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Original Article

Response surface methodology optimization of phenolic and flavonoid recovery from *Ruta montana* and their antioxidant and antibacterial activities

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Abstract:

This study aimed to optimize the extraction conditions of phenolic and flavonoid compounds from the aerial parts of *Ruta montana* L. (Rutaceae) and to evaluate their associated antioxidant and antibacterial activities. Response Surface Methodology (RSM) based on a Central Composite Design (CCD) was employed to investigate the combined effects of extraction temperature, extraction time, solid-to-liquid ratio, and methanol concentration on total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging activity, and antibacterial activity against *Staphylococcus aureus*. Quadratic polynomial models were developed for all responses and demonstrated high predictive accuracy, with coefficients of determination (R^2) exceeding 0.92 and non-significant lack of fit values. Multi-response optimization using a desirability function identified optimal extraction conditions of 68 °C, 3 h extraction time, a solid-to-liquid ratio of 1.53:10 (g/mL), and 80% methanol. Under these conditions, the extracts exhibited high TPC (376.26 µg GAE/g DW), TFC (159.17 µg QE/g DW), strong antioxidant activity (86.72% DPPH inhibition), and notable antibacterial activity (14 mm inhibition zone). Experimental validation confirmed close agreement between predicted and observed values. These findings demonstrate that *R. montana* is a valuable source of bioactive phenolic compounds and highlight the effectiveness of RSM for optimizing extraction processes to enhance both chemical yield and biological activity.

Key words:
Ruta montana L; response surface methodology; central composite design; optimization; DPPH scavenging activity; antibacterial activity.

Apstrakt:

Naslov na srpskom

Cilj ovog istraživanja bio je optimizacija uslova ekstrakcije fenolnih i flavonoidnih jedinjenja iz nadzemnih delova biljke *Ruta montana* L. (Rutaceae) i procena njihove antioksidativne i antibakterijske aktivnosti. Za ispitivanje kombinovanog uticaja temperature ekstrakcije, vremena ekstrakcije, odnosa čvrste i tečne faze i koncentracije metanola na ukupan sadržaj fenola (TPC), ukupan sadržaj flavonoida (TFC), sposobnost neutralizacije DPPH slobodnih radikala i antibakterijsku aktivnost prema *Staphylococcus aureus* primenjena je metodologija odzivne površine (Response Surface Methodology, RSM) zasnovana na centralnom kompozitnom eksperimentalnom planu (Central Composite Design, CCD). Za sve ispitivane odgovore razvijeni su kvadratni polinomni modeli koji su pokazali visoku prediktivnu tačnost, sa koeficijentima determinacije (R^2) većim od 0,92 i statistički neznačajnim odstupanjem modela od eksperimentalnih podataka (lack of fit). Višekriterijumska optimizacija primenom funkcije poželjnosti identifikovala je optimalne uslove ekstrakcije: temperaturu od 68 °C, vreme ekstrakcije od 3 sata, odnos čvrste i tečne faze 1,53:10 (g/mL) i 80% metanol. Pod ovim uslovima ekstrakti su pokazali visok ukupan sadržaj fenola (376,26 µg GAE/g suve mase), ukupan sadržaj flavonoida (159,17 µg QE/g suve mase), izraženu antioksidativnu aktivnost (86,72% inhibicije DPPH radikala) i značajnu antibakterijsku aktivnost (zona inhibicije od 14 mm). Eksperimentalna validacija potvrdila je dobro slaganje između predviđenih i eksperimentalno dobijenih vrednosti. Dobijeni rezultati pokazuju da je *R. montana* značajan izvor bioaktivnih fenolnih jedinjenja i potvrđuju efikasnost metodologije odzivne površine u optimizaciji procesa ekstrakcije radi povećanja prinosa bioaktivnih jedinjenja i njihove biološke aktivnosti.

Ključne reči:
Ruta montana L.; metodologija odzivne površine; centralni kompozitni dizajn; optimizacija; aktivnost neutralizacije DPPH radikala; antibakterijska aktivnost.

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Introduction

Medicinal plant extracts contain phytochemicals with diverse biological activities, including antioxidant, anti-inflammatory, antimicrobial, antiviral, cardioprotective, and neuroprotective properties (Bhanwala et al., 2025). These benefits are largely mediated by multiple molecular mechanisms, including free radical scavenging, transition metal chelation, disruption of microbial cell membranes, inhibition of key enzymatic activities, and modulation of critical cellular signaling pathways (Al-Khayri et al., 2022; Rudrapal et al., 2022). Extracts with higher phenolic concentrations consistently exhibit enhanced bioactivity across various plant species (Takó et al., 2020; Nurzyńska-Wierdak, 2023; El Allaoui et al., 2024; Montri et al., 2025).

The extraction of bioactive compounds from plant matrices presents significant scientific challenges due to complex interactions between extraction parameters and the physicochemical properties of target compounds (Alara et al., 2021). Solvent polarity, temperature, extraction duration, and solid-to-liquid ratio critically influence not only extraction yield but also the chemical profile and biological efficacy of extracts. Solvent polarity determines selectivity toward specific compound classes, with polar solvents efficiently extracting hydrophilic phenolics and flavonoids, whereas solvent mixtures often achieve superior recovery of diverse phytochemical groups (Ghaffar et al., 2025). Temperature plays a dual role: elevated temperatures enhance solubility, reduce viscosity, and improve mass transfer rates, thereby accelerating extraction kinetics; however, excessive temperatures or prolonged exposure may induce thermal degradation of heat-sensitive phenolic compounds, particularly flavonoids and certain phenolic acids (Hobbi et al., 2021). This complexity is especially relevant for plants such as *Ruta montana*, which contains diverse compound classes with varying polarities and thermal stabilities (Szewczyk et al., 2023). The solid-to-liquid ratio also significantly affects extraction efficiency and yield, with optimization studies demonstrating that carefully balanced ratios maximize phenolic recovery while maintaining compound stability (Ramesh et al., 2024).

Ruta montana L., an ethnomedicinal plant endemic to North Africa, plays an important role in Algerian and Moroccan traditional medicine, where it is commonly used to manage inflammatory disorders, microbial infections, and hypertension (El-Ouady et al., 2021). Although this longstanding use highlights its therapeutic relevance, research has only partially explored how extraction conditions influence the recovery of its key bioactive constituents and, consequently, its biological performance.

