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Contour Plot-Based Interpretation of Factor Effects on Total Flavonoid Content in *Morus alba* var. *shalun* Using Box-Behnken Design

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Abstract. *Morus alba* var. *shalun* is recognized as a potential source of flavonoids with important pharmacological properties. However, studies focusing on the statistical interpretation of extraction variables and contour plot analysis for flavonoid extraction from this plant remain limited. This study employed Response Surface Methodology (RSM) using a Box–Behnken design to evaluate the effects of ethanol concentration, extraction temperature, and extraction time on total flavonoid content (TFC). A quadratic regression model was analyzed using ANOVA, residual diagnostics, and contour plot visualization to examine the relationship between extraction variables and response behavior. The results showed that ethanol concentration had the strongest influence on TFC, followed by extraction time and temperature, whereas interaction effects among variables were relatively insignificant. The developed model demonstrated good statistical performance with an R^2 value of 0.9723. Residual and Box–Cox analyses confirmed the adequacy and reliability of the model for describing flavonoid extraction characteristics in *Morus alba* var. *shalun.*

Keywords: *Morus alba* var. *shalun*, Flavonoid extraction, Box–Behnken design, Contour plot, Response surface methodology

Abstrak. *Morus alba* var. *shalun* dikenal sebagai sumber flavonoid yang memiliki aktivitas farmakologis penting. Namun, penelitian yang berfokus pada interpretasi statistik variabel ekstraksi dan analisis contour plot pada ekstraksi flavonoid tanaman ini masih terbatas. Penelitian ini menggunakan Response Surface Methodology (RSM) dengan Box–Behnken design untuk mengevaluasi pengaruh konsentrasi etanol, suhu ekstraksi, dan waktu ekstraksi terhadap total flavonoid content (TFC). Model regresi kuadratik dianalisis menggunakan ANOVA, diagnostik residual, dan visualisasi contour plot untuk mengkaji hubungan antara variabel ekstraksi dan perilaku respons. Hasil penelitian menunjukkan bahwa konsentrasi etanol memberikan pengaruh terbesar terhadap TFC, diikuti oleh waktu ekstraksi dan suhu ekstraksi, sedangkan efek interaksi antarvariabel relatif tidak signifikan. Model yang dikembangkan menunjukkan performa statistik yang baik dengan nilai R^2 sebesar 0,9723. Analisis residual dan Box–Cox mengonfirmasi kecukupan dan reliabilitas model dalam menggambarkan karakteristik ekstraksi flavonoid pada *Morus alba* var. *shalun*.

Kata kunci: *Morus alba* var. *shalun*, Ekstraksi flavonoid, Box-behnken design, Contour plot, Response surface methodology

1. Introduction

Morus alba is widely recognized as a medicinal plant with important pharmacological properties and has been traditionally utilized in many Asian countries for herbal remedies. The biological activities of this plant are strongly associated with the presence of various phytochemical constituents, particularly flavonoids, which exhibit antioxidant, anti-inflammatory, and antidiabetic properties. Because flavonoids contribute substantially to the therapeutic value of *Morus alba*, total flavonoid content (TFC) is frequently used as an important indicator for assessing the medicinal quality and functional potential of the plant [1–2]. Therefore, studies related to flavonoid extraction are essential for improving the utilization and characterization of *Morus alba* based natural products.

The recovery of flavonoid compounds from plant matrices is affected by several extraction variables, including solvent concentration, extraction temperature, and extraction duration. These parameters influence solvent penetration, solubility of bioactive compounds, diffusion efficiency, and compound stability during extraction. Conventional extraction studies commonly rely on single-factor experiments; however, this approach is limited because it cannot effectively describe the interaction effects among variables and often requires extensive experimental trials. Consequently, statistical approaches such as Response Surface Methodology (RSM) have become increasingly important for evaluating extraction systems and describing the combined influence of multiple variables simultaneously [3].

