



Phenolic composition of ultrasound-assisted extracts optimized using response surface methodology based on DPPH scavenging activity, total phenolic content, and total flavonoid content from three aromatic and medicinal plants and their mixture

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ARTICLE INFO

Keywords:

Optimization
RSM
Phenolic compounds
HPLC
Antioxydant activity
Laurus nobilis
Rosmarinus officinalis
Pimpinella anisum
Mixture

ABSTRACT

This study aimed to optimize the most common extraction parameters using Ultrasound-assisted extraction (UAE) to maximize the recovery of phenolic content and the free radical scavenging activity DPPH from *Pimpinella anisum*, *Rosmarinus officinalis*, *Laurus nobilis* using response surface methodology (RSM). UAE was optimized for each plant using a Box-Behnken design, with ethanol/water ratio, extraction temperature, time, and ultrasonic power as independent variables. Therefore, the proportions of the three plant extracts in the mixture and to evaluate the interaction effects among these combinations. For maximum TPC, TFC, and DPPH free radical scavenging activity, the optimized extraction conditions were as follows: for *P. anisum*, 50 °C extraction temperature, 20 min extraction time, 10% ethanol concentration, and 60% ultrasonic power; for *R. officinalis*, 25 °C extraction temperature, 51 min extraction time, 17% ethanol concentration, and 89% ultrasonic power; and for *L. nobilis*, 75 °C extraction temperature, 56 min extraction time, 4% ethanol concentration, and 74% ultrasonic power. The optimized mixture, consisting of 64.9% *P. anisum*, 13.8% *R. officinalis*, and 21.3% *L. nobilis*, exhibited high levels of TPC (34.37 mg GAE/g DW), TFC (130.34 mg CE/g DW), and percentage of inhibition of free radical scavenging activity DPPH (PI = 49.45%). HPLC-DAD analysis of the individual extracts revealed distinct phenolic profiles. *L. nobilis* was particularly rich in hydroxybenzoic acid (279.21 µg/g) and caffeic acid (261.88 µg/g), *P. anisum* was dominated by cinnamic acid (147.28 µg/g) and ferulic acid (100.48 µg/g), while *R. officinalis* exhibited a high content of gallic acid (96.66 µg/g). Compound-dependent interaction effects were observed within the mixture, with enhancement levels for epicatechin gallate (176.75 µg/g), rosmarinic acid (94.53 µg/g) and sinapic acid (36.02 µg/g), whereas other compounds showed additive or reduced levels relative to single extracts.

1. Introduction

Aromatic and medicinal plants represent a rich source of bioactive compounds with diverse applications in pharmacology, nutraceuticals, and the food industry. In the Mediterranean region, *Pimpinella anisum*, *Rosmarinus officinalis*, and *Laurus nobilis* have long been used in traditional medicine to treat digestive disorders, respiratory conditions,

inflammation, and infections (Bontzolis et al., 2024a; Gonçalves et al., 2022; Paparella et al., 2022). Their biological activities are mainly attributed to a wide range of phytochemicals, including phenolic acids (e.g., rosmarinic, caffeic, and gallic acids), flavonoids (e.g., quercetin, catechin, rutin), and volatile compounds such as anethole and 1, 8-cineole (Bontzolis et al., 2024; Donkor et al., 2023).

Despite their potential, the efficient recovery of these bioactive

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<https://doi.org/10.1016/j.jarmap.2026.100732>

Received 6 March 2026; Received in revised form 2 July 2026; Accepted 14 July 2026

Available online 21 July 2026

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compounds remains challenging. Many phytochemicals are thermosensitive, susceptible to oxidation, and poorly soluble in conventional solvents, which can lead to degradation and low extraction yields (Bontzolis et al., 2024a; Chemat et al., 2017). Conventional techniques such as maceration, Soxhlet extraction, and hydrodistillation are still widely used. However these techniques are associated with major limitations, including long extraction times, high solvent consumption, and possible thermal degradation of sensitive compounds (Bontzolis et al., 2024; Chemat et al., 2017).

Ultrasound-assisted extraction (UAE) has emerged as a promising alternative, offering reduced extraction time, lower solvent usage, and improved recovery yields. Its mechanism is based on acoustic cavitation, which enhances mass transfer through cell wall disruption (Khan et al., 2025). However, the efficiency of UAE is highly dependent on operating parameters including ultrasonic power, frequency, sonication time, solvent type, sample-to-solvent ratio, temperature, pressure, and sample features (particle size, moisture content, and pretreatment) (Thangadurai et al., 2025). Among them, solvent concentration, extraction time and temperature, and ultrasound power are widely recognized as key points that are crucial to optimize (Muala et al., 2021).

Response Surface Methodology (RSM) with Box–Behnken Design (BBD) is a widely used statistical tool to optimize the parameters influencing the extraction process, as it evaluates the interaction effects of multiple variables, determines the optimal conditions with a minimal number of experimental runs, and even excludes unrealistic extreme value (Thangadurai et al., 2025).

In parallel, increasing attention has been given to the use of plant combinations. The rationale behind investigating plant mixtures lies in the fact that bioactive compounds do not always act independently, and their combined effects may differ significantly from those observed in single-extract systems. Interactions between phytochemicals can result in synergistic, additive, or antagonistic effects, particularly in terms of antioxidant and antimicrobial activities. Synergistic combinations are of particular interest, as they may enhance biological efficiency, reduce the required dosage of individual extracts, and decrease resistance effects. Although synergistic antimicrobial interactions have been reported in various plant mixtures, these effects are often unpredictable and depend strongly on the composition and relative proportions of the extracts (Donkor et al., 2023; Sharma et al., 2020). In this context, the selection of these three Mediterranean plants *Pimpinella anisum*, *Rosmarinus officinalis*, and *Laurus nobilis* is supported by their complementary phytochemical profiles, particularly differences in phenolic and flavonoid composition (Amaral et al., 2019; Bettaieb Rebey et al., 2020; El Faqer et al., 2024), which may promote interaction effects when combined.

Although *Rosmarinus officinalis* and *Laurus nobilis* have been extensively studied for their antioxidant properties, *Pimpinella anisum* has been comparatively less explored in the context of optimized hydroalcoholic extraction and multi-plant systems. Moreover, to the best of our knowledge, no study has simultaneously (i) optimized UAE conditions for these three Mediterranean plants using a statistical design approach and (ii) systematically evaluated their interactions using an augmented simplex-centroid mixture design and HPLC analysis. Therefore, the aim of this study (1) optimize UAE conditions for each plant using a Box–Behnken design, (2) investigate the interactions of different combinations among their extracts by simple augmented centroid, and (3) characterize their polyphenolic profiles using HPLC-DAD to identify synergistic, additive, and antagonistic interactions.

2. Material and methods

2.1. Chemicals and reagents

Ultrapure water, ethanol–water mixtures, and methanol were used. The reagents used were Folin–Ciocalteu reagent, sodium carbonate, sodium nitrite, vanillin, concentrated hydrochloric acid, 2,2-

diphenyl–1-picrylhydrazyl (DPPH, 95%; Lot Y01H050, Germany, Thermo Scientific), aluminum trichloride, and NaOH.

Gallic acid, chlorogenic acid, ferulic acid, salicylic acid, o-anisic acid, anisic acid, m-anisic acid, hydrocinnamic acid, cinnamic acid, caffeic acid, rosmarinic acid, tannic acid, p-coumaric acid, p-quinic acid, sinapic acid, hydroxybenzoic acid, catechin hydrate, epicatechin gallate, myricetin, naringin, kaempferol, quercetin, quercetin dihydrate, rutin, and rutin hydrate were purchased from Sigma-Aldrich and used as standards for analysis.

2.2. Plant material

Samples were collected from the local market in Mahdia, a city in the sahel of Tunisia, during the winter, specifically in February 2023. The plant materials were shade-dried under controlled conditions, protected from humidity, and kept at room temperature (15 °C) for 21 days until constant weight was achieved. The dried materials were then ground using a Moulinex grinder (Moulinex PerfectMix Cook; reference LM835D10), transferred into 200 g glass vials, and stored at room temperature in the dark until further use.

2.3. Ultrasounds assisted extraction

Antioxidants from *Laurus nobilis*, *Rosmarinus officinalis*, and *Pimpinella anisum* were extracted using an ultrasound bath (Sonorex DIG-IPLUS DL 102 H, Bandelin) operating at 35 kHz. Preliminary tests established an optimal solid-to-liquid ratio of 0.1 g/mL. To optimize the eco-friendly ultrasound-assisted extraction of antioxidants from these plants, a series of experiments was conducted to determine the optimal extraction parameters, including ethanol concentration, ultrasonic power, extraction time, and temperature. The parameter ranges were selected based on a thorough review of previous studies optimizing the extraction of bioactive compounds from the same plant species (Leal et al., 2011; Muñoz-Márquez et al., 2013a; Paniwnyk et al., 2009). Accordingly, specific intervals were defined for each plant to achieve the most effective extraction conditions.

According to a preliminary study conducted to determine the optimal solid-to-liquid ratio, 0.1 g of dried plant material for each plant was extracted in 1 mL of hydroethanolic solvent under the conditions and parameters presented in the corresponding tables (S1, S3 and S5). For *L. nobilis*, the extraction was conducted using ethanol/water solutions (0%, 10%, and 20%) under ultrasonic powers of 20, 60, and 100% at temperatures of 40°C, 60°C and 80°C for 30, 45, and 60 min (Table S1) (Muñoz-Márquez et al., 2013a). Under similar ethanol gradients and ultrasound powers, *R. officinalis* was processed at temperatures of 35°C, 40°C, and 50°C for 30, 45, and 60 min (Table S3) (Paniwnyk et al., 2009); and *P. anisum* was extracted at temperatures of 25°C, 50°C, and 75°C for 10, 20 and 30 min (Table S5) (Soussi et al., 2022).

After sonication, the extracts were centrifuged using a SIGMA 2–16 P universal centrifuge (685 g, 20 min, 4 °C), and the resulting supernatants were collected and stored at 4 °C for further analyses.

2.4. Experimental design and statistical analysis

To carry out the experiment, an experimental design was drawn up using the Nemrodw software. The design adopted is a "Box–Behnken" design comprising 25 experiments, involving four independent variables and four response measures.

The variables studied were extraction temperature (X1), extraction time (X2), ultrasonic power (X3), ethanol percentage (X4).

Linear terms (X_i) describe the direct effect of each independent variable on the response. Interaction terms (X_iX_j) represent the combined influence of two variables acting simultaneously, indicating whether the effect of one factor depends on the level of another factor.

Quadratic terms (X_i^2) were included to evaluate curvature effects and identify possible optimum conditions within the experimental domain.

