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Original Paper

Response surface optimization of extraction parameters for enhanced phenolic extraction and antioxidant activity from *Cassia fistula* flowers

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***Abstract*— *Cassia fistula* is a well-known plant species for the medicinal use of it leaves, barks and flowers. It has been identified as a rich source of phenolics which can be attributed to its medicinal properties. However, these phenolics should be extracted appropriately to effectively utilize them in various applications. This study focused on maximizing the extraction yield of total phenolics and total anthocyanins along with maximum 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity from edible flowers of *C.fistula* as a function of solid:liquid ratio, ethanol concentration, extraction temperature and time. The data was subjected to response surface methodology and the corresponding second order polynomial models were generated. The results showed that the polynomial models for all responses were significant, did not show lack of fit and presented determination of coefficients above 95%. This indicates the suitability of the models for prediction purposes. Using desirability function, the optimum extraction process parameters to obtain maximum values of all responses was found to be 29.56% ethanol, 1:45 solid to liquid ratio at extraction temperature of 31°C and time 24 minutes. Under these optimal conditions, the experimental values were in close agreement with the predicted values and did not show any significant difference ($p<0.05$). The identified optimized conditions could be used to extracts phenolics from *C.fistula* flowers in a cost effective and efficient manner.**

Keywords*—*Cassia fistula, edible flowers, phenolics, response surface methodology

I. INTRODUCTION

The knowledge on disease healing properties of various plant species have exceptionally contributed to the derivation and formulation of various herbal medicines. As cited by herbalists, large number of plant species from genus *Cassia* have been employed in traditional medicine as hepatoprotective, anti-fungal and anti-inflammatory agents [1]. Among them, *Cassia fistula* is an economically prominent plant species which has been used in the management of different ailments.

C.fistula belonging to the family Fabaceae, is considered to be an ornamental plant due to its yellow blossoms and its widely cultivated in the tropics [2]. Different parts of this plant have exhibited numerous distinctive medicinal properties and has been consumed in various forms to treat wide range of diseases. Roots of *C.fistula* has been used as a remedy for rheumatic conditions, hemorrhages and migraine [3], juice of the leaves have been used to treat jaundice, ringworm and dropsy [4], seeds have reported to be effective against abdominal pain, chronic constipation, leprosy and decoctions of edible flowers have been used to control gastro-intestinal disorders and dermal infections [5]. Recently edible flowers of this plant have gained the attention of researchers due to its phytochemical composition, associated bioactivities and potential to be used in therapeutical applications. Phytochemical profiling of *C.fistula* flowers has indicated the presence of specific constituents such as alkaloids, rhein, leucopelargonidin tetramer, fistulin, kaempferol, heptacosanoic acids, hentriacontanoic, triacontanoic, non acosanoic, and gibberellic acid which contribute to the bioactive properties of the flowers such as antioxidant, anti-inflammatory, anti-diabetic, anti-pyretic and anti-tumor potential [6,7,8]. Since scientific knowledge has pointed out that effective utilization of these flowers as a functional ingredient in pharmaceutical as well as food based applications, greatly depend on the phytochemical richness of the extracts obtained from them, it is vital to appropriately extract the phytochemicals from *C.fistula* flowers.

Recovery of phytochemicals from plant matrix can be achieved by various techniques and solid-liquid extraction is one of the widely used convenient and reliable extraction method [9]. The extraction efficiency of phytochemicals from this method is influenced by several parameters such as solvent

concentration, time, temperature and solid:liquid ratio. Thus, in order to achieve maximum extraction quantity, it is important to optimize these parameters. Response surface methodology (RSM) has been widespread as a statistical tool for rational experimental design and optimization of process variables. It is a collection of statistical and mathematical approaches for determining the importance of a number of influencing parameters in the optimum behavior, especially when complex correlations are present [10]. It can be applied to study the individual effects as well as the interactive effects of different independent variables on the responses simultaneously. The present study aims to apply the RSM to simultaneously optimize the process parameters in order to maximize the yield of total phenolics and total anthocyanin content along with maximum antioxidant activity from *C.fistula* flowers.

II. Materials And Methodology

A. Sample collection and extract preparation

Fresh *C.fistula* flowers were collected from Negombo area of Sri Lanka and the voucher specimen was deposited in the herbarium of Wayamba University of Sri Lanka. The flowers were cleaned, appropriately, freeze dried and powdered using a laboratory grinder. The powdered samples were stored in amber glass bottles at -18°C until further analysis.

Sample (1g) was mixed with ethanol (20 to 40 mL), at different concentrations (40% to 100%). The mixture was then homogenized and exposed to temperature in the range of 30 to 60°) for varying time period (30 to 60 minutes). The extracts obtained after filtering through Whatman filter paper # 1 were stored at -4°C until further analysis. The liquid to solid ratio (ml/g), ethanol concentration (%), temperature (°C) and time (minutes) combinations for experiments were based on the experimental design generated by Minitab statistical software version 17. Fig. 1. illustrates the summary of the study discussed above.

