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Original Article

Optimizing Ultrasonic Extraction of Antioxidants and Polyphenols in Five Indigenous Remedial Plants Using Box-Behnken Design Combined with Response Surface Methodology

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ABSTRACT

Aim/Background: This study aimed to optimize the ultrasonic extraction of antioxidants and polyphenols from five indigenous medicinal plants *Cassia auriculata, Celastrus paniculatus, Moringa concanensis, Murraya koenigii,* and *Alternanthera sessilis* using Box-Behnken Design (BBD) and Response Surface Methodology (RSM). The goal was to enhance the extraction efficiency of these bioactive compounds, which are crucial for mitigating oxidative stress and preventing chronic diseases. **Materials and Methods:** The extraction process was optimized by varying key parameters: extraction time (45-60 min), solvent ratio (40-80% ethanol-water mixture), and solid-liquid ratio (1:5 to 1:15 g/mL), with plant source as a categorical factor. The response Variables measured were DPPH scavenging Activity (VCEAC) and Total Phenolic Content (TPC). The study employed BBD and RSM to develop a predictive model for these responses. **Results:** The results indicated that extraction duration, solvent concentration, and solid-liquid ratio significantly impacted antioxidant activity. *Cassia auriculata* exhibited the highest Antioxidant activity (VCEAC of 14.59 µg) under optimal conditions: 56.9 min extraction, 77.54% solvent concentration, and a 1:6.3 g/mL solid-liquid ratio. *Celastrus paniculatus* followed closely. The predictive model showed high accuracy with R^2 values of 0.9551 for antioxidant activity and 0.9415 for TPC. **Conclusion:** The study successfully optimized the ultrasonic extraction parameters for antioxidants and polyphenols from the selected medicinal plants. The findings underscore the importance of selecting appropriate plant sources based on desired medicinal properties and demonstrate the potential for utilizing these extracts in pharmaceuticals, supplements, and cosmetics. The combination of RSM and BBD proved effective in providing a reliable predictive model for extraction efficiency.

Keywords: Antioxidant, Box-Behnken Design, Optimization, Polyphenols, Ultra-sonication.

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INTRODUCTION

Human health depends quite much on antioxidants and phenolic compounds, which neutralize free radicals, therefore reducing oxidative stress related to chronic diseases including cancer, neurological conditions, and heart disease. Thanks to their anti-inflammatory, anti-cancer, cardioprotective, and neuroprotective properties.[1-3] Major classes of plant-based antioxidants and phenolic compounds offer great health benefits; they work via boosting endogenous antioxidant defenses, chelating metal ions starting oxidative processes and scavenging of free radicals.[4,5]

Modern healthcare depends much on herbal medicine, which uses millennia of traditional use to provide several treatment choices. Long essential to medicine, plants offer a vast variety of bioactive chemicals with different pharmacological effects including glycosides, terpenoids, alkaloids and flavonoids.[6] These make them important for creating new medicines and supplements as they feature anti-inflammatory, antibacterial, antioxidant and anticancer properties. Investigating herbal treatments offers alternative or supplementary therapies to traditional medicine in addition to helping to advance medical understanding. Herbal medicines' therapeutic power depends critically on their variety of phytochemicals. Every plant species has special chemicals that interact with biological systems in distinct ways to provide focused benefits for a variety of medical disorders.[7]

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Recent advances in herbal research include improved analytical instruments, better phytochemical profiling, and enhanced extraction procedures using ideal solvents and methodologies. These developments have increased the yield, purity, and potency of bioactive compounds, leading to more effective herbal remedies. However, challenges remain, such as inconsistent yields due to variations in extraction parameters, including solvent type, solid-liquid ratio, particle size, and extraction time. These elements might differ greatly across different plant species, so it is crucial to customize extraction conditions especially for every plant. The demand to balance these factors to optimize yield while preserving the quality of the obtained bioactive compounds generates the need for optimization. Without proper optimization, the extraction process may be ineffective, resulting in poor yields and maybe ignoring important chemicals, therefore influencing the efficacy and financial feasibility of the extraction process.[10]

Featuring great usage in traditional medicine and possible modern therapeutic uses, Indian medicinal plants are important sources of bioactive chemicals with antioxidant, anti-inflammatory, hepatoprotective, and antibacterial effects. The leaves of *Cassia auriculata* (L.), *Celastrus paniculatus* Willd, *Moringa concanensis* Nimmo, *Murraya koenigii* (L.) Spreng and *Alternanthera sessilis* (L.) are rich sources of bioactive chemicals recognized for their hepatoprotective qualities. Traditional medicine has long known these herbs for their ability to preserve liver function. Flavonoids and phenolics included in *Cassia auriculata* help to fight oxidative stress, a main cause of liver damage.[11] Renowned for its anti-inflammatory and antioxidant properties, *Celastrus paniculatus* helps liver regeneration.[12] *Moringa concanensi* is packed with essential minerals and antioxidants to guard against liver poisons.[13] *Murraya koenigii's* great phenolic and alkaloid concentration explains its hepatoprotective and detoxifying qualities.[14] *Alternanthera sessilis* has great antioxidant action, which enables liver cells to be less oxidatively stressed.[15] By means of scientific justification for investigating their synergistic hepatoprotective properties, the extraction of bioactive substances from these leaves offers a possibility for developing efficient herbal medicines.

Alternanthera sessilis (AS), often called "dwarf copperleaf" or "sessile joyweed," is also known as "ponnankanni" in India, hence emphasizing its importance in traditional medicine. *Alternanthera sessilis* is easily identifiable by its wide leaves and adaptation to many surroundings. Lung diseases, including bronchitis and asthma as well as liver diseases and gastrointestinal disorders are treated with AS. It's also drunk in India for fever and snakebites and Malaysia for blood glucose control. Taiwan and Malaysia use AS for renal and cardiovascular health. Polyphenols, flavonoids, carotenoids, terpenes, and alkaloids are just a few of the phytochemicals the plant has. Especially since it contains gallic acid, which is connected to possible anti-cancer effects. *Alternanthera sessilis* has main pharmacological actions as an antioxidant, anti-hyperglycemic, antibacterial, antifungal, and hepatoprotective agent. Furthermore, increasing AS's medicinal potential are its anti-inflammatory and analgesic qualities.[16]

Murraya koenigii (MK), commonly known as curry leaves or "Salam India," is a plant distinguished by its short greyish-brown stem and lush, bipinnate compound leaves, and they emit a distinctive aroma. Traditionally, MK has been valued for its stimulant, stomachic, antipyretic, and analgesic properties. It is used in treating diarrhea, dysentery, and insect bites and as a topical paste to neutralize toxins from animal bites. The plant is rich in phytochemicals, particularly alkaloids such as mahanimbine, murrayanine, and girinimbine. Pharmacologically, MK demonstrates a range of beneficial activities. It has anti-inflammatory and analgesic effects, significant antipyretic activity in fever models, and antidiabetic and anticancer properties due to its alkaloid content. Additionally, it exhibits antimicrobial activity and provides hepatoprotective benefits by protecting the liver and aiding in the regeneration of pancreatic islets.

