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Response Surface Methodology for Extraction of Phenolics from Safflower (*Carthamus tinctorius* L.) Seed Meal

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The present study used the response surface methodology (RSM) using Box-Behnken design to optimize the extraction conditions of phenolics from safflower defatted seed meal. Response surface methodology was applied to optimize and evaluate three independent variables *i.e.*, temperature (60-80 °C), solvent concentration (50-80 %) and extraction time (1-3 h) and their effect on the extraction yield, total phenolic content and DPPH radical scavenging activity of the extract. Minimum concentration of extract to inhibit 50 % activity of DPPH was calculated. ANOVA of the regression model demonstrated that the model for phenolic content of the extract was highly significant and adequate ($p = 0.0054$, $R^2 = 0.888$). The variable with the largest effect on the phenolic content was found to be quadratic term of temperature ($p < 0.01$). For the preparation of safflower seed meal extract, temperature of 60 °C, ethanol concentration, 80 % and time of 2.7 h were found to be optimal which gave 12.50 %, yield of extract, 15.09 g GAE/100 g extract phenolic content and 84.61 µg/mL IC_{50} value.

Keywords: DPPH activity, Phenolic compounds, Response surface methodology, Safflower, Seed meal.

INTRODUCTION

India is the largest producer of safflower (*Carthamus tinctorius* L.) in the world with highest acreage (4.3 lakh hectares) and it is cultivated as an oil seed crop [1]. This species, has sparked the interest of many countries because of its adaptability to different environmental conditions and more specifically, for the quality of its seed oil and meal [2]. After oil extraction, the remaining meal, also referred to as oil cake, is under utilized but could be an excellent source of raw material showing potential applications as nutraceuticals and functional food ingredients. As, many of the antioxidants in oilseeds are not necessarily fat soluble or their extraction into the oil is low [3] and hence they remain in seed meal. Recently, increasing interest has been observed in safflower seeds as material of research because of their antioxidant and anticarcinogenic potential and ability to avert or ameliorate many degenerative ailments [4].

Major phenolic constituents of defatted safflower seeds have been identified as the two serotonin conjugates *viz.* N-(*p*-coumaroyl)serotonin (CS) and N-feruloyl serotonin (FS). They are members of the indole hydroxycinnamic acid amides, with serotonin (5-HT), *p*-coumaric acid (*p*-ca) and ferulic acid (fa) representing components of their structures [5,6]. Serotonin derivatives have been reported to possess antibacterial and free radical-scavenging activities [4,7] and these compounds behave as antioxidants in plasma and on liver HDL-cholesterol and total cholesterol [8]. These compounds have been reported to increase proliferation of fibroblasts [9] and show other benefits against cardiovascular risk [5].

Antioxidants, including phenolic compounds are among phytochemicals that may render their effects *via* antioxidation and relief from oxidative stress and its consequences. Dietary antioxidants also play an important role as nutraceuticals due to their role in protecting the body from free radicals, reactive oxygen species and reactive nitrogen species, which are derived either from normal metabolic processes or from external sources [10-12].This protection is likely to involve number of mechanisms of action, including inhibition of the generation of free radicals, enhancement of the scavenging capacity against free radicals, reducing capacity and metal chelating ability [13,14].

The synthetic antioxidants have been used to control lipid oxidative rancidity in foods, but use of synthetic antioxidants has been restricted due to safety concerns as well as consumer awareness towards health. Therefore, plant sources are rich in antioxidants, including polyphenolic compounds, tocopherols, vitamin C and carotenoids, and are attracting to the food industry as replacements for synthetic ones. Box-Behnken design based on the response surface methodology (RSM) was applied to optimize conditions for extraction of phenolic extract. Response

surface methodology is a statistical mathematical method that uses quantitative data in an experimental design to determine and to solve multivariate equations for the optimization of processes. It is a useful tool to minimize the number of trials and provide multiple regression approach to achieve optimization. Keeping in view the importance of safflower phenolic compounds, the present study was designed for the assessment of safflower seed meal, a potential low-cost feedstock for extraction of antioxidant-rich phenolic extract using response surface methodology.