B. Experimental design and model validation

Optimization of extraction parameters to maximize the yield of total phenolics and anthocyanins from *C.fistula* flowers along with maximum DPPH radical scavenging activity was carried out using RSM. Statistical software Minitab (17) was used for modeling and analysis. The experimental design was carried-out based on four factor- two level design referred to as central composite design. The design consisted of 31 experimental runs including 16 corner points, 7 center points and 8 axial points, to study the effect of four independent variables (X_A= Liquid to solid ratio, X_B = Ethanol concentration, X_C = Temperature, X_D = Time) on the response variables TPC, TAC and DPPH radical scavenging activity. The center points were used to define the experimental error of the data. The experimental values obtained are shown in Table 1. Response surface analysis and Analysis of variance (ANOVA) was used to determine the regression coefficients and the statistical significance of the model terms. The observed values were fitted into a second order polynomial model as shown in (1) and the regression coefficient β generated is shown in Table 2.

$$Y = \beta 0 + \Sigma \beta i Xi + \Sigma \beta i + \Sigma \beta ij Xij \quad (1)$$

Y indicates the response variable, X_i and X_j are independent variables, β_0 is the constant coefficient of the model, β_i, β_{ii} and β_{ij} are regression coefficients of the single effects, quadratic effects and interactive effects of independent variables respectively.

The predictive extraction model was verified by performing experiments using the optimized process parameters. The predicted values from the final response regression equation were compared to the experimental values to determine the validity and adequacy of the model.

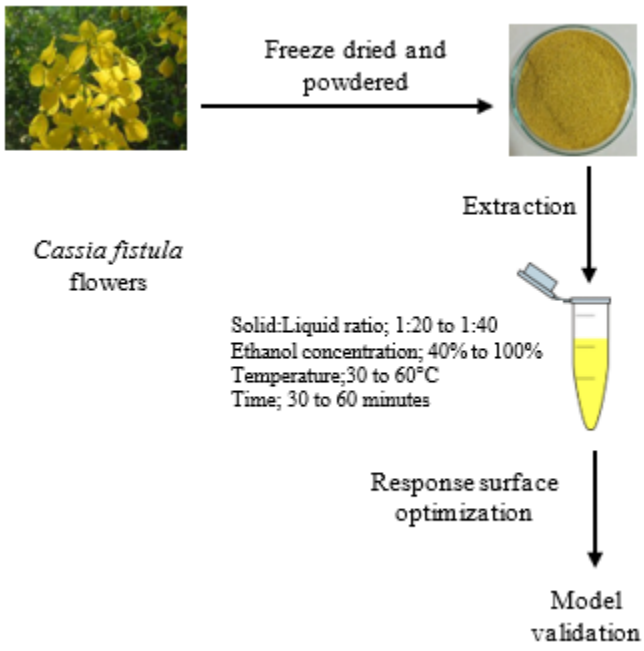

Fig. 1. Graphical representation of the response surface study performed with *Cassia fistula* flowers

C. Determination of total phenolic content (TPC)

The TPC contents of the flower extracts were determined using the Folin-Ciocalteu method [11]. Accordingly, 100 µL of Folin–Ciocalteu (0.5N) reagent was mixed with 500 µL sample extracts and homogenized. The mixture was incubated at room temperature in dark for 15 minutes. Then 2500 µL of sodium carbonate (7.5%, W/V) was added and further incubated for 2 hours in the dark. The absorbance of the incubated mixture was measured at 760 nm using a UV/VIS spectrometer (840-210800 Thermo Fisher Scientific, USA). The concentration of total phenols was expressed as mg gallic acid equivalents (GAE) per g dry weight (DW) of flowers.

D. Determination of total anthocyanin content (TAC)

TAC of the flower extracts was determined based on the pH-differential method [12]. Briefly, 500 µL of sample extract was mixed with 3500 µL potassium chloride buffer (0.025 M,

pH 1) or 3500 µL of sodium acetate buffer (0.025 M, pH 4.5) separately and incubated for 15 min. The absorbance of the resulting mixture was measured at 510 and 700 nm. The difference in the absorbance was calculated as follows:

$$A = [(A_{510} - A_{700})pH_1 - (A_{510} - A_{700})pH_{4.5}]. \quad (2)$$

The concentration of monomeric anthocyanin was calculated using the formula, absorbance × MW × dilution factor ×1000)/(ε ×1), where the molar absorptivity (ε) and molecular weights (MW) of cyanidin-3-glucoside was ε = 26900; MW = 449.2 respectively. Results were expressed as milligrams of cyanidin 3-glucoside equivalents (cy-3-glu) per gram of DW of flowers.

