RJC

RASĀYAN *J. Chem.*
Vol. 18 | No. 3 |1758-1772| July-September | 2025
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

OPTIMIZATION OF POLYPHENOL RECOVERY AND ANTIOXIDANT ACTIVITY FROM *Nelumbo nucifera* LEAF USING BOX-BEHNKEN DESIGN AND LC-HRMS PROFILING

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ABSTRACT

The leaves of *Nelumbo nucifera* are rich in polyphenols, known for their antioxidant and therapeutic properties. This study aimed to optimize extraction conditions to maximize total phenolic content (TPC) and total flavonoid content (TFC) using the Box-Behnken Design (BBD). The relationship between polyphenol content and antioxidant activity was also evaluated. Solid-liquid extraction was employed under various conditions designed using BBD, varying extraction time (X1), ethanol concentration (X2), and solid-to-liquid ratio (X3). The TPC and TFC were quantified using colorimetric methods, while antioxidant activity was assessed through DPPH radical scavenging assays. Polyphenolic compounds were identified using LC-HRMS. Data analysis included ANOVA to validate the model and its predictions. Optimal extraction conditions were determined as 53.87 X1, 69.03% X2, and an X3 of 10.48:1. Under these conditions, the predicted TPC and TFC were 25.894 mg GAE/g and 12.158 mg QE/g, respectively, with a high desirability score of 0.931. Experimental validation confirmed these values with no significant differences ($p = 0.253$). The optimized extract (PRN) exhibited stronger antioxidant activity compared to the non-optimized extract (NCR), achieving up to 84.23% DPPH inhibition at 500 µg/mL. LC-HRMS analysis identified key polyphenolic compounds, including quercetin-3-β-D-glucoside, rutin, kaempferol, and myricetin, which contribute to the extract's antioxidant potential. The findings support SDG 3 (Good Health and Well-being) and highlight the potential of *N. nucifera* as a natural antioxidant source for pharmaceutical, nutraceutical, and cosmetic applications. Future studies should further explore its bioactivity and applications *in vivo*.

Keywords: *Nelumbo Nucifera*, Phenolic, Flavonoid, Antioxidant, Box-Behnken Design, LC-HRMS Analysis
RASĀYAN *J. Chem.*, Vol. 18, No.3, 2025

INTRODUCTION

Lotus (*Nelumbo nucifera* Gaertn.), an aquatic plant, is extensively cultivated in Southeast Asia, particularly in Indonesia.[1] The root, seed, and young lotus leaf are highly favored as vegetables in Asian cuisine.[2] The mature leaf, known for its fibrous texture, is commonly used as a functional food in Asia.[3] Recent studies have shown that the lotus leaf possesses anti-obesity and antioxidant properties, making it a popular ingredient in antioxidant beverages and tea bags in Indonesia.[4] The primary antioxidants in *N. nucifera* leaf are flavonoids and other phenolic compounds, including hyperin, isoquercetin, astragalin, kaempferol, myricetin, and catechin.[5-7] However, many reports have linked plant extracts rich in polyphenol compounds to the potential for an antioxidant agent.[5,8] Consequently, extraction conditions need to be optimized to prepare the polyphenol-rich extract of *N. nucifera* leaf (PRN). Extraction marks the initial step in preparing the PRN, where various techniques are employed to recover polyphenol content from natural sources[9]. These methods include solid-liquid extraction with organic solvents, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), supercritical fluid extraction, and high-pressure processes.[10] However, the efficiency and outcome of the extraction process depend on several critical factors that must be carefully considered during its execution.[11] Notably, previous studies have identified three key parameters in the solid-liquid extraction method: extraction time (X1), ethanol concentration (X2), and

http://doi.org/10.31788/RJC.2025.1839304

solid-to-liquid ratio (X3), all of which significantly influence the extraction outcomes.[12-14] However, the interaction between each extraction factor on polyphenol recovery needs to be devised and studied.
Developing a conventional extraction method to recover polyphenol compounds from *N. nucifera* leaves can present challenges without an appropriate methodological approach. Recently, response surface methodology (RSM) has proven to be a valuable tool for evaluating the interactions between response variables and the various factors that influence them.[15] RSM is particularly effective for optimizing variables and identifying optimal conditions for process improvement when properly applied.[16] It allows researchers to analyze the effects of single or combined independent variables on the process, which cannot be fully understood by examining factors individually.[17] Among the various RSM techniques, the Box-Behnken Design (BBD) is one of the most widely utilized due to its accuracy and efficiency in process design and product optimization.[18] Therefore, the present study employs BBD to investigate the interactions between extraction factors and their effects on polyphenol recovery from *N. nucifera* leaves, facilitating the development of an optimized solid-liquid extraction method. Furthermore, the antioxidant activity of PRN will be assessed to establish the correlation between polyphenol content and antioxidant properties, providing a comprehensive understanding of the extraction's impact on bioactive compound functionality.

EXPERIMENTAL

Materials

The main materials in this experiment included *N. nucifera* leaves collected from the local village of Marelan, Medan, North Sumatra, Indonesia, which are fresh and green. At the same time, those that were too old or rotten were disposed of. The sample was identified as *Nelumbo nucifera* leaf by Prof. Dr. Etti Sartina Siregar, S.Si., M.Si (Botanical expert) in Herbarium Medanese, Universitas Sumatra Utara (Voucher ID: 015/MEDA/2024). In addition, the chemical reagents, including gallic acid, quercetin, Folin-Ciocalteu, aluminum chloride ($AlCl_3$), sodium bicarbonate (Na_2CO_3), and methanol in addition to ethanol (pro analysis), was purchased from Sigma Aldrich.

Preparation of *Nelumbo nucifera* Leaf

Nelumbo nucifera leaves were cleaned and weighed, then dried at 50 °C for 48 h. Approximately 500 g of the final dry matter content was ground into a fine powder and stored in a vacuum bag for preservation.

