Optimizing the Microwave-Assisted Extraction Method to Enhance Extraction Yield and Total Phenolic Content from Moringa Leaves (*Moringa oleifera* Lam.)

Poonam Kumari[1], Anurag Khatkar[2], Pooja Rani[3], Sarita Khatkar[4*], Rimmy Nandal[5*]

[1]Faculty of Pharmaceutical Sciences, Baba Mast Nath University, Asthal Bohar, Rohtak-124021, Haryana, India.

[2]Department of Pharmaceutical Sciences, M.D. University, Rohtak, Haryana, India

[3]Chandigarh Pharmacy College-Jhanjeri, Mohali(Punjab), India. 140307

[4] Geeta Institute of Pharmacy, Geeta University, Panipat-132145, Haryana, India

[5]Shri Baba Mastnath Institute of Pharmaceutical Sciences and Research, Baba Mastnath University, Rohtak-124021

**Corresponding Author:Rimmy Nandal*

Shri Baba Mast Nath Institute of Pharmaceutical Sciences and Research, Baba Mast Nath University, Asthal Bohar, Rohtak-124021, Haryana, India

Email: nandalrimmy17@gmail.com

ABSTRACT

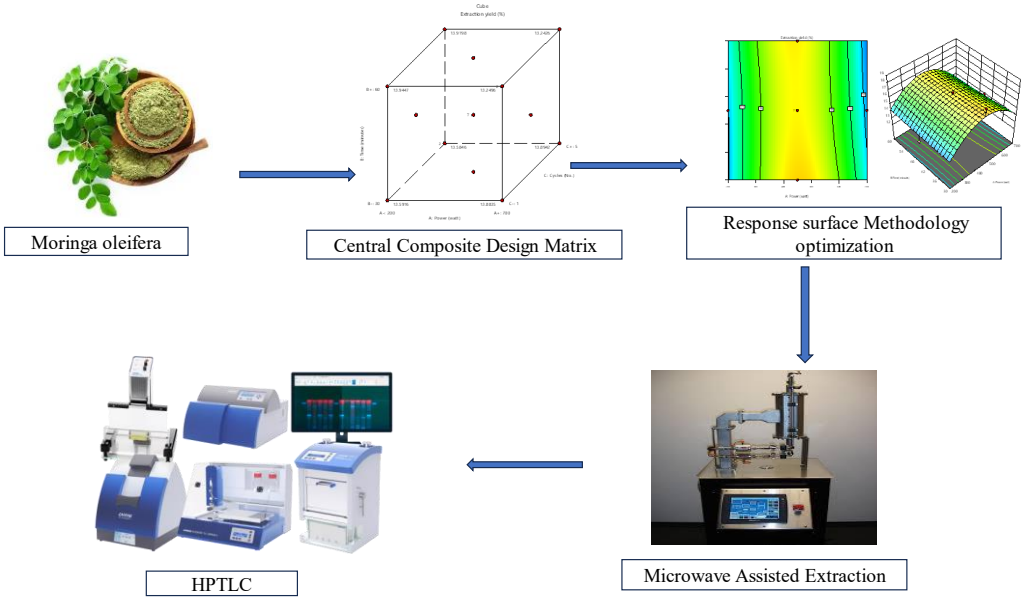
Background: Growing in tropical and subtropical regions of the world, *Moringa oleifera* is a tree whose leaves, seeds, bark, roots, and flowers are used as ingredients in food and medicine. Extracts from *Moringa* leaves have been found to contain antidiabetic compounds like flavonoid molecules; newer extraction methods like ultrasound and microwaves are substitutes to improve the extraction performance of flavonoid compounds while maintaining their antidiabetic activity.

Objective: Microwave-assisted extraction (MAE) of phenolic compounds from *Moringa oleifera* leaves was accomplished by adjusting the process parameters utilizing a Composite Central Design of Response Surface Methodology (CCD-RMS).

Methods: A 3^3 CCD-RMS was used; three independent variables were studied: power(watt), temperature (°C) and no. of cycles in the range of 200-700, 30-60 and 1-5 respectively. The results showed that the optimal MAE conditions were at 30 minutes in 3 cycleswith maximum percentage yield of 18%.