E. Determination of 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

As described by Öztürk [13], 400 µL of flower extract was mixed with 3600 µL of 100 µM ethanolic DPPH solution and incubated at 37°C for 30 minutes in the dark. The absorbance of the remaining DPPH in the resulting mixture was measured at 517 nm against blank using a UV–Visible spectrophotometer. Percentage of DPPH radical scavenging was calculated using the formula:

$$\%\ \text{Scavenging} = (\text{Acontrol} - \text{Asample})/\text{Acontrol} * 100 \quad (3)$$

A_{sample} and $A_{control}$ denotes the absorbance of the samples and control respectively. Control was prepared by replacing the samples with the solvent.

TABLE I. CENTRAL COMPOSITE DESIGN FOR PROCESS VARIABLES AND CORRESPONDING RESPONSE VARIABLES

Runs	Process variables				Response variables		
	Solid : Liquid ratio (X_A-W/V)	Ethanol concentration (X_B-%)	Temperature (X_C-°C)	Time (X_D-minutes)	TPC (mg GAE/g DW)	TAC (mg Cy-3-Glu/g DW)	DPPH (% scavenging/g DW
1	40	40	30	30	41.60	3.42	21.85
2	30	130	45	45	20.06	0.16	20.83
3	30	70	45	45	14.91	0.80	11.40
4	20	40	60	60	25.94	0.43	14.32
5	40	40	60	30	27.89	0.21	29.46
6	20	100	30	60	6.51	1.39	37.01
7	30	70	45	45	38.06	2.89	1.47
8	40	100	30	60	16.69	3.42	24.29
9	30	70	45	45	26.91	0.48	32.02
10	20	40	30	30	22.51	0.53	31.60
11	40	100	60	30	24.69	5.77	24.64
12	40	100	60	60	0.69	1.28	66.03
13	30	70	45	45	30.34	1.60	51.30
14	20	100	60	60	1.94	1.60	41.00
15	30	70	45	45	11.49	2.56	49.74
16	30	70	45	45	15.77	0.96	20.00
17	30	70	75	45	33.77	0.32	48.72
18	40	100	30	30	26.97	0.43	38.10
19	40	40	60	60	39.31	1.50	2.84
20	20	40	60	30	19.09	0.53	4.07
21	10	70	45	45	8.69	0.53	18.00
22	20	100	60	30	3.66	4.06	12.99
23	30	70	15	45	17.49	8.98	47.96
24	20	40	30	60	29.37	1.28	3.33
25	30	10	45	45	28.63	5.45	25.36
26	30	70	45	15	3.77	4.33	38.98
27	50	70	45	45	9.14	5.88	10.59
28	40	40	30	60	35.89	7.48	3.01
29	30	70	45	75	20.91	0.16	7.25
30	30	70	45	45	15.77	5.29	10.12
31	20	100	30	30	13.37	1.82	0.80

III. Results And Discussion

A. Model fitting

RSM is a widely used mathematical and statistical technique for modeling and analyzing a process in which the response of interest is influenced by different independent variables. The main objective of this technique is to simultaneously optimize various responses. The design of experiments is the most important aspect of RSM which determines the most suitable points to examine the response [14]. In the present work, central composite design (CCD) was used to determine the effect of four process parameters (Ethanol percentage, extraction time, temperature and solid to liquid ratio) on TPC, TAC and DPPH radical scavenging activity of *C.fistula* flowers. The levels for each independent variables were selected based on literature. The observed values of 31 experimental runs are presented in Table 1, meanwhile the results of ANOVA are tabulated in Table 2. The CCD consists of three types of points factorial points, a central point, and axial points which are at a distance α from the central point. CCD is appropriate for sequential experimentation and provides a reasonable amount of information for testing lack-of-fit while not involving an unusually large number of experimental runs [15].

TABLE II. Regression coefficients and ANOVA results describing the effect of process variables on the total phenolic content, total anthocyanin content and DPPH radical scavenging activity of Cassia fistula flowers and model adequacy

Factor		TPC	TAC	DPPH
Intercept		48.7	1.8	144
Linear	X_A	2.84	0.17	2.03
	X_B	0.10	0.005	-1.28
	X_C	-0.5	-0.14	-3.83
	X_D	1.69	0.22	-1.24
Quadratic	X_A	-0.02	0.001	-0.03
	X_B	0.001	0.00	-0.001
	X_C	0.006	0.002	0.02
	X_D	-0.008	-0.001	-0.006
Cross product	X_{AB}	-0.001	-0.001	0.01
	X_{AC}	-0.003	-0.003	0.01
	X_{AD}	-0.01	0.002	-0.02
	X_{BC}	-0.002	0.002	0.007
	X_{BD}	-0.008	-0.001	0.02
	X_{CD}	0.002	-0.003	0.02
R^2		0.78	0.81	0.79
Adjusted R^2		0.76	0.79	0.77
p value (model)		0.02	0.01	0.03
p value (Lack of fit)		0.47	2.11	0.81

