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Optimization of Ultrasonic Extraction of Natural Dye from *Calendula Arvensis* Petals by the Response Surface Method

Fatima Hami[a,*], Sarra Aourabi[b], Hala Mghari[a], Fatima Mahjoubi[b] and Amina Bari[a]

[a]*Laboratory of Biotechnology, Environment, Agri-Food and Health (LBEAS), Faculty of Science, USMBA Fez, P. O. Box : 1796 (Atlas), Fez 30000, Morocco*

[b]*Laboratory of Engineering, Modeling and Systems Analysis (LIMAS), Faculty of Science, USMBA Fez, P. O. Box: 1796 (Atlas), Fez 30000, Morocco*

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The purpose of this study was to use hydro-ethanolic solvent to extract natural color extract as effectively as possible from *Calendula arvensis* flower petals. The effects of ethanol volume, extraction time, and solvent/material ratio on the efficacy of *Calendula arvensis* flower petals extractives by ultrasonic extraction at 50 kHz and room temperature were investigated. Total phenol content (TPC) and color power were used to quantitatively describe each extract. Using Response Surface Methodology (RSM), the extraction procedure was optimized, and extraction effectiveness was increased (TPC, color power). Different ethanol: water ratios were employed in the experiments. Through a variance analysis, the effectiveness of the extraction procedure was established (ANOVA). The maximum extraction efficiency of the hydro-ethanolic extraction (28.21%), the polyphenol content (779.11 mg GAE/g extract), and color power (42) were quantified using a 30 min treatment time, a 60:40 ethanol: water ratio, and a 15 mL/g solvent/material ratio. The results obtained show that ultrasonic-assisted extraction is a successful technique for extracting natural color from *Calendula arvensis.*

Keywords: *Calendula arvensis*, Color power, Optimization, Polyphenols content, RSM, Ultrasonic

INTRODUCTION

Since ancient times, natural dyes have been used to color a variety of materials, including wool, cotton, silk, and leather. Additionally, these dyes have been utilized in the production of paints, printing inks, watercolors, and cosmetics [1]. Dyes are different from pigments as they have a higher solubility with finer particles than pigments [2]. The usage of synthetic dyes is being considered with caution as environmental preservation and sustainability problems gain in popularity. The use of natural dyes is thought to be more sensible, environmentally responsible, safe, and easily obtained from renewable sources than synthetic colors [2]. The biodegradability and excellent environmental compatibility of natural dyes are well known [3-4].

Calendula arvensis is a brittle, angular-stemmed annual herbaceous plant that is between 15 and 30 cm long. Depending on the environment, it has either deciduous or evergreen foliage. Although it is indigenous to the Mediterranean region, the species is common throughout many different regions of the globe [5]. The blooms of *C. arvensis* are well known for their wide range of hues, which include golden yellow, orange yellow, and various shades of orange. Marigold petals contain lutein and lutein fatty acid esters, which account for over 90% of the plants' pigment content [6-7]. Lutein $C_{40}H_{56}O_2$, the primary colored pigment in *C. arvensis* flowers, is a member of the carotenoid family [8]. Given its main benefits in solid-liquid extraction, such as improved extraction kinetics and yield, reduced operating temperature allowing the extraction of thermolabile

*Corresponding author. E-mail: 95fatimahami@gmail.com

compounds, low cost of the equipment, and ease of operation compared to other new extraction techniques, such as microwave-assisted extraction, and the ability to use any solvent which allows for intervention in the extraction of a wide variety of compounds, we used the ultrasonic-assisted extraction method in our study.

In our study, we used the ultrasonic-assisted extraction method given its main advantages in solid-liquid extraction including increased extraction kinetics and yield, reduced operating temperature allowing the extraction of thermolabile compounds, low cost of the equipment, and ease of operation compared to other new extraction techniques such as microwave-assisted extraction, the possibility of using any solvent which allows to intervene in the extraction of a wide variety of natural compounds [9]. The traditional method of optimization is laborious and time-consuming since one factor at a time is taken into consideration. In this method, the interactions of various factors are ignored and hence, the chances of obtaining the true optimum conditions are dubious [10]. To overcome this difficulty, usage of statistical optimization procedure in the form of response surface methodology (RSM) is used.