Phytochemical investigations of Moroccan and Algerian populations of *R. montana* consistently report high total phenolic and flavonoid contents, with extracts characterized by phenolic acids (protocatechuic acid, *p*-coumaric acid, salicylic acid), coumarins (hesperidin), and flavonoids (isoquercitrin, kaempferol-3-glucoside, luteolin)—compounds that are repeatedly associated with strong biological activity across multiple bioassays (Merghem et al., 2020; Szewczyk et al., 2023; Bouhenna et al., 2024). Nevertheless, despite increasing clarity regarding chemical profile, the extraction conditions required to simultaneously maximize multiple bioactive compounds and their linked biological activities in *R. montana* have not yet been systematically optimized (Aouzal et al., 2024).

Response Surface Methodology (RSM), particularly when implemented via a Central Composite Design (CCD), provides a statistically robust framework for modeling the combined effects of extraction variables and for identifying optimal operating conditions with a reduced number of experiments (Said et al., 2015). As a multivariate optimization approach, RSM enables simultaneous evaluation of main effects and factor interactions, supports accurate response prediction through fitted second-order polynomial models, and improves experimental efficiency by reducing cost and minimizing experimental error (Reji et al., 2022). Compared with alternative designs such as the Box–Behnken design, CCD can provide a more precise estimation of two-factor interaction terms because it includes axial points, although both designs show strong statistical performance when center points are properly replicated. Moreover, the quadratic models produced by RSM are particularly effective for optimizing multiple responses—such as phenolic yield, antioxidant capacity, and antimicrobial activity—using desirability function approaches that integrate multi-response optimization within a single analytical framework (Weremfo et al., 2023; Almeida et al., 2025; Ponphaiboon et al., 2026).

The objective of this study was to systematically investigate optimal extraction conditions to maximize the total phenolic and flavonoid yields from *R. montana* aerial parts while simultaneously enhancing their antioxidant and antibacterial activities. A CCD-based RSM approach was employed to optimize temperature, extraction time, methanol concentration, and solid-to-liquid ratio.

Materials and Methods

Plant Sample Preparation

Fresh aerial parts of *Ruta montana* (Clus.) L.,

were collected during the peak flowering period from T'kout, Batna province, Algeria (35°30'00"N 6°23'00"E, altitude 950 m). The plant material was taxonomically authenticated by Dr. A. Zeraib, and a voucher specimen (RM-2022-05) was deposited at the Herbarium of the Faculty of Natural and Life Sciences, University of Biskra. The collected samples were washed with tap water and shade-dried at 25 °C for 10 days. The dried material was then cut into small pieces, ground into a fine powder using a blender (IKA Werke, Germany), and sieved to obtain a uniform particle size of 500 µm. Finally, the resulting powder was stored in amber glass containers, protected from light, at ambient temperature until further use.

Experimental design

The optimization of the extraction process of *R. montana* aerial parts was performed using Response Surface Methodology (RSM). The experiment was conducted according to a Central Composite Design (CCD) using Design-Expert software (version 13.0, Stat-Ease Inc., Minneapolis, MN, USA). Extraction temperature (X1), extraction time (X2), solid-to-liquid ratio (X3), and methanol (MeOH) concentration (X4) were selected as independent variables to optimize the total phenolic content, their antioxidant and antibacterial activities. These four factors were selected because they are known to exert the strongest influence on phenolic extraction efficiency, whereas other factors (particle size, pre-soaking) were kept constant based on preliminary experiments. Each factor was operated at three coded levels (-1, 0, and +1), corresponding to minimum, central, and maximum values, respectively **(Tab. 1)**. A Central Composite Design (CCD) with rotatability ($\alpha = 2$) was used. The design comprised 16 factorial points (based on a full 2^4 factorial design), 8 axial points, and 2 replicates of the center point, resulting in 26 randomized experimental runs. This design allows estimation of linear, quadratic, and two-way interaction effects with far fewer experiments than a full 34 factorial (81 runs). The design included two center-point replicates to estimate pure error and assess model adequacy.

To predict the optimal conditions, a second-order polynomial model was developed to describe the relationship between the independent variables and responses using the multiple regression equation:

$$Y_i = \beta 0 + \Sigma\, \beta_i X_i + \Sigma\, \beta_{ii} X_i^2 + \Sigma\, \beta_{ij} X_i X_j$$

Where X_i and Xj represent the independent variables, and $\beta 0$, β_i, β_{ii}, and β_{ij} ($i \neq j$) represent regression coefficients for the intercept (constant), linear, quadratic, and interaction terms, respectively. These regression coefficients were estimated using the ordinary least-squares method. Only terms with $p < 0.05$ were retained in the final reduced quadratic models.

Extraction procedure

The extraction of bioactive compounds from *R. montana* aerial parts was performed by maceration in methanol at varying concentrations (20%, 50%, 80%) using varying solid-to-liquid ratios (0.8:10, 1.2:10, 1.6:10 g/mL), temperatures (30, 50, 70 °C), and extraction times (60, 120, 180 min) according to the experimental design. All extractions were performed in triplicate to ensure reproducibility. After extraction, the mixtures were filtered through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, UK). The resulting filtrates were concentrated to dryness using a rotary evaporator (Büchi R-300, Switzerland) at 40 °C under reduced pressure (150 mbar). The dried extracts were subsequently redissolved in dimethyl sulfoxide (DMSO) to a final concentration of 100 mg/mL. For biological assays, the extracts were further diluted in appropriate solvents to the required concentrations. Pure DMSO served as the negative control in all biological assays to account for potential solvent effects.

Total phenolic content

The total phenolic content (TPC) in the extracts was quantified using the Folin-Ciocalteu assay (Kemegne et al., 2018) with minor modifications. Briefly, 100 µL of the extract (1 mg/mL in methanol) was mixed with 500 µL of Folin-Ciocalteu reagent solution (1:10, v/v in distilled water). After a 2-3 min interval, 1500 µL of 20% sodium carbonate solution (Na_2CO_3) was added. The samples were

Table 1. Coded levels and corresponding actual values for the extraction parameters.