Modelling of the extraction parameters were carried out using second order polynomial equation. The regression coefficients for the investigated responses indicate the significance of the models with a p-value less than 0.05 for the three responses. In addition to this, the results showed that the models could be applied to predict the studied responses reflected by p values for lack of fit ($p > 0.05$). Overall the ANOVA table revealed that all model responses were significant ($p<0.05$) while the lack of fit was insignificant for all model responses ($p>0.05$). The non-significant lack of fit indicates that the model term adequately explains the relationship between the process parameters and model responses. Also the three-dimensional (3D) response surface plots displayed interaction effects between process parameters towards the model responses.

B. Effect of extraction variables on TPC

Based on the obtained responses, after the elimination of all insignificant effects of extraction variables, the final model generated for TPC by fitting the second order polynomial, could be expressed as follows,

$$\text{TPC (mg GAE/g DW)} = 48.7 + 0.106X_B - 0.5\,X_C - 0.027\,X_A^2 - 0.006\,X_C^2 - 0.008\,X_{BD} \quad (4)$$

From the analysis it was observed that the model p value was 0.029 and lack of fit was insignificant with the p value of 0.475, indicating that the proposed model is well fitted. Also, the model displayed a good model prediction with R^2= 0.782 and Adj.R^2= 0.761. The linear effect of ethanol concentration showed a significant ($p<0.05$) positive effect on the yield whereas the quadrative effects of solid:liquid ratio and temperature as well as interactive effects of ethanol concentration and extraction time yielded negative effects on TPC. The response surface plots obtained for the interactive effects of different variables is shown in Fig 1.

Based on the obtained results, The TPC of the flower extracts varied from 0.68 to 41.60 mg GAE/g DW, with the lowest and highest TPC observed in experimental runs 12 and 1 respectively. As observed in Figure 2 (a) and (b) it can be noted that, the extractable phenolic content was high at lower ethanol concentration and was maximized at 30%. With increasing ethanol concentration, the TPC has decreased. Polarity of the solvents play a vital role in extracting polyphenols from the flower matrix. Ethanol is a widely used organic solvent due to its extraction efficacy, low toxicity, and accepted by the US Food and Drug Administration for extraction purposes as well as for food-based applications. Extraction of phenolics depends on the physicochemical parameters of the phenolic compounds as well as solvent [16]. To enhance the yield of extraction the solubility of the compound should be enhanced by using a mixture of water and organic solvent over a limited compositional range rather than using absolute solvent. This is primarily because, cell vacuoles contain predominantly free phenolic compounds, and the cell wall has more lignin, flavonoids and insoluble polyphenols that are conjugated to proteins, sugars, organic acids and cell wall polysaccharides [17]. Combination of water and ethanol facilitates efficient extraction of polyphenols, where water acts as a swelling agent and ethanol breaks down the bonds between the solutes and the flower matrix, thus absolute ethanol shows the poorest yield of TPC [18]. Different matrix requires different percentages of ethanol to maximize the yield depending on their phenolic composition. For example, as reported previously, recovery yield of phenolics from *Vernonia cinerea* leaves [19] was maximized at 40% whereas phenolic content from apple pomace and olive leaves were maximized at 50% [20, 21].