Moringa concanensis Nimmo (MC) (commonly known as Moringa) is a member of the *Moringaceae* family, closely related to *Moringa oleifera*. MC has long been used to treat several ailments and infections. Its varied phytochemical profile lends nutritional advantages. Among the important bioactive substances are carbohydrates, phenols, terpenoids, saponins, alkaloids, and flavonoids. Especially, methanol extracts of the leaves and flowers show phenols and saponins, but lacking are steroids, anthraquinones, tannins, oils and resins. *Moringa concanensis* has antibacterial action pharmacologically because of saponins, antioxidant effects from flavonoids and phenols, and possible anti-inflammatory and anticancer activities. These qualities point to its possibilities for improving health and creating helpful medications.[17]

Celastrus paniculatus Willd (CP), commonly known as the "Intellect Tree" in India, is a climbing shrub belonging to the *Celastraceae* family. Traditionally, its seeds and oil are employed in Ayurveda and Unani medicine to cure neurological problems, including cognitive impairment, paralysis, epilepsy, and sleeplessness. Additionally, it is utilized for joint discomfort, leprosy, and muscular weariness. The oil is valued for massage, the leaf sap serves as an emmenagogue and remedy for opium poisoning, and the bark possesses abortive and depurative qualities. Phytochemically, CP includes a varied array of compounds: alkaloids (celastrin, paniculatin), sesquiterpenes (β-dihydroagarofuranoids), flavonoids, sterols (β-sitosterol, campesterol), fatty acids (palmitic, stearic, oleic), and non-fatty acids (benzoic, cinnamic). The herb is recognized for its nootropic benefits, increasing memory and cognition by elevating brain acetylcholine levels. It also demonstrates neuroprotective qualities and a spectrum of other actions, including anti-Alzheimer,

anticonvulsant, antidepressant, antioxidant, analgesic, anti-inflammatory, antiarthritic, and gastroprotective effects.[18]

Cassia auriculata (CA), commonly known as Tanner's cassia, is a valuable medicinal shrub used in traditional medicine. Traditional uses of *Cassia auriculata* include treatments for rheumatism, conjunctivitis, diabetes, leucorrhoea, skin disorders, scorpion stings, and stomachaches. The leaves are applied as a paste for skin conditions and hair loss, while the juice from the leaves is used for various ailments. Flowers are utilized for treating spermatorrhea and diarrhea. Phytochemically, *Cassia auriculata* is rich in flavonoids, phenols, tannins, terpenoids, alkaloids, quinones, saponins, and steroids. These compounds underpin its therapeutic efficacy. Pharmacologically, the plant exhibits a range of activities: antidiabetic, antioxidant, antibacterial, hepatoprotective, nephroprotective, anticancer, anti-inflammatory, antimicrobial, and antihyperlipidemic.[19]

This study delves into the optimization of sonication extraction to maximize the bioactive component from five indigenous plants. The work intends to improve the production of bioactive chemicals by optimizing parameters like Extraction time, Solvent ratios, and solid-liquid ratio, thereby enhancing extraction efficiency, speed, and quality for medicinal uses.

MATERIALS AND METHODS

Collection of plant materials

Leaves of *Althernanthera sessilis, Murraya koenigii, Cassia auriculata, Moringa concanensis* and *Celastrus paniculatus* were gathered from Bangalore's Foundation for Revitalization of Local Health Tradition (FRLHT) botanical garden grounds. The collected plant materials were sorted, and whole matured leaves of each plant were separated, washed twice with distilled water, followed by overnight shade drying, and dried in the hot air oven at 45°C for 48 hr. The dried leaves were ground to obtain a powder of 40 mesh size and stored in an airtight container until further use.[20]

Procurement of chemicals and reagents

Methanol AR, Gallic acid, Ascorbic acid and Sodium carbonate were purchased from Merck. Folin-Ciocalteu reagent and DPPH (2,2-Diphenyl-1-picrylhydrazyl) was procured from HI media, Ethanol was procured from LOBA chemicals.

Optimization of Ultra sonication process

To optimize the extraction process to maximize the antioxidant and phenolic content, a Box-Behnken Design (BBD) was applied to optimize the extraction conditions, which included extraction time, solvent ratio, solid-liquid ratios and a nominal categoric factor- plant source with five different levels with DPPH scavenging activity (Y_A) in terms of Vitamin-C Equivalent Antioxidant Capacity (VCEAC) and Total Phenolic Content (TPC) (Y_B) in terms of Gallic Acid Equivalent (GAE) as two response variables was created using BBD using a trial version of Design-Expert 13.0 software to produce the model for optimizing the sonication extraction to maximize the antioxidant and polyphenols.[21]

Response Surface Methodology by BBD

A 3-level numeric factor with a five-level nominal category factor BBD methodology was undertaken in accordance with a previous study with slight modification.[21] 3 numeric factors; Extraction Time (A), Solvent ratio (B), Solid-liquid ratios (C) along with the nominal category- plant Source (D), and the DPPH activity (in µg VCEAC) and TPC (in µg GAE) were set as two response variables, the independent variables were fixed at 3 levels namely low (−1), midpoint (0) and high (+1), with A being 30, 45 and 60 min; B being 40%, 60% and 80% v/v of Ethanol in Distilled water; C being 1:5, 1:10 and 1:15 g/mL respectively; and the Nominal category with five levels of D with Level-1 being leaves of *Althernanthera sessilis* (AS); Level-2 being leaves of *Murraya koenigii* (MK); level-3 being leaves of *Moringa concanensis* (MC); Level-4 being leaves of *Celastrus paniculatus* (CP); Level-5 being leaves of *Cassia auriculata* (CA) respectively, as summarized in Table 1. To reduce the usage of excess solvent and plant materials, the optimization was carried out with miniature samples. 5 g of each plant material was extracted with the mentioned solvent system for the specified time as per the model.