The Extraction of *Nelumbo nucifera* Leaf Using a Solid-Liquid Extraction Technique

The dried sample was extracted using a solid-liquid extraction technique, following 17 different conditions that were designed by Design Expert®13 software. RSM with BBD of three factors and their combination was used in this experiment to determine the extraction conditions that provide the most optimal total phenolic content (TPC) and total flavonoid content (TFC). All independent variables, including X_1, X_2, and X_3 were created at three levels in BBD Table-1 for each code value (−1, 0, and 1). The extraction of *N. nucifera* leaves was conducted by dissolving the dry powder of *N. nucifera* leaf samples in a carefully selected solvent over an appropriate extraction period. All extraction conditions were carried out based on the interaction of variables determined by the Design Expert®13 software, as outlined in Table-2. Ultimately, seventeen experimental runs were performed to optimize the recovery of total phenolic content (TPC) and total flavonoid content (TFC) from the *N. nucifera* leaves.

Table-1: Actual Level of Factors used in the Box-Behnken Design

Factors	Symbol	Actual levels		
		-1	0	1
Extraction time (hours)	X_1	24	48	72
Ethanol concentration (%)	X_2	60	70	80
Solid-to-liquid ratio (w/v)	X_3	5	10	15

Table-2: The Design of Extraction Conditions after Plotting the Factors into Software

Run	X_1 (hours)	X_2 (%)	X_3 (w/v)
1	48	70	10
2	24	70	5
3	48	70	10
4	72	70	15

5	72	80	10
6	24	80	10
7	48	70	10
8	72	70	5
9	24	60	10
10	48	70	10
11	48	60	5
12	24	70	15
13	48	80	5
14	72	60	10
15	48	60	15
16	48	70	10
17	48	80	15

Total Phenolic Content of *Nelumbo nucifera* Leaf Extract

The Total Phenolic Content (TPC) of all extracts was determined using a colorimetric method with the Folin-Ciocalteu reagent and gallic acid as the standard. The sample solution was prepared at a concentration of 200 µg/mL and dissolved in distilled water as the solvent. Prior to testing, the Folin-Ciocalteu reagent was diluted in distilled water at a 1:10 ratio. A portion of the sample solution was pipetted and mixed with 0.5 mL of the diluted Folin-Ciocalteu reagent. Subsequently, 1 mL of 10% (w/v) sodium carbonate solution was added. The mixture was then incubated at room temperature for 35 minutes. The absorbance of the resulting solution was measured at a wavelength of 742 nm using a UV-visible spectrophotometer. The TPC was calculated and expressed in terms of gallic acid equivalents (mg GAE/g extract).[19]

Total Flavonoid Content of *Nelumbo nucifera* Leaf Extract

The Total Flavonoid Content (TFC) analysis was conducted using a colorimetric method. To begin, the sample extract was prepared at an appropriate concentration, typically dissolved in distilled water or another suitable solvent. An aliquot of the sample solution was mixed with 0.5 mL of 2% aluminum chloride ($AlCl_3$) solution, which acts as the color-developing reagent. To ensure a complete reaction, the mixture was diluted with distilled water to a final volume, typically 5 mL, and thoroughly vortexed. The solution was then incubated at room temperature for 30 minutes to allow the formation of the flavonoid-$AlCl_3$ complex, which exhibits a yellow coloration. Following incubation, the absorbance was measured at a wavelength of 415 nm using a UV-visible spectrophotometer. A calibration curve was prepared using quercetin as a standard, and the TFC of the sample was calculated based on this curve. Results were expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g extract).[20]

Data Analysis for Optimization of Total Phenolic and Flavonoid Contents Using Response Surface Methodology (RSM)

Based on the obtained responses, a contour plot was generated to visualize the relationships between variables. The data from the response analysis were evaluated for significance and model suitability using Analysis of Variance (ANOVA). The appropriate ANOVA model was selected based on program recommendations, favoring the model with the highest level of significance and a significant ANOVA value. The model selection process involved three stages. First, models were evaluated using the Sequential Model Sum of Squares, with a probability value (P-value) less than the significance threshold ($P \leq 0.05$). Second, suitability was assessed through Lack of Fit testing, where a non-significant P-value ($P \geq 0.05$) was preferred. Third, the statistical summary was reviewed, prioritizing models with an R^2 value close to 1.00 to identify the optimum point. The final model was selected based on its ability to achieve statistical significance in ANOVA while demonstrating non-significance in the Lack of Fit test.[21] Additionally, the Design Expert®13 software provided a normal plot of residuals, which was used to assess the normality of the data. Residuals closely aligning with the normal (straight) line indicated that the data were normally distributed, suggesting that the actual results closely matched the predicted values provided by the program. Subsequently, the correlation between the trial data and the response was assessed using the coefficient of determination (R^2), adequacy precision, and the adjusted coefficient of determination (R^2 adjusted). For a statistically robust model, the R^2 value should closely align with the adjusted R^2 value, indicating minimal

disparity and a reliable fit. In this analysis, the R^2 value was found to be highly comparable to the adjusted R^2, reflecting the model's reliability. Furthermore, the model's significance was validated at a probability level of 0.0001%, with R^2, adjusted R^2, and predicted R^2 values all exceeding 90%, confirming the model's strong predictive and explanatory power.[22]

Phytochemical Analysis Using LC-HRMS

The examination of polyphenol compounds in PRN was performed using liquid chromatography-high-resolution mass spectrometry (LC-HRMS). The analysis utilized a TSQ Exactive (Thermo) instrument at the BRIN facility in Indonesia. For the mobile phase, 0.1% formic acid in water was used as phase A, while 0.1% formic acid in acetonitrile served as phase B. A gradient system was applied throughout the experiment. The separation was carried out on a Hypersil GOLD aQ column (50 × 1 mm, 1.9 µm) with a flow rate of 40 µL/min over a 70-minute run time. The data were analyzed using Compound Discoverer software with the support of the mzCloud database for compound identification and characterization.