Independent variable	Unit	Symbol	Coded level		
Temperature	°C	X_1	30	50	70
Time	h	X_2	1	2	3
Solid-to-liquid ratio	g/mL	X_3	0.8:10	1.2:10	1.8:10
MeOH concentration	%	X_4	20	50	80

incubated for 2 h in the dark at room temperature. The absorbance of the reaction mixtures was measured at 765 nm with a UV spectrophotometer (Shimadzu UV-1800, Japan). A calibration curve was established using gallic acid as a standard under the same conditions as the extracts, and the results were expressed as µg of gallic acid equivalent per 1 g of dry weight (µg GAE/g DW). All measurements were performed in triplicate.

Total flavonoid content

The total flavonoid content (TFC) of *R. montana* extracts was measured using the aluminum colorimetric assay (Djeridane et al., 2006) with minor modifications. 1 mL of each extract (1 mg/mL in methanol) was mixed with 1 mL of 2% $AlCl_3$ solution (w/v in methanol). After 30 min of incubation at 25 °C, absorbance was read at 430 nm. Quercetin (0-100 µg/mL) was used as the standard for the calibration curve. TFC was expressed as µg of quercetin equivalent per 1 g of dry weight (µg QE/g DW). All measurements were performed in triplicate.

DPPH radical scavenging activity

The free radical scavenging activity of the extracts was evaluated according to the protocol described by Boudjedjou et al. (2019), with minor modifications. Briefly, 100 µL of each extract (1 mg/mL in methanol) was added to 900 µL of a 0.1 mM DPPH solution in methanol, and the mixture was mixed well. The mixtures were incubated for 30 min in the dark at room temperature. The absorbance was then measured at 517 nm. Ascorbic acid (0-100 µg/mL) was used as a standard positive control. All measurements were performed in triplicate.

The antioxidant activity of the extracts is expressed as a percentage of inhibition (% inhibition) calculated as follows:

$$\text{Antioxidant activity (\%)} = [(\text{Control Abs} - \text{Extract Abs}) / \text{Control Abs}] \times 100$$

Antibacterial activity

The antibacterial activity of *R. montana* aerial parts extracts was evaluated against *Staphylococcus aureus* (ATCC 25923) obtained from the American Type Culture Collection (Manassas, VA, USA) using the disc diffusion method (Zeraib et al., 2021) with minor modifications. Bacterial cultures were maintained on Mueller-Hinton agar (MHA) at 4 °C and sub-cultured every two weeks. For the assay, a 100 µL aliquot of bacterial suspension, adjusted to 1×10^8 CFU/mL (0.5 McFarland standard), was spread onto Mueller-Hinton agar plates. Sterile paper discs (6 mm diameter) were impregnated with 20 µL of each extract dissolved in DMSO at 200 mg/mL, and then placed on Petri dishes. Gentamicin (10 µg/disc) was used as a positive control, and pure DMSO served as a negative control. After incubation at 37 °C for 24 h, the inhibition zone diameters were measured using ImageJ (https://imagej.net/ij/). All experiments were carried out in three independent biological replicates, each with three technical replicates. Antibacterial activity was expressed as inhibition zone diameters (mm). Bacterial sensitivity to the extracts was classified as follows according to Ponce et al. (2003): not sensitive (–) (inhibition zone diameter < 8 mm); sensitive (+) (inhibition zone diameter 9–14 mm); very sensitive (++) (inhibition zone diameter 15–19 mm); and extremely sensitive (+++) (inhibition zone diameter > 20 mm).

Statistical analysis

All experiments were conducted in triplicate (n=3). The statistical significance of regression models was assessed using analysis of variance (ANOVA) with a 95% confidence interval ($p<0.05$). Model suitability was evaluated through predicted R^2, adjusted R^2, and lack-of-fit tests. The quality of the fitted models was assessed using the coefficient of determination (R^2) and the adjusted coefficient of determination (R^2adj). The significance of each coefficient was determined using Fisher's F-test and Student's t-test. The lack-of-fit test was used to verify the adequacy of the models. Statistical analyses were performed using Design-Expert software (version 13.0) and OriginPro®2024. Multi-responses optimization was performed using the desirability function approach to identify global optimal conditions that simultaneously maximized all responses.

Results and Discussion

Optimization of phenolic compounds extraction conditions from R. montana aerial parts

RSM using a Central Composite Design (CCD) was employed to optimize total phenolic content (TPC) extraction from *R. montana* aerial parts (**Fig. 1**). Experimental results and ANOVA are presented in **Tab. 2** and **Tab. 3**, and the predicted TPC was modeled using the following quadratic polynomial equation:

$$Y_{tp}C = 258.67 + 10.33\ X_2 - 24.33\ X_3 + 26.04\ X_4 + 9.75\ X_2X_3 + 14.59\ X_1X_4 + 21.91\ X_2X_4 + 12.09\ X_3X_4 + 20.72\ X_1^2 + 27.05\ X_2^2 + 17.43\ X_3^2 - 26.05\ X_4^2$$

The fitted polynomial model showed excellent performance ($R^2 = 0.97$), explaining 97% of the variability in total phenolic content. The adjusted R^2 (0.93) closely agreed with the R^2 value, indicating