In order to optimize the extraction conditions, including concentration of solvent, extraction time, and solvent-to-material ratio, response surface methodology (RSM) has been widely used and exploring the relationship between multiple variables and a response of interest [11], specifically the Box-Behnken method, aims to quantitatively determine variations in the response function concerning significant influencing factors [12]. Using experimental mathematical models [13], an approximate relationship between output responses and input variables can be determined so that process parameters can be optimized to achieve desirable responses.

We employed RSM to optimize the extraction conditions for our investigation, which included the solvent concentration, extraction time, and the solvent to *C. arvensis* material ratio, which was measured by ultrasonication. The Box-Behnken approach, which forms the foundation of the experimental design, allows for the primary variables that affect the extraction yield to be varied. In the present work, we used RSM to optimize the ultrasonication of phenolic compounds and color power from *C. arvensis*. Our work aimed to establish the optimized parameters of ultrasonication for the phenolic compounds extract and color power from *C. arvensis*, and offer scientific reference for quality assay and utilization of the resource.

MATERIAL AND METHODS

Plant Material

The collection of Calendula arvensis was carried out in March 2021 close to the Dhar El Mahraz Faculty of Science as well as in a field of the Sefrou road. Afterward, the single petals were collected and dried in the open air for three weeks [14].

Extraction Method

Ultrasonic assisted extraction. In a 250 mL beaker, the plant material powder and solvent are introduced into an ultrasonic bath extractor-Wise Clean (45 Hz, 50 W), which was performed according to the Box-Behnken design to maximize ultrasound extraction of the petals. The extraction time, ethanol percentage (%), and solvent/material ratio were the three variables that were modified. After extraction, the obtained extracts were filtered and evaporated (Table 1).

Phytochemical screening. A series of tests known as phytochemical screening are performed either on the powder or on the infusion. These tests provide a good indication of whether or not a plant has particular primary and secondary metabolites. Total polyphenols (total tannins, gallics, catechics, flavonoids, *etc.*), terpenic chemicals (saponosides), nitrogenous compounds (alkaloids), and glucosides are the molecules that have been identified [15-16].

Response surface methodology. The ethanol percentage (%), extraction time (min), and solvent/material amount (ml g^{-1}) were all optimized using RSM for *C. arvensis* by

Table 1. Factors Controlled by Ultrasonic Method

Parameter	-1	0	+1
X1: Ethanol (%)	40	60	80
X2: Time (min)	20	30	40
X3: Solvent/material (m g^{-1})	5	10	15

ultrasonication. To prevent the deterioration of the thermosensitive compounds, the temperature in this experiment was kept constant at room temperature. The experimental design chosen is based on the Box-Behnken method allowing varying the main factors that influence the extraction yield and the polyphenol content. In the study, the experiments were performed on the Box-Behnken Design (BBD). The optimal extraction variable combination for *C. arvensis* yield, total phenol content, and coloring power was optimized using three variables.

The complete design was carried out in random order and consisted of 17 experiments including five replicates at the central point (Table 2). The data from BBD were analyzed by multiple regressions to fit the following quadratic polynomial model Eq. (1):

$$\boldsymbol{Y} = \beta_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j \quad (1)$$

Where Y is the predicted response, $ß_0$ is a constant b_i, b_{ii} and b_{ij} are the linear, quadratic, and interactive coefficients of the model, respectively. Accordingly, X_i and X_j represent the levels of the independent variables, respectively. The quality of the fitted model was expressed by the coefficient of determination (R^2).

To calculate the yield of extraction (Y_1) encoded by the independent variables; ethanol volume (X_1), solvent-to-material ratio (X_2), and extraction time (X_3), we used the quadratic multinomial regression equation as follows (2):

$$Y_1 = 35.800 + 0.894\, X_1 - 1.533\, X_2 + 0.939\, X_3 - 1.808\, X_1^2 - 4.792\, X_2^2 - 5.600\, X_3^2 \quad (2)$$

Determination of the total phenolic content (TPC). The Folin-Ciocalteu technique was used to determine the phenol contents of the different extracts using gallic acid as a reference [17]. We put 100 µl of the plant extract, which had been well diluted, into a test tube with 6 ml of distilled water. Then, while stirring, 500 µl of the Folin reagent was added. After 5 min, 1.5 ml of a 20% Na_2CO_3 solution is added. The solution is completed to a 10 ml volume using distilled water. The absorbance was measured with a blank made from