A total of Eighty-five independent experiments were carried out in randomized order and are shown in Table 1. Experimental procedures were conducted in accordance with the predetermined sequence and the polynomial equation was employed to accurately represent the experimental data of the variables. An ANOVA (Analysis of Variance) confirmed the statistical importance. Statistically insignificant variables ($p>0.05$) were eliminated, and only the experimental data were fitted to parameters that were statistically significant ($p<0.05$). In this model, a great prediction efficiency is shown by a coefficient of determination (R^2) value that is near to 1. The correctness of the model was further tested by estimating modified R^2 values.

The F-value and Lack-of-Fit (LOF) in the regression model were used to calculate the probability. The responses obtained from the regression models were then visually depicted through contour plots and 3-dimensional graphs. For model validation, the experimental results were compared with the predicted values ,and the optimal extraction conditions for achieving maximum DPPH activity and TPC were determined.

Estimation of Antioxidants by DPPH free radical scavenging activity

The free radical scavenging activity of extracts was assessed by using the method followed by Annegowda *et al.*,[22] with some minor modifications. Standard vitamin C of 1 mg/mL concentration was prepared using methanol; the stock solutions

were further diluted to obtain various required concentrations ranging between 1 and 10 µg. 2 mL of 0.2 mM DPPH reagent was added to the 1 mL of the various concentrations of standard/500 µL of menstruum and vortexed; the control consisted of 1 mL of methanol and 2 mL of DPPH reagent. The reaction mixture was incubated in the dark at room temperature for 30 min; further the decrease in the intensity of the reaction mixture was measured using a UV-vis spectrometer at 517 nm. This decrease in the intensity was used to evaluate the percentage of inhibition using the formula.

$$Percentage\ of\ inhibition = \frac{(Absorbance\ of\ Control - Absorbance\ of\ reaction\ mixture)}{Absorbance\ of\ Control} X100$$

All the measurements were done in triplicate; the obtained percentage of inhibition of the standard was used to construct a calibration curve and the antioxidant activity of the samples from the different models was expressed in terms of VCEAC in µg.[23]

Estimation of polyphenol contents

Determination of TPC is evaluated by using Folin-Ciocalteu (FC) reagent. TPC was evaluated by the traditional colorimetric method as mentioned by Annegowda *et al.*,[20] with slight modifications. In this procedure, 2 mL of FC reagent, diluted tenfold with distilled water, was added to a 500 µL aliquot of the sample solution (menstruum/standard) containing different concentrations of standard gallic acid (1-10 µg) or 500 µL of menstruum. After 5 min, 1.5 mL of 7.5% w/v sodium carbonate was added and the mixture was vortexed. The resulting solution was incubated in the dark for 60 min at room temperature. Absorbance of the blue complex was then measured at 765 nm against a blank. A calibration curve was constructed using gallic acid standards of various concentrations (1-10 µg), and the TPC results were expressed as micrograms of Gallic Acid Equivalents (GAE) in µg. All measurements were performed in triplicate.[20]

Statistical analysis

The experimental data was processed using Microsoft excel. ANOVA for the model, the colour plots and the surface 3D graphs were obtained using the Trial version of Design experiment 13.0.

RESULTS

Model verification for DPPH and TPC response variables

Antioxidant activity in terms of DPPH scavenging activity and polyphenols in terms of TPC from 500 µL of *Cassia auriculata, Celastrus paniculatus, Moringa concanensis, Murraya koenigii,* and *Alternanthera sessilis* leaves menstruum as obtained from five nominal categories of a total of 85 runs in BBD are summarized in Table 2. Antioxidant activity in terms of VCEAC and TPC in terms of GAE ranged from 0.04 to 13.86 µg and 0.19 to 14.78 µg respectively. The highest values of antioxidant and polyphenol contents for the leaves of five different plants for 500 µL of menstruum are summarized in Table 2. The experimental data obtained for the responses were fitted into a second-order polynomial model equation, and the resulting regression coefficients and *p*-values indicated the significance of the individual factors with the response.

ANOVA for Quadratic model obtained by BBD

Response 1: DPPH free radical scavenging activity

The current model produced significant results for DPPH activity with the Model F-value of 50.24 indicating significance of the model and there is only a 0.01% chance that an F-value this large could occur due to noise. The ANNOVA results showed a significance of $p<0.0001$ for linear effect A-Extraction time, B-Solvent Concentration, C-Solid-Liquid ratio and D-Plant Source for antioxidant activity and also showed significant interaction between AB ($p<0.001$) AC ($p<0.001$), AD ($p<0.001$), BC ($p<0.05$), BD ($p<0.001$) and CD ($p<0.001$). Similarly, we observed a significant quadratic effect of A^2 on the antioxidant activity in terms of VCEAC.

The Lack of Fit F-value of 5.89 suggests that the Lack of Fit is significant ($p<0.0001$), indicating that the model appropriately fits the data. There is only a 0.01% probability that such a high Lack of Fit F-value could be due to random noise. The R^2 value demonstrated a strong correlation between the response and the independent variables. The Predicted R^2 of 0.8859 closely aligns with the Adjusted R^2 of 0.9361, with a difference of less than 0.2. Adequate Precision, which evaluates the signal-to-noise ratio, is desirable when above 4. In this case, the ratio of 33.123 indicates a strong signal, confirming that the model is suitable for navigating the design space. The following equation encompasses the model indicating the relationship between Antioxidant content and the extraction variables used.

$$Y=3.65+0.9762A+1.13B-1.44C-2.03D[1]-1.23D[2]-1.26D[3]+0.2706D[4]+0.4668AB-0.4851AC-0.3124AD[1]-0.2082AD[2]-0.3586\ AD[3]-0.1022AD[4]-0.3999BC-0.5298BD[1]-0.6847BD[2]-0.5444BD[3]+0.1580BD[4]+0.8456CD[1]+0.6292CD[2]+0.3447CD[3]-0.0688CD[4]-0.3449A^2-0.2498B^2-0.1302C^2$$

Note: The equation expressed in terms of coded factors allows predictions about the response based on specific levels of each factor. Typically, the high levels of the factors are coded as +1, while the low levels are coded as -1. This coded equation is helpful in assessing the relative influence of each factor by comparing the respective factor coefficients.