Conclusion: This optimization will make it possible to assess better use of *M. oleifera* leaves as a suitable antidiabetic agent.

Graphical Abstract:



1. INTRODUCTION

In recent years, there has been a growing interest in extracting biomolecules from plant sources. These plant-derived compounds are not only cost-effective and healthy but also serve as promising alternatives to chemical drugs, which often come with harmful side effects for both humans and animals. Secondary metabolites from various plants have been reported to offer a wide range of medicinal, pharmaceutical, and nutraceutical benefits. Research has shown that natural antioxidants, in particular, can help prevent diseases related to oxidative stress, obesity, and even certain types of cancer[1-3].

Among the many plant species, *Moringa oleifera* stands out as a rich source of bioactive compounds, particularly in its leaves and extracts. Studies have indicated that dried or desiccated Moringa leaves exhibit higher antioxidant capacity than their fresh counterparts. *Moringa oleifera*, native to the Cis-Himalayan regions of India, Afghanistan, Bangladesh, and Pakistan, has since been widely cultivated around the world. This is due to its unique blend of phenolic compounds, flavonoids, antioxidants,

and its ability to thrive in diverse soil types, particularly in tropical and subtropical climates[4-5].

In addition to its antioxidant properties, Moringa is packed with essential nutrients such as iron, potassium, calcium, and vitamins A, C, and E, as well as amino acids. The plant is an important source of dietary supplements for treating malnutrition and is also widely studied for its medicinal properties. *Moringa oleifera* leaves contain a variety of bioactive compounds with health benefits, including antioxidant, antimicrobial, anti-inflammatory, anticancer, antidiabetic, hepatoprotective, and cardioprotective effects. Recent studies have shown that consumption of Moringa leaf extracts leads to improved antioxidant enzyme activity, reduced body weight, lower cholesterol, triglycerides, and blood glucose levels, as well as improved histological conditions in the heart and liver of rat models[6-9].The method of extraction plays a significant role in maximizing the recovery of phenolic, flavonoid compounds from Moringa leaves[10]. Microwave-assisted extraction (MAE) has been shown to yield higher amounts of bioactive compounds with less solvent and shorter extraction times compared to conventional methods. This is because ultrasound waves disrupt plant cell walls, making it easier for the solvent to penetrate the tissue and extract its valuable components. However, more research is needed to optimize the variables involved in MAE, such as frequency, temperature, time, and power levels, to achieve the best results [11-13].

The aim of this study was to optimize the yield of phenolic compounds and their antioxidant capacity during MAE from *Moringa oleifera* leaves using a Central Composite Design (CCD) approach within Response Surface Methodology (RSM). By applying this statistical method, the study sought to evaluate and optimize the interactions of key factors—such as amplitude, temperature, and time—with dependent variables like extraction yield, total phenolic content (TPC), and total flavonoid content (TFC). This approach aims to identify the optimal conditions for maximizing the extraction of

bioactive compounds from Moringa leaves, ensuring a higher recovery yield and better antioxidant capacity.

2. MATERIALS AND METHODS

Plant collection and identification Fresh leaves of *Moringa oleifera* were collected from the area around Rohtak. The collected parts of *Moringa oleifera* were authenticated at the Maharshi Dayanand University, Rohtak.

2.1. Chemicals and reagents

Ethanol(70%) often used as a solvent for extracting bioactive compounds due to its polarity and ability to dissolve a wide range of compoundswere sourced from Hi-Media Laboratories. Analytical reference standard were obtained from Sigma-Aldrich.

2.2. Preparation of Plant material

Moringa leaves were harvested from twenty-year-old trees at MDU, Rohtak (Haryana), India, at 45-day intervals for analysis. The freshly harvested leaves were thoroughly rinsed twice with clean water. The leaflets were then separated from the rachis and dried at 40 ± 2°C for 4 hours using a cabinet solar dryer. After drying, the leaves were ground into a fine powder using a semi-automatic pulverizer (commercial-grade) and passed through a 250 µm sieve. The milling process and sieving helped increase the interaction between the solvent and the plant material. The resulting Moringa leaf powder was stored in moisture-free conditions at 4°C for further analysis[14,15].