Table-3: Total Phenolic and Total Flavonoid Contents of *Nelumbo nucifera* Leaf Extract Under Different Extraction Conditions

X_1	X_2	X_3	Response 1	Response 2
			Total phenolic content	Total flavonoid content
Hours	%	Ratio	mg GAE/g sample	mg QE/g sample
48	70	10	26.34	12.46
24	70	5	19.21	7.31
48	70	10	24.46	11.56
72	70	15	18.35	10.35
72	80	10	17.46	9.45
24	80	10	16.23	7.56
48	70	10	25.67	11.64
72	70	5	19.54	9.57
24	60	10	15.45	5.57
48	70	10	25.57	11.43
48	60	5	22.34	4.57
24	70	15	15.57	10.39
48	80	5	17.82	5.93
72	60	10	22.67	8.57
48	60	15	20.46	9.45
48	70	10	26.87	12.58
48	80	15	19.35	6.78

Conventional Extraction of *Nelumbo nucifera* Leaf

The conventional extraction of *N. nucifera* leaf was carried out using reflux. Briefly, 100 g of *N. nucifera leaf* dry powder was soaked with 1 L of 96% ethanolic in a round-bottom flask. A set of reflux tools was assembled and run under 70°C for 2 hours. The mixture was filtered to gain the filtrate and evaporated using a rotary evaporator (PRIO, RE-2000-VN). The extract (non-optimized) was collected in a dry container and kept at 2 - 8°C before being used in the experiment.[23]

Antioxidant Activities of the Polyphenol-Rich Extract of *Nelumbo nucifera* Leaf

The measurement of antioxidant activities from the PRN was established by observing the effect of the sample on inhibiting the radical DPPH and ABTS. The effect of the sample to inhibit the radical DPPH was obtained by mixing a 0.5 mL sample with a 3.5 mL solution of DPPHº (0.2 mM DPPHº solution diluted in ethanol of pro analysis) and incubating at room temperature for 30 min. The absorbance values of the sample, ethanol pro analysis, and distilled water were determined at a wavelength of 517 nm. Additionally, ascorbic acid was used as a positive control to validate the assay results. The sample absorbance (As), solvent absorbance (Ae), and blanko absorbance (Aw). The antioxidant activity was calculated by employing the following formula (Eq.1):[24]

$$Scavenging\ capicity\ (\%) = \left(1 - \frac{As - Ae}{Aw}\right) x\ 100 \quad (1)$$

Statistical Analysis

The data obtained from the antioxidant activity experiments were analyzed using statistical methods to ensure the reliability and accuracy of the results. The analysis involved calculating the mean and standard deviation of at least three replicates for each experimental condition. Statistical significance between groups was determined using a one-way Analysis of Variance (ANOVA), followed by post hoc tests (Tukey's HSD) to identify significant differences between specific treatments, with a significance level set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Total Phenolic and Flavonoid Contents of *Nelumbo nucifera* Leaf Extract

The present study demonstrates the effect of different extraction conditions on the total phenolic content (TPC) and total flavonoid content (TFC) of *Nelumbo nucifera* leaf, which is presented in Table-3. Table-3 indicates that the extraction conditions significantly influenced the TPC and TFC of *N. nucifera* leaf extract. Among the tested conditions, the highest TPC (26.87 mg GAE/g sample) and TFC (12.58 mg QE/g sample) were observed at an extraction time (X_1) of 48 hours, ethanol concentration (X_2) of 70%, and a solid-to-liquid ratio (X_3) of 1:10 w/v. Meanwhile, lower TPC and TFC recovery were associated with different combinations of X_1, X_2, and X_3. For example, an X_1 of 24 hours, X_2 of 60%, and an X_3 of 10 resulted in the lowest TPC (15.45 mg GAE/g sample) and TFC (5.57 mg QE/g sample), demonstrating the importance of sufficient time and solvent polarity for effective extraction. However, a statistical analysis was conducted to evaluate the significance of the effects of X_1, X_2, and X_3 on TPC and TFC. Analysis of variance (ANOVA) revealed significant differences ($p < 0.05$) among the various extraction conditions, confirming the critical influence of these factors on the extraction efficiency. Post hoc tests further indicated that the combination of 48 hours of X_1, 70% of X_2, and 1:10 w/v of X_3 yielded significantly higher TPC and TFC than other conditions. The optimal condition, which involved 48 hours of X_1, 70% X_2, and an X_3 of 10, demonstrated the highest TPC and TFC.

This outcome can be attributed to the sufficient interaction time for the solvent to penetrate the plant matrix and solubilize phenolic and flavonoid compounds effectively. The ethanol concentration of 70% was ideal for balancing the polarity to dissolve both hydrophilic and lipophilic compounds.[25] These findings align with previous studies reporting that a mid-range ethanol concentration is often most effective for extracting phenolic and flavonoid compounds.[26-28] Moreover, this study provides critical insights into the extraction process for *N. nucifera* leaf extracts, emphasizing the importance of optimizing conditions to enhance the recovery of phenolic and flavonoid compounds. These compounds are known for their strong antioxidant properties, which have potential applications in food preservation and pharmaceuticals. Therefore, a more in-depth discussion on the influence of extraction factors and their interactions in optimizing phenolics and flavonoids in *N. nucifera* extract will be explained using the RSM approach.

RSM Application in Optimizing TPC and TFC of *Nelumbo nucifera* Leaf Extract

Table-4 demonstrates the fit summary of the optimization model for TPC and TFC, indicating that the quadratic model provides the best fit for both responses. For TPC, the quadratic model showed a sequential p-value of less than 0.0001 and a lack of fit p-value of 0.1960, suggesting that the model is statistically significant and that the lack of fit is not significant, supporting its adequacy. The adjusted R^2 value of 0.9100 and predicted R^2 value of 0.5661 further indicate a strong model performance, with the adjusted R^2 showing that the model explains a significant portion of the variability in the data, although the predictive capability (predicted R^2) is lower, indicating room for improvement. Similarly, for TFC, the quadratic model was suggested based on its sequential p-value of 0.0001 and lack of fit p-value of 0.1074. The adjusted R^2 value of 0.8921 indicates a robust fit to the experimental data, while the predicted R^2 of 0.4155 reflects moderate predictive capability.