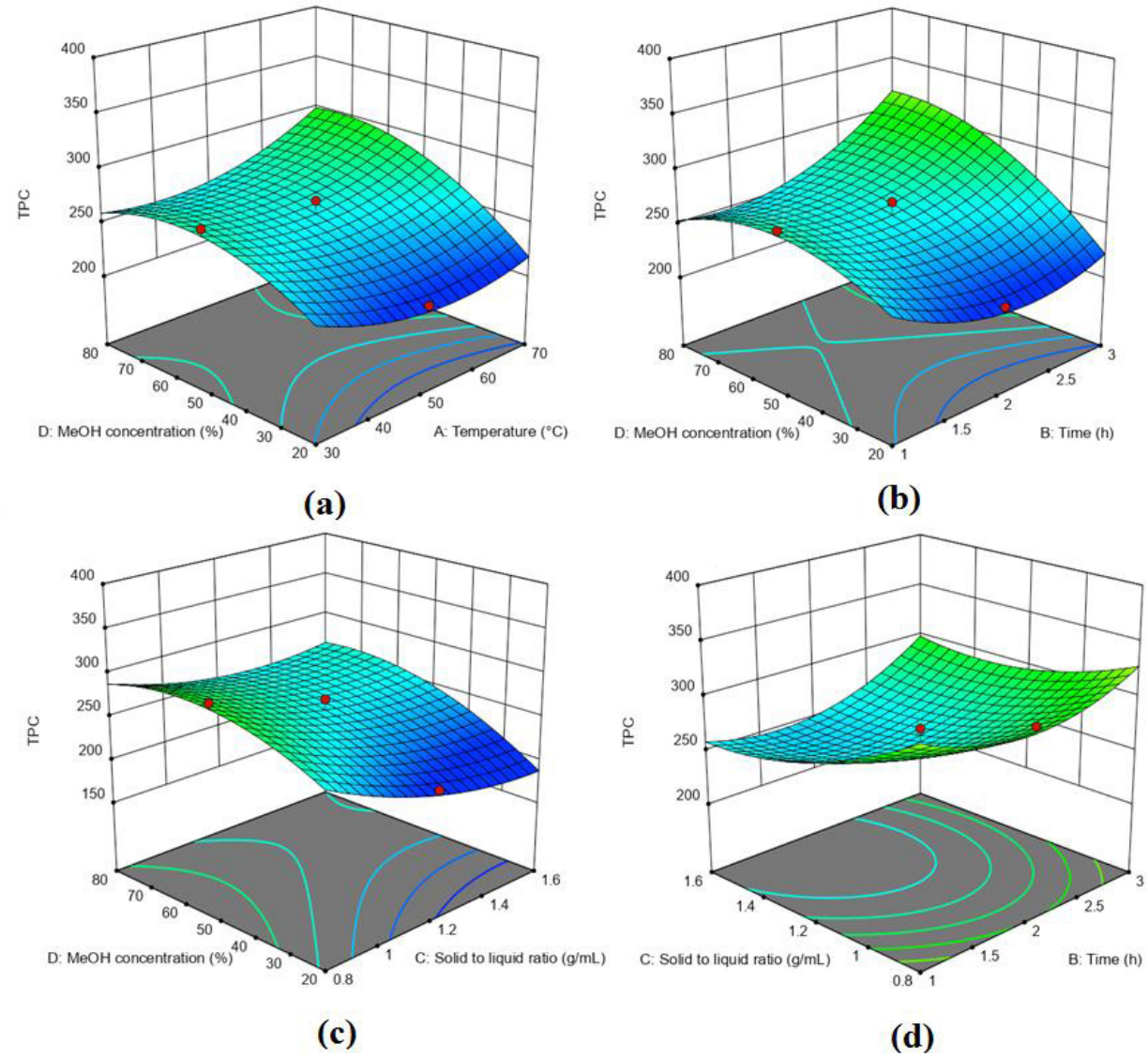

Fig. 1. 3D plots showing the effects of interaction between the independent factors, (**a**) extraction temperature and MeOH concentration, (**b**) extraction time and MeOH concentration, (**c**) solid-to-liquid ratio and MeOH concentration, and (**d**) extraction time and solid-to-liquid ratio, on the extraction yield of TPC from *R. montana* aerial parts.

good model robustness and agreement between predicted and experimental values. The model was highly significant ($p < 0.0001$), while the lack of fit was not significant ($p = 0.6478$), confirming the adequacy of the model for predicting phenolic yield and describing the extraction process (**Tab. 2** and **3**).

Experimental TPC values showed notable variability across extraction conditions, with recovery rates ranging from 212.1 to 388.94 µg GAE/g DW **(Tab. 2)**. The highest phenolic content (388.94 µg GAE/g DW) was achieved in experiment 26 (70 °C, 3 h, 1.6:10 g/mL, 80% MeOH, whereas the lowest (212.1 µg GAE/g DW) was obtained in experiment 5 (50 °C, 2 h, 1.2:10 g/mL, 20% MeOH).

The linear effects of extraction time, solid-to-liquid ratio, and methanol concentration significantly influenced TPC ($p < 0.05$), consistent with previous reports indicating that these variables govern phenolic extraction efficiency (Alara et al., 2021; Cacace et al., 2003).

Methanol concentration exhibited a significant positive linear effect, indicating improved solubility and mass transfer of phenolics in hydro-organic media; however, a significant negative quadratic effect ($p = 0.0049$) demonstrated that excessively high MeOH concentrations become detrimental. This suggests an optimum at moderate methanol levels (approximately 60–80%), whereas methanol concentrations >80% may reduce recovery by limiting the extraction of more polar phenolics and

Table 2. Experimental design and responses for total phenolic content, total flavonoid content, antioxidant and antibacterial activities

Run	X_1 (°C)	X_2 (h)	X_3 (g/10 mL)	X_4 (%)	TPC (µg GAE/g DW)	TFC (µg QE/g DW)	AOX (%)	ABA (mm)
1	50	3	1.2	50	288.86	105.23	89.89	9.33
2	50	2	1.2	50	254.55	80.29	81.33	10
3	30	1	0.8	80	300.24	135.92	88.1	13
4	70	1	1.6	20	227.72	72.39	78.85	7
5	50	2	1.2	20	212.1	61.09	70.12	10
6	30	2	1.2	50	280.2	78.06	90.84	10.33
7	50	1	1.2	50	279.84	107.31	89.21	8
8	70	2	1.2	50	275.85	78.63	85.97	9.67
9	50	2	1.2	80	250.41	115.29	83.53	16.33
10	30	3	0.8	20	291.36	42.19	78.51	8
11	50	2	0.8	50	306.87	90.85	71.32	10.33
12	70	1	0.8	80	321.22	124.79	86.34	14
13	30	3	0.8	80	334.85	157.2	87.73	11.67
14	30	1	1.6	80	260.93	157.63	80.35	10
15	50	2	1.2	50	270.97	77.9	85.03	9.33
16	70	3	0.8	20	276.1	76.91	72.03	7
17	30	3	1.6	80	319.37	140.34	85.5	15
18	70	3	0.8	80	388.28	145.27	72.67	13.67
19	70	1	0.8	20	326.75	128.99	72.18	8.33
20	30	1	1.6	20	241.38	38.81	50.66	9.67
21	50	2	1.6	50	242.6	84.17	77.26	8.67
22	30	1	0.8	20	331.95	80.58	74.74	10.33
23	70	3	1.6	20	221.22	65.62	89.45	8.33
24	70	1	1.6	80	285.28	128.45	87.33	8.33
25	30	3	1.6	20	252.27	42.17	69.71	8
26	70	3	1.6	80	388.94	173.86	92.04	16.33