2.3. Microwave-assisted extraction

In this optimization study, microwave-assisted extraction (MAE) was performed using an (Ragatech,India) 5g sample of dried Moringa leaves was microwaved with 70% ethanol at a 1:10 ratio using pulse mode (5 seconds on, 5 seconds off). The extraction was carried out under ice-cold conditions to prevent degradation of the bioactive compounds. After the microwave treatment, the Moringa extracts were centrifuged at 6000 rpm for 10 minutes. The supernatant was then concentrated using a rotary evaporator at 40°C and

lyophilized. The freeze-dried Moringa extracts were stored at deep freeze temperatures for further analysis [16].

2.4. Estimation of moringa leaf extract recoveryyyield

The yield of the Moringa leaf extract was calculated based on the mass of the lyophilized extract obtained after the complete removal of the solvent, relative to the amount of leaf sample used for extraction. The entire hydroethanolic extract was first concentrated under vacuum using a rotary evaporator at 40°C and then lyophilized. The dried extracts were weighed, and the results were expressed as a percentage of the initial leaf sample weight[17].

2.5. Estimation of total phenolic contents in moringa extracts

The total phenolic content (TPC) in the Moringa leaf extract was quantified spectrophotometrically using the modified Folin-Ciocalteau method, following the procedure outlined by Chavan et al. (2013) and Rakesh et al. (2021). The TPC was calculated by referencing a calibration curve generated with gallic acid as the standard[18].

2.6 Estimation of totalflavonoid content in moringa extracts

The total flavonoid content in the Moringa leaf extract was determined using an aluminum chloride method with slight modifications as recommended by Rakesh et al. (2021). The flavonoid content was calculated based on a calibration curve using quercetin as the reference standard.

Standard preparation

A suitable quantity (5 mg) each of GA, QT, and RT was weighed accurately and transferred to separate 10 mL volumetric flasks, 5 mL of methanol was added followed by sonication for 10 min, and the volume was made up to 10 mL with methanol. The resulting solutions were filtered through Whatman filter paper and suitable volumes were applied to TLC plates for further analysis.

2.7 HPTLC instrumentation and chromatographicconditions

A Camag HPTLC system was utilized, which included a UV chamber, a twin trough chamber, a saturation pad, a TLC scanner 3, and win CATS 1.2.2 software (Camag, Muttens, Switzerland). A CamagLinomat V (Hamilton, Broadus, Switzerland) sample applicator was used to spot the standards and samples in the shape of bands 6 mm wide using a microliter syringe on aluminum plates that had been precoated with silica gel 60 F254 (10×10 cm with 0.2 mm thickness, E. Merck, Germany). A consistent slit size of 5 mm × 0.45 mm and a scanning speed of 20 mm/s were maintained. The chromatograms were produced up to 80 mm in length using linear ascending development in the twin trough glass chamber. The TLC plates developed by this technique were dried with the help of an air dryer.

2.8 Preliminary HPTLC analysis

Solvents such as toluene, isopropanol, n-butanol, ethanol, methanol, ethyl acetate, formic acid, dioxane, and acetic acid in different ratios were used as the mobile phase in the first HPTLC experiments. However, issues were noted, such as low RF values for sample. The RF values of QT improved as a result of the methanol addition. However, the RF value of QT and sample was significantly impacted by changes in the amount of methanol (>3 and <2). After a number of permutations and combinations, a mixture was chosen as the mobile phase because it provided a rather decent separation of sample and QT. The experiments revealed that the chromatographic technique conditions had a significant impact on the three biomarkers' RF value and peak area [20].

2.9 RSM experimental modelling and statisticalanalysis

The optimization of Moringa leaf extraction using the microwave-assisted method was modeled and simulated through Response Surface Methodology (RSM) with the help of Design Expert 13 software. RSM is a combination of statistical and mathematical techniques used to improve, design, and optimize processes. The optimization study was carried out through 21 experimental trials, which included 7 central points and 14 non-central points, following the Central Composite Design (CCD). In these experiments,

three independent variables—Power, Time and No. of cycleswere varied (Table 1), while two dependent variables—Extraction Yield(Table1)were measured. The relationship between the independent and dependent variables was analyzed using the least-squares multiple regression method, and a second-order polynomial equation was derived from the experimental data.