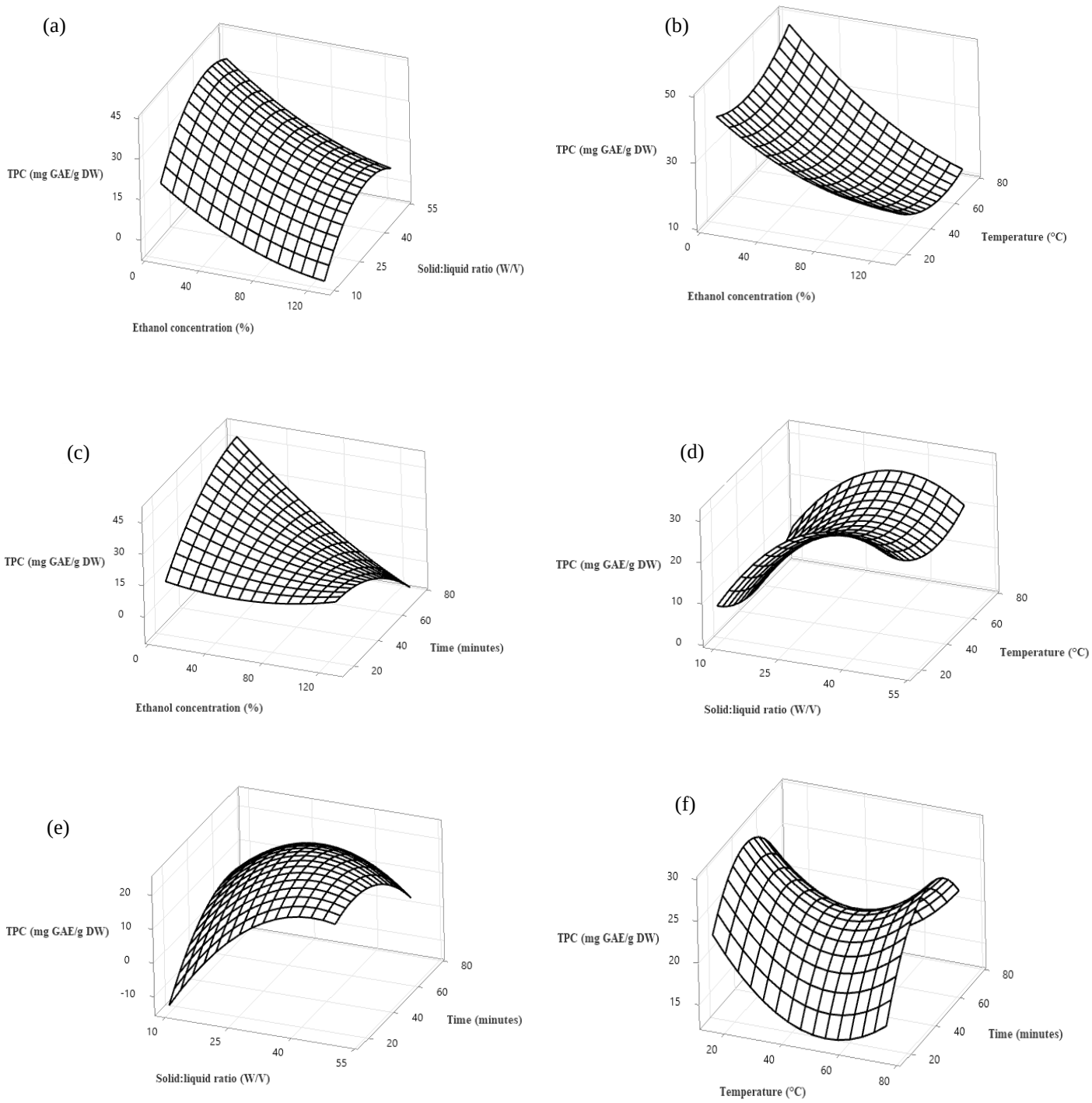

Fig. 2. Response surface plots of phenolic extraction (mg GAE /g DW) extraction from Cassia fistula flowers as a function of solid to liquid ratio, ethanol concentration, extraction temperature and time. Values were kept constant as follows, Temperature 45°C, ethanol concentration 70%, Extraction time 45 minutes and solid to liquid ratio 1:30.

As illustrated in Fig. 2 (c), when ethanol concentration was interacted extraction time, the highest possible yield was obtained at lower ethanol concentration and higher extraction times. However, as a single factor, increasing extraction time increased the yield up to 45 minutes and prolonged extraction time gradually decreased the yield. Extraction time is another crucial factor in solvent extraction of phenolics. This is primarily due to the fact, that appropriate time is required for the equilibrium to be reached between the solution in the flower matrix and extraction solvent. The equilibrium concentration is important for the efficient diffusion of phenolics from the flower matrix and the time required to reach the equilibrium concentration varies depending on the phenolic composition of the matrix [22]. Increasing the extraction time after the equilibrium will not cause a significant increase in the yield.