Contour and 3D surface plot for DPPH response for optimized model

The equation based on actual factors can be used to predict the response for specific levels of each factor. However, this equation is not suitable for evaluating the relative effect of each factor, as

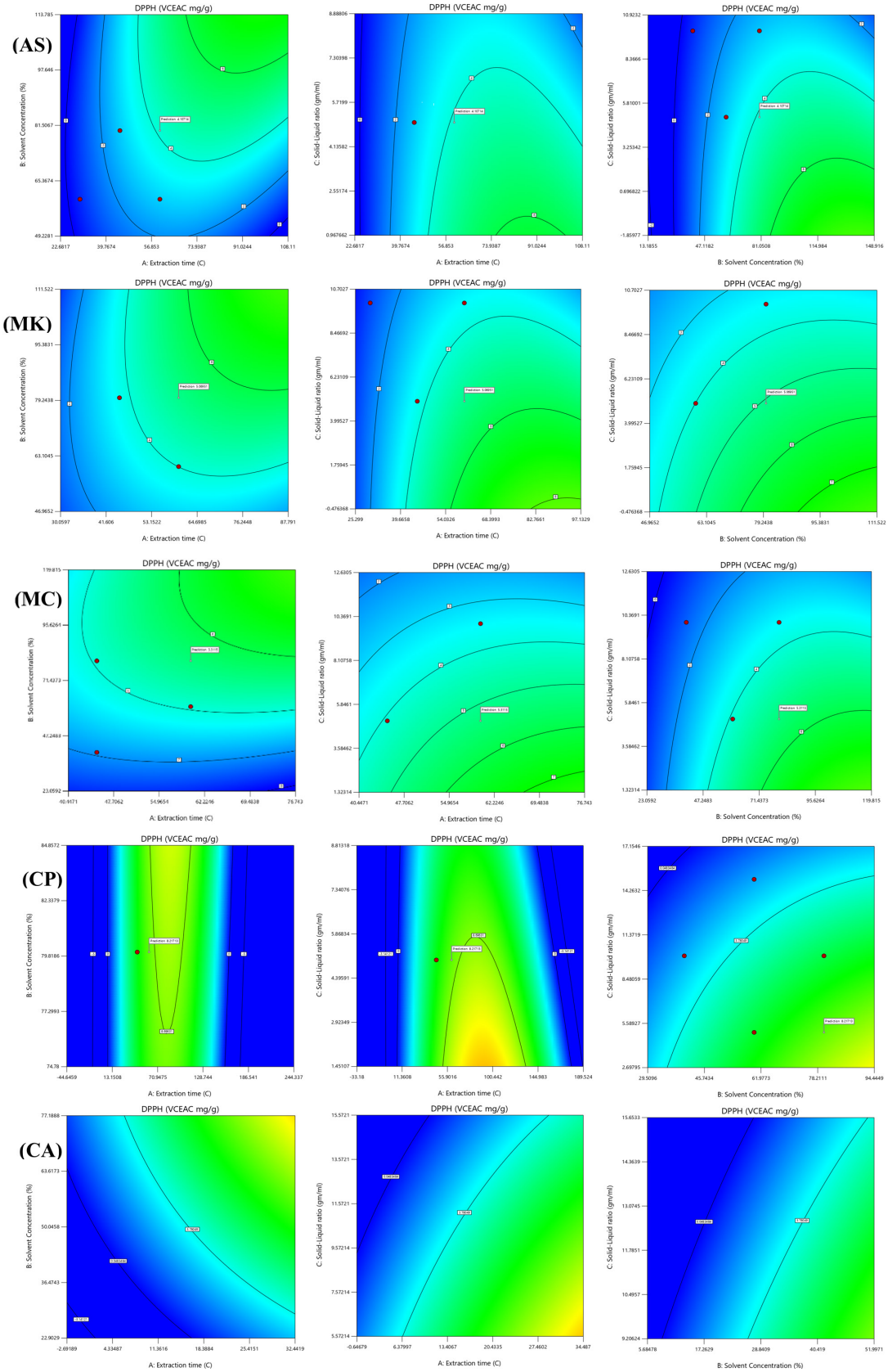

Figure 1: Contour plots for DPPH free radical scavenging capacity of *Alternanthera sessilis* (AS), *Murraya koenigii* (MK), *Moringa concanensis* (MC), *Celastrus paniculatus* (CP) and *Cassia auriculata* (CA) leaves by optimized ultrasonication extraction as per numerical optimization.