These metrics confirm that the quadratic model is statistically significant and adequately captures the relationship between the extraction parameters and the TFC. Meanwhile, the linear and two-factor interaction (2FI) models for both TPC and TFC showed poor performance, as indicated by higher sequential p-values ($p > 0.05$), significant lack of fit ($p < 0.05$), and negative adjusted R^2 and predicted R^2 values. This suggests that these models do not adequately describe the data and fail to capture the complexity of the system.

Table-4: Fit summary for optimization model of TPC and TFC

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Response 1: Total phenolic content					
Linear	0.5822	0.0030	-0.0648	-0.3015	
2FI	0.8755	0.0017	-0.2959	-1.0436	
Quadratic	< 0.0001	0.1960	0.9100	0.5661	Suggested
Cubic	0.1960		0.9456		Aliased
Response 2: Total flavonoid content					
Linear	0.4376	0.0025	-0.0061	-0.3173	
2FI	0.8586	0.0014	-0.2163	-1.2682	
Quadratic	0.0001	0.1074	0.8921	0.4155	Suggested
Cubic	0.1074		0.9527		Aliased

The results indicate that a quadratic model effectively captures the nonlinear relationships between extraction parameters and the responses (TPC and TFC). The statistical significance of the quadratic model reflects the importance of optimizing these parameters to maximize phenolic and flavonoid content recovery.[29] The relatively lower predicted R^2 values, however, highlight potential limitations in the model's predictive capability, which could be addressed by incorporating more experimental data or refining the model.[30] In addition, the lack of fit analysis shows that the quadratic model fits well with the experimental data, reinforcing its suitability for describing the system[31]. However, the lower predictive R^2 values for TFC compared to TPC suggest that flavonoid extraction might involve additional complexities, such as interactions not fully accounted for in the current model. Ultimately, these findings provide a solid foundation for optimizing the extraction process, emphasizing the critical role of statistical modeling in understanding and enhancing bioactive compound recovery from *N. nucifera*. Furthermore, the current study presents the analysis of variance (ANOVA) for the quadratic model and provides critical insights into the effects of various extraction parameters on TPC and TFC, which is presented in Table-5. For TPC, the model is statistically significant, as evidenced by a p-value of 0.0004 and an F-value of 18.96. Among the individual factors, extraction time (X_1) and ethanol concentration (X_2) were significant contributors to TPC ($p = 0.0100$ and $p = 0.0187$, respectively), while the solid-to-liquid ratio (X_3) showed no significant effect ($p = 0.1609$). Significant interaction effects were observed between X_1 and X_2 ($p = 0.0374$), indicating that the combined influence of X_1 and X_2 plays a crucial role in optimizing phenolic compound recovery. The quadratic terms (X_1^2, X_2^2, X_3^2) were all highly significant ($p < 0.01$), suggesting that the relationships between these parameters and TPC are nonlinear. This underscores the need for careful optimization to identify the best combination of factors for maximum phenolic extraction. The lack of fit was not significant ($p = 0.1960$), indicating the model adequately describes the data. The high R^2 value (0.9606) and adjusted R^2 value (0.9100) confirm the model's robustness, while a predicted R^2 of 0.5661 suggests moderate predictive capability.

Table-5: ANOVA Results with Various Treatments on the Response of TPC and TFC

Source	Sum of Squares	df	Mean Square	F-value	P-value	
Response 1: Total phenolic content						
Model	232.95	9	25.88	18.96	0.0004	significant
X_1	16.70	1	16.70	12.24	0.0100	
X_2	12.65	1	12.65	9.27	0.0187	
X_3	3.35	1	3.35	2.46	0.1609	
X_1X_2	8.97	1	8.97	6.57	0.0374	
X_1X_3	1.50	1	1.50	1.10	0.3292	
X_2X_3	2.91	1	2.91	2.13	0.1878	
X_1^2	98.12	1	98.12	71.89	< 0.0001	
X_2^2	37.95	1	37.95	27.81	0.0012	
X_3^2	32.71	1	32.71	23.97	0.0018	
Residual	9.55	7	1.36			
Lack of Fit	6.25	3	2.08	2.53	0.1960	not significant
Pure Error	3.30	4	0.8251			

Cor Total	242.50	16				
Std. Dev.	1.17		R^2	0.9606		
Mean	20.79		Adjusted R^2	0.9100		
C.V. %	5.62		Predicted R^2	0.5661		
			Adeq Precision	11.5173		
Response-2: Total Flavonoid Content						
Model	94.59	9	10.51	15.70	0.0007	significant
X_1	6.32	1	6.32	9.44	0.0180	
X_2	0.3042	1	0.3042	0.4544	0.5219	
X_3	11.50	1	11.50	17.17	0.0043	
X_1X_2	0.3080	1	0.3080	0.4601	0.5194	
X_1X_3	1.32	1	1.32	1.98	0.2027	
X_2X_3	4.06	1	4.06	6.06	0.0433	
X_1^2	2.13	1	2.13	3.19	0.1173	
X_2^2	49.67	1	49.67	74.18	< 0.0001	
X_3^2	13.90	1	13.90	20.76	0.0026	
Residual	4.69	7	0.6695			
Lack of Fit	3.51	3	1.17	3.99	0.1074	not significant
Pure Error	1.17	4	0.2936			
Cor Total	99.28	16				
Std. Dev.	0.8182		R^2	0.9528		
Mean	9.13		Adjusted R^2	0.8921		
C.V. %	8.96		Predicted R^2	0.4155		
			Adeq Precision	12.1945		