EXPERIMENTAL

Procurement of safflower seed: Seeds of Safflower variety PBNS 12 were procured from Directorate of Oilseed Research, Hyderabad, India. The seeds were ground and oil was extracted using solvent.

Preparation of safflower seed meal: For preparation of seed meal, 10 g of ground seed was weighted and each was set in a Soxhlet apparatus with eight sample holder and then continuously extracted for 8 h at one time using *n*-hexane. After extraction, the solvent was evaporated. The defatted safflower meal was used for further studies.

Extraction of antioxidant phytochemicals from safflower seed meal: Finely ground powdered safflower seed meal (50 g) was mixed with various fractions of ethanol and incubated under different time and temperature conditions. After incubation, the sample was centrifuged at 2000 ×g for 10 min to separate the insoluble fractions and total phenol and antioxidant potential was estimated in the supernatant.

Response surface modeling: Response surface methodology was used to evaluate the effects of three independent variables, temperature (T, 60-80 °C), solvent concentration (C, 50-80 %) and time (t, 1-3 h) on the extraction efficiency of phenolic, which was reflected by three dependent variables including extraction yield (Y_1, %), phenolic content based on dry weight of the extract (Y_2, g gallic acid equivalents (GAE)/ 100 g) and DPPH radical scavenging activity IC_{50} value (Y_3, µg/mL). The coded and uncoded independent variables used in the RSM design are listed in Table-1. The levels of the independent parameters were based on preliminary experimental results.

A second-order polynomial equation was used to express the yield (Y) as a function of the independent variables:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

where Y represents the response variable, β_0 is a constant, β_1, β_2, β_3 are the linear coefficients, β_{11}, β_{22}, β_{33} are the quadratic coefficients, β_{12}, β_{13}, β_{23} are the linear-by-linear interaction coefficients and X_1, X_2, X_3 are the coded values of independent variables. The model was built based on the variables with confidence levels of 95 %.

Experimental design, data analysis and quadratic model building were conducted using the software 'Design Expert 8' (Version 8.0.2, Stat-Ease, Minneapolis, MN). Five replicates at the center of the design were used to allow for the estimation of a pure error sum of squares. Differences between variables were tested for significance using the one-way ANOVA analysis procedure.

Optimization: Optimum values of the processing variables were obtained with the help of the numerical optimization technique of the Design-Expert software (Version 8.0.2, Stat-Ease, Minneapolis, MN).The software can be used to assign goals to the processing variables and the responses. The software was used to generate optimum processing conditions and to predict the corresponding response.

Determination of total phenolic content (TPC): Total phenolic content in safflower seed meal extract was estimated using a Folin-Ciocalteu method [15]. To 0.1 mL of the extract, 3.9 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent were added. The tubes were incubated at room temperature for 5 min. To this 1.5 mL of 20 % sodium carbonate was added and tubes were kept at room temperature for 0.5 h. The blue colour developed was read at 760 nm. The measurement was compared to a standard curve of gallic acid concentrations and expressed as grams of gallic acid equivalents (GAE) per 100 g extract.

Determination of DPPH radical scavenging activity: The antioxidant activity of the extracts was measured on the basis of the scavenging activity of the stable 2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical as described by Alasalvar *et al.* [16], with slight modifications. A known aliquot of extract was added to 3 mL of 0.1 mM methanolic solution of DPPH. Absorbance at 517 nm was determined after 10 min. The percent inhibition activity was calculated by the formula:

$$1 - \frac{\text{Optical density of sample}}{\text{Optical density of control}} \times 100$$

RESULTS AND DISCUSSION

Model fitting: As a collection of statistical techniques, response surface methodology (RSM) has been widely used to analyze or to optimize the independent factors which influence the extraction yield or extract profiles of bioactive components from natural materials. In present study to assess the effects of three independent variables including extraction time (t), temperature (T) and ethanol concentration (C) on different dependent variables, RSM was applied and the coefficients R^2, adjusted R^2, standard deviation (SD), mean and CV % were computed (Table-2).