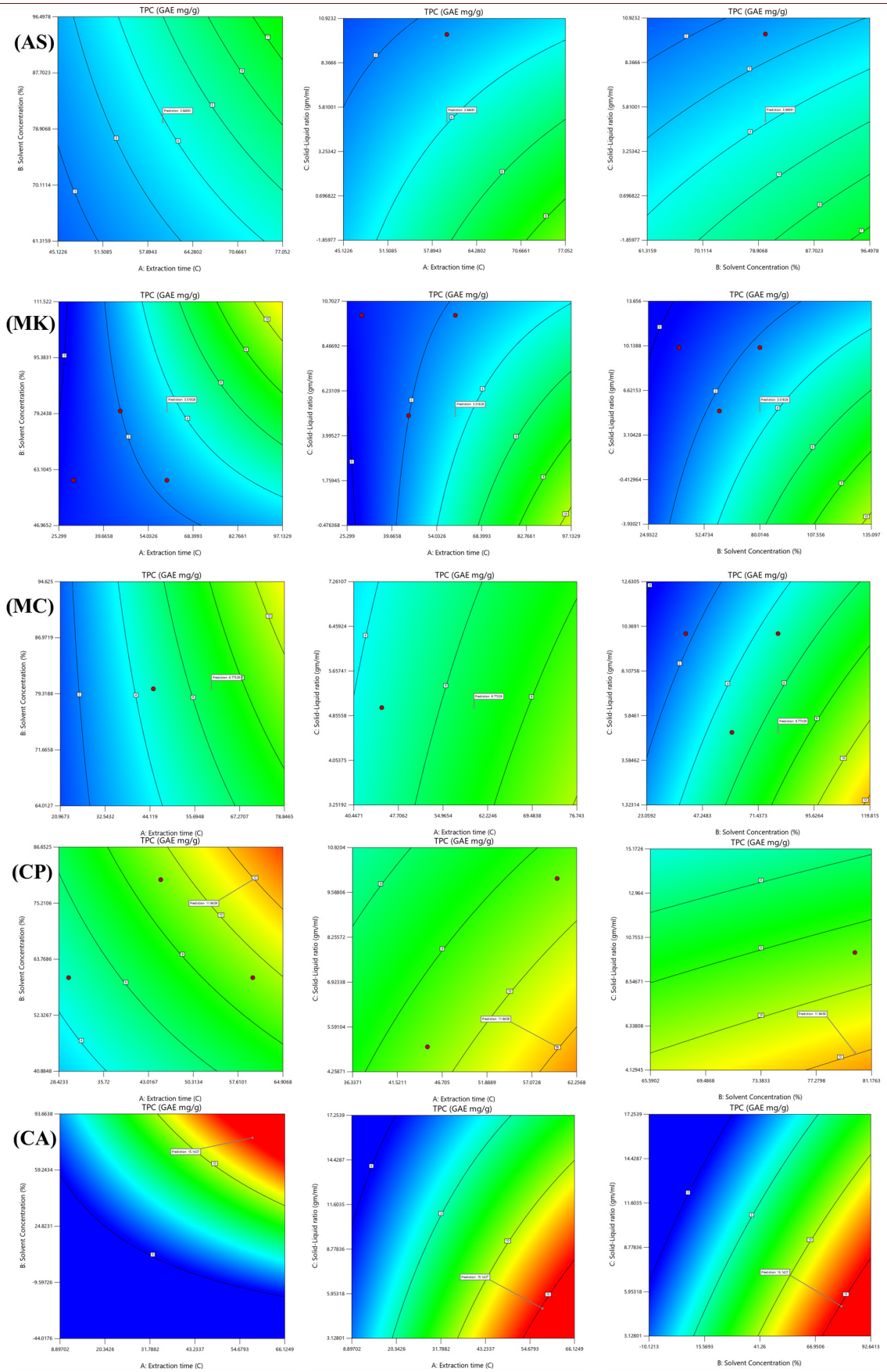

Figure 2: Contour plots for total phenolic contents of *Alternanthera sessilis* (AS), *Murraya koenigii* (MK), *Moringa concanensis* (MC), *Celastrus paniculatus* (CP) and *Cassia auriculata* (CA) leaves by optimized ultrasonication extraction as per numerical optimization.

the coefficients are adjusted to match the units of the factors, and the intercept does not lie at the center of the design space. The 3D surface responses and contour plots are elaborated to optimize the extraction variables to maximize the antioxidants extraction, and the combined effect of the extraction variables on the Antioxidant activity of each level of the Nominal category is depicted in Figure 1.

Model Validation

The model estimated that the optimal extraction for achieving the maximum antioxidant values from five indigenous leaves is summarized in the Table 3.

Response 2:Total Phenolic Content

The current model produced significant results for TPC, with the Model F-value of 36.99 indicating the significance of the model, and there is only a 0.01% chance that an F-value this large could occur due to noise. The ANOVA results showed a significance of $p<0.0001$ for linear effect A-Extraction time, B-Solvent Concentration, C-Solid-Liquid ratio and D-Plant Source for antioxidant activity and also showed significant interaction between AB ($p<0.01$); AC ($p<0.001$), AD ($p<0.001$), and BC ($p<0.05$).
BD ($p<0.001$), and CD ($p<0.001$). Similarly, we observed a significant quadratic effect of A^2 on the antioxidant activity in terms of VCEAC.

The Lack of Fit F-value of 49.91 indicates a significant Lack of Fit ($p<0.0001$), suggesting that the model effectively fits the data. The probability of obtaining such a large Lack of Fit F-value due to noise is only 0.01%. The R^2 value demonstrates a strong correlation between the response and the independent variables. The Predicted R^2 of 0.8377 aligns well with the Adjusted R^2 of 0.9146, with a difference of 26.71. Adequate Precision, which measures the signal-to-noise ratio, shows a desirable value above 4, with a ratio of 26.712, indicating a strong signal. Therefore, this model is suitable for navigating the design space. The following equation encompasses the model indicating the relationship between Antioxidant content and the extraction variables used.

$$3.29+1.21A+1.13B-1.31C-2.12D[1]-1.98D[2]-0.7127D[3]+1.74D[4]+0.6144AB-0.6885AC 0.6661AD[1]-0.9833AD[2]-0.4254AD[3]+0.3531AD[4]-0.2966BC-0.7893BD[1]-0.8958BD[2]-0.2600BD[3]+0.6627BD[4]+0.8872CD[1]+1.05CD[2]+0.2407CD[3]-0.7692CD[4]+0.0798A^2-0.2647B^2-0.0798C^2$$

Note: The equation, expressed in terms of coded factors, allows for predictions about the response based on specific levels of each factor. Typically, the high levels of the factors are assigned a code of +1, while the low levels are coded as -1. This coded equation is particularly useful for determining the relative influence of each factor by comparing their respective coefficients.

Contour and 3D surface plot for TPC response for optimized model

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. The 3D surface responses and contour plots are elaborated in order to optimize the extraction variables to maximize the antioxidants extraction, and the combined effect of the extraction variables on the Antioxidant activity of each level of the Nominal category is depicted in Figure 2.

Model Validation

The model estimated that the optimal extraction parameters for achieving the maximum polyphenols from five indigenous leaves is summarized in the Table 3.

Numerical optimization of the extraction method for achieving maximum response factors

For achieving the maximum antioxidant activity and Total phenolic content, optimization of the extraction parameters was validated again by setting the range of parameters and maximizing the response for each level of the Nominal categories. The optimized model for achieving the maximum response is depicted in Figure 3.

To validate the models, the experiments were performed as per the predicted optimal conditions mentioned in Figure 3. The experiments were performed as per the optimal conditions and were compared with the predicted values. Table 4 summarizes the predicted DPPH and the TPC contents and the results obtained by experimentation by following the optimal conditions. The experimentally obtained values for the response factors were in accordance with the predicted response values as per the BBD, resolving the significance of the RSM model and its success in the development of a suitable model for the extraction of antioxidant and polyphenolic rich menstruum from *Alternanthera sessilis, Murraya koenigii, Moringa concanensis* and *Celastrus paniculatus,* and *Cassia auriculata* leaves of indigenous importance.

DISCUSSION

Aiming to optimize the extraction parameters for extraction of plant materials by ultrasonication and to maximize antioxidant activity and TPC of the menstruum thus obtained, five indigenous medicinal plants-*Cassia auriculata, Celastrus paniculatus, Moringa concanensis, Murraya koenigii,* and *Alternanthera sessilis*-were subjected to optimization by BBD. This present work particularly sought to maximize the antioxidant capacity and phenolic content obtained by the extraction process, which are generally seen as important markers with numerous health advantages. These plants are well-known for their possible therapeutic characteristics.[3,12,16,18,19] The study used a statistical method called RSM and BBD to find the best conditions for extracting a menstruum with these bioactive compounds.