Meanwhile, for TFC, the model is also statistically significant, with a p-value of 0.0007 and an F-value of 15.70. Among the factors, extraction time (X_1, p = 0.0180) and solid-to-liquid ratio (X_3, p = 0.0043) were significant, while ethanol concentration (X_2) was not (p = 0.5219). Interaction effects were not significant except for X_2X_3 (p = 0.0433), suggesting that the interplay between X_2 and X_3 marginally affects TFC. Quadratic terms X_2^2 and X_3^2 were highly significant ($p < 0.01$), while X_1^2 was not (p = 0.1173), indicating that the effects of X_2 and X_3 on TFC are more complex. The lack of fit was not significant (p = 0.1074), further supporting the model's adequacy. The high R^2 value (0.9528) and adjusted R^2 value (0.8921) demonstrate the model's ability to explain variability in the data, though the predicted R^2 (0.4155) indicates a weaker predictive performance. However, the ANOVA results highlight the critical role of X_1 and X_2 in optimizing TPC and the importance of X_3 for TFC. The nonlinear effects of these factors, as revealed by significant quadratic terms, underscore the need for precise control and optimization during extraction processes. The lack of fit analysis supports the adequacy of the quadratic model for both responses, indicating it can reliably represent the experimental data.

Total phenolic content
$$+25.78 + 1.44X_1 - 1.26X_2 - 0.6475X_3 - 1.50X_1X_2 + 0.6125X_1X_3 + 0.8525X_2X_3 - 4.83X_1^2 - 3.00X_2^2 - 2.79X_3^2 \quad (2)$$

Total flavonoid content
$$+11.93 + 0.8887X_1 + 0.1950X_2 + 1.20X_3 - 0.2775X_1X_2 - 0.5750X_1X_3 - 1.01X_2X_3 - 0.7120X_1^2 - 3.43X_2^2 - 1.82X_3^2 \quad (3)$$

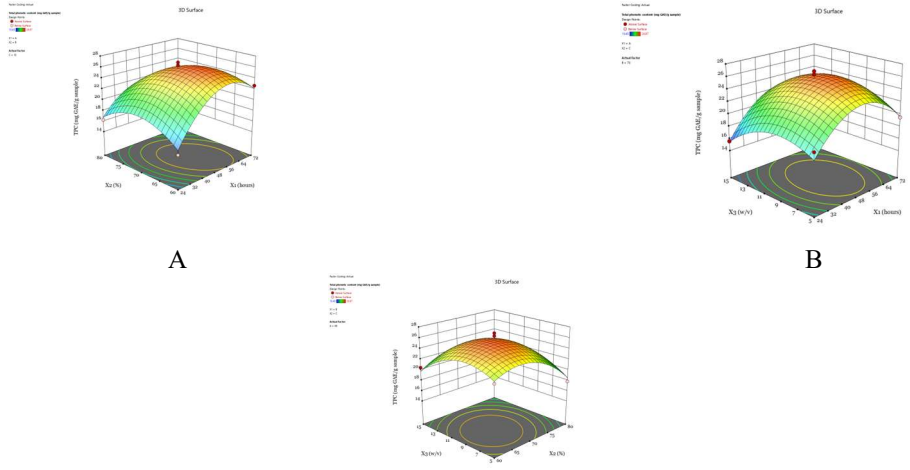
The final equations (Eq. 2 and Eq 3) in terms of coded factors provide a mathematical representation of the effects of extraction parameters on TPC) and TFC. These equations are crucial for predicting responses under various extraction conditions and understanding the relative impact of each factor. Equation 2 revealed that X_1 has a positive linear effect (+1.44), indicating that increasing X_1 enhances TPC. X_2 has a negative linear effect (-1.26), suggesting that higher X_2 may reduce phenolic extraction efficiency. X_3 also has a small negative effect (-0.6475), implying a minimal decrease in TPC with higher ratios. Additionally,

interaction terms like X_1X_2 (-1.50) indicate that combined effects of X_1 and X_2 negatively influence TPC, while X_1X_3 (+0.6125) and X_2X_3 (+0.8525) interactions are less impactful.

A B

Fig.-1: The Diagnostic Plot for Total Phenolic Content (A) and Total Flavonoid Content (B)

Afterward, quadratic terms (X_1^2, X_2^2, X_3^2) are all negative, demonstrating significant nonlinear relationships. Notably, X_1^2 (-4.83) has the strongest influence, reflecting a diminishing return on TPC with prolonged X_1. Related to Equation 2, Equation 3 presents that X_1 and X_3 positively affect TFC (+0.8887 and +1.20, respectively), highlighting their importance in enhancing flavonoid recovery. Meanwhile, X_2 has a minimal positive effect (+0.1950), indicating limited influence on TFC under the tested conditions. Additionally, interaction terms such as X_2X_3 (-1.01) suggest that the combined effect of X_2 and X_3 negatively impacts TFC, while X_1X_2 (-0.2775) and X_1X_3 (-0.5750) interactions are less pronounced. Whereas, quadratic terms (X_1^2, X_2^2, X_3^2) are negative, with X_2^2 (-3.43) having the largest coefficient, demonstrating the significant nonlinear effect of ethanol concentration on TFC. However, Equations 2 and 3 demonstrate that TPC and TFC are influenced by both linear and nonlinear relationships with X_1, X_2, and X_3. While X_1 and X_3 have a generally positive impact on both responses, X_2 shows a more complex behavior, with potential optimal ranges rather than a linear increase or decrease. The significance of interaction and quadratic terms underscores the necessity of optimizing these parameters to achieve maximum recovery. These equations provide a predictive tool for tailoring extraction processes and highlight the relative importance of each parameter and their interactions.