X1: temperature; **X2**: extraction time; **X3**: solid-to-liquid ratio; **X4**: MeOH concentration; **TPC**: total phenolic content; **TFC**: total flavonoid content; **AOX**: antioxidant activity; **ABA**: antibacterial activity; **GAE**: gallic acid equivalent; **QE**: quercetin equivalent; **DW**: dry weight.

increasing co-extraction of non-phenolic compounds, highlighting the value of RSM for capturing non-linear behavior. A significant time–methanol interaction **(Fig. 1)** further demonstrated that longer extraction times increased TPC primarily at higher methanol levels (70–80%), reaching an optimum near 3 h, whereas low methanol concentrations (20–40%) yielded low TPC even with extended time, underscoring the need for joint factor optimization. These trends agree with studies reporting reduced TPC in more water-rich solvents and greater co-extraction of non-phenolic constituents (Do et al., 2014; Anis et al., 2022).

The solid-to-liquid ratio significantly affected TPC through both its linear and interaction effects with MeOH concentration. Its negative linear coefficient suggests that increasing the ratio initially reduces extraction, likely by lowering the concentration gradient that drives mass transfer (Cacace et al., 2003; Tan et al., 2014). However,

Table 3. Coefficients of regression and *p*-values of the all-response variables

Source	TPC	*p*-value	TFC	*p*-value	AOX	*p*-value	ABA	*p*-value
Lack of fit	0.65		0.09		0.52		0.31	
Intercept	258.67		82.78		83.27		10.08	
(X_1) Temperature	5.45	0.0756	6.78	0.064	1.71	0.0616	– 0.19	0.487
(X_2) Time	10.33	**0.003**	– 1.44	0.668	1.65	**0.0688**	0.48	0.0888
(X_3) Solid-to-liquid ratio	–24.33	**<0.0001**	– 4.40	0.208	0.42	0.6201	– 0.28	0.3048
(X_4) MeOH concentration	26.04	**<0.0001**	37.22	**<0.0001**	5.96	**<0.0001**	2.32	**<0.0001**
(X_1)*(X_2)	3.14	0.313	2.38	0.509	– 1.64	0.0874	0.50	0.095
(X_1)*(X_3)	– 0.29	0.9223	0.08	0.9819	5.46	**<0.0001**	– 0.17	0.553
(X_1)*(X_4)	14.59	**0.0005**	– 9.93	**0.0160**	– 2.64	**0.011**	0.50	0.095
(X_2)*(X_3)	9.75	**0.0073**	4.59	0.215	3.12	**0.0043**	1.12	**0.0017**
(X_2)*(X_4)	21.91	**<0.0001**	10.23	**0.0137**	– 2.34	**0.021**	0.96	**0.0049**
(X_3)*(X_4)	12.09	**0.002**	9.17	**0.0236**	1.19	0.196	– 0.13	0.6536
(X_1)^2	20.72	**0.018**	– 5.65	0.5307	5.11	**0.039**	– 0.21	0.7628
(X_2)^2	27.05	**0.004**	22.27	**0.0270**	6.25	**0.015**	– 1.55	**0.0450**
(X_3)^2	17.43	**0.039**	3.51	0.6952	– 9.01	**0.002**	– 0.71	0.3206
(X_4)^2	– 26.05	**0.005**	4.19	0.6405	– 6.47	**0.013**	2.95	**0.0012**

TPC: total phenolic content; **TFC**: total flavonoid content; **AOX**: antioxidant activity; **ABA**: antibacterial activity.

a positive quadratic effect indicates the presence of an optimum, highlighting the need to balance solvent volume, since excessive solvent dilutes the driving force and complicates processing, whereas insufficient solvent limits recovery (Płotka-Wasylka et al., 2017). Overall, the model's quadratic and interaction terms provide a predictive framework for multi-factor optimization beyond one-factor-at-a-time approaches.

Optimization of flavonoid extraction conditions from R. montana aerial parts

RSM coupled with a CCD was applied to optimize total flavonoid content (TFC) extraction from *R. montana* aerial parts **(Fig. 2**). The experimental results are presented in **Tab. 2**, and the predicted TFC is described by the following quadratic polynomial model:

$$YTFC = 82.78 + 37.22\ X_4 - 9.93\ X_1X_4 + 10.24\ X_2X_4 + 9.17\ X_3X_4 + 22.27\ X_2^2$$

The results showed that the model was statistically robust (R^2=0.94, adjusted R^2=0.86, $p < 0.0001$) and showed no significant lack of fit ($p = 0.0896$), confirming its suitability for prediction.

The TFC ranged from 42.19 µg QE/g DW to 173.86 µg QE/g DW. The highest value (173.86 µg QE/g DW) was obtained at 70 °C, an extraction time of 3 h, a solid-to-liquid ratio of 1.6:10 g/mL, and 80% methanol. However, the lowest flavonoid content (42.19 µg QE/g DW) was observed at 30 °C, an extraction time of 3 h, a solid-to-liquid ratio of 0.8:10 g/mL, and 20% methanol.

ANOVA and regression analysis (**Tab. 2** and **3**) indicated that MeOH concentration had a highly significant linear effect on TFC (p <0.0001), and its interactions with the other factors were also significant. The RSM response surfaces highlighted clear MeOH–time (p = 0.0236) and MeOH–temperature (p = 0.0160) interactions, with TFC increasing markedly as MeOH percentage rose. Maximum flavonoid recovery was achieved at 70–80% MeOH, an extraction time of 3 h, and a temperature range of 60–70 °C.