Table 1: BBD to optimize the extraction process of antioxidant and polyphenols. Real values adopted for each factor and coded values are shown.

	Factors								
Run	A: Extraction time (Min)	B: Solvent Concentration (%)	C: Solid-Liquid ratio (gm/mL)	D: Plant Source	Run	A: Extraction time (Min)	B: Solvent Concentration (%)	C: Solid-Liquid ratio (gm/mL)	D: Plant Source
1	0	0	0	CP	44	-1	1	0	MK
2	0	0	0	MC	45	0	1	-1	MC
3	0	0	0	AS	46	0	0	0	CP
4	-1	-1	0	MC	47	-1	0	-1	AS
5	0	1	1	AS	48	0	0	0	MK
6	1	0	1	MC	49	-1	0	1	CA
7	0	0	0	MC	50	1	1	0	CP
8	-1	-1	0	AS	51	1	-1	0	MK
9	-1	1	0	CP	52	1	1	0	MK
10	1	0	1	MK	53	0	1	-1	CP
11	0	-1	-1	CP	54	0	-1	1	MC
12	-1	1	0	MC	55	0	0	0	MC
13	1	1	0	CA	56	-1	0	1	CP
14	1	0	-1	AS	57	1	1	0	AS
15	0	0	0	CA	58	0	0	0	MK
16	0	1	1	CA	59	-1	-1	0	CP
17	0	0	0	AS	60	1	-1	0	MC
18	0	0	0	CA	61	-1	0	-1	CP
19	0	-1	-1	CA	62	1	0	-1	CA
20	1	-1	0	CA	63	-1	1	0	AS
21	-1	-1	0	CA	64	1	0	-1	CP
22	0	0	0	MC	65	0	-1	1	AS
23	0	0	0	MC	66	0	0	0	CP
24	0	0	0	AS	67	1	0	1	AS
25	-1	1	0	CA	68	1	-1	0	CP
26	0	1	1	MC	69	0	0	0	AS
27	0	-1	-1	MC	70	1	0	-1	MK
28	0	0	0	CP	71	1	-1	0	AS
29	0	1	1	MK	72	0	-1	-1	AS
30	0	1	-1	CA	73	0	0	0	CA
31	0	0	0	CP	74	-1	0	-1	MC
32	-1	0	1	AS	75	-1	0	-1	CA
33	0	-1	1	CP	76	-1	-1	0	MK
34	0	0	0	AS	77	1	0	1	CP
35	0	-1	1	CA	78	1	1	0	MC
36	0	-1	-1	MK	79	0	1	-1	MK
37	0	0	0	MK	80	1	0	-1	MC
38	-1	0	1	MC	81	0	0	0	CA
39	0	0	0	MK	82	0	0	0	MK

40	1	0	1	CA	83	0	-1	1	MK
41	0	1	-1	AS	84	0	0	0	CA
42	-1	0	1	MK	85	-1	1	0	MK
43	0	1	1	CP					

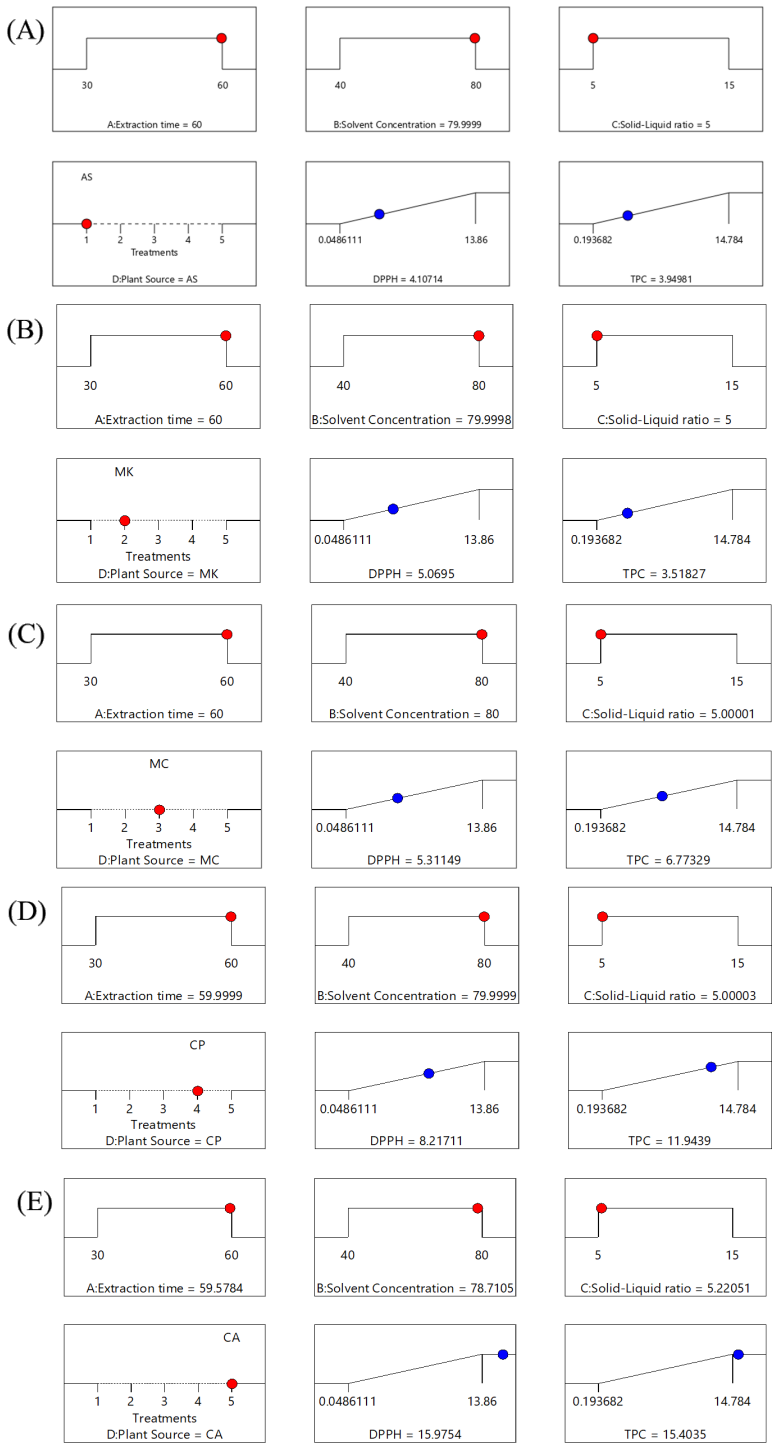

Figure 3: Solution for achieving maximum antioxidant and TPC from (A) *Alternanthera sessilis*, (B) *Murraya koenigii*, (C) *Moringa concanensis*, (D) *Celastrus paniculatus* and (E) *Cassia auriculata* leaves by optimized ultrasonication extraction as per Numerical optimization.