Table 2. BBD Experimental Design with the Independent Variables

Experiment	Ethanol (%)	Time (min)	Solvent/material (ml g^{-1})	Yield (%)	TPC (mg EAG/g extract)	Color power -
1	40	20	10	30.00	312.04	31.00
2	80	20	10	31.00	607.22	35.00
3	40	40	10	26.00	554.36	33.00
4	80	40	10	22.00	552.23	34.00
5	40	30	5	29.00	604.22	40.00
6	80	30	5	27.00	614.34	41.00
7	40	30	15	28.00	629.11	34.00
8	80	30	15	25.00	611.23	33.00
9	60	20	5	26.00	753.22	35.00
10	60	40	17	32.00	779.11	42.00
11	60	20	15	18.00	729.12	34.00
12	60	40	15	38.00	612.11	32.00
13	60	30	10	36.00	610.11	33.00
14	60	30	10	35.00	609.23	33.00
15	60	30	10	36.00	602.11	33.00
16	60	30	10	36.00	601.00	33.00
17	60	30	10	36.00	603.00	33.00

distilled water, after 2 h of incubation at room temperature, using a SELECTA-type spectrophotometer at a wavelength of 765 nm. A calibration curve was created at various gallic acid concentrations. At least three times each test was run. The amount of phenol in an extract is measured in milligram equivalents of gallic acid (mg GAE/g extract).

Determination of coloring power. The coloring power of a color is influenced by the type of pigment used, the quantity, and the degree of grinding fineness. With further grinding, the intensity of the color pigment becomes strength. The higher the color power of a paint, the less pigment is needed to affect other colors [18]. The connection that influences coloring power is as follows:

Color power = $\lambda \times 250$
λ: absorbance

Statistical analysis. The experimental results of the response surface design were analyzed by NemrodW. The modeling technique was based on a quadratic model including linear, squared, and interaction elements. Significant terms in the model for each answer were found using analysis of variance (ANOVA). The experimental data were evaluated using descriptive statistical analyses like the *p*-value. If the *p*-values were 0.05 or below, they were considered statistically significant. All experiments were carried out in triplicate unless otherwise noted in the text. Statistical analysis is used to establish the statistical significance of the issues under research and to establish the various parameters (determination coefficient R^2 and the impacts of the components).

RESULTS AND DISCUSSION

Phytochemical Screening

The presence of polyphenolic chemicals (tannins, flavonoids, and saponosides) is more evident than that of the other components, according to the results of the phytochemical screening of *C. arvensis* employing ultrasonic extraction of plant petals. There were no Alcaloïds anywhere in the tubes. These findings are similar to those from studies on *C. arvensis* flowers carried out in [5] (Table 3).
All experiments were carried out in triplicate unless otherwise noted in the text.

Adjustment of the Model

It was critical to investigate the process elements in order to obtain a more realistic mode. Preliminary testing indicated the ethanol percentage range (40-80%), extraction time (20-40 min), and solvent-to-material ratio (10-30 ml g^{-1}). As shown in Table 4, the analysis of variance (ANOVA) of extraction yield, polyphenols, and color power of *C. arvensis*, indicated that the experimental data had a coefficient of determination (R^2) of 0.997, 0.980, and 0.990 respectively, which indicates that only 0.3% of total variations were not explained by the model for extraction yield, 2% for polyphenols indicating good representation of parameter variability by the models and 1% for dye strength.

A good statistical model should have an adjusted coefficient of determination (R^2adj) near to R^2. As demonstrated in Table 4, R^2adj for *C. arvensis* polyphenols (0.955), extraction yield (0.993), and R^2adj for coloring power was (0.980) were close to R^2. Furthermore, the model's high significance is supported by the fact that R^2pred (0.995 for extraction yield, 0.943 for polyphenols, and 0.970 for color power) is substantially compatible with R^2adj.

Table 5 demonstrates that ethanol (X_1) ($p < 0.05$) and solvent/material (X_3) had the largest influence on extraction yield, followed by extraction time (X_2) ($p < 0.05$). All of the quadratic parameters (X_1^2, X_2^2, and X_3^2), as well as their interaction parameters (X_1X_2, X_1X_3), were clearly significant at the $p < 0.05$ level, although (X_2X_3) was not ($p > 0.05$).