Among the available RSM models, the Box–Behnken design (BBD) is considered advantageous because it provides efficient experimental arrangements with fewer runs while still allowing evaluation of linear, interaction, and quadratic effects among variables [4]. In addition, graphical analyses generated from RSM, particularly contour plots, provide clearer visualization of the relationship between extraction variables and response values. Contour plots are useful for interpreting variable interactions and identifying response distribution tendencies that may not be easily observed through numerical analysis alone [5]. Nevertheless, studies specifically discussing contour plot interpretation for total flavonoid extraction from *Morus alba* var. *shalun* are still limited. Furthermore, previous reports have generally emphasized extraction outcomes, whereas detailed statistical interpretation involving residual diagnostics, regression coefficient behavior, and response surface characteristics has not been extensively explored. This limitation highlights both the research gap and analytical gap in the statistical evaluation of flavonoid extraction from *Morus alba* var. *shalun*. Therefore, this study was conducted to analyze the effects of ethanol concentration, extraction temperature, and extraction time on the total flavonoid content of *Morus alba* var. *shalun* using a Box–Behnken experimental design. In addition, contour plot interpretation, regression coefficient analysis, and residual diagnostic evaluation were performed to examine the interaction behavior among variables and to assess the adequacy of the developed quadratic model in describing flavonoid extraction characteristics.

2. Method and Experimental

The extraction of total flavonoid content (TFC) from *Morus alba* var. *shalun* was evaluated using Response Surface Methodology (RSM) based on a Box–Behnken Design (BBD). The BBD approach was selected because it is efficient for evaluating quadratic response surfaces while requiring a relatively small number of experimental runs. This design is commonly used to investigate the effects of multiple variables and their interactions in extraction studies, as well as to develop predictive statistical models [6]. In this study, three independent variables were investigated, namely ethanol concentration (A), extraction temperature (B), and extraction time (C), each examined at three levels. The experimental design consisted of 17 runs, including several center point replicates to estimate experimental error and evaluate model reliability. The extraction process was carried out using ethanol as the extraction solvent due to its effectiveness in extracting flavonoid compounds. For each experimental run, the extraction conditions were adjusted according to the Box–Behnken design matrix with different combinations of ethanol concentration, extraction temperature, and extraction time. The obtained extracts were subsequently analyzed for total flavonoid content, and the measured TFC values were used as the response variable for statistical analysis. The experimental results showed that TFC values ranged from 19.90 to 27.70 mg QE/g, indicating that the extraction conditions influenced flavonoid recovery. The experimental data were fitted to a quadratic polynomial model and analyzed using analysis of variance (ANOVA) to evaluate the significance and adequacy of the developed model. The model exhibited an F-value of 27.29 with a p-value of 0.0001, indicating that the model was statistically significant and that the probability of the result occurring due to random error was very low [6–11]. The coefficient of determination (R^2) was 0.9723, demonstrating that 97.23% of the variability in total flavonoid content could be explained by the model.

In addition, the adjusted R^2 value of 0.9367 indicated good agreement between predicted and experimental data. The adequate precision value of 18.722, which was greater than 4, confirmed that the model had an adequate signal-to-noise ratio and could adequately describe the experimental design space. The regression analysis showed that ethanol concentration (A), extraction temperature (B), and extraction time (C) significantly affected total flavonoid content ($p < 0.05$). Among the investigated factors, ethanol concentration showed the strongest influence, followed by extraction time and temperature. Furthermore, the quadratic terms A^2 and C^2 were statistically significant, indicating curvature effects within the experimental range. In contrast, the interaction terms between variables were statistically insignificant, suggesting that the individual effects of the factors were more dominant than their interaction effects. The regression coefficients demonstrated that the linear terms positively contributed to the response, whereas the negative coefficients of the quadratic terms suggested a decrease in TFC values beyond certain experimental conditions. The variance inflation factor (VIF) values were equal to 1, indicating the absence of multicollinearity among the independent variables and confirming the robustness of the developed model [7]. Although the predicted R^2 value (0.5566) was lower than the adjusted R^2 value, indicating moderate predictive capability, the model was still considered adequate for describing the experimental data. To facilitate interpretation of the results, contour and response surface plots were generated from the quadratic model. These plots were used to visualize the relationship between extraction variables and total flavonoid content, as well as to illustrate the distribution of the response within the studied experimental range. Response surface analysis is widely used to interpret factor effects and interactions in extraction studies [8].