Analysis of variance (ANOVA) showed that the resultant second order polynomial model adequately represented the experimental data with the coefficient of multiple determi-

TABLE-1
UNCODED AND CODED LEVELS OF INDEPENDENT VARIABLES USED IN THE RESPONSE SURFACE METHODOLOGY DESIGN

Independent variable	Symbol	Level		
		Low (–1)	Middle (0)	High (+1)
Temperature (°C)	X_1	60	70	80
Percentage of solvent (%)	X_2	50	65	80
Time (h)	X_3	1	2	3

TABLE-2
EXPERIMENTAL DATA FOR YIELD OF EXTRACT (%), PHENOLIC CONTENT (g GAE/100 g EXTRACT) AND DPPH ACTIVITY IC_{50} (µg/mL) OBTAINED FROM SAFFLOWER SEED MEAL

Standard	Run	Temperature (°C)	Percentage of solvent (%)	Time (h)	Yield of extract (%)	Phenolic content (g GAE/100 g extract)	DPPH activity IC_{50} (µg/mL)
		X_1	X_2	X_3	Y_1	Y_2	Y_3
17	1	70.00	65.00	2.00	13.18	13.41	101.67
10	2	70.00	80.00	1.00	11.93	12.62	105.00
7	3	60.00	65.00	3.00	12.65	14.18	84.33
16	4	70.00	65.00	2.00	13.18	13.41	101.67
5	5	60.00	65.00	1.00	14.08	13.36	89.67
12	6	70.00	80.00	3.00	10.93	14.88	108.67
2	7	80.00	50.00	2.00	12.07	19.91	122.00
8	8	80.00	65.00	3.00	12.13	17.04	120.67
11	9	70.00	50.00	3.00	14.18	11.71	109.67
15	10	70.00	65.00	2.00	13.18	13.41	101.67
13	11	70.00	65.00	2.00	13.18	13.41	101.67
6	12	80.00	65.00	1.00	13.33	13.04	127.67
14	13	70.00	65.00	2.00	13.18	13.41	101.67
4	14	80.00	80.00	2.00	11.68	19.03	115.67
3	15	60.00	80.00	2.00	14.25	14.02	83.00
1	16	60.00	50.00	2.00	13.05	16.64	97.33
9	17	70.00	50.00	1.00	10.80	14.94	101.33
Mean					12.79	14.61	104.32
Std. Dev.					0.86	1.14	5.05
C.V. (%)					6.72	7.82	4.84
R-Squared					0.7189	0.8884	0.9276
Adj R-Squared					0.3575	0.7449	0.8346

nations (R^2) for the responses of phenolic content and DPPH radical scavenging IC_{50} yields being 0.888 and 0.924, respectively.

ANOVA was used to evaluate the significance of the coefficients of the models (Table-3). For any of the terms in the model, a large regression coefficient and a small *p*-value would indicate a more significant effect on the respective response variables [17].Thus, the variable with the largest effect on the phenolic content was the quadratic term of temperature ($p < 0.01$). All quadratic effects as well as interaction effect of percentage of solvent and time, affected the phenolic content significantly (Table-3). The results revealed that in linear terms, only temperature had the significant ($p < 0.05$) effect on the phenolic content g GAE/100 g extract (Y_2) response as compared to other independent variables studied. However, the variable with the largest effect on the DPPH activity (IC_{50}) was the linear term of temperature ($p < 0.001$). The results of response surfaces for yield (Y_1, %), phenolic content based on dry weight of the extract (Y_2, g GAE/100 g extract) and DPPH radical scavenging IC_{50} (Y_3, µg/mL) were in the range of 10.8-14.25 %, 12.62 -19.91 g GAE/100 g extract and 83-122 µg/mL, respectively.