Table 3. Results of Phytochemical Screening of *C. Arvensis* Petals

			Compounds	Screened			
Species	Extraction	Tanins	Flavonoïds	Saponosids	Steroïds/ terpenes	Alcaloïds	Cardiac Glycosids
C.arvensis	Ultrasonds	+++	+++	+++	-	-	+

Table 4. Analysis of Variance for the Fitted Quadratic Polynomial Model of Extraction Yield, Polyphenols, and Dye Strength of *C. Arvensis*

Source	SS	DF	MS	*p-value*
Regression	446.1853	9	49.5761	< 0.01 ***
Residual	76.0500	7	10.8643	
Validity	75.2500	3	25.0833	0.0207 ***
Pure error	0.8000	4	0.2000	
Total	522.2353	16		
R^2 = 0.997, R^2 adj = 0.993, R^2 pred = 0.995				
Source	SS	DF	MS	*p-value*
Regression	9.14061E+0004	9	1.01562E+0004	< 0.01 ***
Residual	1.84935E+0003	7	2.64193E+0002	
Validity	1.84621E+0003	3	6.15402E+0002	< 0.01 ***
Pure error	3.14552E+0000	4	7.86380E-0001	
Total	9.32554E+0004	16		
R^2 = 0.980, R^2 adj = 0.955, R^2 pred = 0.943				
Source	SS	DF	MS	*p-value*
Regression	2.14061E+0004	9	1.02562E+000	< 0.02 ***
Residual	1.94935E+0003	7	1.64193E+0002	
Total	3.32554E+0004	16		

R^2 = 0.990, R^2 adj = 0.980, R^2 pred = 0.970. SS: Sum of squares; DF: Degree of freedom; MS: Mean square. Where ***: significant.

Table 5. Estimated Regression Model of the Relationship between the Response Variable (Yield, Total Phenols, and Dye Strength of *C. Arvensis*) and the Independent Variables (X_1, X_2, and X_3)

	p-value		
Variable	Yield	TPC	Coloring power
X_0	< 0.01 ***	< 0.01 ***	< 0.01 ***
X_1	< 0.01 ***	< 0.01 ***	0.03***
X_2	0.0963 ***	< 0.01 ***	0.047 ***
X_3	0.0689 ***	< 0.01 ***	0.047 ***
X_1^2	0.0167 ***	< 0.01 ***	0.0140 ***
X_2^2	0.0317 ***	< 0.01 ***	0.0369 ***
X_3^2	< 0.01 ***	< 0.01 ***	1.57 *
X_1-X_2	< 0.01 ***	< 0.01 ***	31.8
X_1-X_3	0.0864 ***	< 0.01 ***	15.1
X_2-X_3	0.257 **	< 0.01 ***	1.01

According to the ANOVA findings, the first-order terms of the independent variables (X_1, X_2, and X_3) of the quadratic terms (X_1^2, X_2^2, and X_3^2) and the interaction terms (X_1X_2, X_1X_3, and X_2X_3) substantially impacted the polyphenols content of *C. arvensis* ($p < 0.05$). Furthermore, Table 5 shows that the solvent/material (X_3) had the biggest impact on color power (< 0.05).

Analysis of the Response Surface

Effect of extraction parameters on performance. The effect of independent components and their interactions on the extraction yield of *C. arvensis* may be shown by drawing the regression equation from the two-dimensional response surface curves shown in Figs. 1A, 1B, and 1C using the 2D+3D response surface. According to Fig. 1A, which depicts the influence of the interaction between ethanol concentration (X_1) and extraction duration (X_2) on the yield, the yield of *C. arvensis* extractions can reach a maximum value (28.21%) with an ethanol concentration of 88% and an extraction period of 30 min.

When the extraction time was 31 min and the solvent-to-material ratio was 17 ml g^{-1}, the highest yield (23.66%) was obtained (see Fig. 1B), demonstrating the interaction between the two factors. The highest yield (27.52%) was observed at