2.10 Central Composite Design (CCD) for Optimization

With Design Expert® 13 software, *Moringa oleifera* were optimized using a CCD response surface methodology design. Y_1 ranged from 13 to 18, Equationsexplain the effect of different component proportions on the response Y_1[21].

Table 1: Optimization trials of central composite design (where Apower(watt), BTime (min), CNo. of cycles from spotting to chromatography

Runs	**A** **Power (Watt)**	**B** **Time** **(min)**	**C** **No. of cycles**	**Y_1** **Extraction Yield** **(%)**
1	700	30	1	13
2	700	60	1	14
3	450	45	1	16
4	450	45	3	17
5	200	60	5	15
6	450	45	3	17
7	200	60	1	14
8	450	60	3	15
9	450	45	3	17
10	200	30	1	14
11	450	45	3	17
12	**450**	**30**	**3**	**18**

13	700	60	5	13
14	450	45	3	17
15	450	45	3	17
16	450	45	5	16
17	700	30	5	14
18	200	30	5	13
19	700	45	3	14
20	450	45	3	17
21	200	45	3	13

3. Results and discussion

The results obtained for total flavonoid content, in the extracts of dried leaves of M. oleifera were 18%. The batch 12 showed the highest extraction of flavonoids of moringa extraction. Derived from these results and using the CCD-RMS, it was possible to optimize the extraction conditions individually for phenols and flavonoids and perform a simultaneous optimization that included the two response variables: total phenols and total flavonoids. From the response values of phenolic compounds for the different combinations of variables, it was statistically demonstrated that they fit a second-order polynomial model. We found that the ethanol concentration in linear and quadratic terms and the interaction of the ethanol concentration and temperature had negative and significant effects ($p=0.0001$), which indicated that lower ethanol concentration favored the extraction of phenolic compounds. The temperature in a linear term had positive and significant effect, showing that a higher temperature favors the extraction of phenolic compounds. The model had a satisfactory level of adequacy with an R^2 of 0.8420 and the adjusted R^2 was 0.7128, which indicated a strong agreement between the observed values and those predicted by the quadratic equation. The location of the stationary point represented the optimal extraction conditions according to the response surface, 450

power, 30 minutes, to obtain a maximum concentration of total flavonoidcontent of 18% extraction by MAE. The values obtained in the prediction were confirmed experimentally; the results obtained under the same process conditions were 18% extraction by MAE of total flavonoid content, which confirmed the adequacy of the model (Table 1). MAE was an efficient method to maximize the extraction from *M. oleifera* leaves. This result is greater than the value of MAE of total flavonoid found in extract of leaves of M. oleiferacollected by using an optimization.

$$\sqrt{Y_1} = 0.8076 - 0.0017A \quad + 0.0010B \quad - 2.285E-06C \quad - 0.0089A \quad B \quad +$$
$$2.856E-06A \quad C \quad \text{-}2.856\text{E}-06B \quad C \quad - 0.3787A^1 \text{ -}0.0005B^2 \text{ -}0.0053C^3 \quad (1)$$

3.1. The impact of amplitude, temperature and extraction time on extraction yield

Using numerical data acquired from Design expert, quadratic regression models were developed through response surface methodology to assess the influence of independent variables of MAE on the extraction yield of moringa leaves. From Table 3 these, constructedmodel exhibited significance of the experimental process with an F value of 6.2for power, extraction time and no. of cycles. The well-fitted model, significant demonstrates a strong fit for the factors mentioned above. Model terms with p-values (0.0001) indicate their significance. The analysis of variance (ANOVA) for the quadratic regression model reveals that the predicted R^2 of 0.8420 agrees with value of adjusted R^2 of 0.7128.

3.2 The impact of power, time and no. of cycles

The experimental and projected values for the total phenol content in the moringa leaf extract obtained through microwave-assisted extraction, encompassing various combinations of independent variables were presented in Table 1. The projected values are juxtaposed with the actual experimental results, revealing a high degree of concordance.The table 2delineates the statistical analysis and its respective interpretation.