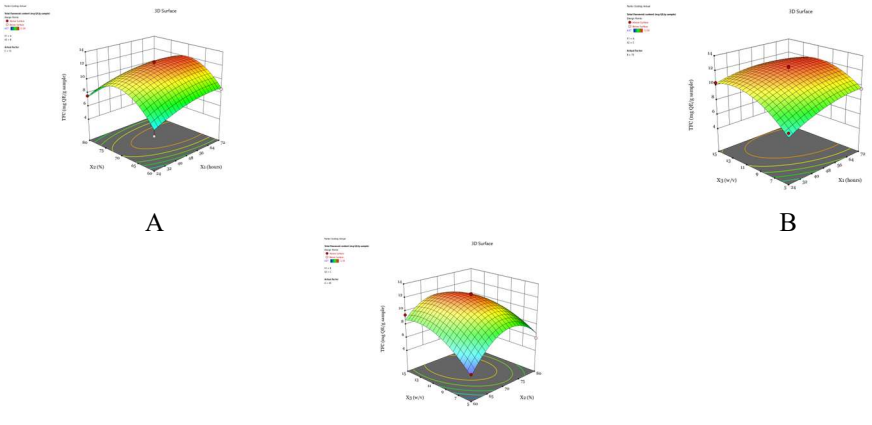
A B

C

Fig.-2: The Interaction of Each Extraction Factor on Total Phenolic Content Recovery. Interaction Between X_1 and X_2 (A). Interaction between X_1 and X_3 (B). Interaction between X_2 and X_3

Moreover, a recent study showed the diagnostic plot for TPC and TFC appears to be a residual versus predicted values plot in Fig.-1. The diagnostic plot for TPC and TFC illustrates the relationship between predicted and observed values, providing an evaluation of the regression model's performance. Figure-1A presents the alignment of most data points along the diagonal red line, suggesting that the model predictions closely match the observed values, indicating a good fit. The residuals appear to be evenly distributed around the line, without systematic patterns, confirming that the model satisfies the assumption of homoscedasticity.[32] The color gradient, ranging from blue (lower TPC values) to red (higher TPC values), demonstrates consistent model performance across the entire range of TPC data. The absence of significant

outliers or clusters further supports the robustness of the model. This aligns with the statistical findings that the quadratic regression model for TPC is significant and has strong fit metrics, as indicated by high R^2 and adjusted R^2 values from prior analyses. Meanwhile, Fig.-1B reveals the regression model for TFC demonstrates a strong fit, as most data points align closely with the diagonal line, indicating accurate predictions relative to observed values. The residuals are randomly distributed without discernible patterns, confirming the assumptions of homoscedasticity and linearity. Furthermore, the absence of significant outliers underscores the robustness of the model. The smooth color gradient from blue to red across the plot reflects the model's consistency in predicting TFC across its entire range, from lower to higher values. Overall, the quadratic regression model is reliable for interpreting TPC and TFC responses under the tested conditions.



A B C

Fig.-3: The Interaction of Each Extraction Factor on Total Flavonoid Content Recovery. Interaction Between X_1 and X_2 (A). Interaction between X_1 and X_3 (B). Interaction between X_2 and X_3

To illustrate the interaction of each extraction factor on the recovery of TPC and TFC, the current study refers to the data presented in Fig.-2 and 3. Figure-2 presents the interaction of each extraction factor on TPC recovery. The surface plot in Fig.-2A illustrates the interaction effects between X_1 and X_2 on the TPC. The curved shape of the surface indicates a nonlinear relationship, with the TPC values varying significantly depending on the levels of X_1 and X_2. The highest TPC value, approximately 26.87 mg GAE/g, is observed at intermediate levels of X_1 (around 48 hours) and X_2 (approximately 70%), as represented by the peak of the surface. This suggests an optimal combination of these two factors that maximizes phenolic recovery. At lower levels of X_1 (e.g., 24 hours) and X_2 (e.g., 60%), the TPC decreases significantly, with values as low as 15.45 mg GAE/g. Similarly, at higher levels of X_1 (e.g., 72 hours) and X_2 (e.g., 80%), the TPC also declines, with values dropping to around 17.46 mg GAE/g. This decline at extreme conditions may be due to insufficient compound solubilization at shorter extraction times or possible degradation of phenolics during prolonged exposure to higher concentrations.[33] In addition, the interaction between X_1 and X_3 on the TPC is presented in Fig.-2B. Figure-2B reveals a nonlinear relationship, with the highest TPC, approximately 26.87 mg QE/g, observed at an intermediate X_1 (around 48 hours) and a moderate X_3 (approximately 1:10). This highlights the importance of maintaining a balanced extraction duration and solvent-to-sample ratio to optimize phenolic recovery. At lower X_1 (e.g., 24 hours) and lower X_3 (e.g., 1:5), the TPC decreases significantly, likely due to insufficient solvent interaction and incomplete phenolic compound extraction. Similarly, at higher X_1 and higher X_3, the TPC also declines, potentially due to solvent oversaturation and degradation of phenolic compounds over extended durations.[34] Moreover, Fig.-2C illustrates a similar interaction between X_2 and X_3 with the previous interactions. However, the optimum TPC was gained at particular points of X_1, X_2, and X_3, respectively. The TPC will decrease if the crucial point of each factor leads to a decrease or an increase. Subsequently, the interaction between extraction factors was observed in TFC recovery. Figure-3A illustrates the interaction of X_1 and X_2 on the recovery of TFC. Overall, the result is not significantly different from the previous explanation. The TFC varies depending on the levels of X_1 and X_2. The highest TFC, approximately 12.58 mg QE/g, is observed at intermediate levels of X_1 (around 48 hours) and X_2 (around 70%), as represented by the peak of the surface.