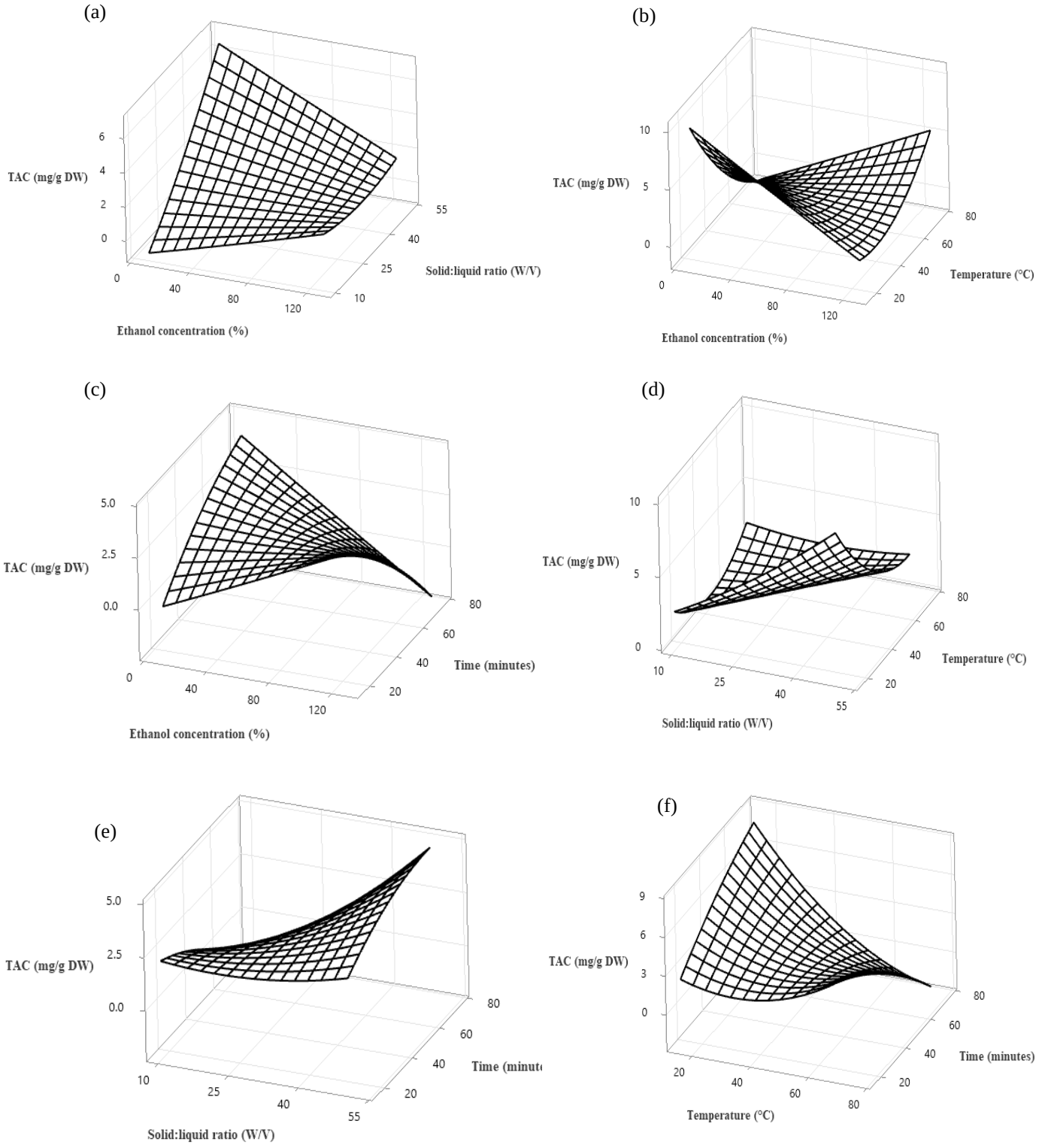

Fig. 3. Response surface plots of anthocyanin (mg cyanidin-3-glucoside /g DW) extraction from Cassia fistula flowers as a function of solid to liquid ratio, ethanol concentration, extraction temperature and time. Values were kept constant as follows, Temperature 45°C, ethanol concentration 70%, Extraction time 45 minutes and solid to liquid ratio 1:30.

However, previous studies have documented that prolonged extraction time could reduce the TPC due to photodegradation, structural deformation due to chemical and enzymatic reaction and oxidation of phenolics [23]. For example, phenolics extracted from grape seeds and pomegranate flowers have decreased with increasing extraction time [16,24].