Table 2: Model summary statistics

		Models			Lack of fit	
Factors	**R^2**	**Adjusted R^2**	**PredictedR^2**	**Adequacy precision**	**F-value**	**p-value**
Y_1	0.8420	0.7128	0.7272	5.9441	6.52	0.0001

3.3. The impact of power, time and no. of cycles on total flavonoid content

The quadratic curve derived from the Multivariate regression analysis utilizing response surface methodology (RSM) of total flavonoid content, considering the independent factors of Amplitude, Temperature, and Time. To assess the significance of the experimentalmodel, an analysis of variance (ANOVA) was conducted.

3.4. Data Validation

Data validation was conducted using one-way analysis of variance (ANOVA) along with the F test. Coefficients deemed significant ($p<0.0001$) were utilized in developing the polynomial equations. To further assess the model's fit, the lack of fit and correlation coefficients (r^2) was analyzed (Tables 4 and 6). For response surface analysis, 2D contour plot and 3D response surface plot was utilized (Figure 1). The entire diagnostic plots for the model, including the normal probability plot, run plot, residual plot (Figure 3), and histogram plots (Figure 4), were also used to assess the adequacy of the data fit. To eliminate any measurement bias, all the experimental runs were conducted in a random sequence.

HereTable 3, 4 displays the ANOVA results for Y1. The **Predicted R^2** of 0.7128 is not as close to the **Adjusted R^2** of 0.8420 as one might normally expect; i.e. the difference is more than 0.2. This may indicate a large block effect or a possible problem with your model and/or data. Things to consider are model reduction, response transformation, outliers, etc. All empirical models should be tested by doing confirmation runs. **Adequate Precision** measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio of 12.471 indicates an adequate signal. This model can be used to navigate the

design space.Factor coding used is **Coded**, Sum of squares used is **Type III – Partial.** The **Model F-value** of 6.52 implies the model is significant. There is only a 0.26% chance that an F-value this large could occur due to noise.P-values less than 0.0500 indicate model terms are significant. In this case A^2 is a significant model term. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

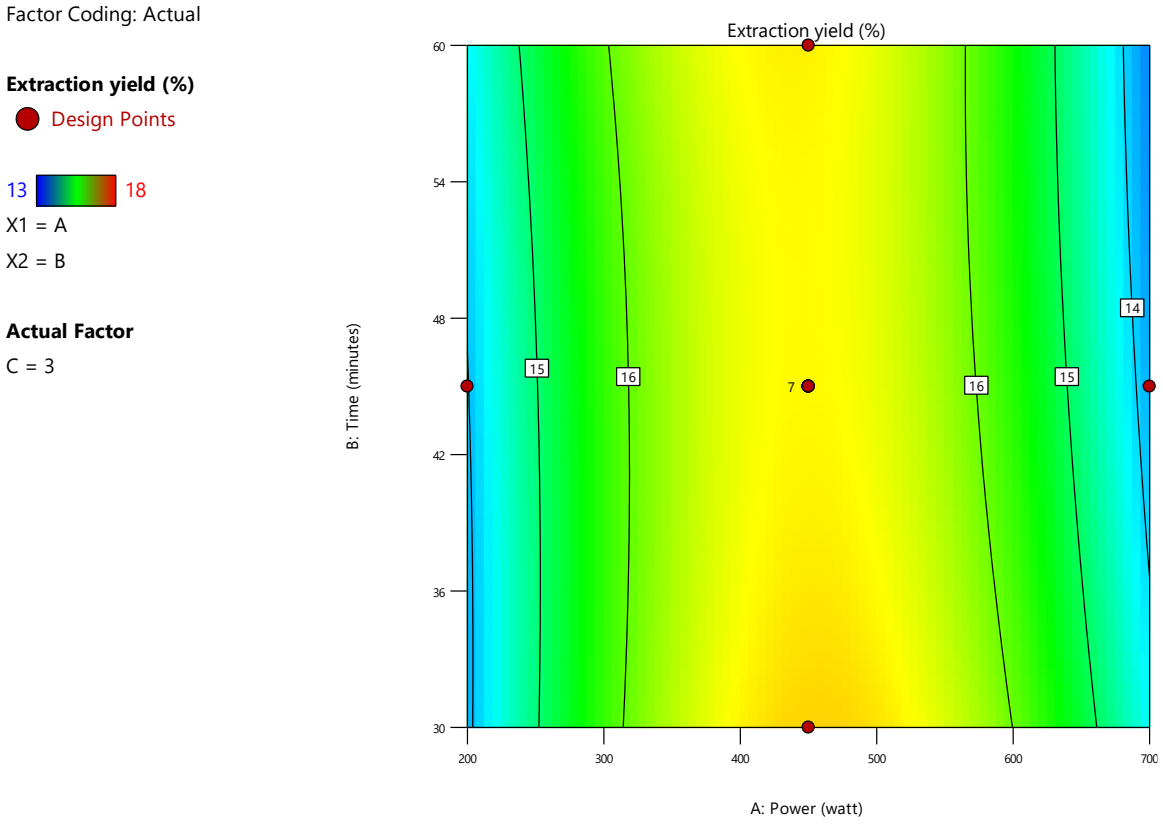
Table 3: Summary of Fit Statistics for response Y1