3. Results and Discussion

The optimization of total flavonoid content (TFC) from *Morus alba* var. *shalun* was successfully evaluated using a quadratic model developed through the Box–Behnken design. The experimental results demonstrated a substantial variation in TFC values, ranging from 19.90 to 27.70, indicating that the selected process variables significantly influenced flavonoid extraction efficiency. The highest TFC value (27.70) was observed under specific experimental conditions, while the lowest value (19.90) suggested suboptimal extraction parameters. To assess the adequacy of the developed model, an analysis of variance (ANOVA) was performed, and the results are presented in Table 1. The model exhibited a high F-value of 27.29 with a p-value of 0.0001, confirming that the quadratic model was statistically significant. This indicates that the probability of the model being affected by random noise is extremely low. Furthermore, the coefficient of determination ($R^2 = 0.9723$) suggested that more than 97% of the variability in the response could be explained by the model, demonstrating excellent model fit. The adjusted R^2 value (0.9367) was also in good agreement with R^2, indicating the reliability of the model in explaining the experimental data.

Table 1 ANOVA for Quadratic Model of Total Flavonoid Content

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	77.19	9	8.58	27.29	0.0001
A (Ethanol concentration)	33.62	1	33.62	106.97	<0.0001
B (Temperature)	8.82	1	8.82	28.06	0.0011
C (Time)	21.78	1	21.78	69.30	<0.0001
AB	0.0000	1	0.0000	0.0000	1.0000
AC	0.0100	1	0.0100	0.0318	0.8635
BC	0.4900	1	0.4900	1.56	0.2519
A^2	4.64	1	4.64	14.77	0.0063
B^2	1.42E-14	1	1.42E-14	~0	1.0000
C^2	7.12	1	7.12	22.64	0.0021
Residual	2.20	7	0.3143	–	–

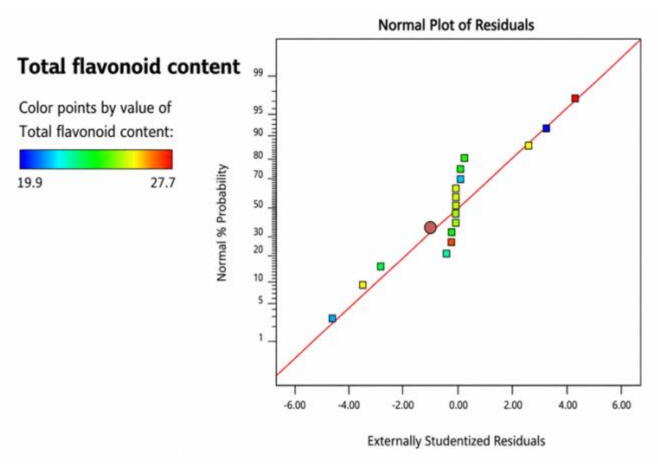
The statistical analysis revealed that all linear factors (A, B, and C) significantly affected TFC ($p < 0.05$). Among them, ethanol concentration (A) had the most dominant effect, as indicated by the highest F-value (106.97), followed by extraction time (C) and temperature (B). This finding suggests that solvent polarity plays a crucial role in the solubility and extraction of flavonoids, which is consistent with previous studies reporting ethanol concentration as a key factor in phytochemical extraction [9]. In addition to linear effects, the quadratic terms of ethanol concentration (A^2) and extraction time (C^2) were also significant, indicating the presence of curvature in the response surface. This implies that increasing these variables beyond certain levels may lead to a decrease in TFC, possibly due to degradation of flavonoid compounds at extreme conditions. On the other hand, the interaction terms (AB, AC, and BC) were not statistically significant, suggesting that the combined effects of variables were relatively weak compared to their individual contributions. The regression coefficients of the model further support these findings and are presented in Table 2. The positive coefficients of the linear terms indicate that increasing ethanol concentration, temperature, and time initially enhances flavonoid extraction. However, the negative coefficients of A^2 and C^2 confirm the existence of optimal conditions beyond which the response declines.

Table 2 presents the regression coefficients of the quadratic model generated from the Box–Behnken Design (BBD) for predicting total flavonoid content (TFC). The regression analysis showed that the intercept value was 25.30, indicating the average predicted response of TFC under the experimental conditions. Among the linear terms, factor A (ethanol concentration) exhibited the highest positive coefficient (2.05), followed by factor C (extraction time) with a coefficient of 1.65, and factor B (temperature) with a coefficient of 1.05. These positive coefficient values indicated that increasing ethanol concentration, extraction temperature, and extraction time tended to increase the total flavonoid content.

