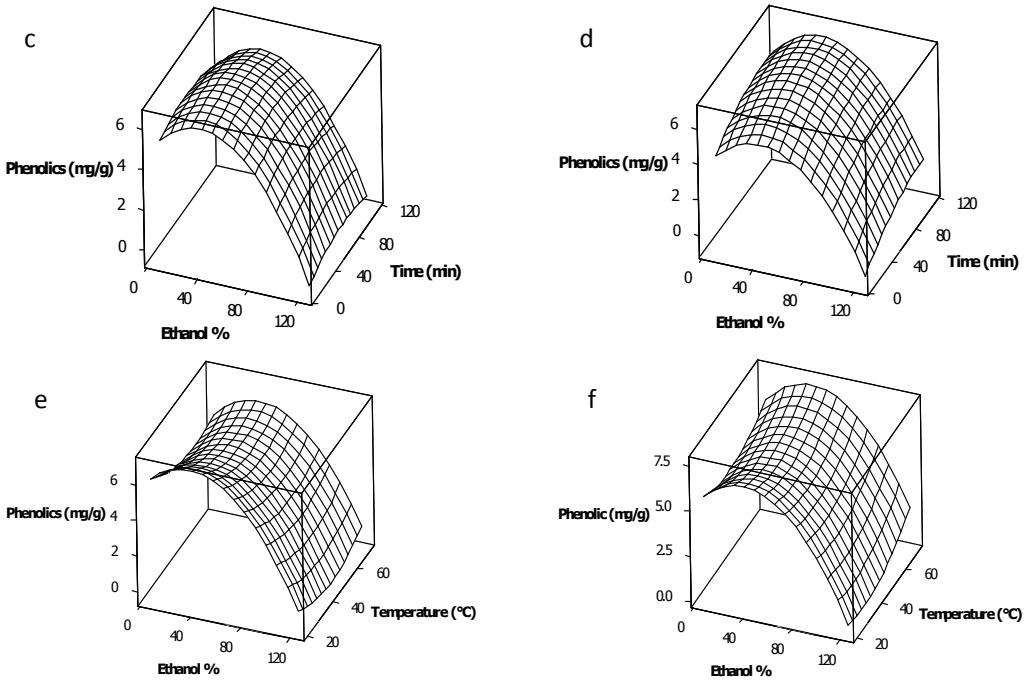
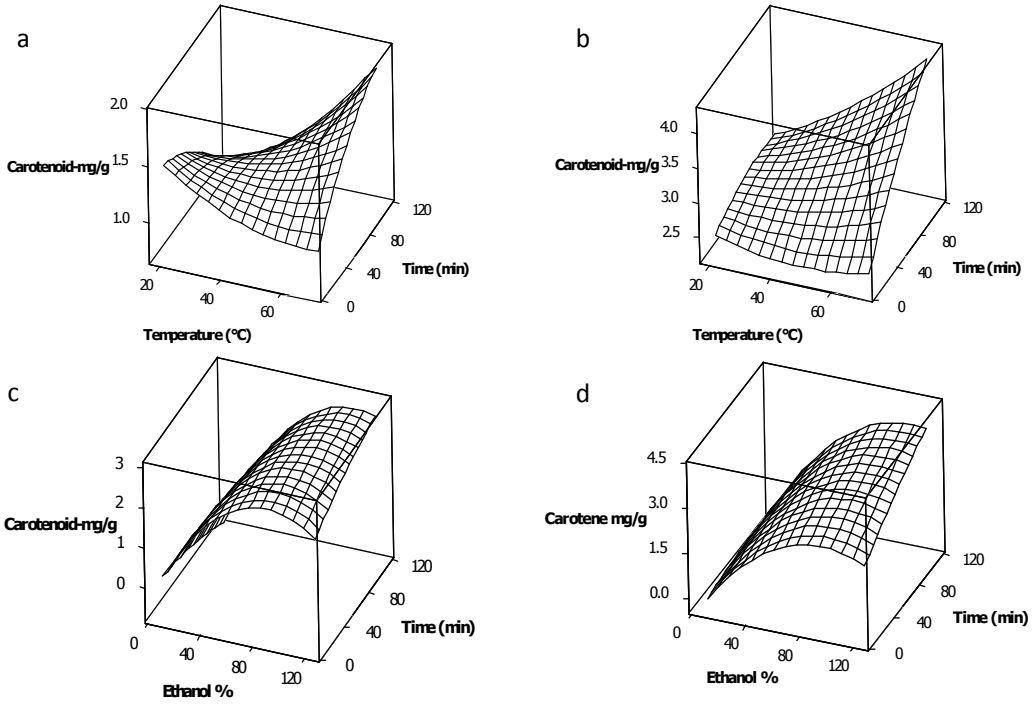