The findings demonstrated that 3 main factors-extraction duration, solvent content, and the solid-liquid ratio-significantly impacted the antioxidant activity of the plant extracts.[24,25] ANOVA supported this by demonstrating that the interplay among these elements also played a critical part in controlling the antioxidant output.[26] This is consistent with previous research, which has highlighted how extraction factors such as time and solvent concentration may influence the effectiveness of bioactive chemical extraction from plant materials.

In comparison with previous studies, the optimized extraction parameters in this research provide a significant advancement in the field of pharmacognosy. Previous studies have often focused on the evaluation of phytochemical content without optimizing the extraction process. For instance, researchers analyzed the phytochemical content of *Alternanthera sessilis* leaf extracts without using RSM or BBD for optimization.[27,28] Another study focused on *Murayya koenigii* leaves, optimizing 3 extraction methods (solvent-assisted, microwave-assisted and ultrasonic-assisted) for phytochemical analysis and antioxidant potential, without the use of RSM and BBD.[29] Despite fluctuations in the phytochemical contents, the ultrasonication method yielded the highest antioxidant content. Our study addresses this gap by providing a systematic approach to optimize extraction

Table 2: DPPH scavenging activity (µg VCEAC) and TPC (µg GAE) of experimental runs.

Run	Response		Run	Response		Run	Response	
	DPPH VCEAC in µg	TPC GAE in µg		DPPH VCEAC in µg	TPC GAE in µg		DPPH VCEAC in µg	TPC GAE in µg
1	3.98	5.31	30	12.66	9.24	59	2.08	1.11
2	2.34	2.66	31	3.98	5.29	60	1.00	1.29
3	1.62	1.04	32	0.26	0.24	61	1.09	1.30
4	0.58	0.65	33	1.92	2.56	62	3.75	4.82
5	1.09	0.70	34	1.62	1.02	63	13.86	14.78
6	1.36	1.62	35	4.03	2.94	64	0.77	0.72
7	2.34	2.62	36	2.01	1.08	65	6.44	9.65
8	0.21	0.19	37	2.96	1.56	66	0.78	0.50
9	3.71	4.77	38	0.72	0.81	67	3.98	5.31
10	1.20	0.63	39	2.08	1.11	68	1.05	0.78
11	3.86	5.14	40	4.63	4.94	69	1.72	2.58
12	2.13	2.39	41	2.46	1.58	70	0.05	1.62
13	13.73	14.65	42	0.66	1.66	71	3.59	1.89
14	3.13	2.30	43	2.69	3.58	72	0.05	0.84
15	8.36	6.10	44	2.00	1.08	73	1.57	1.01
16	5.65	4.12	45	1.98	1.07	74	8.36	6.10
17	1.62	1.04	46	3.54	4.03	75	2.15	2.42
18	8.36	6.09	47	3.98	5.24	76	7.82	5.73
19	8.10	5.91	48	0.78	0.72	77	0.53	0.29
20	3.71	3.95	49	2.08	1.11	78	2.16	3.23
21	2.09	1.53	50	2.61	1.91	79	4.02	4.80
22	2.34	2.61	51	6.39	9.56	80	3.14	1.68
23	2.34	2.66	52	2.96	1.56	81	4.06	4.85
24	1.62	1.05	53	3.56	1.87	82	8.36	6.10
25	7.75	5.67	54	6.03	8.03	83	2.08	1.11
26	0.05	1.58	55	1.13	1.28	84	1.00	0.54
27	2.26	2.58	56	2.34	2.59	85	8.36	6.10
28	3.98	5.31	57	1.25	1.61			
29	1.40	0.75	58	3.10	2.31			

conditions, thereby enhancing the yield and efficacy of bioactive compounds.

For example, extraction time plays a dual function in determining antioxidant activity. On the one hand, increasing the extraction duration improves the solubilization of antioxidant chemicals derived from plant sources. However, longer extraction durations may lead to the breakdown of sensitive components like phenolics, diminishing total antioxidant efficacy.[30,31] The best extraction time reported in this study was roughly 45-60 min for most of the plant species, which fits with earlier research findings.[32] Solvent concentrations also appeared as a key factor impacting antioxidant activity. Ethanol-water combinations have been commonly employed in extraction methods due to their efficacy in dissolving phenolic chemicals.[33] In this investigation, a solvent concentration range of 40-80% was determined to be optimum for enhancing antioxidant production. This is consistent with past observations where the combination of ethanol and water allows for the selective extraction of polar molecules such as phenolics.[34]

It is important to note that antioxidant and polyphenolic content in *Cassia auriculata* and *Murraya koenigii* have been studied using RSM.[35,36] However, our study is the first to find the best way to extract antioxidants and polyphenols from the leaves of *Celastrus paniculatus, Moringa concanensis,* and *Alternanthera sessilis*. Our study primarily focused on optimizing the extraction process for total phenolic content and antioxidant properties (DPPH) using a hydroalcoholic solvent. Previous studies on *Murraya koenigii* using the RSM employed different methods; for example, one study used a different solvent, methanol, with the aim of maximizing the flavonoid content.[37] Previous studies optimized *Cassia auriculata* using RSM, but they used slightly different parameters, particularly in terms of solvent concentration. They used 60% hydroalcoholic as the maximum solvent ratio and found the TPC at this maximum solvent ratio.[35] However, we acknowledge the limitations of not optimizing other phytoconstituents, such as flavonoids and tannins, or using different organic solvents. Future studies could explore these aspects to provide a more comprehensive understanding of the phytochemical profiles of these plants. This is in line with the findings of Lefebvre, T., *et al.* (2021), who highlighted the importance of solvent selection in extraction processes.[38]

The solid-liquid ratio, another key component, was revealed to have a considerable influence on the antioxidant extraction process. Higher solid-liquid ratios often enhance the concentration of extracted chemicals in the solvent, resulting in increased antioxidant action. However, at very high ratios, the solvent may become saturated, hence lowering extraction efficiency.[39] The best solid-liquid ratio identified in this investigation varied depending on the plant type but generally ranged from 1:5 to 1:15 g/mL.