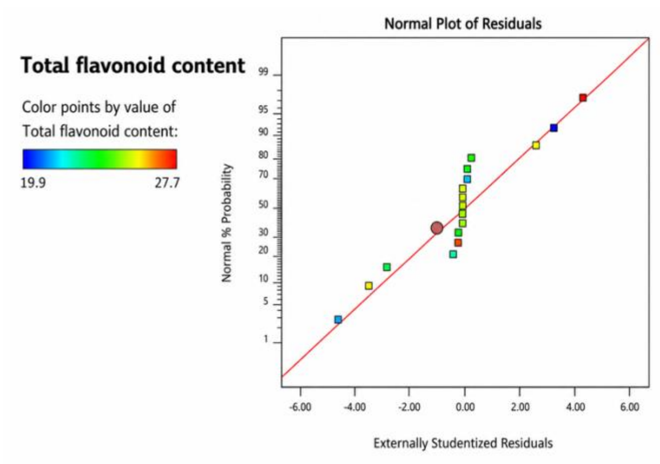
Table 2 Regression Coefficients of the Quadratic Model

Factor	Coefficient	Standart Error	95% CI Low	95% CI High
Intercept	25.30	0.2507	24.71	25.89
A	2.05	0.1982	1.58	2.52
B	1.05	0.1982	0.5813	1.52
C	1.65	0.1982	1.18	2.12
AB	0.0000	0.2803	-0.6628	0.6628
AC	0.0500	0.2803	-0.6128	0.7128
BC	0.3500	0.2803	-0.3128	1.01
A^2	-1.05	0.2732	-1.70	-0.4040
B^2	0.0000	0.2732	-0.6460	0.6460
C^2	-1.30	0.2732	-1.95	-0.6540

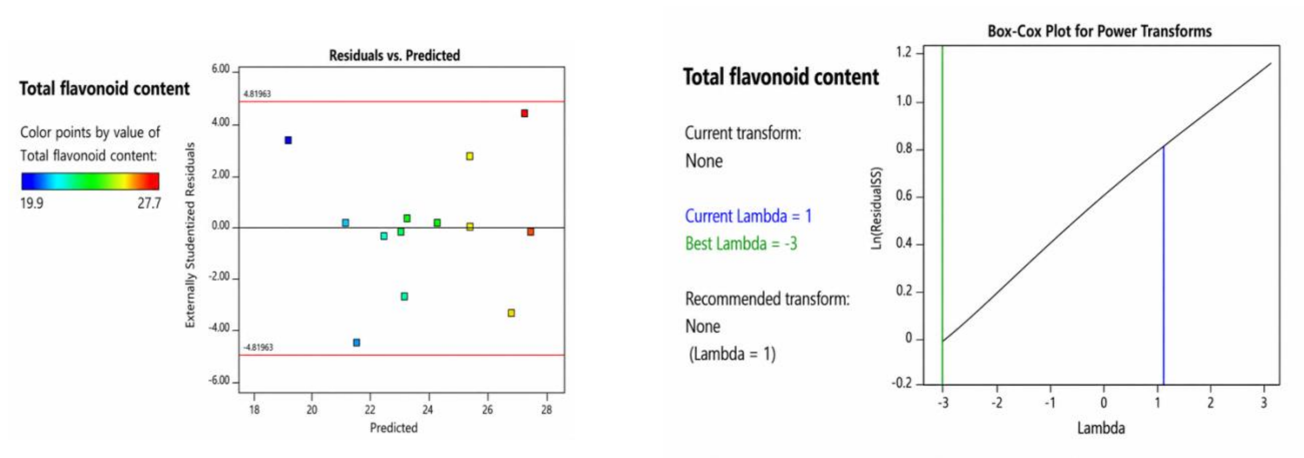
The interaction terms AB, AC, and BC showed relatively small coefficient values compared to the linear factors. The AB interaction coefficient was 0.0000, indicating no interaction effect between ethanol concentration and temperature on TFC. Meanwhile, the AC and BC interaction terms showed minor positive effects, suggesting limited interaction contributions to the response variability. For the quadratic terms, negative coefficient values were observed for A^2 (−1.05) and C^2 (−1.30), indicating the presence of curvature effects in the model. This suggested that excessively high ethanol concentration and extraction time could reduce the extraction efficiency of flavonoid compounds after reaching optimum conditions. In contrast, the quadratic term B^2 showed a coefficient value of 0.0000, indicating that temperature did not exhibit a strong quadratic effect within the studied range. The 95% confidence intervals (CI) further supported the reliability of the developed model. The confidence interval ranges for the significant linear and quadratic terms did not cross zero, indicating that these variables contributed meaningfully to the prediction of total flavonoid content. Overall, the regression coefficient analysis demonstrated that ethanol concentration and extraction time were the most influential factors affecting flavonoid extraction efficiency, while the interaction effects among variables were relatively weak.