Responses Y1, Y2, Y3, and Y4 correspond respectively to the DPPH radical scavenging inhibition percentage (IP), the total polyphenol content expressed as gallic acid equivalents per gram of dry weight (mg GAE/g DW), the flavonoid content expressed as catechin equivalents per gram of dry weight (mg CE/g DW), and the tannin content expressed as catechin equivalents per gram of dry weight (mg CE/g DW).

The model chosen for the analysis of the results is a box behken response surface model.

With 10 coefficients and represented with the equation Eq. (1):

$$Y = b_0 + b_1 * X_1 + b_2 * X_2 + b_3 * X_3 + b_4 * X_4 + b_{11} * (X_1 * X_1) + b_{22} * (X_2 * X_2) + b_{33} * (X_3 * X_3) + b_{44} * (X_4 * X_4) + b_{12} * (X_1 * X_2) + b_{13} * (X_1 * X_3) + b_{23} * (X_2 * X_3) + b_{14} * (X_1 * X_4) + b_{24} * (X_2 * X_4) + b_{34} * (X_3 * X_4) \quad (1)$$

The coefficients in the equation represent the fundamental effects and interactions between the variables under study namely ("Goupy, 2006 Les plans d'expériences. Revue MODULAD, No. 34, 74–116. - References - Scientific Research Publishing," n.d.):

b0: The intercept or response value when all variables are zero.

b1, b2, b3 and b4: The main effects associated respectively with the variable X1(extraction temperature), variable X2(extraction time), variable X3 (ultrasonic power) and variable X4 (percentage of ethanol) respectively. These coefficients indicate the linear influence of each variable on the response in question.

b11, b22 and b33: These coefficients reflect the quadratic effects of X1, X2, X3 and X4.

respectively, showing the curvature of the response with respect to each variable and.

revealing a non-linear relationship between variable and response.

b12, b13 and b23: These coefficients describe the interaction effects between pairs of.

variables, respectively. They indicate whether the impact of one variable on the response.

depends on the value of the other variable.

2.5. Experimental design and statistical analysis of the mixture of the studied plants

To carry out the experiment, an experimental design was drawn up using the Nemrodw software. The design adopted is the augmented simplex centroid design comprising 10 experiments involving 3 components and 3 response measures.

The components studied were *Pimpinella anisum* (X1), *Rosmarinus officinalis* (X2), and *Laurus nobilis* (X3), including individual extracts, binary combinations, and ternary combinations.

The responses Y1, Y2, Y3, and Y4 correspond, respectively, to the DPPH radical scavenging inhibition percentage and the total polyphenol content expressed as gallic acid equivalents per gram of dry weight (mg GAE/g DW), as well as the flavonoid content expressed as catechin equivalents per gram of dry weight (mg CE/g DW). A second-degree model was chosen and represented by the equation Eq. (2):

$$Y = b_1X_1 + b_2X_2 + b_3X_3 + b_{12}(X_1 \cdot X_2) + b_{13}(X_1 \cdot X_3) + b_{23}(X_2 \cdot X_3) \quad (2)$$

Where:

b1, b2 and b3: The main effects associated with the variables X1 (percentage of *Pimpinella anisum*), X2 (percentage of *Rosmarinus officinalis*), and X3 (percentage of *Laurus nobilis*), respectively. These coefficients indicate the linear influence of each variable on the response

("Goupy, 2006 Les plans d'expériences. Revue MODULAD, No. 34, 74–116. - References - Scientific Research Publishing," n.d.).

b12, b13, and b23: Interaction effects between pairs of variables. These coefficients describe how the impact of one variable on the response depends on the value of the other variable.

2.6. Evaluation of the antioxidant activity

The antioxidant activity by DPPH assay was determined according to Samee Ullah et al. (2026) with slight modifications. A DPPH solution (2.36 mg/100 mL ethanol) was prepared 2 h before use and incubated in the dark. One milliliter of each extract was mixed with DPPH solution in equal volume (v/v) and then incubated in the dark for 30 min.

The activity of the extracts is assessed against the 100% assay (100% DPPH solution without extract). The percentage inhibition of free DPPH radical formation is calculated as follows following formula Eq. (3):

$$PI = (1 - DO_{\text{extract}} / DO_{100\%}) * 100 \quad (3)$$

where DO_{extract} represents the absorbance of the reaction mixture containing the extract, and $DO_{100\%}$ corresponds to the absorbance of the control solution containing only the DPPH reagent without extract, considered as 100% of the initial DPPH radicals.

The IC50 value, defined as the concentration of extract required to inhibit 50% of the DPPH radicals, was determined by plotting the percentage inhibition against extract concentration and calculating the concentration corresponding to 50% inhibition from the regression equation. The IC50 was calculated only for the final optimized extract.

2.7. Determination of total phenolic, flavonoids and condensed tannins contents

Total phenolic content (TPC) was determined by the Folin-Ciocalteu assay according to Khajeh et al. (2026), and expressed in mg gallic acid equivalents per gram of dry weight (mg GAE/gDM). Total flavonoids content (TFC) was measured according to the method described by Khajeh et al. (2026) and expressed in mg catechin equivalents per gram of dry weight (mg GAE/gDW). Condensed tan All the analyses were performed in triplicate.

2.8. HPLC analysis of phenolic compounds

The optimized hydro-ethanolic extracts of *Pimpinella anisum*, *Rosmarinus officinalis*, *Laurus nobilis*, as well as their mixture, were prepared and then concentrated using a SpeedVac before the direct injection of 10 µL into the high-performance liquid chromatography system (HPLC-DAD) (Waters Alliance™ e2695 XC HPLC System), equipped with a diode-array detector (DAD) and a non-polar C18 column (100 × 4.6 mm, 4 µm).

Polyphenolic compound analysis was performed in gradient mode, with a flow rate of 0.7 mL/min and a column temperature maintained at 40 °C. The mobile phase consisted of 10% methanol containing 0.1% phosphoric acid (v/v) as eluent A, and the same composition for eluent B. The applied gradient program was as follows: 0 min (0% A, 100% B), 20 min (80% A, 20% B), 56 min (75% A, 25% B), 86 min (72% A, 28% B), 88 min (65% A, 35% B), 93 min (30% A, 70% B), and 94–99 min (0% A, 100% B) (Skendi et al., 2017). Chromatographic peaks were recorded at 250, 280, 320, 370, and 510 nm.

Compound identification was performed by comparison with authentic analytical standards including gallic acid, caffeic acid, catechin, syringic acid, ferulic acid, coumarin, rutin, vanillin, and quercetin based on retention times and UV–Vis spectra, and by comparison with literature data. Quantification was carried out using calibration curves generated from their respective authentic standards, including rosmarinic acid, carnosol, carnosic acid, carvacrol, rutin, quercetin, hypericin, and hyperforin. All calibration curves were obtained within a range of 20–200 mg/L, analyzing four concentrations (20, 50, 100, 200 mg/L) in

duplicate. Compound detection was performed at the specified wavelengths, and the linear equations obtained showed an $R^2 > 0.998$.

2.9. Statistical analysis

NemrodW (version 2000, LPRAI, Marseille, France) software was used for the experimental design and statistical analysis. The coefficients of determination (R^2) and analysis of variance (ANOVA) were used to assess the quality of fit of the regression models. The three-dimensional RSM approach and desirability were used to determine the best extraction conditions for the three independent variables and each dependent variable. In addition, a heatmap correlation analysis was performed to visualize and evaluate the relationships between the different studied parameters and bioactive properties.

3. Results and discussion

3.1. Optimization of *Laurus nobilis* extract

3.1.1. Fitting the second order polynomial models for the four responses

Experimental data were analyzed using Nemrod W software (LPRAI, version 2000) according to a Box–Behnken experimental design, and the responses were modeled using a second-order polynomial (quadratic) regression model. The adequacy of the models was evaluated using the coefficients of determination (R^2 and adjusted R^2 (R^2_A)).

The extraction efficiency of bioactive compounds from plant material is influenced by multiple factors, including extraction temperature and time, ultrasonic power, and proportion of hydroethanolic solvent. In this study, the models for the percentage inhibition of DPPH radical scavenging activity (PI, Y1), total phenolic content (TPC, Y2), total flavonoid content (TFC, Y3), and condensed tannin content (CTC, Y4) were developed using regression analysis with coefficients presented in Table 1:

For the Extraction temperature (X1), a synergistic effect was observed on TFC (Y3) and CTC (Y4), suggesting enhanced solubility and release of these compounds at higher temperatures. Conversely, temperature had an antagonistic effect on DPPH activity (Y1) and TPC (Y2), which may reflect the degradation of thermosensitive compounds under

Table 1
Regression coefficient and fit statistics of the predicted second order polynomial models for phenolic compounds and antiradical activity of *Laurus nobilis*.

Factor	DPPH	TPC	TFC	CTC
Intercept	52.62***	11.84*	96.08***	87.62
Linear				
X1	−3.76	−0.82	0.41	1.09
X2	2.56	0.22	13.31*	−64.42*
X3	−0.80	1.00	1.35	−30.45
X4	4.20	0.95	5.19	1.86
Cross product				
X1 × 2	−9.51*	−2.04	−4.92	−10.58
X1 × 3	−0.66	−5.25*	−7.12	−3.85
X1 × 4	2.20	−0.26	−4.05	0.77
X2 × 3	−1.13	−3.11	−16.92	81.16
X2 × 4	2.57	1.53	3.08	−11.15
X3 × 4	2.07	−1.07	−9.62	−4.80
Quadratic				
X1 ²	−7.08	0.15	7.09	21.70
X2 ²	−10.67	−0.23	27.86*	74.77
X3 ²	0.79	−2.53	−8.74	14.39
X4 ²	−2.56	−0.46	2.61	−34.85
R2	0.689	0.754	0.771	0.668
R2A	0.554	0.654	0.551	0.602

X1: Extraction temperature (°C); X2: extraction time (min); X3: Ultrasonic power (%); X4: Ethanol percentage (%); *: indicates significance level (p -value ≤ 0.05); **: indicates highly significant level (p -value ≤ 0.01); ***: indicates remarkably significant level (p -value ≤ 0.001). TPC: total polyphenol content; TFC: Total flavonoid content; CTC: condensed tannins content; DPPH: 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity.

elevated thermal conditions, consistent with previous reports (Dobroslavic et al., 2022). For extraction time (X2), while synergistic effects were noted for Y1, Y2 and Y3 (13.31*), a negative effect was observed on CTC (−64.42*), indicating that prolonged extraction may lead to tannin degradation. Notably, TFC initially increased with extraction time, reaching a maximum at 48 min, before declining with further extension. This pattern likely reflects the rapid initial release of flavonoids into the solvent, followed by gradual degradation due to prolonged exposure to extraction conditions (Dobroslavic et al., 2022).