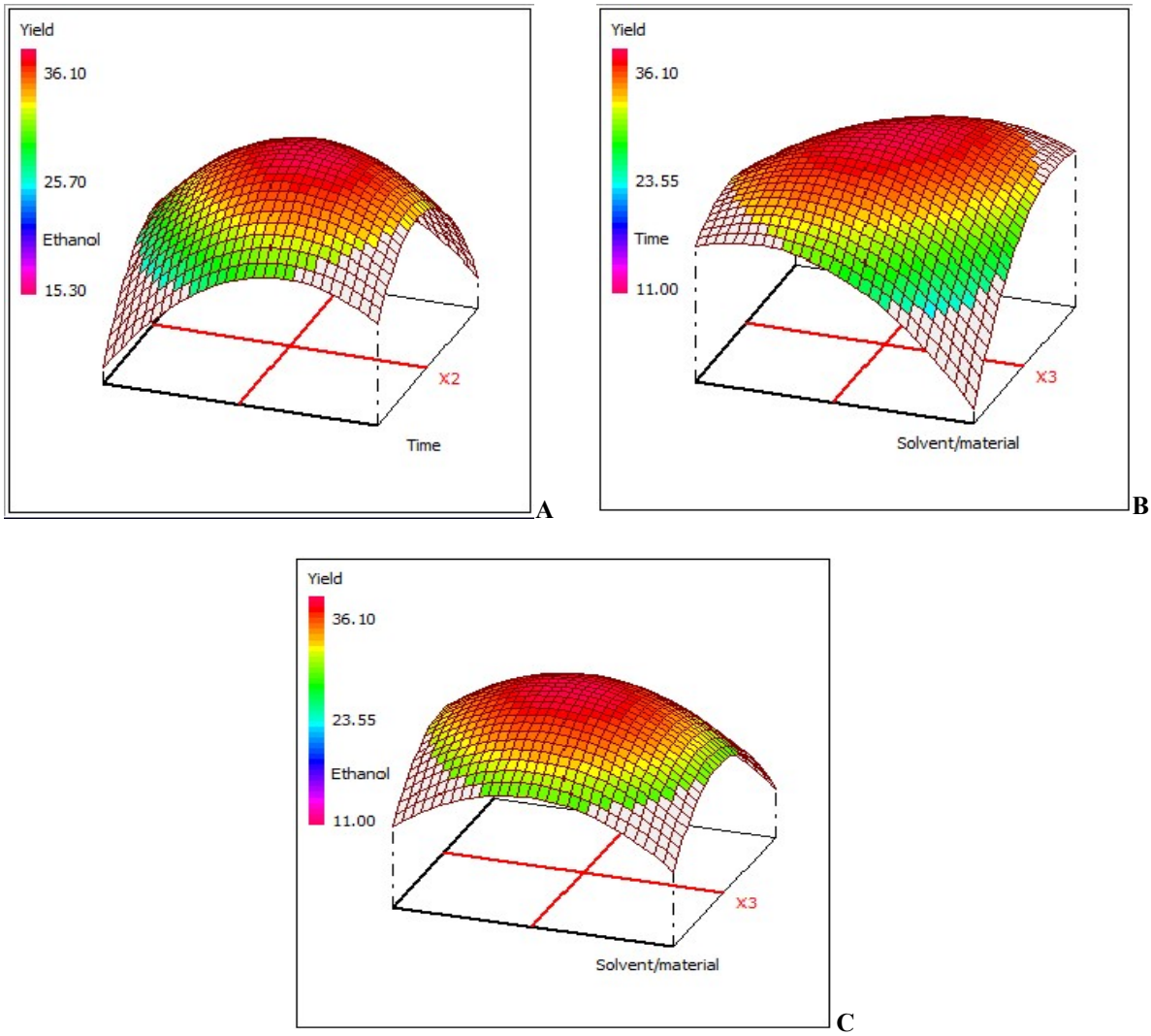

Fig. 1. Response surface graph of ethanol and extraction time (A), extraction time and solvent/material (B), of ethanol and solvent/material (C) and their interactions with the yield of *C. arvensis.*

an ethanol concentration of 88% and a solvent/material ratio of 17 ml g^{-1}, as shown in Fig. 1C, which also shows the impact of ethanol concentration (X_1) and solvent/material ratio (X_3) on C. arvensis extraction yield.

Effect of extraction parameters on total phenol content. Figures 2A, 2B and 2C illustrate the response surface curves and plots for the influence of extraction parameters on total phenol content. According to Fig. 2A, the maximal extraction of total phenols (773.53 mg EAG/g extract) is possible when the ethanol percentage and extraction time are 60% and 31 min, respectively. Because the extraction of phenolic compounds is mostly dependent on the polarity of solvents and compounds, a single solvent may not be efficient for the extraction of a bioactive chemical [14, 19]. Water and alcohol work better combined than alcohol alone to remove phenolic compounds [14,20]. However, when the ethanol percentage exceeded 75.30% in this study, the total phenol yield decreased. The amount of total phenol generated increased from 20 to 40 min of extraction time. This conclusion made sense since when the extraction procedure is extended, phenolic compounds are more likely to be removed. Figure 2B depicts the link between extraction time and solvent-to-material ratio. We observed that the highest yield of total phenols (775.43 mg GAE/g extract) was