Response surface analysis: The best way to visualize the effect of the independent variables on the dependent ones

TABLE-3
REGRESSION COEFFICIENTS OF THE FITTED QUADRATIC EQUATION AND STANDARD ERRORS FOR THE YIELD OF EXTRACT (%), PHENOLIC CONTENT (g GAE/100 g EXTRACT) AND DPPH ACTIVITY IC_{50} (µg/mL)

Regression coefficient	Yield of extract (%) (Y_1)		Phenolic content g GAE/100 g extract (Y_2)		DPPH activity IC_{50} (µg/mL) (Y_3)	
	Regression coefficient	Standard error	Regression coefficient	Standard error	Regression coefficient	Standard error
β_0	13.18	0.38	13.41	0.51	101.67	2.26
Linear						
β_1	-0.65	0.30	1.35*	0.40	16.46***	1.78
β_2	-0.16	0.30	-0.11	0.40	-2.25	1.78
β_3	-0.081	0.30	0.48	0.40	-0.041	1.78
Quadratic						
β_{11}	0.38	0.42	2.43**	0.56	1.12	2.46
β_{22}	-0.80	0.42	1.56*	0.56	1.71	2.46
β_{33}	-0.42	0.42	-1.43*	0.56	2.79	2.46
Interaction						
β_{12}	-0.40	0.43	0.87	0.57	2.00	2.52
β_{13}	0.16	0.43	0.79	0.57	-0.41	2.52
β_{23}	-1.09*	0.43	1.37*	0.57	-1.17	2.52

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; β_0 is a constant, β_{ii} and β_{ii} are the linear, quadratic and interactive coefficients of the second order polynomial equation, respectively.

is to draw surface response plots of the model, which was done by varying two variables within the experimental range and holding the one constant at the central point. The results showed that significant models were found for the two dependent variables phenolic content based on dry weight of the extract (Y_2, g GAE/100 g extract) and DPPH radical scavenging IC_{50} (Y_3, µg/mL). In present model, yield (Y_1, %) of the extract showed minor variations under different conditions and was not affected significantly. However, quadratic effect of temperature and interaction effect of temperature and percentage of solvent significantly affected the yield of extract.

Multiple regression coefficients were determined by the least-squares technique in order to predict quadratic polynomial models for the tested response variables and the regression equations were obtained as shown below:

$$Y_1 = +13.18 - 0.65.\,T - 0.16.\,C - 0.081.\,t - 0.40.\,T.\,C + 0.16.\,T.\,t - 1.09.\,C\,t + 0.38.\,T^2 - 0.80.C^2 - 0.42.\,t^2$$

$$Y_2 = +13.41 + 1.35.\,T - 0.11.\,C + 0.48.\,t + 0.87.\,T.\,C + 0.79.\,T.\,t + 1.37.\,C.\,t + 2.43.\,T^2 + 1.56.\,C^2 - 1.43.\,t^2$$

$$Y_3 = +101.67 + 16.46.\,T - 2.25.\,C - 0.041.\,t + 2.00.\,T.\,C - 0.41.\,T.\,t - 1.17.\,C.\,t + 1.12.\,T^2 + 1.71.\,C^2 + 2.79.\,t^2$$

and regression coefficients have been shown in Table-3.

Fig. 1 represents response surface plots showing the effect of percentage of solvent used time and temperature, respectively on the phenolic content g GAE/100 g extract (Y_2). Fig. 1a showed that by increasing the temperature and solvent fraction, total phenolic content decreased. This could be due to the degradation of polyphenols and decrease in the polarity of solvent at higher temperatures [18,19].