Ultrasonic power (X3) positively affected TPC and TFC, which can be attributed to cavitation-induced mechanical and thermal effects that disrupt cell walls and enhance solvent penetration, facilitating the release of bioactive compounds (Dobroslavic et al., 2022). However, it negatively impacted DPPH activity and CTC, highlighting the differential stability and extractability of various phytochemicals under sonication.

Ethanol concentration (X4) exhibited synergistic effects across all responses (Y1–Y4). Hydroethanol mixtures were more effective than either water or absolute ethanol in extracting phenolics and flavonoids, with 35% ethanol identified as optimal for TPC which is consistent with previous findings (Muñiz-Márquez et al., 2013a).

Interactions between variables were also significant. The interaction of temperature and ultrasonic power ($X1 \times 3$) negatively affected TPC (−5.25*), suggesting that high temperature combined with strong sonication may accelerate degradation of phenolics. Similarly, the interaction between temperature and extraction time ($X1 \times 2$) had a negative effect (−9.51*) on DPPH scavenging activity, likely due to thermally induced breakdown of flavonoids and other antioxidants at higher extraction temperatures.

3.1.2. Optimization using the response surface method (RSM)

By setting the ultrasound percentage at 60% and the ethanol percentage (10%), Fig. 1 shows that increasing extraction temperature enhanced flavonoid and tannin contents but reduced total polyphenol content and inhibition percentage, with optimal values at 56°C.

The increase in flavonoid and tannin contents with rising temperature can be attributed to enhanced molecular motion, diffusion, penetration, and dissolution at higher temperatures, which facilitate improved extraction. However, flavonoid content (TFC) increased only up to 70°C, after which it begins to decline. This behavior aligns with findings of Dobroslavic et al. (2022) who confirmed that high temperatures ($>150^\circ\text{C}$) during pressurized lipid extraction led to the degradation and denaturation of flavonoid compounds.

Similarly, temperatures above 75°C promoted the degradation of key bioactive compounds, as reported in studies on environmentally friendly extraction methods for flavonoids (Rodríguez-Pérez et al., 2015). Regarding extraction time, it was observed that increasing time enhanced the percentage inhibition of DPPH, total polyphenols, and flavonoid contents but reduced tannin content, with optimal values recorded at 48 min. These results are consistent with findings by Muñiz-Márquez et al. (2013), who reported that the extraction time maximizing phenolic compound recovery ranged between 40 and 60 min.

The quadratic terms (X_1^2 , X_2^2 , X_3^2 , X_4^2) describe the curvature of the response surfaces and indicate non-linear relationships between the extraction variables and the studied responses. These terms are essential in response surface methodology to identify the presence of optimal operating conditions within the experimental domain.

A negative quadratic coefficient indicates a concave response surface, suggesting the existence of a maximum point, while a positive coefficient reflects a convex shape, indicating a minimum or more complex non-linear behavior.

In this study, significant quadratic effects were observed particularly for X_2^2 in the TFC response (27.86*) and for several terms in the CTC response, notably X_2^2 (74.77) and X_4^2 (−34.85). These results confirm that extraction time (X_2) and ethanol percentage (X_4) exert strong non-

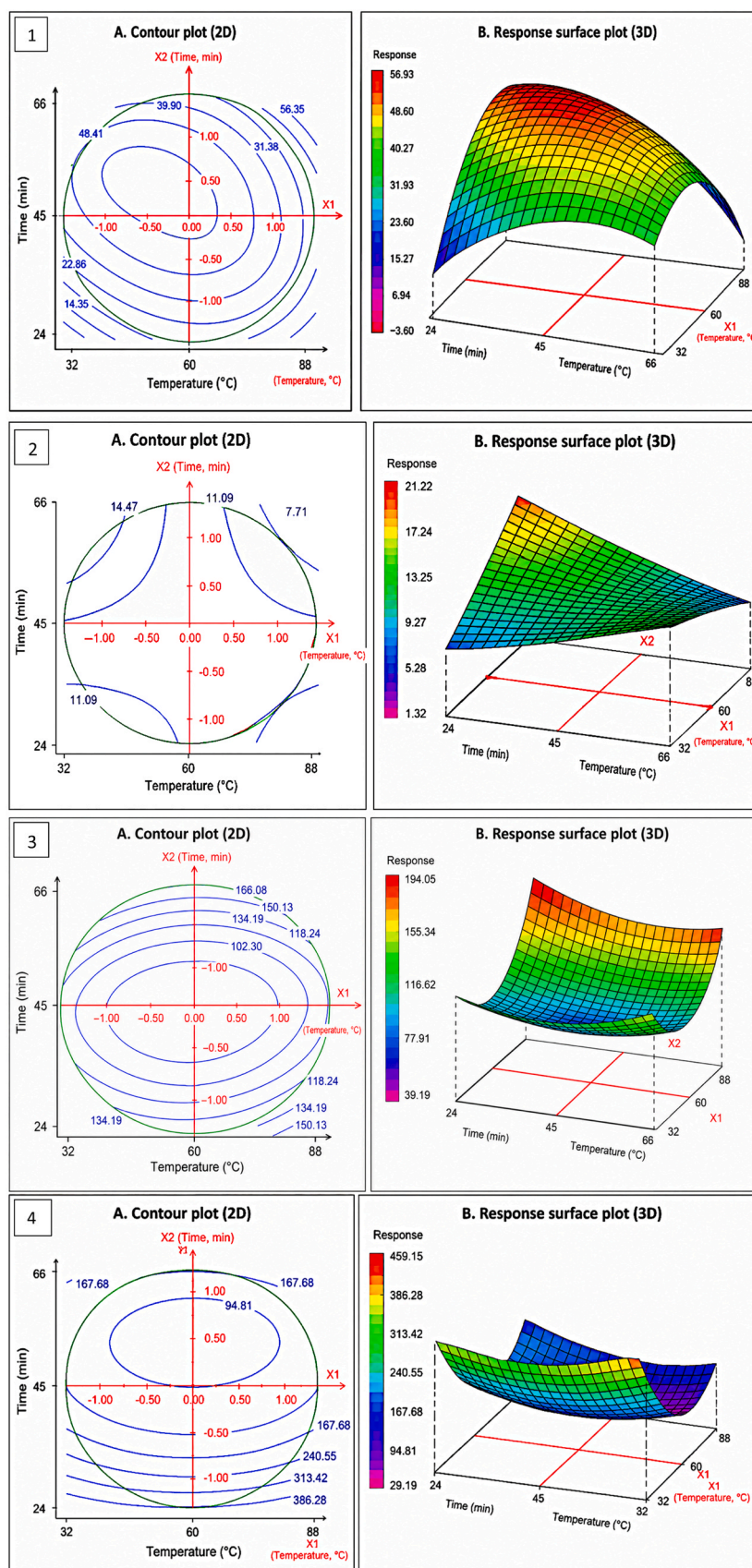


Fig. 1. 2D and 3D model of the response surface method for DPPH (1), total polyphenols content (2), flavonoids content (3) and condensed tannins content (4) of *Laurus nobilis*.

linear effects on bioactive compound extraction.

3.1.3. Determination of optimal conditions using the desirability method

The desirability method provided optimal Eco-extraction conditions and estimated values for total polyphenols, flavonoids, tannins, and the percentage of inhibition of DPPH (PI), as well as percentage desirability. A compromise between the four responses (Y1, Y2, Y3, and Y4) allowed us to determine the optimal conditions.

The optimum combination of factors and levels was obtained using the Response Surface Methodology (RSM). The optimal extraction conditions for maximum TPC (7.01 mg GAE/gDW), TFC(115.71 mgCE/gDW), CTC(91.39 mgCE/gDW) and percentage of inhibition of DPPH (30.40%), generated by Design-Expert software, were as follows: an extraction temperature of 75°C, an extraction time of 56 min, an ethanol concentration of 4%, and an ultrasonic power of 74% (Refer to Table S8 and Table S9 in the Supplementary Material).

3.2. Optimization of Rosmarinus officinalis extract

3.2.1. Fitting the second order polynomial models for the four responses

The models proposed for the selected responses: The percentage of inhibition of DPPH (PI:Y1), total phenolic content (TPC, Y2), total flavonoid content (TFC, Y3), and condensed tannin content (CTC, Y4) were represented by equations Y1–Y4, with regression coefficients which are presented in Table 2:

Extraction temperature showed a synergistic effect on CTC, while it exhibited an antagonistic effect on DPPH activity, TPC, and TFC.

Higher temperatures reduced solvent viscosity, facilitating mass transfer and enhancing the diffusion of bioactive compounds through plant cell membranes; however, excessive heating can accelerate the degradation of thermolabile compounds (Liu et al., 2024). Khan et al. (2025) also reported that temperatures above 50 °C significantly reduced TPC. Generally, elevated temperatures improved phenolic extraction through enhanced solubility, matrix disruption, and increased solvent diffusion, but prolonged exposure can compromise compound stability. The extraction process can be divided into a rapid “washing phase” during the first 10–20 min, accounting for most of the phenolic release, followed by a slower diffusion-controlled phase (Khan

Table 2
Regression coefficient and fit statistics of the predicted second order polynomial models for phenolic compounds and antiradical activity of *Rosmarinus officinalis*.

Factor	DPPH	TPC	TFC	CTC
Intercept	24.37	44.29**	105.31***	89.92***
Linear				
X1	−5.31	−0.77	−5.37	1.22
X2	−4.26	8.63*	4.42	0.32
X3	−10.29*	−0.62	−4.74	−2.89
X4	6.89	5.44	25.09**	−4.55
Cross product				
X1 × 2	4.91	0.36	−3.27	1.92
X1 × 3	−1.02	9.24	12.50	−1.35
X1 × 4	−9.97	−3.22	7.25	−5.39
X2 × 3	−4.88	−2.83	10.00	3.46
X2 × 4	−2.42	−3.72	−1.92	0.58
X3 × 4	−0.95	7.86	3.27	10.00
Quadratic				
X1 ²	8.71	−6.49	−9.92	0.80
X2 ²	16.60	6.13	6.55	−1.51
X3 ²	11.47	−2.47	12.60	−4.97
X4 ²	1.59	9.20	14.40	−0.74
R2	0.673	0.725	0.656	0.656
R2A	0.656	0.540	0.600	0.600

X1: extraction temperature (°C); X2: extraction time (min); X3: ultrasonic power (%); X4: ethanol percentage (%);*: indicates significance level (p-value ≤ 0.05); **: indicates highly significant level (p-value ≤ 0.01); ***: indicates remarkably significant level (p-value ≤ 0.001). TPC: total polyphenol content; TFC: total flavonoid content; CTC: condensed tannins content; DPPH: 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity.

et al., 2025).