At shorter X_1 (e.g., 24 hours) and lower X_2 (e.g., 60%), the TFC is significantly reduced, with values as low as 4.57 mg QE/g. Similarly, at longer X_1 (e.g., 72 hours) and higher X_2 (e.g., 80%), the TFC also declines. The decrease at longer extraction times may be due to flavonoid degradation, while higher ethanol concentrations could cause a polarity mismatch, reducing flavonoid solubility.[35] Similar conditions were observed in the other interaction between extraction factors and are presented in Fig.-3B and 3C. However, the bell-shaped surface indicates that a balance between extraction factors is critical. Excessively short or prolonged extraction times, higher or lower ethanol concentration, and very low or high solid-to-liquid ratios lead to suboptimal flavonoid yields.[36] These results emphasize the importance of carefully optimizing the interaction between extraction factors to maximize the recovery of phenolics and flavonoids.

The Solution of Extraction Conditions to Optimize of TPC and TFC from *Nelumbo nucifera*

Table-6 presents a solution for the optimization of extraction conditions for maximizing TPC and TFC using the BBD, providing a statistically robust solution for identifying optimal parameters. The proposed optimal extraction conditions include an X_1 of 53.87 hours, an X_2 of 69.03%, and an X_3 of 1:10.48 w/v. yielding a predicted TPC of 25.894 mg GAE/g and a TFC of 12.158 mg QE/g. These conditions achieve a high desirability score of 0.931, indicating the effectiveness of the optimization process in balancing both responses. However, this solution was performed to produce an *N. nucifera* extract in real experiments. Verification of extraction models was carried out by comparing the TPC and TFC in real experiments with prediction values. Table-6 demonstrates the TPC and TFC of *N. nucifera* extract in predicted values and real experiments under optimal conditions, and then conventional extraction (reflux). Upon experimental validation, the observed TPC and TFC values closely matched the predicted results, confirming the reliability of the model. T-test analysis exhibited that the TPC and TFC in real experiments are not significantly different from the predicted values ($p = 0.253$). Furthermore, the present study showed that the TPC and TFC of *N. nucifera* leaf extract under optimal conditions were significantly higher than the TPC and TFC of *N. nucifera* leaf extract under reflux (NCR) with $p < 0.05$, respectively. The higher TPC and TFC observed in the optimal extraction conditions suggest that this technique may be slightly more effective under the tested conditions, potentially due to better solvent penetration and prolonged contact with the plant matrix. In contrast, the reflux method yielded lower results, which might be due to the thermal degradation of sensitive compounds over extended heating periods.[12,37]

Table-6: Verification of Extraction Model

X_1 (hours)	X_2 (%)	X_3 (w/v)	TPC (mg GAE/g)			TFC (mg QE/g)			Desirability	
			P	E	R	P	E	R		
53.87	69.02	10.48	25.89	26.35 ± 2.04	15.78 ± 1.40	12.15	13.67 ± 1.82	10.34 ± 0.81	0.93	Selected

P means predicted value; E is means experimental value; R is means reflux method

The Antioxidant Activity of *Nelumbo nucifera* Leaf Extract

The antioxidant activity of *N. nucifera* leaf extracts is revealed by inhibiting the radical DPPH. Figure-4 demonstrates the % inhibition of DPPH after administration of PRN and NCR in various concentrations. The results indicate a concentration-dependent increase in DPPH scavenging activity for all samples ($p = 0.0021$). At the lowest concentration (31.25 µg/mL), NCR exhibited a mean inhibition of 16.51 ± 0.85%, PRN showed 30.89 ± 2.41%, and ascorbic acid achieved the highest inhibition of 56.14 ± 1.84%, demonstrating its significantly superior antioxidant activity. Statistical analysis using ANOVA revealed significant differences ($p < 0.05$) in DPPH inhibition between the three samples at all tested concentrations, indicating the distinct efficacy of each sample. Post hoc tests further confirmed that ascorbic acid consistently exhibited significantly higher scavenging activity than both NCR and PRN. Additionally, PRN demonstrated significantly greater inhibition compared to NCR at all concentrations, highlighting its stronger antioxidant potential. At the highest concentration (500 µg/mL), the inhibition values reached 71.49 ± 1.25% for NCR, 84.23 ± 1.64% for PRN, and 92.16 ± 1.50% for ascorbic acid. These results underline the superior radical scavenging ability of ascorbic acid, which served as the positive control. The statistical findings validate the effectiveness of PRN as a promising antioxidant, with performance significantly better than NCR, though slightly less potent than ascorbic acid. The differences between the

samples are statistically robust, highlighting the potential application of PRN as a natural antioxidant source.

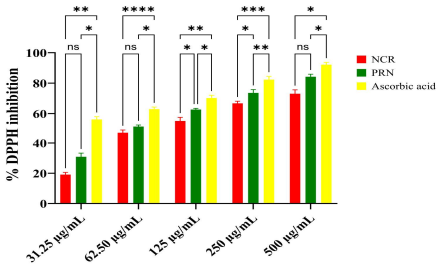
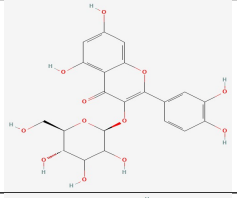
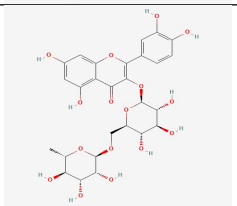
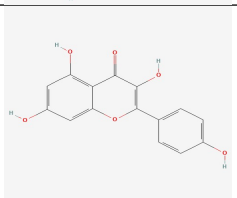
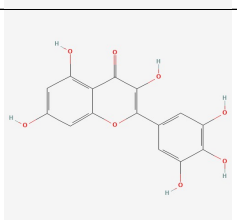
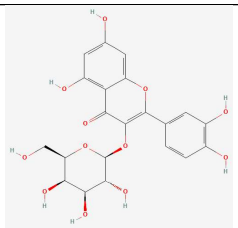
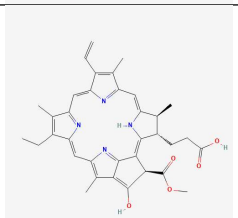
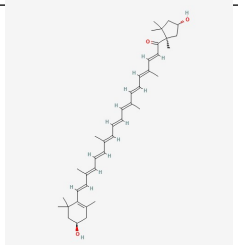