Std. Dev.	0.1174	**R^2**	0.8420
Mean	3.90	**Adjusted R^2**	0.7128
C.V. %	3.01	**Predicted R^2**	-0.7272
		Adeq Precision	5.9441

Table 4: Summary of statistical ANOVA for response Y_1

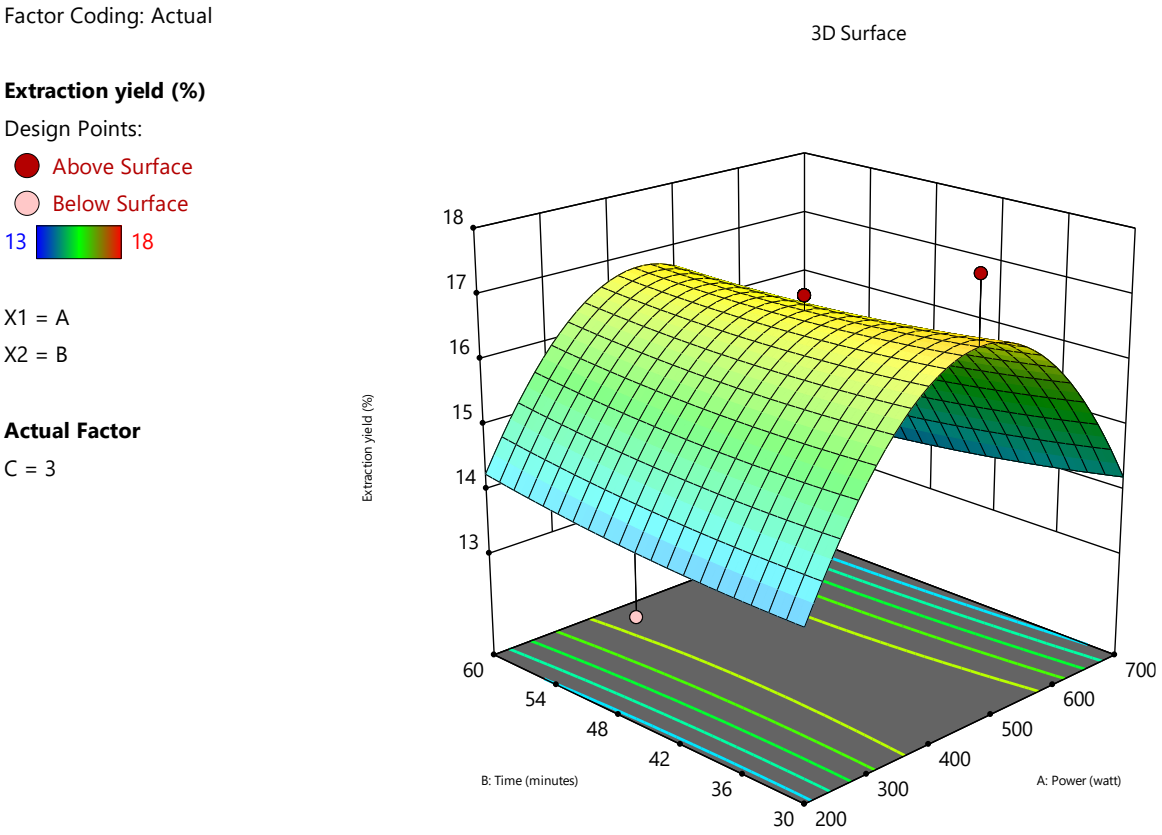
Source	**Sum of Squares**	**Df**	**Mean Square**	**F-value**	**p-value**	
Model	0.8076	9	0.0897	6.52	0.0026	Significant
A-X1	0.0017	1	0.0017	0.1252	0.7301	
B-X2	0.0010	1	0.0010	0.0759	0.7881	
C-X3	2.285E-06	1	2.285E-06	0.0002	0.9900	
AB	0.0089	1	0.0089	0.6491	0.4375	
AC	2.856E-06	1	2.856E-06	0.0002	0.9888	
BC	2.856E-06	1	2.856E-06	0.0002	0.9888	
A^2	0.3787	1	0.3787	27.50	0.0003	
B^2	0.0005	1	0.0005	0.0397	0.8458	
C^2	0.0053	1	0.0053	0.3842	0.5480	