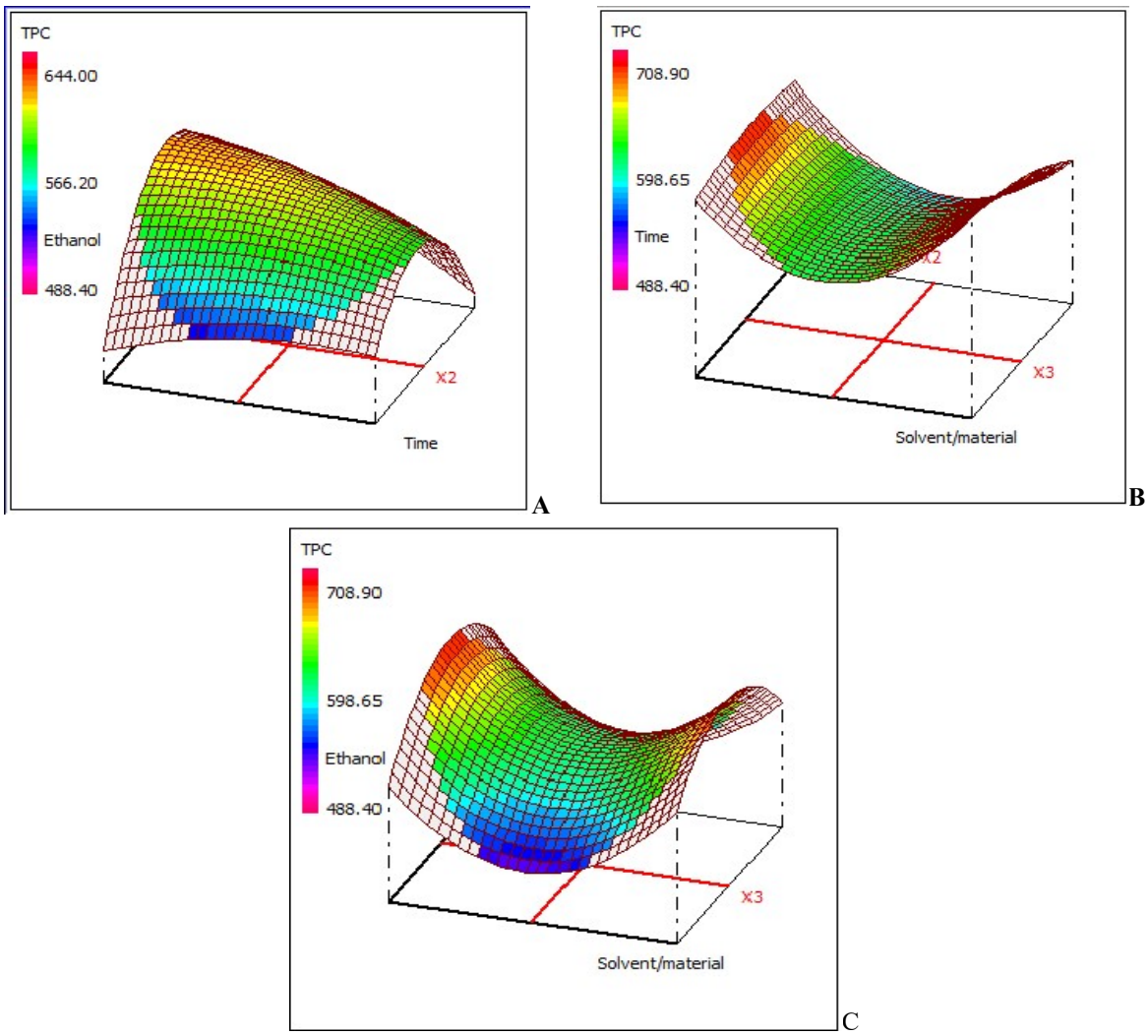

Fig. 2. surface response graph of ethanol and extraction time (A), extraction time and solvent/material (B), ethanol and solvent/material (C), and their interactions with the phenols of *C. arvensis.*

obtained when the extraction time was 31 min and the solvent-to-matter ratio was 10 ml g^{-1}. Figure 2C depicts the connection between ethanol concentration and solvent-to-material ratio. The graph demonstrates that the greatest extraction of total phenols (779.11 mg GAE/g extract) was possible at ethanol concentrations of 61% and 10 ml g^{-1}.