IC_{50} is the concentration of extract which is required to inhibit DPPH activity. For the extract to be more active IC_{50} should be low. The more potent the antioxidant activity of the

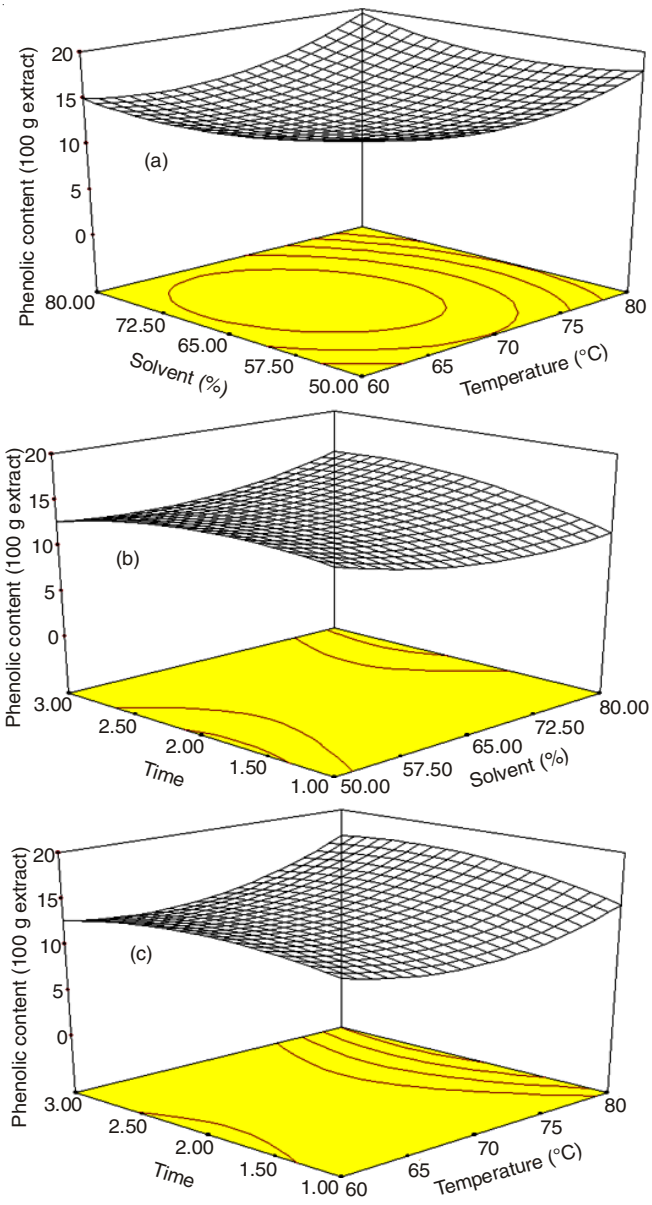

Fig. 1. Surface plot of the phenolic content g GAE/100 g extract (Y_2) as a function of temperature and percentage of solvent (a), percentage of solvent and time (b) and temperature and time (c)