Extraction time positively influenced TPC (8.63*), TFC, and CTC, while its effect on DPPH activity was antagonistic, suggesting that extended extraction promoted accumulation of phenolics and flavonoids but may lead to the degradation of some antioxidants (Liu et al., 2019).

Ultrasonic power showed an antagonistic effect on all responses. In fact, moderate ultrasonic intensity enhanced cell wall disruption and improves extraction, but excessive power causes cavitation bubbles that reduced energy transfer and extraction efficiency (Khan et al., 2025).

Ethanol percentage had synergistic effects on DPPH, TPC, and TFC (25.09**), but an antagonistic effect on CTC. Hydroethanolic mixtures are generally more effective than water or absolute ethanol in extracting phenolics and flavonoids (Dobrosłavić et al., 2022), with water/ethanol mixtures demonstrating optimal extraction across various temperatures and plant materials (Menhem et al., 2021). Additionally, ultrasound-assisted extraction using ethanol or water has been shown to yield significantly higher TPC compared to Soxhlet extraction, highlighting the combined effect of solvent and ultrasonic energy on compound recovery (Bontzolis et al., 2024).

The quadratic effects showed different behaviors depending on the response.

X1² exhibited inconsistent values across the responses, indicating a weak and non-systematic curvature effect. X2² was predominantly positive, suggesting a convex response surface and a tendency toward a minimum or less pronounced optimum. X3² showed mixed but relatively strong values, indicating an important non-linear effect with response-dependent behavior. X4² was mostly positive and relatively high for some responses, suggesting that ethanol percentage strongly influences the curvature of the models and contributes to non-linear response behavior.

3.2.2. Optimization using the response surface method

By setting the ultrasound percentage at 60% and the ethanol percentage (10%) at the central point of the plane, the 2D and 3D response surface graphs (Fig. 2) shows that, as extraction temperature was elevated, tannin content increased while total polyphenol content, flavonoid content, and inhibition percentage decreased with optimal values recorded at 30°C.

Extraction time increased the contents of total polyphenols, flavonoids, and tannins and decreases the inhibition percentage of DPPH with optimal values recorded around 24 min. These results confirm those of Leal et al. (Leal et al., 2011) who demonstrate that sonication is a more effective extraction technique when compared to thermal methods within 15 min at 50°C. Also, Paniwnyk et al. (2009) has shown that at 35°C, short-time (30 min) is more efficient than long-time extraction (60 min).

3.2.3. Determination of optimal conditions using the desirability method

The optimal extraction conditions were generated by the Design-Expert software. Extraction conditions for maximum TPC (51.43 mg GAE/mgDW), TFC(129.88 mg CE/gDW), TCC(89.64 mg CE/gDW), and percentage of inhibition of DPPH (36.94%) were as follows: extraction temperature of 25°C, extraction time of 51 min, ethanol concentration of 17%, and ultrasonic power of 89% (refer to table S11 and table S12 in the Supplementary Material).

In this context, ullah et al., (2026b) reported that the optimal extraction conditions for dandelion, as determined by RSM, were a sonication time of 30 min, an ultrasound amplitude of 70%, and an extraction temperature of 40 °C.

3.3. Optimization of Pimpinella anisum extract

3.3.1. Fitting the second order polynomial models for the four responses

The models proposed for the selected responses: th percentage of inhibition of DPPH scavenging activity (PI: Y1), total phenolic content (TPC, Y2), and total flavonoid content (TFC, Y3) are represented by

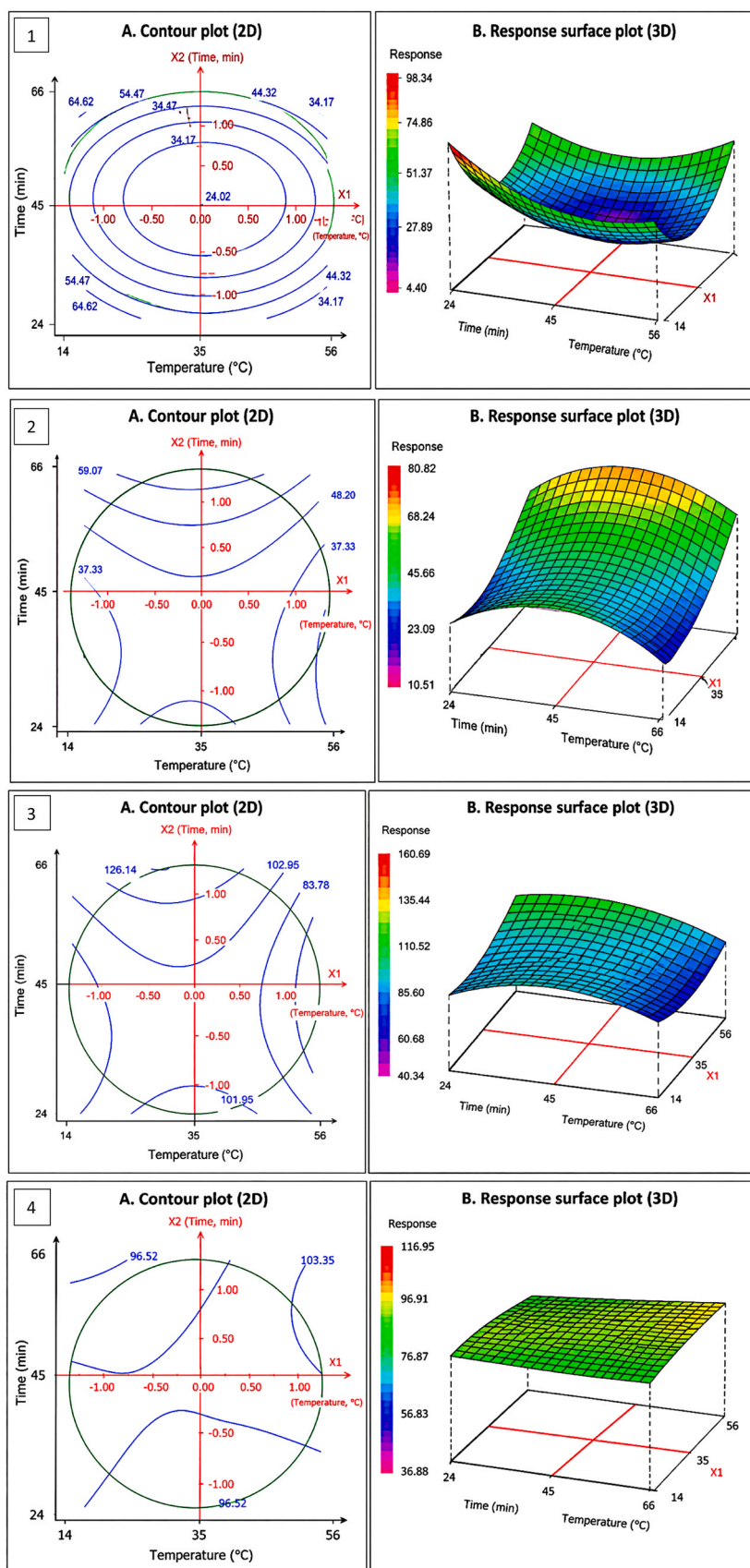


Fig. 2. 2D and 3D model of the response surface method for DPPH (1), total polyphenols Content (2), flavonoids content (3) and condensed tannins content (4) of *Rosmarinus officinalis*.

equations Y1–Y3, with regression coefficients calculated using Nemrod W software (LPRAI, version 2000) and presented in Table 3.

Extraction temperature (X1) showed a synergistic effect on the percentage of inhibition of DPPH (PI:Y1), indicating enhanced antioxidant activity at higher temperatures, while it exhibited an antagonistic effect on TPC and TFC, suggesting that elevated temperatures can promote flavonoid dissolution but may also accelerate their degradation because of thermal instability.

Maceration time (X2) had a synergistic effect on TPC and TFC, which enhanced solvent penetration and ultrasonic energy transfer leading to facilitate the release of phenolic compounds. However, prolonged extraction negatively affected DPPH activity, likely due to the degradation of sensitive antioxidant molecules (Liu et al., 2019; Khan et al., 2025).

For ultrasonic power (X3), a positive effect was observed on TPC, reflecting the role of cavitation and mechanical disruption in improving phenolic extraction, whereas DPPH activity and TFC were negatively impacted, possibly due to excessive cavitation leading to reduced energy transfer efficiency and compound degradation.

Ethanol concentration (X4) positively influenced TFC which is in agreement with previous reports on the effectiveness of hydroethanolic mixtures in enhancing flavonoid solubility, while the percentage inhibition of DPPH and TPC showed antagonistic responses, which may be attributed to the differential solubility of specific compounds and the sensitivity of phenolics to higher alcohol concentrations.

The most pronounced quadratic effect was observed for X_2^2 , particularly for the third response (−23.00), indicating a strong concave curvature and confirming that extraction time has a major influence on the response with a clear optimal point beyond which the response decreases.

Moderate quadratic effects were observed for X_1^2 and X_4^2 , while X_3^2 showed a weak but positive curvature, suggesting a limited non-linear effect on the studied responses.

3.3.2. Optimization using the response surface method

By setting the ultrasound percentage at 60% and the ethanol percentage (10%) at the central point of the plane, the 2D and 3D response surface graphs (Fig. 3) shows that as extraction temperature increased,

Table 3

Regression coefficient and fit statistics of the predicted second order polynomial models for phenolic compounds and antiradical activity of *Pimpinella anisum*.

Factor	DPPH	TPC	TFC
Intercept	22.02	21.63	124.51
Linear			
X1	0.21	−0.25	−6.22
X2	−6.70	0.37	7.18
X3	−2.78	1.16	−1.54
X4	−1.91	−0.36	9.94
Cross product			
X1 × 2	2.52	5.77	4.23
X1 × 3	−9.95	−1.33	21.54
X1 × 4	−6.05	3.78	1.35
X2 × 3	−0.60	0.92	4.81
X2 × 4	−0.45	−4.54	−3.66
X3 × 4	−2.36	−1.94	−5.96
Quadratic			
X1 ²	6.91	1.92	−0.21
X2 ²	−7.21	−6.58	−23.00
X3 ²	0.26	0.15	8.16
X4 ²	2.85	1.35	−5.02
R ²	0.633	0.694	0.657
R ² A	0.519	0.566	0.576

X1: extraction temperature (°C); X2: extraction time (min); X3: ultrasonic power (%); X4: ethanol percentage (%);*: indicates significance level (p -value ≤ 0.05); **: indicates highly significant level (p -value ≤ 0.01); ***: indicates remarkably significant level (p -value ≤ 0.001). TPC: total polyphenol content; TFC: total flavonoid content; CTC: condensed tannins content; DPPH: 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity.

the inhibition percentage increases while total polyphenol content and flavonoid content decreased, with optimal values recorded around 15°C. In this context, the optimized UAE conditions for extraction of total phenolics from fennel are as follow: 25°C of temperature, methanol proportion (80:20) of hydroalcoholic solvent, 20 min of processing time (Leal et al., 2011).