The plant source was a very significant factor influencing antioxidant activity, showing that inherent variations in the phytochemical makeup of the plants had a prominent role in determining antioxidant capacity.[40] Among the five plants investigated, *Cassia auriculata* consistently demonstrated the greatest antioxidant activity, followed by *Celastrus paniculatus*. This discovery is confirmed by literature, as both plants are known to be rich in phenolic compounds, flavonoids and tannins, which are powerful antioxidants.

The variance in antioxidant activity across various plant species may be related to changes in their secondary metabolite profiles. While all the plants analyzed have known antioxidant characteristics, the number and kinds of phenolics, flavonoids, and other antioxidants might vary greatly. This underscores the necessity of selecting the appropriate plant species for extraction depending on the intended medicinal characteristics.[41]

The combination of RSM and BBD allows for a systematic analysis of the interaction between different extraction factors.[42] This technique offered a predictive model with excellent accuracy (R^2=0.9551 for antioxidant activity and R^2=0.9415 for TPC), enabling precise modification of the extraction procedure. One of the primary characteristics of RSM is its capacity to examine both the individual and combined impacts of several variables

Table 3: Condition of the independent factors for achieving maximum antioxidant and polyphenolic content.

Factors				Responses	
Plant Source	Extraction time (Min)	Solvent Concentration (%)	Solid-Liquid ratio (gm/mL)	DPPH VCEAC in µg	TPC GAE in µg
C. auriculata	60 min	60%	1:5	13.86	14.78
C. paniculatus	60 min	60%	1:5	6.44	9.65
M. concanensis	60 min	60%	1:5	4.05	4.84
M. koenigii	60 min	60%	1:5	3.59	1.88
A. sessilis	60 min	60%	1:5	3.12	2.30

Table 4: Predicted and experimentally obtained actual response values for the optimal conditions designed by BBD.

Factors				DPPH VCEAC in µg		TPC in GAE µg	
Plant Source	Extraction time (Min)	Solvent Conc. (%)	Solid-Liquid ratio (gm/mL)	Actual	Predicted	Actual	Predicted
A. sessilis	60min	80%	1:5	4.05	4.10	3.86	3.94
M. koenigii	60 min	79.99%	1:5	4.98	5.06	3.47	3.51
M. concanensis	60 min	79.99%	1:5	5.27	5.31	6.58	6.77
C. paniculatus	60 min	80%	1:5	8.17	8.21	11.86	11.94
C. auriculata	58.30 min	79.15%	1:5.3	15.85	15.93	5.08	5.14

on a given response. The significant interaction effects identified between variables such as extraction duration and solvent concentration (AB) and solid-liquid ratio and plant source (CD) underline the complexity of the extraction process. These interactions are crucial for fine-tuning the extraction parameters to obtain optimal yield, particularly when dealing with diverse plant species with variable chemical compositions.

Additionally, we have considered potential biases and limitations in the extraction process to ensure the reliability of the results. We have considered factors such as the stability of phenolic compounds during prolonged extraction times and the potential saturation of solvents at high solid-liquid ratios. This makes the analysis more reliable and is in line with what Alara, O. R., *et al.* (2021) found when they looked into how different extraction conditions affect the stability of phenolic compounds.[43]

The optimization procedure produced various optimal conditions for each plant species, reflecting the plants' unique phytochemical profiles. For example, *Cassia auriculata* needed an extraction duration of 56.9 min, a solvent concentration of 77.54%, and a solid-liquid ratio of 1:6.3 g/mL to get the greatest antioxidant activity (14.59 µg VCEAC). In contrast, *Celastrus paniculatus* attained its highest antioxidant activity under somewhat different settings. This highlights the need for adjusting extraction parameters based on the unique plant material being employed. The results of this study have significant practical implications for the extraction of bioactive chemicals from medicinal plants. By adjusting the extraction conditions, researchers and producers may increase the yield of antioxidants and phenolic chemicals, hence boosting the medicinal potential of plant extracts. These optimized extracts can be employed in numerous applications, including nutritional supplements, medicines, and cosmetics.

Furthermore, the results provide a strong framework for the creation of polyherbal formulations that mix the bioactive chemicals from several plants. Given that different plants offer varying kinds and amounts of antioxidants and phenolics, mixing extracts from many species may result in synergistic effects, boosting the overall efficacy of the product. Such polyherbal formulations might be particularly beneficial for tackling complicated health issues, such as oxidative stress, inflammation and metabolic disorders.

Future research should focus on confirming the *in vivo* effectiveness of these tailored extracts, particularly in models of oxidative stress-related illnesses. Additionally, large-scale extraction experiments might be done to determine the feasibility of industrial-scale manufacturing of these bioactive chemicals. Exploring new extraction procedures, such as supercritical fluid extraction or ultrasound-assisted extraction, may further increase the efficiency and yield of antioxidants and phenolics from medicinal plants.

CONCLUSION

In conclusion, the study effectively improved the extraction conditions for antioxidant activity and TPC from five indigenous medicinal herbs using RSM and BBD. The results demonstrate the importance of extraction duration, solvent concentration and solid-liquid ratio in determining the yield of bioactive chemicals. *Cassia auriculata* and *Celastrus paniculatus* were identified as the most effective sources of antioxidants and phenolics, making them good candidates for further study in therapeutic applications. The results of this study lay the groundwork for future research aimed at exploiting the therapeutic potential of these plant species through improved extraction techniques.

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ABBREVIATIONS

AS: *Alternanthera sessilis*; **MK:** *Murraya koenigii*; **MC:** *Moringa concanensis*; **CP:** *Celastrus paniculatus*; **CA:** *Cassia auriculata*; **TPC:** Total Phenolic Content; **DPPH:** 2,2 Diphenyl 1 picrylhydrazyl; **VCEAC:** Vitamin C Equivalent Antioxidant Capacity; **GAE:** Gallic Acid Equivalent; **FC:** Folin Ciocalteu; **ANOVA:** Analysis of Variance; **BBD:** Box Behnken Design; **R^2:**

Coefficient of Determination; **LOF:** Lack of Fit; **RSM:** Response Surface Methodology; **UV-vis:** Ultraviolet-Visible Spectroscopy.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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