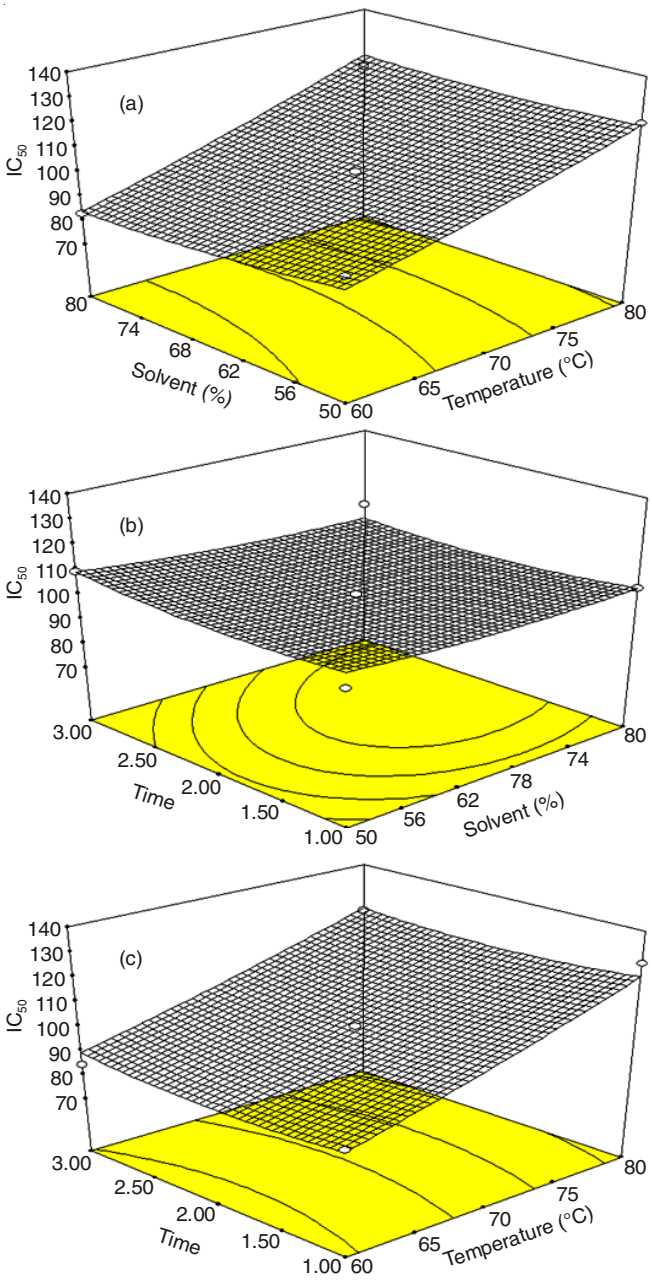

Fig. 2. Surface plot of the DPPH activity IC_{50} (µg/mL) (Y_3) as a function of temperature and percentage of solvent (a), percentage of solvent and time (b) and temperature and time (c)

extract less is the IC_{50} value. Fig. 2 represents response surface plots showing the effect of percentage of solvent used time and temperature, respectively on the DPPH radical scavenging activity IC_{50} (Y_3). From a 3D surface plot (Fig. 2), it was observed that radical scavenging ability decreased with the increase in temperature in these parameters and the percentage of inhibition of DPPH radicals started declining. Liu *et al*. [20] and Singh *et al.* [21] also observed the inhibition of DPPH radical scavenging activity at higher temperature. Table-3 represents that all quadratic effects and interaction effect of temperature and percentage of solvent had significant effect on DPPH radical scavenging activity IC_{50} (Y_3).

Optimization of extraction conditions: Response optimization is conducted to predict the optimum levels of independent variables leading to the desired response goal [22]. In order to check the exact optimum points of independent variables resulting in the optimized conditions, a numerical optimization was employed. The numerical optimization result showed that the overall optimum area was predicted to be obtained by extraction at the combined level of 60 °C, solvent percentage of 80 % and time of 2.70 h with desirability of 0.700 by response surface plots and response optimizer. The predicted optimum response values for the yield of extract, phenol content and IC_{50} value were selected as 12.50 %, 15.09 g GAE/100 g extract and of 84.61 µg/mL.

Conclusion

The present study optimized an extraction procedure using response surface methodology and assessed the potential antioxidant activity for phenolics from safflower defatted seed meal. ANOVA implied that the quadratic term of temperature ($p < 0.01$) was the most significant factor affecting the phenolic content. For the preparation of safflower seed meal extract optimum values of temperature, ethanol concentration and time were 60 °C, 80 % and 2.7 h, respectively and the yield of extract, phenolic content and IC_{50} value were found to be 12.50 %, 15.09 g GAE/100 g extract and of 84.61 µg/mL, respectively.

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