Fig. 1. Pair wise response surface plots of the phenolics (mg GAE/g DW) extraction from *Sesbania grandiflora* leaves as a function of ethanol %, extraction temperature and time: ethanol % was kept constant at 30% (a) and 100% (b); temperature of extraction was kept constant at 30°C (c) and 60°C (d); the time of extraction was kept constant at 30 min (e) and 90 min (f)



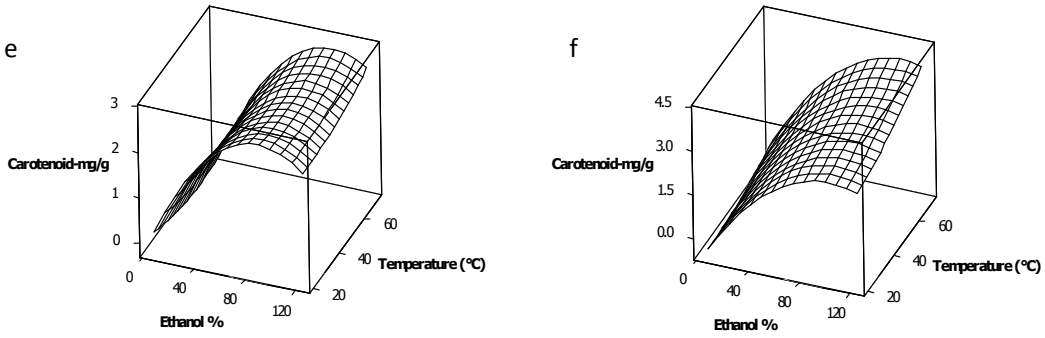


Fig. 2. Pair wise response surface plots of the carotenoids (mg/g DW) extraction from *Sesbania grandiflora* leaves as a function of ethanol %, extraction temperature and time: ethanol % was kept constant at 30% (a) and 100% (b); temperature of extraction was kept constant at 30°C (c) and 60°C (d); the time of extraction was kept constant at 30 min (e) and 90 min (f)

Table 5. Predicted values and experimental values of total phenolics and carotenoids at the optimum extraction conditions for *Sesbania grandiflora*

Optimum extraction conditions		Predicted values (mg/g)		Experimental values (mg/g)	
Phenolics	**Carotenoids**	**Phenolics**	**Carotenoids**	**Phenolics**	**Carotenoids**
ETOH:46.6%	ETOH:100%	7.74	4.32	8.09±1.35	5.12±0.93
Temp:70.2°C	Temp:70.2°C				
Time:110.5 min	Time:110.5 min				

3.4 Optimization of Phenolics and Carotenoids and Verification of the Model

Multiple graphical and numerical optimizations were run for determining the optimum levels of studied extraction conditions with desirable levels of phenolics and carotenoids contents. Optimum ethanol concentration, extraction temperature, extraction time were developed for the two responses and they were 46.6%, 70.2°C and 110.5 min for phenolics and 100%, 70.2°C and 110.5 min for carotenoids, respectively (above Table 5). For these optimum extraction conditions, the corresponding predicted response values for phenolics and carotenoids were 7.74 mg GAE/g DW and 4.32 mg/g DW, respectively. An experiment was run in accordance with the recommended optimum conditions for two responses, phenolics and carotenoids. More interestingly, in this study, the values obtained experimentally for both response variables are near to the predicted values, indicating a satisfactory model. The experimental values for total phenolics were 8.09 ± 1.35 mg GAE g extract and 5.12 ± 0.93 mg/g DW carotenoids and no significant difference ($p > 0.05$) was found between the experimental and predicted values of the extractable phenolics and carotenoids from leaves of *S. grandiflora* extract. Therefore, the data confirm the validity of the optimized model.

4. CONCLUSIONS

RSM was successfully implemented to optimize the total phenolics and carotenoid extraction from leaves of *S. grandiflora*. Overall, phenolic extraction prefers low ethanol concentration and the effect of temperature and extraction time was insignificant ($p>0.05$) for total phenolic extraction. Higher carotenoid recovery was observed at higher ethanol concentrations and lower extraction temperatures. Optimum ethanol concentration, extraction temperature and time were 46.6%, 70.20°C, 110.45 min for phenolics and 100%, 70.2°C and 110.5 min for carotenoids respectively. Results revealed that there were no significant differences between the predicted values for studied responses and experimental values obtained with optimum extraction conditions.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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