Extraction time increased the content of total polyphenols and flavonoids and decreased the inhibition percentage of DPPH with optimal values recorded at 11 min. These findings are confirmed by Leal et al. (2011) who reported that the extraction time that maximized recovery of phenolic compounds ranged 10 min. In fact, the content of total polyphenols and flavonoids increases until 10 min of extraction and then decreases. Moreover, they demonstrated that 10 min present the highest yield between different extracts obtained by Soxhlet, Ultrasound, Percolation, Centrifugation, and Steam Distillation (Leal et al., 2011).

3.3.3. Determination of optimal conditions using the desirability method

The optimal extraction conditions were generated by the Design-Expert software. Extraction conditions for maximum TPC (21.62 mgGAE/gDW), TFC (124.51mgCE/DW), and percentage of inhibition of DPPH (22.02%) were as follows: extraction temperature of 50°C, extraction time of 20 min, ethanol concentration of 10%, and ultrasonic power of 60% (Refer to table S14 and Table S15 in the Supplementary Material). In this context, Khan et al. (2026) reported that the optimal extraction conditions for caffeine in black tea were 175 W ultrasonic power, 50 °C extraction temperature, 8 min extraction time, and a solvent-to-solid ratio of 35 mL/g.

Previous studies have employed RSM to improve the extraction of phenolics from plant materials. However, none of them have used this tool to simultaneously optimize conditions for total polyphenols, total flavonoids (TFC), condensed tannins contents and antioxidant activity of *Laurus nobilis*, *Rosmarinus officinalis*, *Pimpinella anisum* and their mixture. Therefore, this contribution extends the current knowledge and/or application of RSM.

3.4. Optimization of the mixture of the three plants

3.4.1. The second order polynomial models for the responses depending to variables

The models postulated for the selected responses: the percentage of inhibition of DPPH, total phenolic content, and total flavonoid content were given by equations (Y1, Y2 and Y3) whose regression coefficients are calculated by the Nemrod W software (LPRAI, version 2000) and represented in Table 4.

The models included linear, interaction, and quadratic terms, and the significance of each term was evaluated by analysis of variance (ANOVA). Significant effects are indicated as follow: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The obtained regression equations are as follows:

$$Y1 = 39.743 * X1 + 59.486 * X2 + 54.859 * X3 + 8.693 * (X1 * X2) - 26.880 * (X1 * X3) + 1.407 * (X2 * X3)$$

$$Y2 = 29.184 * X1 + 38.131 * X2 + 23.986 * X3 - 12.863 * (X1 * X2) + 15.206 * (X1 * X3) + 14.299 * (X2 * X3)$$

$$Y3 = 122.891 * X1 + 156.527 * X2 + 113.464 * X3 + 28.201 * (X1 * X2) - 18.001 * (X1 * X3) - 18.414 * (X2 * X3)$$

With:

X1: percentage of *Pimpinella anisum*; X2: percentage of *Rosmarinus officinalis*; X3: percentage of *Laurus nobilis*.

The obtained models showed good predictive ability, with coefficients of determination (R^2) ranging from 0.783 to 0.980, and adjusted R^2 (R^2A) values between 0.580 and 0.954, indicating an acceptable to excellent fit between experimental and predicted values.

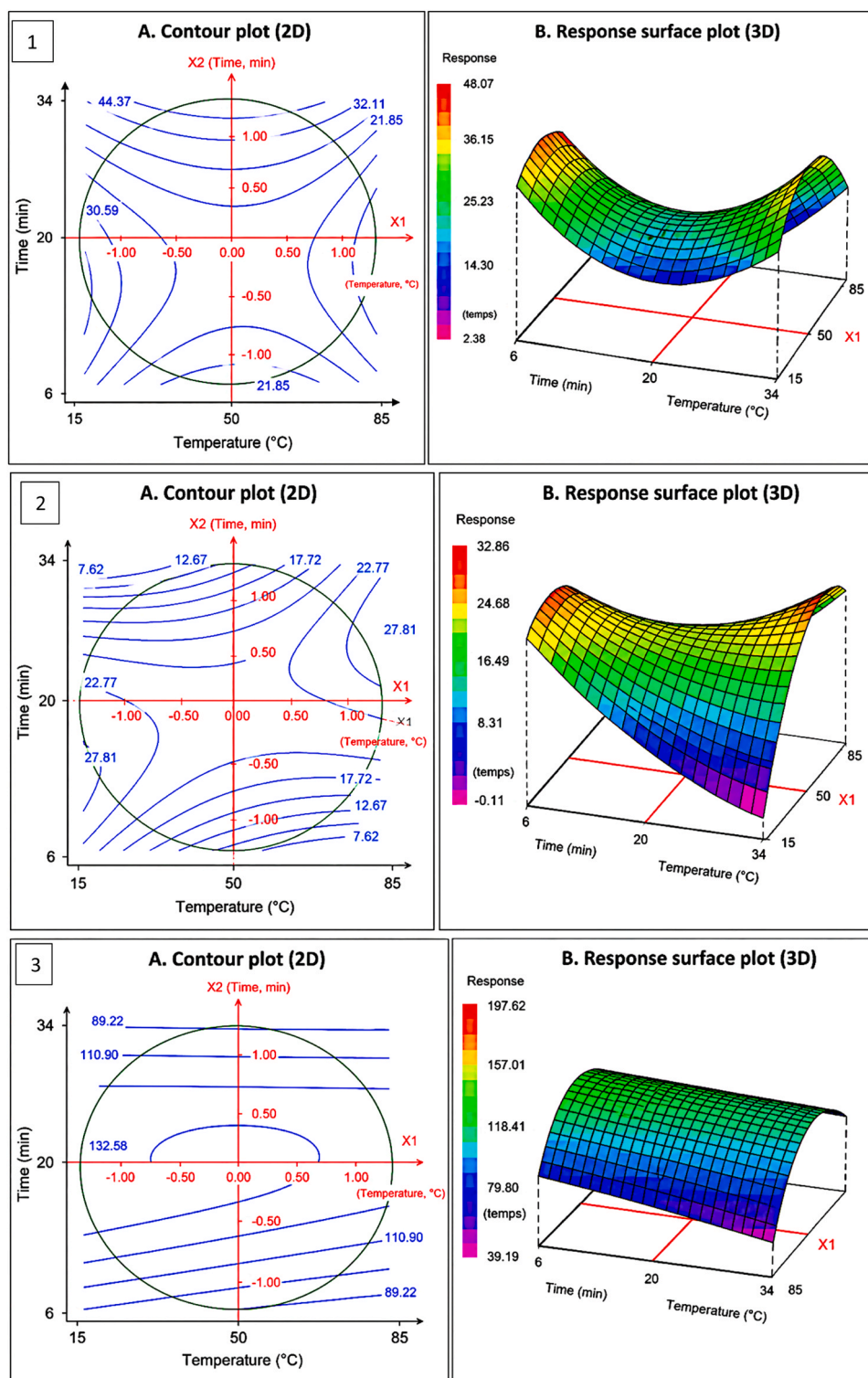


Fig. 3. 2D and 3D model of the response surface method for DPPH (1), total polyphenols Content (2), flavonoids content (3) of *Pimpinella anisum*.

3.4.1.1. Effects of linar (individual plants) on the responses studied. The linear terms represent the individual contribution of each factor (X1, X2, X3) on the studied responses.

For DPPH inhibition (Y1), all three factors exhibited positive effects, with X2 showing the highest significant contribution (59.49***), indicating that *Rosmarinus officinalis* plays a major role in enhancing antioxidant activity.

For total phenolic content (Y2), X1 (29.18**) and X2 (38.13**) showed significant positive effects, suggesting that *Pimpinella anisum* and *Rosmarinus officinalis* contribute strongly to phenolic accumulation.

Similarly, for total flavonoid content (Y3), all three factors showed strong positive and significant effects, particularly X2 (156.58***), confirming its major influence on flavonoid enrichment.

3.4.1.2. Effects of the binary combination (X1 × X2) on the responses studied. According to Table 4, the effect of the combination X1 × X2 on

Table 4

Regression coefficient and fit statistics of the predicted second order models for phenolic compounds and antiradical activity of the mixture.

Individual product			
X1	+ 39.74***	+ 29.18 **	+ 122.89***
X2	+ 59.49***	+ 38.13**	+ 156.58***
X3	+ 54.86***	+ 23.99**	+ 113.46***
Cross product			
X1 × 2	+ 8.69	−12.86	+ 28.20
X1 × 3	−26.88 *	+ 15.21	−18.00
X2 × 3	+ 1.41	+ 14.30	−18.41
R ²	0.980	0.783	0.888
R2A	0.954	0.580	0.748

X1: *P. anisum* extract percentage; X2: *R. officinalis* extract percentage; X3: *L. nobilis* extract percentage; *: indicates significance level (p -value ≤ 0.05); **: indicates highly significant level (p -value ≤ 0.01); ***: indicates remarkably significant level (p -value ≤ 0.001). TPC: total polyphenol content; TFC: total flavonoid content; DPPH: 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity.

the Y1 and Y3 responses was synergistic. This suggests that mixing *Rosmarinus officinalis* with *Pimpinella anisum* leads to an increase in the studied responses, namely the percentage of inhibition of DPPH and total flavonoid content.

In contrast, the effect of the combination X1 × 2 on the Y2 response was antagonistic. This indicates that adding *Rosmarinus officinalis* to *Pimpinella anisum* results in a decrease in total phenolic content.

These results suggest that the antioxidant activity of the X1 × 2 extract combination could be primarily attributed to its flavonoid content.

3.4.1.3. Effects of the binary combination (X1 × 3) on the responses studied. The effect of the combination X1 × 3 on the Y2 response (TPC) was synergistic (p -value ≤ 0.05) (Table 4). This suggests that mixing *Laurus nobilis* with *Pimpinella anisum* leads to an increase in total phenolic content.

In contrast, the effect of the combination X1 × 3 on the Y1 (PI) and Y3 (TFC) responses was antagonistic. This indicates that the addition of *Laurus nobilis* to *Pimpinella anisum* results in a decrease in these studied responses.

3.4.1.4. Effects of the binary combination (X2 × 3) on the responses studied. According to Table 4, the effect of the combination X2 × 3 on the Y1 (PI) and Y2 responses (TPC) was synergistic. This suggests that mixing *Laurus nobilis* and *Rosmarinus officinalis* leads to an increase in total phenolic content.

On the other hand, the effect of the combination X2 × 3 (*R. officinalis* and *L. nobilis*) on the Y3 response (TFC) was antagonistic. This indicates that the addition of *Laurus nobilis* to *Rosmarinus officinalis* results in a decrease in flavonoid content.