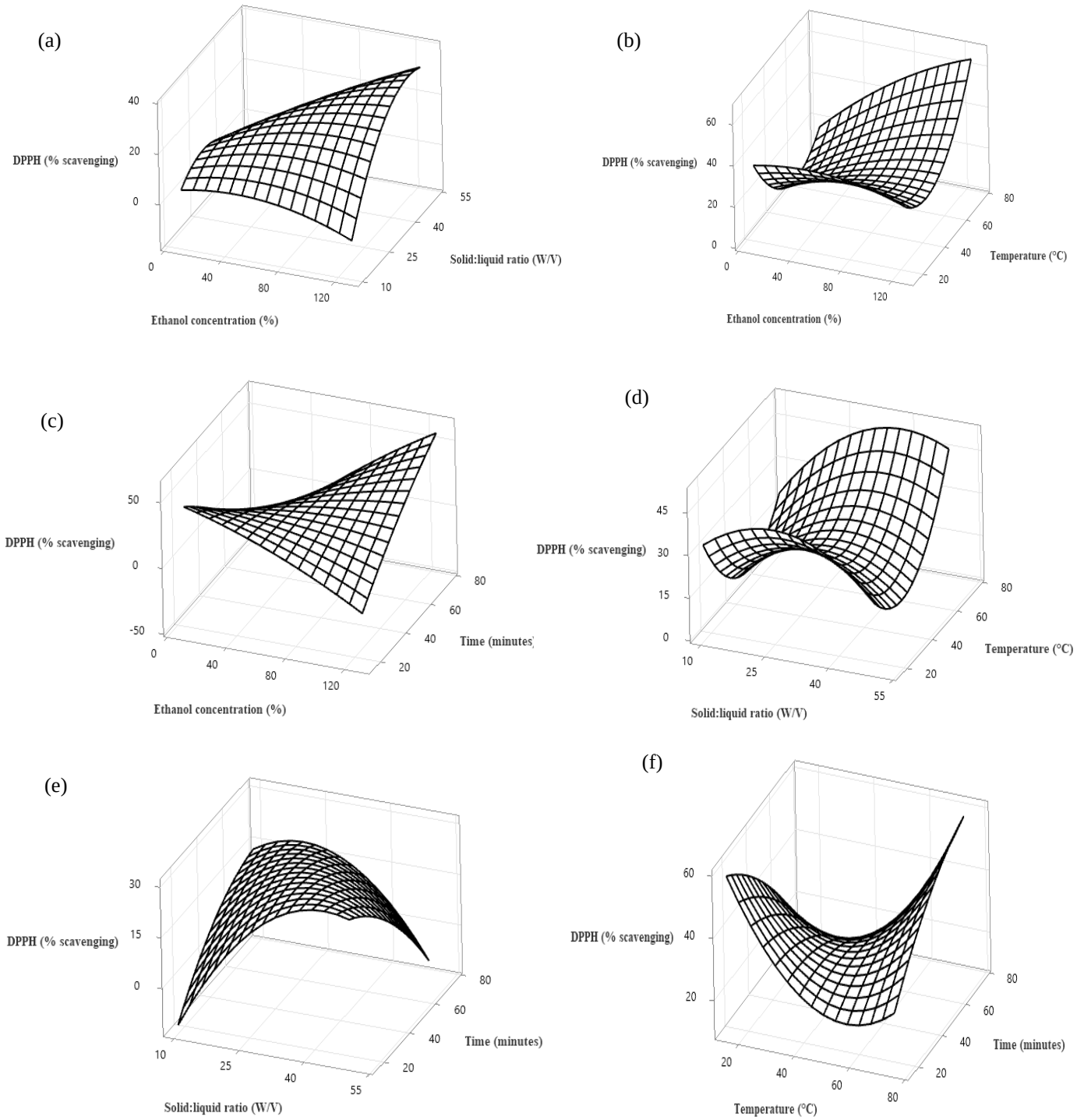

Fig. 4. Response surface plots of DPPH radical scavenging activity (% scavenging) of Cassia fistula flowers as a function of solid to liquid ratio, ethanol concentration, extraction temperature and time. Values were kept constant as follows, Temperature 45°C, ethanol concentration 70%, Extraction time 45 minutes and solid to liquid ratio 1:30.

C. Effect of extraction variables on TAC

The proposed model for TAC shown below, had a p value of 0.010 and the lack of fit was insignificant (p=2.11) with the predication of terms (R^2=81.23 and Adj.R^2=79.42) indicating that the model is well-fitted and reliable to explore further. Considering the linear effects, solid:liquid ratio had a significant ($p<0.05$) positive effect on TAC whereas temperature had a significant negative effect. Based on the quadratic model, a significant ($p<0.05$) positive effect of temperature was observed for the yield of TAC.

$$\text{TAC (mg cy} - 3 - \text{glucoside/g DW)} = 1.81 + 0.17X_A - 0.142\,X_C + 0.002\,X_C^2 + 0.002\,X_{BC} - 0.003X_{CD} \quad (5)$$

The response surface plots obtained for the interactive effects of different variables is shown in Figure 3. The interactive effect between ethanol concentration and extraction temperature expressed a positive effect whereas interactive effects between temperature and extraction time had a significant ($p<0.05$) negative effect on the yield of TAC.

Data from the present study shows that TAC of the flower sample varied from 0.16 to 8.98 mg cy-3-glu/g DW. Considering the effect of ethanol concentration, a steady reduction on TAC was observed with increasing ethanol concentration as observed in Figure 3 (b). The anthocyanin composition in the flower matrix determines the percentage of ethanol required to maximize the yield. If the matrix is rich in hydrophilic anthocyanins, then extraction is favored in the presence of higher amount of water in the binary solvent system. In the present study use of lower concentration of ethanol has increased the yield of TAC, indicating presence of higher amount of hydrophilic anthocyanins [25].