The extraction yield of total phenols was found to increase when the solvent-to-matter ratio was reduced from 20 to 10. This might be because higher amounts of solvent can enter the cells, yet more phenolic compounds can enter the solvent under low-material ratio solvent conditions [19,21].

Effect of extraction parameters on color power. Figures 3A, 3B and 3C show the response surface curves and contour plots for the influence of extraction parameters on the dye strength of *C. arvensis*. As illustrated in Fig. 3A, a maximum color power value of 40 may be attained with a 70% ethanol solution and a 20-min extraction duration. Figure 3B depicts the link between extraction time and solvent-to-matter ratio. We can observe that the staining power may reach a maximum of 42 in 35 min with a solvent/matter ratio of 6 ml g^{-1}. Figure 3C depicts the connection between ethanol concentration and solvent-to-material ratio. The greatest coloring power of 41 was

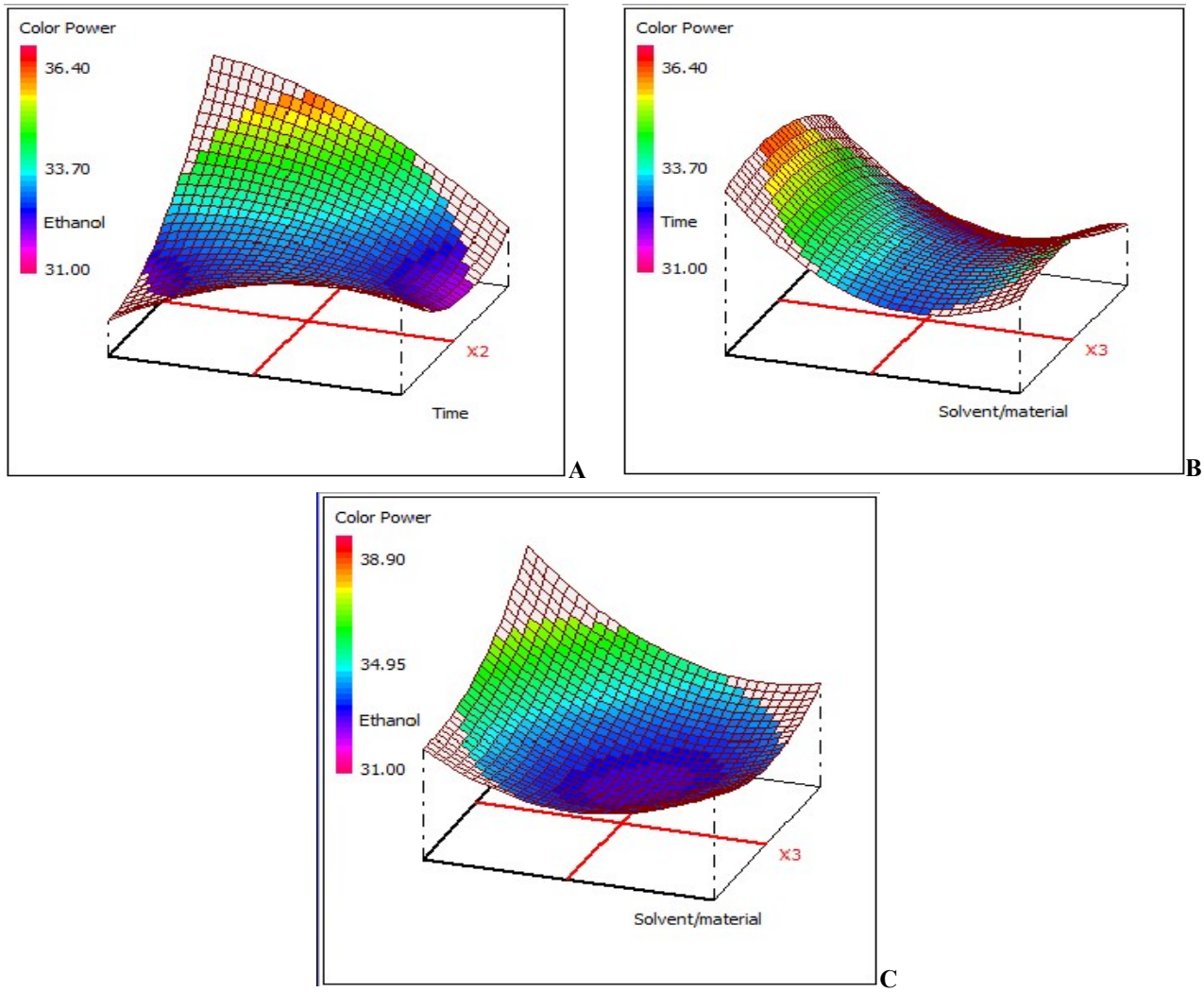

Fig. 3. Response surface graph of ethanol concentration and extraction time (A), extraction time and extraction time, and solvent/material (B) and their interactions with the staining power of *C. arvensis*

obtained with an ethanol concentration of 72% and a solvent-to-material ratio of 6 ml g^{-1}.