These findings suggest that the antioxidant activity of the X2 × 3 combination is primarily driven by other phenolic compounds rather than flavonoids which aligns with Sharma et al. (2020). These authors observed that synergistic interactions in binary combinations of essential oils (*O. gratissimum* with *C. flexuosus* and *V. negundo*) were associated with phenolic compounds, as evidenced by low IC₅₀ values (0.79 and 0.76, respectively). Conversely, antagonistic interactions in combinations with *Citronella java* and *M. longifolia* (IC₅₀ = 1.02 and 1.26, respectively) highlight the importance of appropriate compound concentrations and specific interactions.

The observed synergistic and antagonistic effects in our study may similarly result from the complex interplay between phenolic monoterpenes, phenylpropanoids, and other antioxidant compound. These results emphasize the need to optimize concentrations and combinations of antioxidants to achieve maximum bioactivity.

3.4.1.5. Quadratic effects. Quadratic terms describe curvature effects

and are essential for identifying optimal extraction conditions. Several significant quadratic effects were observed, particularly for Y2 and Y3, indicating the presence of non-linear behavior in the extraction system. These results confirm that the responses do not vary linearly with the factors and that optimal conditions exist within the experimental domain.

3.4.2. Optimization using the response surface method

Fig. 4 shows that, for the percentage of inhibition of DPPH, the binary combinations producing the highest total antioxidant activity were those between *Pimpinella anisum* and *Laurus nobilis* (48.68% and 52.35% of *L. nobilis*) and between *Laurus nobilis* and *Rosmarinus officinalis* (56.01% of *L. nobilis*).

Similarly, for polyphenol content, the binary combinations of *Pimpinella anisum* with *Laurus nobilis* and *Laurus nobilis* with *Rosmarinus officinalis* resulted the highest content (35.20 and 37.76 mg eqGA/g DW).

For flavonoid content, the binary combinations of *P. anisum* with *L. nobilis* and *L. nobilis* with *R. officinalis* also exhibited significant levels (114.33 and 123.14 mgC/g DW, respectively). However, the tertiary combinations showed the best recorded flavonoid contents (140.67 and 149.57 mgC/g DW).

These findings are consistent with those reported by De Souza Vieira et al. (2019), who observed that rosemary extract exhibited a synergistic effect when combined in equal concentrations with thyme and oregano extracts in a tertiary mixture (33.3 mg GAE/kg oil per herb) or in a binary mixture with thyme or oregano (50 mg GAE/kg oil per herb). The enhanced activity observed in the tertiary combinations suggests that the synergy between bioactive components can optimize antioxidant effects when combined in appropriate proportions.

This is supported by Sharma et al. (2020), who highlighted that bioactive components often act synergistically due to their multiple mechanisms of action and diverse target sites. This synergy broadens the spectrum of activity and results in greater efficacy compared to the effects of individual components used independently. In contrast to these reports, the Zhourat extracted by Menhem et al. (2021), did not demonstrate a synergistic effect at the level of the whole mixture, as its free radical scavenging activity was not higher than that of the individual plants. On the contrary, the antioxidant activity was even lower than that of most single plant extracts when water or ethanol were used as solvents. This divergence suggests that, while localized synergistic or antagonistic interactions may have occurred among certain plant combinations within the Zhourat blend, they were not sufficient to translate into a global synergistic effect for the mixture.

3.4.3. Determination of optimal conditions using the desirability method

For maximum TPC (30.68 mgGAE/gDW), TFC (130.34 mg CE/gDW) and percentage of inhibition of DPPH (42.78%), the optimal combination was constituted of 64.9% of *P. anisum* 13.8% of *R. officinalis* and 21.3% of *L. nobilis* (See Table 17 and Table 18 in the Supplementary Material).

Some other studies have employed a mixture of plants to study the antimicrobial activities of their combinations (Donkor et al., 2023). However, none of them have used a mixture design and RSM. Our study is the first attempt to simultaneously optimize conditions for total polyphenols, total flavonoids (TFC) and antioxidant activity in the combinations of these three aromatic and medicinal plant extracts by an Augmented simplex centroid design.

3.5. Polyphenolic profile of *Laurus nobilis*, *Rosmarinus officinalis*, *Pimpinella anisum* extracts and their mixture

The results of phenolic compound quantification using HPLC analysis based on multiple standards, presented in Table 5, demonstrated good to excellent repeatability, with most CV values below 10%, indicating high analytical precision.

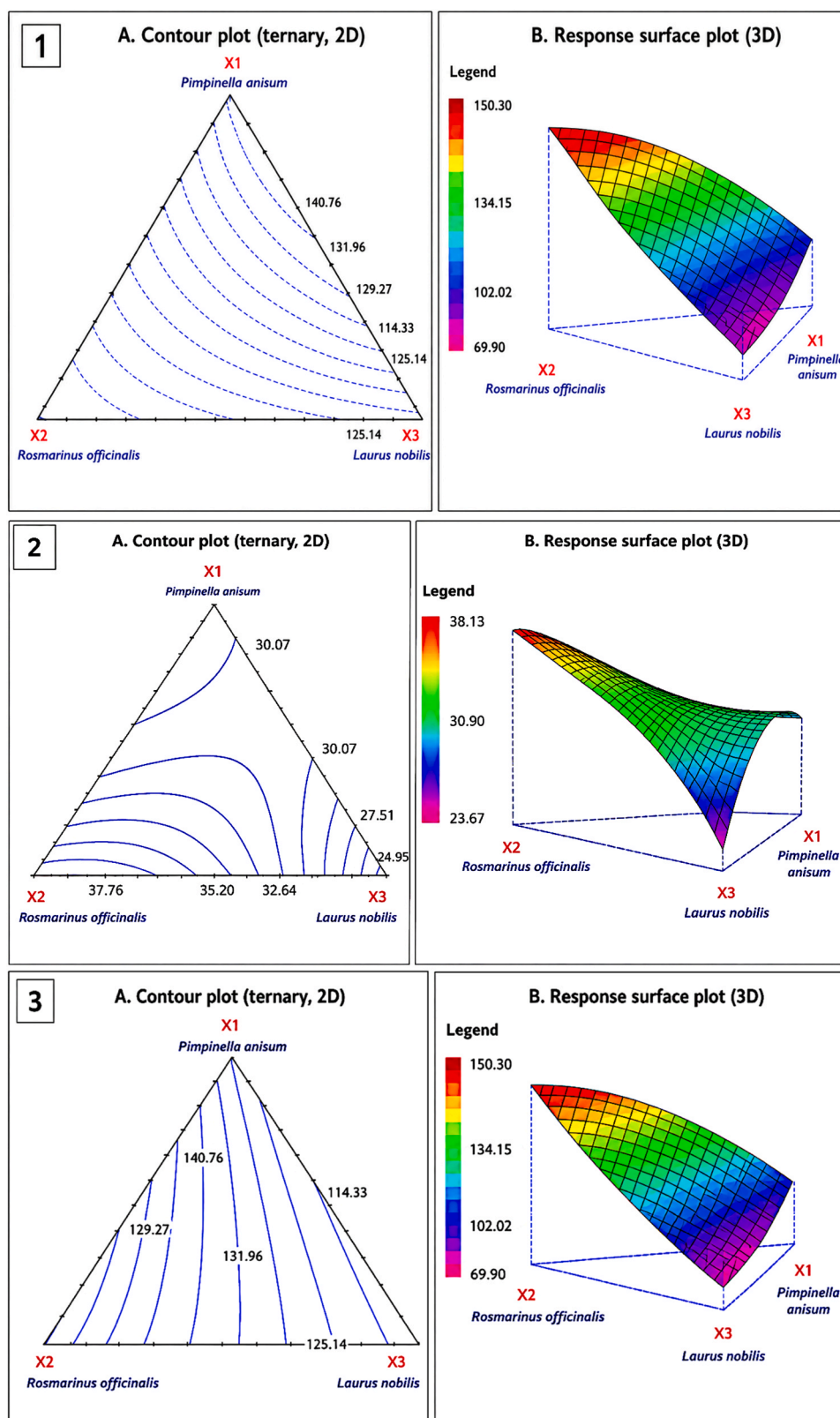


Fig. 4. 2D and 3D model of the response surface method for DPPH (1), total polyphenols content (2) and flavonoids content (3) of the mixture.

Table 5 shows that for *Laurus nobilis*, the most abundant phenolic compounds were caffeic acid ($261.88 \pm 7.46 \mu\text{g/g}$), hydrobenzoic acid ($279.21 \pm 4.90 \mu\text{g/g}$), cinnamic acid ($117.65 \pm 8.45 \mu\text{g/g}$), p-coumaric acid ($100.10 \pm 2.76 \mu\text{g/g}$), p-quinic acid ($109.15 \pm 7.82 \mu\text{g/g}$), and rosmarinic acid ($88.72 \pm 10.21 \mu\text{g/g}$) while intermediate compounds

included salicylic acid ($35.16 \pm 1.51 \mu\text{g/g}$) and anisic acid ($31.43 \pm 2.74 \mu\text{g/g}$), and compounds detected at moderate concentrations included sinapic acid ($27.01 \pm 2.33 \mu\text{g/g}$), m-anisic acid ($21.24 \pm 0.92 \mu\text{g/g}$), hydrocinnamic acid ($15.71 \pm 0.64 \mu\text{g/g}$), o-anisic acid ($19.25 \pm 0.68 \mu\text{g/g}$), ferulic acid ($15.32 \pm 0.94 \mu\text{g/g}$), chlorogenic acid

Table 5Phenolic composition of *Pimpinella anisum*, *Rosmarinus officinalis*, *Laurus nobilis*, and their combined extract (mean $\pm$ SD).