(a)



(b)



(c) (d)

Fig 1. Diagnostic plots of the developed Box–Behnken Design (BBD) model for total flavonoid content (TFC): (a) normal plot of residuals, (b) residuals versus predicted values, (c) externally studentized residuals distribution, and (d) Box–Cox plot for power transformation analysis.

Figure 1a presents the normal plot of residuals for total flavonoid content generated from the 17 experimental runs of the Box–Behnken Design (BBD). The residual points were distributed relatively close to the diagonal reference line, indicating that the residuals followed an approximately normal distribution. This result confirmed that the developed quadratic model satisfied the normality assumption required in response surface methodology (RSM). No significant deviation or extreme outlier was observed, demonstrating the adequacy and reliability of the model for predicting total flavonoid content. Figure 1b shows the residuals versus predicted values plot of total flavonoid content. The residual points were randomly scattered around the zero line without forming a clear systematic pattern, indicating constant variance and good agreement between experimental and predicted values. Most residuals were located within the acceptable limit range (±4.81963), suggesting that the model did not exhibit significant heteroscedasticity and was statistically suitable for optimization analysis. Figure 1c further illustrates the distribution of externally studentized residuals against predicted values. The random dispersion of residual points and the absence of funnel-shaped or curved patterns indicated that no serious model inadequacy was detected.

In addition, all observations remained within the upper and lower residual limits, confirming that the developed BBD model possessed satisfactory predictive performance and stability for evaluating the extraction conditions affecting total flavonoid content. Figure 1d presents the Box–Cox plot for power transformation of total flavonoid content obtained from the Box–Behnken Design (BBD) model. The Box–Cox analysis was performed to determine whether transformation of the response data was necessary to improve model normality and variance stability. Based on the plot, the current lambda value was 1, indicating that the response data were analyzed using the original scale without transformation. Although the best lambda value was identified at −3, the software recommended no transformation because the current lambda ($\lambda = 1$) was still within the acceptable confidence interval range. This result suggests that the experimental data already exhibited adequate normality and homoscedasticity, consistent with the residual diagnostic plots shown previously. Therefore, transformation of the total flavonoid content data was not required, and the developed quadratic model was considered statistically adequate for response surface methodology (RSM) analysis and optimization. The Box–Cox plot further confirmed that the model predictions were reliable and suitable for evaluating the effects of ethanol concentration, extraction temperature, and extraction time on total flavonoid content.

4. Conclusion

The present study demonstrated that ethanol concentration, extraction temperature, and extraction time significantly influenced the total flavonoid content (TFC) of *Morus alba* var. *shalun*. Analysis using the Box–Behnken design successfully described the relationship between extraction variables and flavonoid response through a quadratic regression model with satisfactory statistical performance. The developed model showed high reliability, as indicated by significant ANOVA results, high coefficient of determination (R^2), and acceptable residual diagnostic characteristics. Among the investigated variables, ethanol concentration exhibited the strongest effect on total flavonoid content, followed by extraction time and extraction temperature.

The regression analysis further revealed that the quadratic terms of ethanol concentration and extraction time contributed significantly to the response behavior, indicating the presence of curvature effects within the studied experimental range. In contrast, the interaction effects among variables were relatively weak compared to the individual contributions of each factor. Contour plot analysis and residual diagnostic evaluation provided clearer interpretation of the interaction behavior and statistical adequacy of the developed model. The residual plots confirmed that the model fulfilled the assumptions of normality and homoscedasticity, while the Box–Cox analysis indicated that transformation of the response data was unnecessary. Overall, this study demonstrated that the Box–Behnken design combined with statistical visualization analysis can effectively be used to evaluate flavonoid extraction behavior in *Morus alba* var. *shalun* and provide comprehensive interpretation of extraction-related variables.

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