Optimization of antioxidant activity

The polynomial model for radical scavenging activity was:

$$YAOX = 83.27 + 5.96\ X_4 + 5.46\ X_1X_3 + 3.12\ X_2X_3 - 2.64\ X_1X_4 - 2.34\ X_2X_4 + 5.11\ X_1^2 + 6.25X_2^2 - 9.01\ X_3^2 - 6.47\ X_4^2$$

The model fit the data well (R^2 = 0.92, adjusted R^2 = 0.82) and was highly significant ($p < 0.0001$)

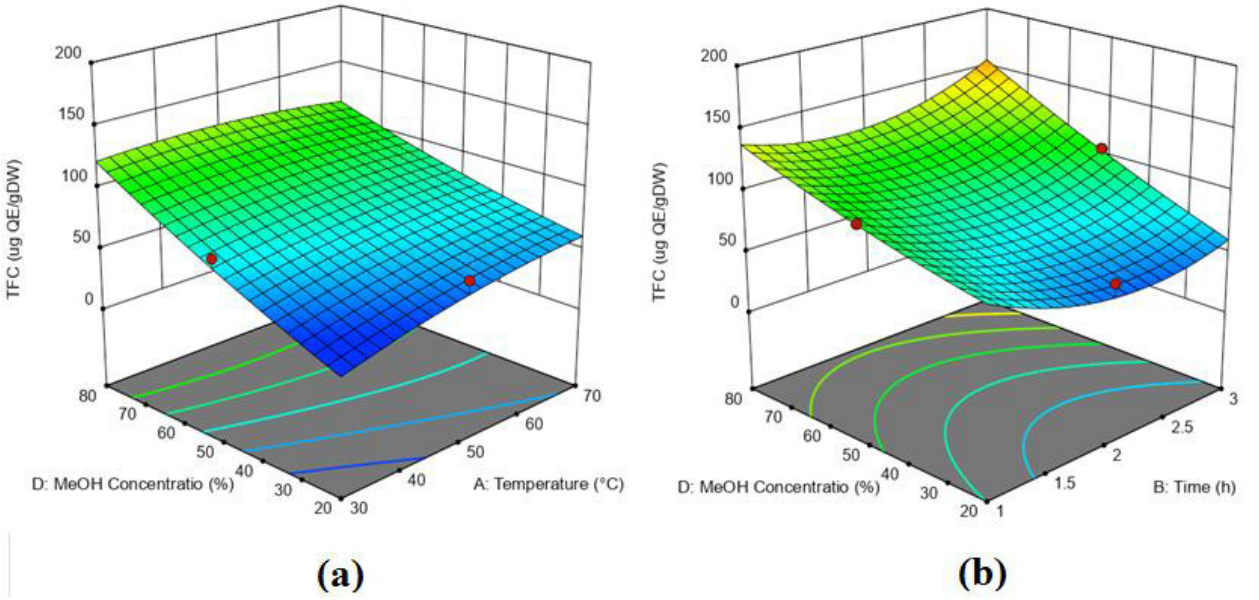

Fig. 2. 3D plots of the interactive effects of extraction temperature (**a**), extraction time (**b**) vs. MeOH concentration on the extraction yield of TFC from *R. montana* aerial parts.

with a non-significant lack of fit ($p > 0.05$), supporting its use for prediction across extraction conditions.

DPPH inhibition ranged from 50.66% to 92.04%, with the minimum at 30 °C, 1 h, 0.8:10 g/mL, and 20% MeOH (run 20) and the maximum at 70 °C, 3 h, 1.6:10 g/mL, and 80% MeOH (run 26). The strongest activity coincided with the highest TPC and TFC and correlated with both (r = 0.87 for TPC vs AOX; r = 0.82 for TFC vs AOX), consistent with the antioxidant role of phenolics/flavonoids (Roby et al., 2013; Ciupei et al., 2024).

Multi-response optimization indicated that the response maxima were not fully aligned: although high MeOH/temperature/time favored TFC, MeOH showed negative interactions with temperature and time for antioxidant activity **(Fig. 3)**, leading to reduced AOX at extreme conditions. This likely reflects degradation and transformation reactions (e.g., oxidation, loss of thermolabile flavonoid glycosides, and polymerization) reported for plant phenolics under severe processing (Cotas et al., 2020; Muala et al., 2021), while still supporting phenolic/flavonoid enrichment as a practical proxy for improving antioxidant potential in *R. montana*.

Optimization of antibacterial activity

The antibacterial activity of *R. montana* extracts against *S. aureus* was evaluated using the disc diffusion assay and modeled by RSM as:

$$YABA = 10.08 + 2.32\ X_4 + 1.12\ X_2X_3 + 0.96\ X_2X_4 - 1.55\ X_2^2 + 2.95\ X_4^2$$

The model's adequacy for predicting the response as a function of extraction conditions was confirmed by a high R^2 (0.93), a high Adj-R^2 (0.83), and a non-significant lack of fit ($p = 0.3139$).

ANOVA and regression analysis (**Tab. 2** and **Tab. 3**) indicated that antibacterial activity was mainly driven by interaction effects rather than strong linear terms, except for MeOH concentration, highlighting the need for RSM, as one-factor-at-a-time optimization could overestimate the role of solvent concentration alone **(Fig. 4)**.

The extracts inhibited *S. aureus* with zones of 7–16.33 mm, likely due to the presence of phenolic compounds and flavonoids, which are known to exert antimicrobial effects through multiple mechanisms, including increasing cell membrane permeability, interfering with nucleic acid synthesis, disrupting cell wall formation, and chelating essential metal ions required for microbial enzymatic processes (Xie et al., 2014; Lima et al., 2023). While disc diffusion provides a useful measure of antibacterial activity, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays are needed to fully quantify the potency of these extracts. Our results should therefore be considered as preliminary screening. Furthermore, antibacterial activity was evaluated only against *S. aureus*; further studies should include additional Gram-positive (e.g., *Bacillus subtilis*, *Enterococcus faecalis*) and Gram-negative (e.g., *Escherichia coli*, *Pseudomonas aeruginosa*) strains to establish a broader antimicrobial profile.