Fig.-4: The Percentage Inhibition of the Sample Against DPPH Radical in Various Concentrations between 31.25 to 500 µg/mL. * Indicates the Result is Significantly Different with $p < 0.05$, ** Indicates the Result is Significantly different with $p < 0.01$, *** Indicates the Result is Significantly Different with $p < 0.001$, **** Indicates the Result is Significantly Different with $p < 0.0001$, and ^{ns} Indicates the Result is not Significantly different with $p > 0.05$

These findings align with previous studies that reported the strong antioxidant activity of ascorbic acid due to its ability to donate electrons and neutralize free radicals efficiently.[38,39] Similarly, research on phenolic-rich plant extracts, such as PRN, has demonstrated significant antioxidant activity attributed to the presence of bioactive compounds like flavonoids and phenolic acids. Previous studies confirmed that *N. nucifera* extract has exhibited antioxidant activity.[40-42] Methanol extracts showed significant antioxidant properties, including free radical scavenging, metal binding, and reducing power.[43] Various extracts exhibited dose-dependent DPPH radical scavenging activity.[44-46] The acetone extract of *N. nucifera* seeds displayed the highest total phenolic content and antioxidant activity.[47] The leaf extract also protected erythrocytes against oxidative stress-induced hemolysis.[48] Additionally, *N. nucifera* extracts showed hepatoprotective effects in vivo, likely due to their antioxidant properties.[49] However, the antioxidant activity of *N. nucifera* extracts has been attributed to the presence of phenolic compounds.[50]

Table-7: Polyphenol Profile of Polyphenol-Rich Extract of *Nelumbo nucifera* Leaf

No	Name	Formula	Retention time (RT)	Chemical structure
1	Quercetin-3-β-D-glucoside	$C_{21}H_{20}O_{12}$	5.741	
2	Rutin	$C_{27}H_{30}O_{16}$	5.551	
3	Kaempferol	$C_{15}H_{10}O_6$	7.617	
4	Myricetin	$C_{15}H_{10}O_8$	5.138	

5	Hyperoside	$C_{21}H_{20}O_{12}$	5.287	
6	Pheophorbide A	$C_{35}H_{36}N_4O_5$	14.944	
7	Capsanthin	$C_{40}H_{56}O_3$	16.813	

Polyphenol Compound Contained in Polyphenol-Rich Extract of *Nelumbo nucifera*

The LC-HRMS analysis of PRN identified a diverse range of polyphenolic compounds with significant bioactive potential, which is presented in **Table-7**. Key polyphenols detected include quercetin-3-β-D-glucoside ($C_{21}H_{20}O_{12}$, RT 5.741 min), rutin ($C_{27}H_{30}O_{16}$, RT 5.551 min), kaempferol ($C_{15}H_{10}O_6$, RT 7.617 min), myricetin ($C_{15}H_{10}O_8$, RT 5.138 min), and hyperoside ($C_{21}H_{20}O_{12}$, RT 5.287 min). Similar to previous reports presented *N. nucifera* leaf extract contains various polyphenol compounds involving kaempferol, quercetin, myrcetin, and hyperoside.[51,52] Additionally, these compounds are well-known for their antioxidant, anti-inflammatory, and therapeutic properties.[53,54] The identification of cianidanol ($C_{15}H_{14}O_6$, RT 4.528 min), dihydroxymandelic acid ($C_8H_8O_5$, RT 1.158 min), and 4-coumaric acid ($C_9H_8O_3$, RT 3.790 min) further supports the extract's potential as a source of bioactive molecules. These phenolic acids and flavonoids are recognized for their ability to scavenge free radicals, inhibit lipid peroxidation, and modulate signaling pathways associated with oxidative stress.[55-57] Several other compounds, such as pheophorbide A ($C_{35}H_{36}N_4O_5$, RT 14.944 min) and capsanthin ($C_{40}H_{56}O_3$, RT 16.813 min), add to the complexity of the extract, potentially contributing to its bioactive profile. These chlorophyll derivatives and carotenoids are associated with anti-inflammatory and photoprotective activities.[58,59] The LC-HRMS analysis reveals that PRN is a rich source of polyphenols, flavonoids, and related bioactives, underscoring its potential for pharmaceutical, nutraceutical, and cosmetic applications.

CONCLUSION

This study successfully optimized the extraction conditions of *N. nucifera* leaf using BBD to enhance the recovery of polyphenols and flavonoids, which are responsible for its antioxidant activity. The optimal conditions, 53.87 hours extraction time, 69.03% ethanol concentration, and a 1:10.48 solid-to-liquid ratio, yielded significantly higher total phenolic and flavonoid contents compared to conventional reflux methods. The antioxidant activity of the optimized extract showed dose-dependent radical scavenging capacity, outperforming the non-optimized extract, as confirmed by DPPH inhibition assays. Furthermore, LC-HRMS profiling identified key bioactive compounds such as quercetin-3-β-D-glucoside, rutin, kaempferol, and myricetin, supporting the extract's potent antioxidant properties. These findings highlight the value of optimizing extraction processes to enhance the functionality of natural products. Importantly, this research supports Sustainable Development Goal (SDG) 3, Good Health and Well-being, by promoting the utilization of plant-based antioxidants as safer alternatives to synthetic agents, contributing to preventive healthcare and nutraceutical development.

ACKNOWLEDGMENTS

The authors would like to thank you Universitas Sumatera Utara and Institut Kesehatan Helvetia for providing a laboratory for this test. Additionally, the authors would like to thank the National Research and Innovation Agency (BRIN, Indonesia) for helping to analyze the polyphenol compounds using LC-HRMS.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3: Good Health and Well-being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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