Considering the impact of temperature, when interacted with ethanol concentration, increasing temperature has increased the yield of anthocyanins. This could justify the fact that, temperature plays an important role in the extraction of anthocyanins. Increasing temperature activates the molecules and increase their diffusion from the flower matrix into the solvent. Also, higher temperature weakens the intercellular interactions and soften the plant tissues facilitating diffusion of molecules [26]. Since anthocyanins are thermolabile compounds, after particular threshold temperature, degradation and transformation of anthocyanins could possibly lower the TAC [27]. Investigations on extraction of anthocyanins from, blueberry wine pomace and raspberries has also revealed that increasing temperature has decreased the yield of anthocyanins [28,29].

Fig. 2 (d) indicates that increasing solid:liquid ratio has steadily enhanced the yield of anthocyanins. When solid to liquid ratio increases it establishes a higher gradient of mass transfer between the solid and liquid which facilitates the diffusion of compounds. Additionally, it also increases the contact area between the matrix and the solvent and penetrates further in to extract higher quantity of anthocyanins [30]. A lower solid to liquid ratio could not yield higher TAC due to the fact that, lower volume of solvent is insufficient to fill up the flower matrix and the hypertonic environment could not be created, holding up the color in the vacuole of the material. Once the solvent reaches a certain volume, the cell rapidly absorbs water swells and bursts out releasing the anthocyanins within the vacuole. In the present study anthocyanin content was maximized at 1:40 and various authors have reported values closer to that obtained in the current study. In the optimization studies conducted on sweet potato and blue butterfly pea flowers it was documented that optimum TAC was observed at a solid:liquid ratio of 1:32 and 1:23 respectively [25].

D. Effect of extraction variables on DPPH radical scavenging activity

As per the results obtained from ANOVA, the model for DPPH scavenging activity was significant ($p=0.03$) and the model could be used to predict the responses. The generated model with insignificant lack of fit ($p=2.11$) is represented as follows,

$$\text{DPPH scavenging activity (\%)} = 144 - 1.281\,X_B + 2.03\,X_A + 0.022\,X_C^2 + 0.021X_{BD} - 0.026\,X_{CD} \quad (6)$$

DPPH radical scavenging activity varied from 0.80% to 66.03% and was mainly affected by the ethanol concentration and solid to liquid ratio in the linear model, and by extraction temperature in the quadratic model. For interactive effect, ethanol concentration and time positively affected the activity whereas extraction temperature and time negatively affected the DPPH radical scavenging activity ($p<0.05$) and the response surface plots obtained for the interactive effects of different variables is shown in Figure 4. Interestingly in the present study it was noted that the radical scavenging activity has not shown any significant increase with increasing ethanol concentration. As observed in the response surface plots, with increasing extraction time the radical scavenging activity has decreased.

E. Optimization of process variables and model validation

The determination of optimum conditions for the simultaneous extraction of phenolics and anthocyanins and optimum DPPH radical scavenging activity was carried out using the desirability function in the scale of 0-1. The desirability value of 1 represents the ideal case and 0 indicates that one or more responses fall outside the desirable range. The generated optimum conditions for maximum TPC and TAC with maximum radical scavenging activity was 29.56% ethanol, 1:45 solid to liquid ratio at extraction temperature of 31°C and time 24 minutes. An experimental run was conducted with the recommended optimum conditions and the obtained responses for TPC, TAC and radical scavenging activity were compared with the predicted values to study the appropriateness of the response models. The obtained values are presented in Table 3. The was no statistically significant ($p>0.05$) difference between the predicted and experimental values at 95% confidence interval. The outcomes indicate the reliability of the parameters for the extraction of phenolics and anthocyanins with maximum antioxidant activity from *C.fistula* flowers.

TABLE III. PREDICTED AND EXPERIMENTAL VALUES OF RESPONSES UNDER OPTIMUM CONDITIONS FOR SIMULTANEOUS OPTIMIZATION OF RESPONSES. EXPERIMENTAL VALUES ARE EXPRESSED AS MEAN±STANDARD DEVIATION

Responses	Predicted values	Experimental values
TPC (mg GAE/ g DW)	35.89	34.21±1.8
TAC (mg cy-3-glucoside)	9.92	8.83±2.5
DPPH (% scavenging)	66.63	66.19±1.23

IV. Conclusion

The current study investigated the optimized conditions to enhance the recovery of phenolics and anthocyanins from *C.fistula* flowers along with optimum radical scavenging activity. The RSM based second order polynomial models were adequate to optimize the ethanol-based extraction of antioxidant compounds due to satisfactory ANOVA and descriptive statistics parameters. The optimum conditions obtained were: 29.56% ethanol, 1:45 solid to liquid ratio at extraction temperature of 31°C and time 24 minutes. The findings of the current study is applicable for the efficient extraction of phenolics from *C.fistula* flowers and to be used in food industry and pharmaceutical products to reduce the cost and labor intensive process.

Acknowledgement

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