While prior work emphasized the antibacterial activity of *R. montana* essential oils (Mohammedi et al., 2019) and identified chloroform fractions as particularly active (Merghem et al., 2020), our results

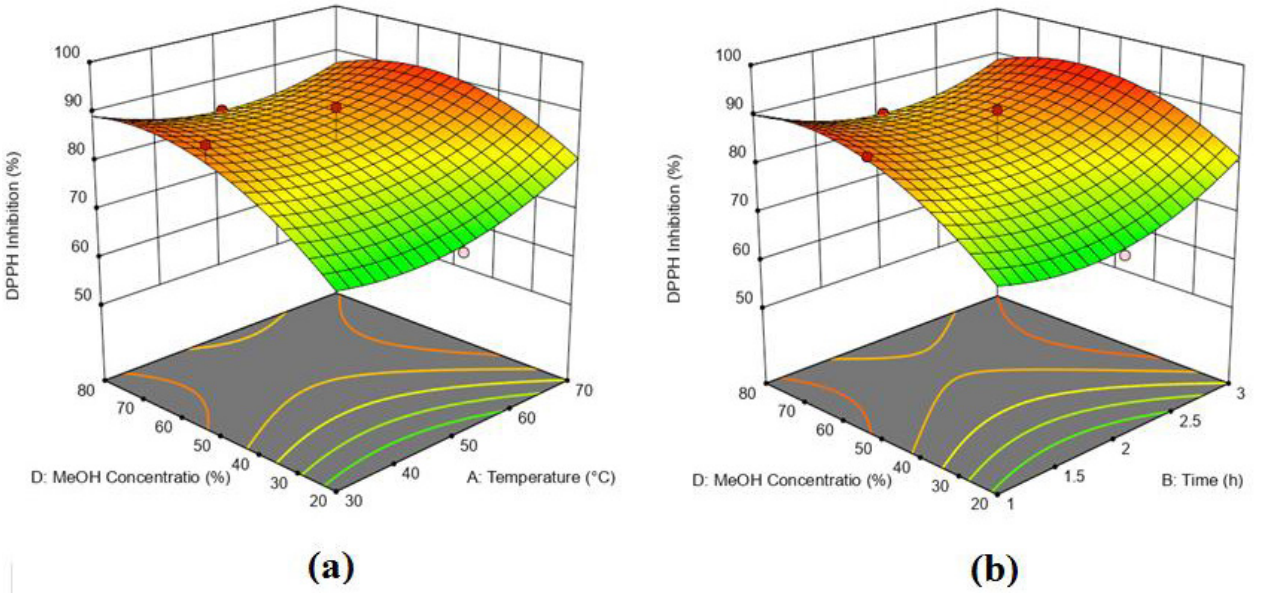

Fig. 3. 3D plots of the interactive effects of extraction temperature (**a**) and extraction time (**b**) vs. MeOH concentration on antioxidant activity of *R. montana* aerial part extracts.

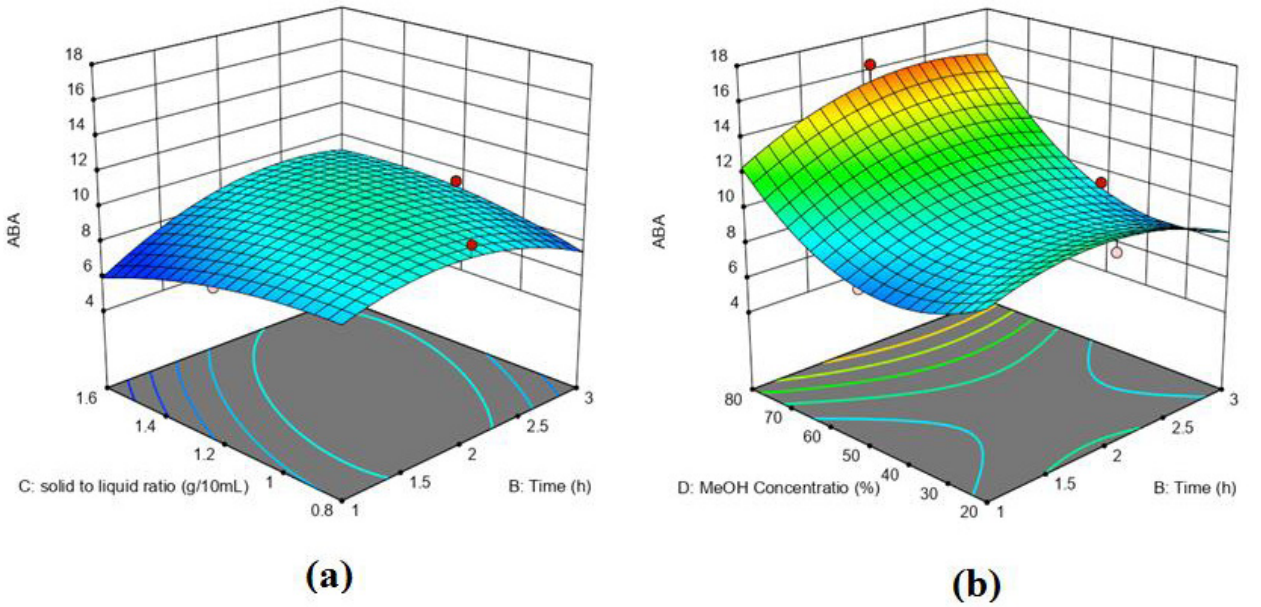

Fig. 4. 3D plots of the interactive effects of extraction time (**a**) and solid-to-liquid ratio (**b**) vs. MeOH concentration on antibacterial activity of *R. montana* aerial part extracts.

indicate that phenolic-rich methanolic extracts can also be effective, particularly under optimized conditions. Overall, the response patterns suggest that optimal extraction yields a balanced mixture of phenolic/flavonoid classes, and identifying the compounds behind this synergy should be prioritized in future fractionation studies.

Validation of optimal conditions and integrated discussion

The quadratic polynomial model identified optimal extraction conditions (desirability = 0.89) of 68 °C, 3 h, 1.528:10 (g/mL) solid-to-liquid ratio, and 80% MeOH. Validation experiments conducted under these conditions yielded experimental values in excellent agreement with model predictions **(Fig. 5):** TPC, 376.26 vs. 368.59 µg GAE/g DW (2.08% error); TFC, 159.17 vs. 157.23 µg QE/g DW (1.23% error); DPPH radical-scavenging activity, 86.72% vs. 93.61% (7.36% error); and antibacterial activity, 14 mm vs. 15.87 mm (11.77% error). All percentage errors remained well below the 15% threshold