Compound	Retention time (RT) min	<i>P. anisum</i> $\mu\text{g/g}$	<i>R. officinalis</i> $\mu\text{g/g}$	<i>L. nobilis</i> $\mu\text{g/g}$	Mixture $\mu\text{g/g}$
Gallic acid	2.97	37.92 $\pm$ 1.56 ^c	96.66 $\pm$ 3.32 ^a	57.81 $\pm$ 1.87 ^b	64.69 $\pm$ 4.86 ^b
Chlorogenic acid	10.87	9.70 $\pm$ 1.41 ^c	20.14 $\pm$ 1.23 ^a	19.53 $\pm$ 0.82 ^a	15.56 $\pm$ 1.80 ^b
Ferulic acid	19.72	100.48 $\pm$ 5.73 ^a	8.41 $\pm$ 1.24 ^c	15.32 $\pm$ 0.94 ^c	97.62 $\pm$ 6.53 ^a
Salicylic acid	23.48	17.03 $\pm$ 1.50 ^b	14.44 $\pm$ 3.74 ^b	35.16 $\pm$ 1.51 ^a	15.79 $\pm$ 1.86 ^b
o-Anisic acid	17.25	18.17 $\pm$ 1.10 ^b	39.66 $\pm$ 1.77 ^a	19.25 $\pm$ 0.98 ^b	17.24 $\pm$ 1.38 ^b
Anisic acid	25.02	8.78 $\pm$ 1.56 ^c	2.27 $\pm$ 0.13 ^c	31.43 $\pm$ 2.74 ^a	13.52 $\pm$ 0.56 ^b
m-Anisic acid	26.79	18.89 $\pm$ 1.31 ^b	1.48 $\pm$ 0.34 ^c	21.24 $\pm$ 0.92 ^a	16.82 $\pm$ 0.73 ^b
Hydrocinnamic acid	31.44	20.00 $\pm$ 2.22 ^b	35.16 $\pm$ 2.25 ^a	15.71 $\pm$ 0.04 ^b	35.10 $\pm$ 0.48 ^a
Cinnamic acid	38.61	147.28 $\pm$ 0.67 ^a	12.73 $\pm$ 0.11 ^c	117.65 $\pm$ 8.45 ^b	38.17 $\pm$ 0.80 ^c
Caffeic acid	11.50	23.14 $\pm$ 1.07 ^c	17.41 $\pm$ 2.41 ^c	261.88 $\pm$ 7.64 ^a	30.36 $\pm$ 0.85 ^b
Rosmarinic acid	38.13	58.30 $\pm$ 7.90 ^b	29.10 $\pm$ 0.06 ^c	88.72 $\pm$ 10.21 ^a	94.53 $\pm$ 4.971 ^a
Tannic acid	3.09	138.87 $\pm$ 0.83 ^a	55.29 $\pm$ 0.60 ^b	57.59 $\pm$ 5.71 ^b	11.54 $\pm$ 0.64 ^c
p-Coumaric acid	16.81	31.92 $\pm$ 0.54 ^b	11.05 $\pm$ 1.02 ^c	100.10 $\pm$ 2.76 ^a	30.38 $\pm$ 1.04 ^b
p-Quinic acid	90.32	33.36 $\pm$ 0.32 ^c	92.21 $\pm$ 0.84 ^b	109.15 $\pm$ 7.82 ^a	67.98 $\pm$ 0.95 ^b
Sinapic acid	21.37	31.58 $\pm$ 3.56 ^b	13.45 $\pm$ 0.45 ^c	27.01 $\pm$ 2.33 ^b	36.02 $\pm$ 1.78 ^a
Hydrobenzoic acid	8.09	22.78 $\pm$ 1.17 ^c	38.46 $\pm$ 0.49 ^b	279.21 $\pm$ 4.90 ^a	38.44 $\pm$ 1.70 ^b
Catechin hydrate	8.46	5.39 $\pm$ 0.61 ^c	3.76 $\pm$ 0.34 ^c	13.34 $\pm$ 0.55 ^b	19.17 $\pm$ 0.54 ^a
Epicatechin gallate	12.6	17.87 $\pm$ 0.55 ^c	13.14 $\pm$ 0.43 ^c	154.83 $\pm$ 3.51 ^b	176.75 $\pm$ 1.71 ^a
Myricetin	35.54	4.49 $\pm$ 0.24 ^b	0.35 $\pm$ 0.04 ^c	12.97 $\pm$ 0.21 ^a	3.18 $\pm$ 0.29 ^b
Naringin	31.10	41.03 $\pm$ 0.40 ^a	3.15 $\pm$ 0.24 ^c	6.22 $\pm$ 0.08 ^c	17.08 $\pm$ 0.46 ^b
Kaempferol	66.87	24.06 $\pm$ 0.06 ^a	4.59 $\pm$ 0.31 ^c	8.16 $\pm$ 0.57 ^b	3.15 $\pm$ 0.17 ^c
Quercetin	53.99	16.63 $\pm$ 0.66 ^a	2.12 $\pm$ 0.27 ^c	6.15 $\pm$ 0.08 ^b	5.65 $\pm$ 0.43 ^b
Quercetin dihydrate	53.75	55.19 $\pm$ 0.35 ^a	35.69 $\pm$ 0.32 ^b	19.57 $\pm$ 0.11 ^c	34.87 $\pm$ 1.46 ^b
Rutin	42.00	12.58 $\pm$ 0.33 ^b	1.97 $\pm$ 0.02 ^c	5.36 $\pm$ 0.46 ^c	14.56 $\pm$ 0.29 ^a
Rutin hydrate	31.13	13.49 $\pm$ 0.87 ^b	21.99 $\pm$ 0.04 ^a	5.41 $\pm$ 0.44 ^c	25.44 $\pm$ 0.73 ^a

(19.53 $\pm$ 0.82 $\mu\text{g/g}$), quercetin dihydrate (19.57 $\pm$ 0.11 $\mu\text{g/g}$).

These results are in great consistence to those found by [El Faqer et al. \(2024\)](#) who prepared both ethanolic and aqueous extracts of *Laurus nobilis* from Morocco and detected peaks corresponding to ferulic acid, caffeic acid, syringic acid, and rutin in both extracts, but they did not observe peaks for gallic acid, catechin, vanillin, or p-coumaric acid. On the other hand, [Hassan et al. \(2024\)](#) identified six molecules in bay laurel by LC-MS of ethanolic extracts obtained by maceration, including two octahydroxy-2,7'-cycloignans (R- and S-isomers) and four kaempferol 3-glycosides with different cinnamoyl substitutions. This comparison could clearly demonstrate that the polarity of solvent, the geographical origin of the plant, the extraction method, and the analytical technique for phenolic compounds identification could strongly influence the phenolic profile detected in bay laurel.

According to [Table 5](#), the optimized hydro-methanolic extract of the Tunisian medicinal plant *Rosmarinus officinalis* contained the following compounds: The most abundant were gallic acid (96.66 $\pm$ 3.32 $\mu\text{g/g}$), tannic acid (55.29 $\pm$ 0.60 $\mu\text{g/g}$), p-quinic acid (92.21 $\pm$ 0.84 $\mu\text{g/g}$), the intermediate compounds were rutin hydrate (21.99 $\pm$ 0.04 $\mu\text{g/g}$), quercetin hydrate (35.65 $\pm$ 0.32 $\mu\text{g/g}$), rosmarinic acid (29.10 $\pm$ 0.06 $\mu\text{g/g}$), o-anisic acid (39.66 $\pm$ 1.77 $\mu\text{g/g}$), chlorogenic acid (20.14 $\pm$ 1.23 $\mu\text{g/g}$), hydrocinnamic acid (35.16 $\pm$ 2.25 $\mu\text{g/g}$) and hydrobenzoic acid (38.46 $\pm$ 0.49 $\mu\text{g/g}$). The compounds detected at lower concentrations were sinapic acid (13.45 $\pm$ 0.45 $\mu\text{g/g}$), epicatechin gallate (13.14 $\pm$ 0.43 $\mu\text{g/g}$), caffeic acid (17.41 $\pm$ 2.41 $\mu\text{g/g}$), cinnamic acid (12.73 $\pm$ 0.11 $\mu\text{g/g}$), salicylic acid (14.44 $\pm$ 3.74 $\mu\text{g/g}$) and p-coumaric acid (11.05 $\pm$ 1.02 $\mu\text{g/g}$).

These findings are consistent with those reported by [Skendi et al. \(2017\)](#), who observed similar compounds in a 70% hydro-methanolic extract of rosemary leaves, with rosmarinic acid being predominant (4941.5 $\mu\text{g/g}$), followed by quercetin, myricetin, rutin, and luteolin. Caffeic acid, vanillic acid, and p-coumaric acid were among the most abundant simple phenolic acids. In aqueous extracts, rosmarinic acid remained dominant (518.7 $\mu\text{g/g}$), confirming its stability and solubility across different solvent systems.

In ethanolic extracts prepared at 100 °C for 30 min in a water bath, [Mahgoub et al. \(2025\)](#) reported particularly high levels of rosmarinic acid (612.39 $\mu\text{g/mL}$) in Egyptian rosemary, representing more than two-thirds of the total detected phenolics, followed by chlorogenic acid

(35.77 $\mu\text{g/mL}$). Other compounds, such as vanillin, caffeic acid, methyl gallate, rutin, ellagic acid, coumaric acid, quercetin, gallic acid, catechin, syringic acid, ferulic acid, and kaempferol, were also identified. These results highlight the chemical diversity and synergistic potential of rosemary phenolics and demonstrate that the extraction method, solvent type, and plant variety significantly influence the phenolic profile.

[Amaral et al. \(2019\)](#), confirmed by using HPLC the presence of gallic, chlorogenic, caffeic, rosmarinic, quercetin, and carnosic acids in different solvent fractions of Brazilian *Rosmarinus officinalis*, with rosmarinic acid (12.381 mg/g in crude extract) and carnosic acid (89.83 mg/g in crude extract) as the dominant compounds. Differences in concentration can be attributed to plant variety, physiology, environmental conditions, and the method and parameters of extraction.

[Table 5](#) presents the concentrations of phenolic compounds identified in *Pimpinella anisum* extract using HPLC and quantified through calibration with authentic standards. According to the quantitative results, the most abundant phenolic compounds were ferulic acid (100.48 $\pm$ 5.73 $\mu\text{g/g}$), cinnamic acid (147.28 $\pm$ 0.67 $\mu\text{g/g}$), tannic acid (138.87 $\pm$ 0.83 $\mu\text{g/g}$), rosmarinic acid (58.30 $\pm$ 7.90 $\mu\text{g/g}$), quercetin dihydrate (55.19 $\pm$ 0.35 $\mu\text{g/g}$). The intermediate-level compounds included naringin (41.03 $\pm$ 0.40 $\mu\text{g/g}$), gallic acid (37.92 $\pm$ 1.56 $\mu\text{g/g}$), p-coumaric acid (31.92 $\pm$ 0.54 $\mu\text{g/g}$), p-quinic acid (33.36 $\pm$ 0.32 $\mu\text{g/g}$), sinapic acid (31.58 $\pm$ 3.56 $\mu\text{g/g}$), caffeic acid (23.14 $\pm$ 1.07 $\mu\text{g/g}$). The Compounds detected at lower concentrations were rutin hydrate (13.49 $\pm$ 0.87 $\mu\text{g/g}$), rutin (12.58 $\pm$ 0.23 $\mu\text{g/g}$), quercetin (16.63 $\pm$ 0.66 $\mu\text{g/g}$), kaempferol (279.21 $\pm$ 4.90 $\mu\text{g/g}$), epicatechin gallate (17.87 $\pm$ 0.55 $\mu\text{g/g}$), salicylic acid (17.03 $\pm$ 1.30 $\mu\text{g/g}$), o-anisic acid (18.17 $\pm$ 1.10 $\mu\text{g/g}$), m-anisic acid (18.89 $\pm$ 1.31 $\mu\text{g/g}$), anisic acid (8.78 $\pm$ 1.56 $\mu\text{g/g}$).