Residual	0.1515	11	0.0138		
Lack of Fit	0.1515	5	0.0303		
Pure Error	0.0000	6	0.0000		
Cor Total	0.9591	20	-		



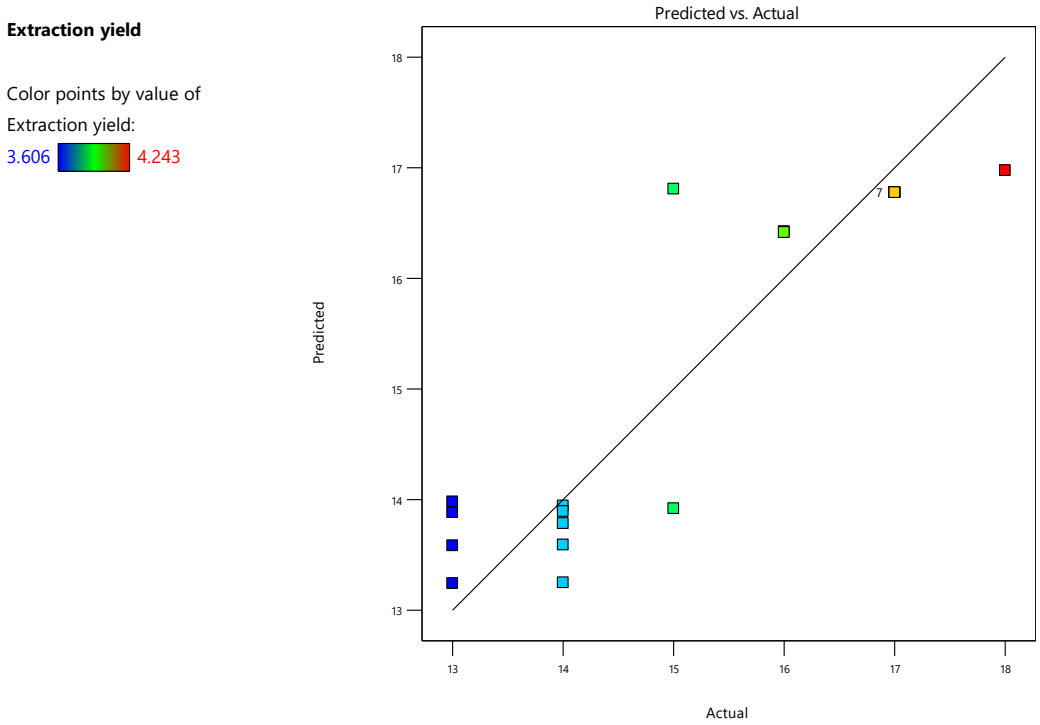
(a)

Figure 1: Depicts contour plots (2-Dimensional) that demonstrate the effect of A, B and Cvariables on Y_1 response.