Verification of the Predictive Model

An optimization study was carried out based on our experimental results and using the desirability function available on the software to identify the optimum operating conditions for obtaining the highest yield, coloring power, and polyphenol content as shown in Table 6.

The three most successful parameters for the three responses were determined to be a solvent/extract amount ratio of 15 ml g^{-1}, an extraction time of 30 min, and an ethanol concentration of 60%. Under optimal circumstances, the estimated yield, polyphenol levels, and staining power are 28.21%, 779.11 mg GAE/g extract, and 42, respectively. The experiments were carried out under the indicated optimal circumstances for the three responses to determine whether the mathematical model was appropriate for forecasting the ideal response values. The results obtained from the trials under the optimal conditions are yield: 27.45%, polyphenol content: 778.97 mg EAG/g extract, and color power: 40.

It was possible to confirm the effectiveness of the model in predicting responses by comparing theoretical and experimental data. The actual and predicted values for polyphenol content, yield, and color strength did not differ significantly, confirming that the model used is significant.

Precision

The Intraday and interday precision was carried out through replicating analysis (n = 6) for one concentration (15 ml g^{-1}). For the intraday precision assay, the analysis was carried out by using one concentration at different time intervals in a day and interday precision was carried out for three consecutive days at the same concentration level as used in intraday precision (Table 7). From Table 7 we have observed that the intraday and interday values are very close to the values obtained from the experience plan software, which indicates good method precision.

Accuracy

To determine the accuracy of the method, the recovery experiment was carried out using the standard addition

Table 6. Optimum Conditions and the Predicted and Experimental Value of Response at the Optimum Conditions

	Ethanol	time	Material ratio	Yield (%)	(mg EGA/g extract)	Color power
Optimum conditions	60%	30 min	15 ml g^{-1}	28.21	779.11	42
Modified conditions	60%	30 min	15 ml g^{-1}	27.45	778.97	40

Table 7. Intraday and Interday Precision Data

Intraday precision data			
Conc. (15 m**l** g^{-1})	Yield (%)	(mg EGA/g extract)	Color power
Mean	27.92	778.98	41.73
SD	0.220	0.150	0.378
% RSD	0.787	0.020	0.905
Interday precision data			
Conc. (15 m**l** g^{-1})	Yield (%)	(mg EGA/g extract)	Color power
Mean	27.63	778.62	41.55
SD	0.654	0.575	0.591
%RSD	2.366	0.073	1.422

Table 8. Accuracy Data

	Yield %	(mg EGA/g extract)	Color power
Accuracy	1.712	0.0012	4.325

method. For the optimum concentration we obtained (15 g), a volume of 100 ml (60% ethanol) was added to the sample and we carried out the ultrasonic extraction for 30 min. For the previously analyzed sample (15 ml g^{-1}), The contents were re-analyzed with the above-described procedure (Table 8). According to Table 8, the accuracy values for yield, total phenols, and dye strength do not exceed 10% [22], so the model is acceptable.

CONCLUSION

Our research intends to improve existing extraction techniques by implementing new, more effective methods, as well as to assess the dyeing potential of various *C. arvensis* extracts.

This investigation included the determination of polyphenol content and dyeing power, as well as the optimization of ultrasonic-aided extraction of *C. arvensis* hydro-ethanolic extracts. Three effective parameters were discovered based on the Response Surface Methodology (RSM) results: 60% ethanol percentage, 30 min of extraction time, and 15 ml g^{-1} of solvent/material. The anticipated yield, polyphenol concentrations, and color power under optimized conditions were 28.21%, 779.11 mg GAE/g extract, and 42, respectively. We may deduce that the extraction method affects the amount of chemical components found in plant extracts. Because the extraction procedure has a significant impact on the solubility of the bioactive molecule, it is vital to understand the chemical properties and kind of molecule that must be extracted from each plant.

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