These results clearly show that the major constituents are dominated by phenolic acids and flavonoids characteristic of anise, particularly rutin and anisic-type acids, which are well-known contributors to antioxidant capacity. [Bettaieb \(2020\)](#) reported that acetone extracts of *P. anisum* from Tunisia obtained by maceration and analyzed via RP-HPLC contained comparable phenolic acids such as gallic, chlorogenic, syringic, p-coumaric, rosmarinic, and ellagic acids as well as several flavonoids, including epicatechin-3-O-gallate, coumarin, rutin, quercetin, naringin, and apigenin. Moreover, [Bettaieb Rebey et al. \(2020\)](#) identified naringin (41.04–43.76%), chlorogenic acid (23.13–27.19%),

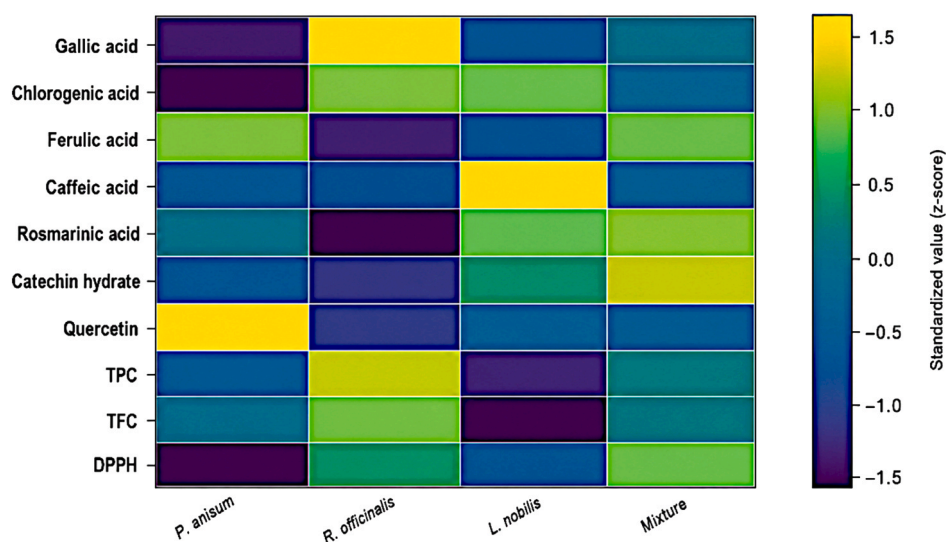


Fig. 5. Heatmap visualization of phenolic composition and antioxidant parameters in the four plant extracts.

and rosmarinic acid (12.26–15.95%) as the predominant compounds, highlighting their central role in antioxidant activity. Similarly, Fayed et al. (2024), using UPLC-ESI-MS/MS on aqueous extracts from Egypt, identified a broad range of phenolics including protocatechuic acid, ethyl caffeate, neochlorogenic acid, feruloylquinic acid derivatives, quercetin-3-O-rhamnoside, kaempferol, and naringenin. Ghazy et al. (2021), also demonstrated that ethanolic extracts of anise contain 25 phenolic compounds that contribute to antioxidant potential, with anethole (272.6 µg/mg), naringenin (153.5 µg/mg), and taxifolin (97.0 µg/mg) as dominant constituents, alongside ellagic acid, catechin, cinnamic acid, rutin, methyl gallate, syringic acid, coumaric acid, caffeic acid, vanillin, and kaempferol.

These results indicate that extraction method, solvent polarity, and geographical origin significantly affect the phenolic composition of *P. anisum*.

Table 5 shows that the optimized mixture derived from the three plant extracts contains several major phenolic constituents. The dominant compounds included epicatechin gallate (116.75 ± 2.33 µg/g), ferulic acid (97.62 ± 6.53 µg/g), gallic acid (64.69 ± 4.86 µg/g), p-quinic acid (67.68 ± 0.95 µg/g). The intermediate-level compounds included cinnamic acid (38.17 ± 0.80 µg/g), sinapic acid (36.02 ± 1.78 µg/g), quercetin dihydrate (34.87 ± 1.46 µg/g), rutin hydrate (25.44 ± 0.73 µg/g) and caffeic acid (30.36 ± 0.85 µg/g). The compounds detected at lower concentrations were rutin (14.56 ± 0.29 µg/g), naringin (17.08 ± 20.46 µg/g), catechin hydrate (19.17 ± 1.71 µg/g), chlorogenic acid (15.56 ± 1.80 µg/g), anisic acid (13.52 ± 0.56 µg/g), m-anisic acid (16.82 ± 0.73 µg/g), o-anisic acid (17.24 ± 1.38 µg/g).

Distinct patterns were observed between phenolic acids and flavonoids when comparing the phenolic composition of the mixture with the sum of individual extracts. Quantitatively, phenolic acids including gallic acid, caffeic acid, chlorogenic acid, ferulic acid, rosmarinic acid, and cinnamic acid were generally more abundant than flavonoids in all individual extracts and in the mixture, reaching concentrations up to several hundred µg/g in certain extracts. Flavonoids, such as catechin, epicatechin gallate and rutin were present at lower concentrations, often under 50 µg/g. Despite their lower abundance, flavonoids displayed additive behavior, with concentrations in the mixture close to the sum of individual extracts, indicating high stability, minimal interaction, and efficient detectability under analytical conditions (Pinelo et al., 2004).

In contrast, most phenolic acids exhibited concentrations in the mixture lower than the theoretical sum, reflecting antagonistic interactions. These effects likely result from post-extraction molecular

interactions, formation of non-covalent complexes with other phenolics or matrix components, partial degradation through oxidation or hydrolysis, and matrix effects that reduce detectability in HPLC analysis (Pinelo et al., 2004). In some cases, certain compounds in the mixture showed higher than the theoretical sum, where the combination of extracts enhanced stability or solubility, producing higher measured concentrations than observed in individual extracts (Saura-Calixto, 1998).

These results indicate that the behavior of phenolic compounds in mixtures is compound-dependent: flavonoids tend to be more stable and additive, phenolic acids are more abundant but prone to antagonistic losses, and a subset of compounds may experience synergistic stabilization. Such distinctions are crucial for interpreting the bioactive potential and functional properties of combined plant extracts.

Consequently, these results showed that extracts obtained with a low ethanol concentration contain higher amount of phenolic compounds than the purely alcoholic extracts. Moreover, the use of ultrasound-assisted extraction with alternative, eco-friendly solvents appear to be a promising approach for obtaining high-quality extracts while minimizing the use of conventional organic solvents.

Certain compounds in the mixture were present at higher levels than in each individual plant. These findings confirm the results of Section 3.4 which showed that the polyphenol and flavonoid contents are higher in the combined extracts. This effect suggests that it would be interesting to purify each compound and evaluate its antioxidant activity individually, to establish a precise correlation between each compound and its antioxidant effect.

Since no previous studies have characterized the phenolic profile of this specific combination of plants, our work represents the first contribution to the detailed characterization of a three-extract mixture.

3.6. Phenolic composition and antioxidant relate patterns

Heatmap visualization (Fig. 5) highlighted pronounced differences in phenolic composition and antioxidant-related parameters among the four studied plant extracts, reflecting plant-specific phenolic profiles. *Rosmarinus officinalis* exhibited generally higher total phenolic and flavonoid contents, whereas *Laurus nobilis* was characterized by elevated levels of specific phenolic acids, particularly caffeic and rosmarinic acids. Therefore, *Pimpinella anisum* showed a profile dominated by cinnamic and ferulic-acid derivatives with comparatively lower antioxidant activity. Notably, the mixture displayed an intermediate phenolic composition but the highest DPPH radical scavenging activity, suggesting potential synergistic interactions among the combined extracts.

The phenolic compounds exhibiting the strongest correlations with antioxidant activity were not necessarily the most abundant ones, indicating that antioxidant capacity is influenced not only by concentration but also by compound-specific reactivity, structural features and synergistic interactions within complex plant matrices. Although causality cannot be inferred from correlation analysis, these findings provide valuable insight into phenolic compounds that may contribute to antioxidant capacity when comparing different plant extracts.

4. Conclusion

This study established optimal ultrasound-assisted extraction (UAE) conditions for *Laurus nobilis* (75 °C, 56 min, 74% power, 4% ethanol), *Rosmarinus officinalis* (25 °C, 51 min, 89% power, 17% ethanol), and *Pimpinella anisum* (50 °C, 20 min, 60% power, 10% ethanol), maximizing recovery of bioactive compounds and antioxidant activity. HPLC-DAD analysis identified gallic acid, caffeic acid, tannic acid, salicylic acid, p-coumaric acid, catechin, kaempferol, and rutin in *L. nobilis*; rosmarinic acid, quercetin, myricetin, rutin, and caffeic acid in *R. officinalis*; and gallic acid, chlorogenic acid, caffeic acid, rosmarinic acid, naringin, quercetin, myricetin, and rutin in *P. anisum*. The optimal mixture (64.9% *P. anisum*, 13.8% *R. officinalis*, 21.3% *L. nobilis*) achieved the highest polyphenolic contents, the greatest percentage of inhibition of free radical scavenging activity DPPH (TPC = 34.37 mg GAE·g⁻¹ DW, TFC = 130.34 mg CE·g⁻¹ DW, PI = 49.45%) and an enriched polyphenolic profile dominated by rosmarinic acid, gallic acid, ferulic acid, caffeic acid, quercetin dihydrate, p-coumaric acid, epicatechin gallate, sinapic acid p-quinic acid and rutin hydrate demonstrating synergistic interactions among the three plants. These results highlight the importance of optimizing multi-plant formulations in agroalimentary, nutraceutical, and pharmaceutical applications. As perspectives, an edible bioactive film can be formulated and enriched with these extracts and this mixture, as well as with a purification of the major compounds of the extracts, such as tannic acid, cinnamic acid and quercetin dihydrate from *Pimpinella anisum*, gallic acid and p-quinic acid from rosemary and laurel, and epicatechin gallate and rosmarinic acid from the mixture.

CRedit authorship contribution statement

Yosra Bannour: Methodology, Investigation, Formal analysis. **Ibtissem Hamrouni Sellami:** Validation, Methodology, Investigation, Funding acquisition, Conceptualization. **Manon Genva:** Writing – review & editing. **Marie-Laure Fauconnier:** Writing – review & editing. **Ayadi Mohamed:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jarmap.2026.100732](https://doi.org/10.1016/j.jarmap.2026.100732).

Data availability

Data will be available on request.

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