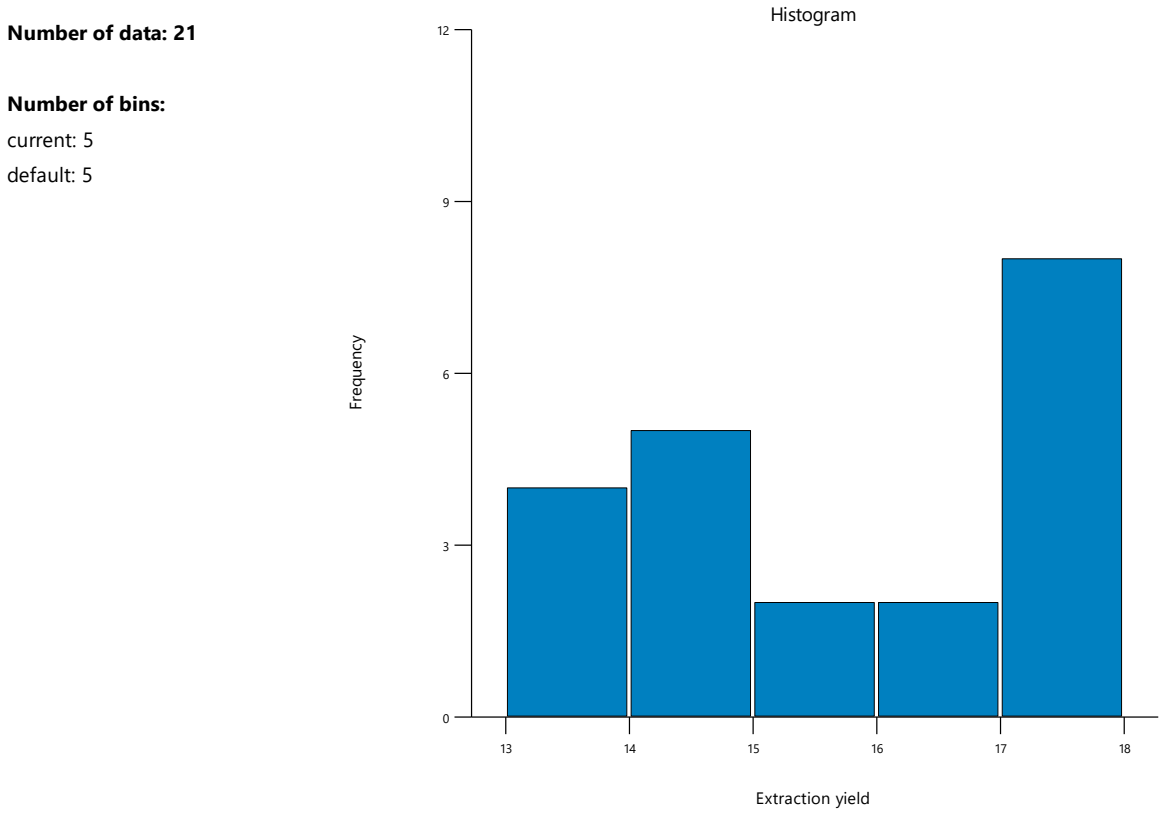
(a)

Figure 2: Depicts three-dimensional response plots that demonstrate the effect of A, B and Cvariables on Y_1response.



(a)

Figure 3: Depicts the predicted vs Actual value of A,B and Cvariables on Y_1response.



(a)

Figure 4: Depicts the Histogram on Y_1 response.

Conclusion

This study utilized the Response Surface Methodology experimental design to concentrate on optimizing Microwave-Assisted Extraction with 70% ethanol. The objective was to attain maximum recovery of extract, total f

lavanoid content, and total flavonoid content from *Moringa oleifera*. Microwave Assisted Extraction stands out as an effective approach within a relatively brief timeframe and this extraction technique reduces both processing time and temperature, making it a valuable method for extracting thermolabile biomolecules found in moringa leaves. Based on the outcome of the optimization model, at 450 watt and 17 min of extraction time under 70%

ethanolic solvent phase system maximum biomolecules from *Moringa oleifera* leaves can be extracted in a very short span with higher yield and quality. These products could be a boon to various food-based industries to fortify moringa biomolecules in wide range of food products along with its nutraceutical properties by adapting micro or nano encapsulation techniques.

CONTRIBUTIONS FROM AUTHORS

Authors contributed to analysis of data, drafting or revising the article, agreed on journal to be submitted, provided final approval to the version published and agreed to accept responsibility for all elements of work.

DISCLOSURE

There is no conflict of interest, according to the authors

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