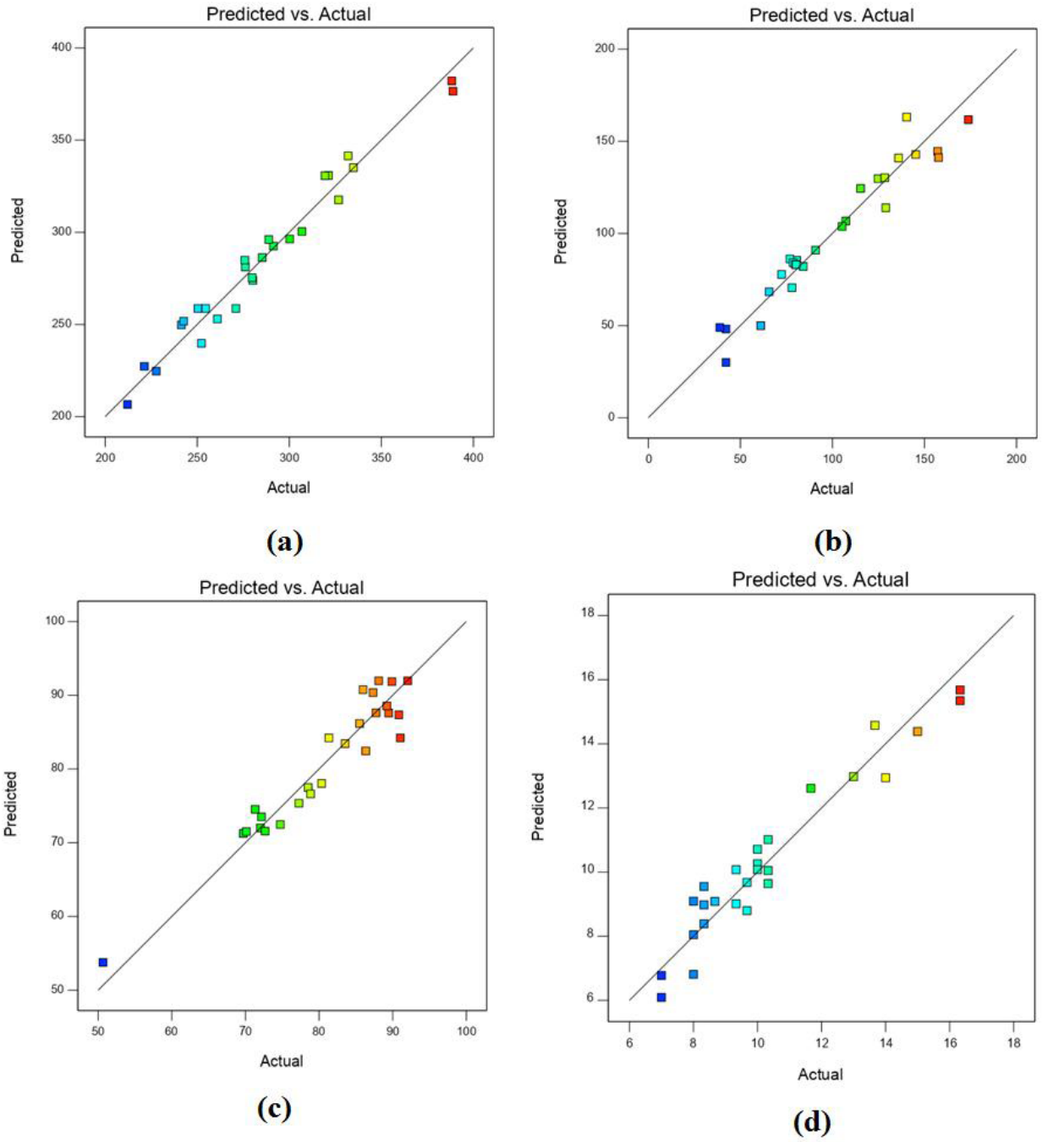

Fig. 5. The predicted data versus actual values of the (**a**) total phenolic content, (**b**) total flavonoid content, (**c**) DPPH radical scavenging activity, and (**d**) Antibacterial activity.

commonly accepted for model validation, with two of four responses exhibiting errors <5%, thereby confirming the model's high predictive accuracy and robustness across disparate bioassays.

The desirability value of 0.89 (on a 0–1 scale, where 1.0 represents perfect simultaneous optimization) indicates that RSM successfully identified an optimal balance among competing multi-response objectives. Rather than independently maximizing TPC, TFC, antioxidant activity, or antibacterial activity—each of which would require different extraction parameters—the model identified conditions that simultaneously yielded high phenolic and flavonoid contents (376 µg GAE/g, 159 µg QE/g), strong radical-scavenging capacity (86.72%), and clinically relevant antibacterial efficacy (14 mm inhibition zones). This integrated outcome demonstrates that well-rounded bioactive extracts can be achieved through coordinated parameter optimization, underscoring the methodological

superiority of RSM over sequential or independent single-factor optimization approaches.

Conclusions

This study systematically investigated optimal extraction conditions for *Ruta montana* aerial parts using a central composite design and response surface methodology to simultaneously maximize phenolic yield, antioxidant activity, and antibacterial efficacy across four extraction variables (temperature, time, MeOH concentration, and solid-to-liquid ratio). Quadratic models for total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging, and *Staphylococcus aureus* inhibition all demonstrated high predictive power ($R^2 > 0.92$, $p < 0.0001$, non-significant lack of fit), with experimental validation confirming prediction errors <15% across all responses.

Optimal conditions were identified as 68 °C, 3 h, 1.528:10 g/mL, and 80% MeOH—yielding 376.26 µg GAE/g DW phenolics, 159.17 µg QE/g DW flavonoids, 86.72% DPPH inhibition, and 14 mm antibacterial zones. Methanol concentration proved most influential, though significant interaction effects revealed competing optimization objectives: conditions that maximize TFC partially conflicted with those that preserve antioxidant activity at extreme parameter combinations. The desirability approach (0.89) successfully resolved this multi-response trade-off, identifying compromise conditions that simultaneously delivered high bioactivity across all four endpoints.

Study limitations

The work establishes *R. montana* as a valuable source of natural phenolic antioxidants and screening-level antimicrobial agents under optimized *in vitro* extraction conditions. However, this study assessed antioxidant activity only via DPPH radical scavenging, which reflects hydrogen-donating ability, but not other antioxidant pathways (e.g., metal chelation, lipid peroxidation inhibition). Future studies should include complementary assays, such as ABTS, FRAP, and ORAC. Additionally, the antibacterial findings reported here are preliminary and require confirmation by MIC and MBC assays in future studies. Compared to previously reported volatile preparations of *R. montana*, optimized methanolic extracts may offer a different but potentially valuable phenolic-rich profile; however, direct comparative studies are needed to establish any superiority. Further *in vivo* validation, toxicity assessment, and safety profiling are necessary before any pharmaceutical or nutraceutical application can be considered.

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